BIOPESTICIDES REGISTRATION ACTION DOCUMENT

Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 Proteins and the Genetic Material Necessary for Their Production (PHP17662 T-DNA) in Event DAS-59122-7 Corn (OECD Unique Identifier: DAS-59122-7)

PC Code: 006490

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

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I. OVERVIEW

A. Background

On August 31, 2005, the Environmental Protection Agency (EPA) issued time-limited, conditional registrations to Mycogen Seeds c/o Dow AgroSciences LLC (EPA Reg. No. 68467-5) and Pioneer Hi-Bred International, Incorporated (EPA Reg. No. 29964-4) for event DAS-59122-7 corn, a plant-incorporated protectant expressing the active ingredient, *Bacillus thuringiensis* (*Bt*) Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (PHP17662 T-DNA) in event DAS-59122-7 corn (Organization for Economic Cooperation and Development (OECD) Unique Identifier: DAS-59122-7). Event DAS-59122-7 corn, at the time of 2005 registration, expressed only the second plant-incorporated protectant (PIP) active ingredient to offer protection against corn rootworm, and expectations were that adaptation of this new technology would result in further reduction of conventional insecticide use by growers attempting to control the highly destructive corn rootworm and maintain their crop yields. Prior to registration and after extensive review of copious amounts of data/information submitted by the applicants, the Agency determined that the use of this pesticide was in the public interest and that it would not cause any unreasonable adverse effects on the environment during the period of time-limited (5 year), conditional registration.

Subsequent to registration of the single-trait products in 2005, the Agency registered stacked and/or pyramided plant-incorporated protectants (PIPs), expressing Cry34/35Ab1 along with other proteins, and two seed blends for either commercial or limited breeding purposes. A complete list of the currently registered products expressing Cry34/35Ab1—including their respective registration numbers, product names, registrants, initial dates of registration, proteins (or active ingredients) expressed, and any limitations/special notes—can be found in Appendix A. In conjunction with the 2010 evaluation (explained in the paragraphs that follow), the Agency attempted to better detail and describe product characterization, human health, environmental effects, and insect resistance management data that were submitted to support the registrations of the following stacked and/or pyramided products expressing Cry34/35Ab1:

(1) Herculex® XTRA Insect Protection Corn (EPA Reg. Nos. 68467-6 and 29964-5)
(2) 1507 x 59122 x MON 810 (EPA Reg. No. 29964-8)
(3) 59122 x MON 810 (EPA Reg. No. 29964-9)

When the first products containing Cry34/35Ab1 were registered by the Agency, Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International, Incorporated were issued time-limited, conditional registrations under section 3(c)(7)(C) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Along with several requirements for further product characterization, environmental effects, and insect resistance management data, the registration notices also clearly established an absolute expiration date of September 30, 2010. The follow-on stacks and/or pyramids, specifically mentioned above, all had the same expiration date with the exception of 1507 x 59122 x MON 810 and 59122 x MON 810, which are set to expire on October 31, 2010. The registrants for the Cry34/35Ab1

single-trait and combination PIPs, due to expire on either September 30, 2010 or October 31, 2010, formally requested that the Agency amend their associated products to extend the expiration dates.

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 Cry34/35Ab1 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 Cry34/35Ab1 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 Cry34/35Ab1 registrations by extending their expiration dates in accordance with the scheme explained in section III(C) of this Biopesticides Registration Action Document (BRAD). The basis for this decision can be found in both the risk assessment for the Cry34/35Ab1 corn products, which is characterized throughout this BRAD, and the Agency's response to comments document.

All data and findings for the Cry34/35Ab1 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 Cry34/35Ab1 corn products, there are other *Bt* corn PIPs, expressing different proteins effective in controlling corn borers 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 - September 2010)
- Cry3Bb1 BRAD (Draft - July 2010; Final - September 2010)
- mCry3A BRAD (Draft - July 2010; Final - September 2010)
- Cry1A.105 and Cry2Ab2 BRAD (Draft - August 2010; Final - September 2010)

[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*.

- Optimum® AcreMax™ *B.t.* Seed Blends BRAD (Draft - August 2010; Final - September 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 Corn Rootworm Monitoring Reviews (June 2010)
- Public Comments on EPA Docket Number EPA-HQ-OPP-2010-0607
- EPA's Response to Comments

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 time frames, 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. Use Profile

Pesticide Name: *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (PHP17662 T-DNA) in event DAS-59122-7 corn (OECD Unique Identifier: DAS-59122-7)

Trade and Other Names: Event DAS-59122-7 Corn, Herculex® Rootworm Insect Protection, Herculex® RW Insect Protection, Herculex® RW Rootworm Protection, Herculex® RW

OPP Chemical Code: 006490

Basic Manufacturers: Mycogen Seeds c/o Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268-1054

Pioneer Hi-Bred International, Incorporated
7100 N.W. 62nd Avenue
P.O. Box 1000
Johnston, IA 50131-1000

Type of Pesticide: Plant-Incorporated Protectant

Use: Field Corn

Target Pests: western corn rootworm (*Diabrotica virgifera virgifera*), northern corn rootworm (*Diabrotica barberi*), and Mexican corn rootworm (*Diabrotica virgifera zeae*)

Products Expressing This Pesticide: See complete list in Appendix A.

C. Regulatory History

Date	Action Type	Description
March 7, 2003	Federal Register Publication (Notice of Filing)	Notice of Filing summarizing information submitted and cited by Mycogen Seeds c/o Dow AgroSciences LLC in support of a request for establishment of a temporary exemption from the requirement of a tolerance for residues of the plant-incorporated protectant, *Bacillus thuringiensis* Cry34/35Ab1 insecticidal crystal protein and the genetic material necessary for its production in corn. (68 Federal Register (FR) 11100)
March 13, 2003	Federal Register Publication (Notice of Receipt)	Notice announcing receipt of applications 68467-EUP-3, 68467-EUP-5, 68467-EUP-T, 68467-EUP-I, 29964-EUP-1, 29964-EUP-3, 29964-EUP-U, and 29964-EUP-L from Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International, Incorporated requesting experimental use permits and experimental use permit amendments for the following: (1) *Bacillus thuringiensis* Cry34/35Ab1protein and the genetic material necessary for its production (from the insert of plasmid PHP 14352) in corn (2) *Bacillus thuringiensis* Cry34/35Ab1 protein and the genetic material necessary for its production (from the insert of plasmid PHP 12560) in corn (3) *Bacillus thuringiensis* Cry34/35Ab1 protein and the genetic material necessary for its production (from the insert of plasmid PHP 17662) in corn (4) *Bacillus thuringiensis* Cry34/35Ab1 protein and the genetic material necessary for its production (from the insert of plasmid PHP 17658) in corn (68 FR 12073)

Date	Action Type	Description
July 7, 2003	Federal Register Publication (Final Rule)	The following temporary exemption from the requirement of a tolerance was established under 40 Code of Federal Regulations (CFR) § 180.1242: "*Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production in corn are temporarily exempted from the requirement of a tolerance when used as plant-incorporated protectants in the food and feed commodities of field corn, sweet corn and popcorn. This temporary exemption from the requirement of a tolerance will permit the use of the food commodities in this paragraph when treated in accordance with the provisions of the experimental use permits 68467-EUP-3, 68467-EUP-5, 68467-EUP-T(7), 68467-EUP-I(8), 29964-EUP-1, 29964-EUP-3, 29964-EUP-U(4), and 29964-EUP-L(5) which may be issued and amended/extended under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), as amended (7 U.S.C. 136). This temporary exemption from the requirement of a tolerance expires and is revoked April 30, 2006. This temporary exemption from the requirement of a tolerance may be revoked at any time if the experimental use permit is revoked or if any experience with or scientific data on this pesticide indicate that the tolerance is not safe." (68 FR 40178)
January 6, 2004	Federal Register Publication (Notice of Issuance)	Notice announcing issuance of experimental use permits to Mycogen Seeds c/o Dow AgroSciences LLC (68467-EUP-7) and Pioneer Hi-Bred International, Incorporated (29964-EUP-5). Each permit allowed for use of *Bacillus thuringiensis* Cry34/35Ab1 proteins and the genetic material necessary for their production (from the insert of plasmid PHP17662) in corn on 624 acres of field corn and was effective from June 24, 2003 to May 1, 2004. (69 FR 658)
March 10, 2004	Federal Register Publication (Notice of Receipt)	Notice announcing receipt of applications from Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International, Incorporated requesting amendments/extensions to their experimental use permits (68467-EUP-7 and 29964-EUP-5, respectively). (69 FR 11431)
August 31, 2004	Federal Register Publication (Notice of Filing)	Notice of Filing summarizing information submitted and cited by Mycogen Seeds c/o Dow AgroSciences LLC in support of a request for the establishment of a permanent exemption from the requirement of a tolerance for residues of the plant-incorporated protectant, *Bacillus thuringiensis* Cry 34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production in corn. (69 FR 53060)

Date	Action Type	Description
September 1, 2004	Federal Register Publication (Notice of Receipt)	Notice announcing receipt of the following applications for products containing new active ingredients: (1) File Symbol: 29964-U. Applicant: Pioneer Hi-Bred International, A DuPont Company, 7250 N.W. 62nd Ave., P.O. Box 552, Johnston, IA 50131-0552. Product Name: Pioneer Brand *B.t.* Cry34/35Ab1 Insect Resistant Corn Seed. Plant-incorporated protectant. Active Ingredient: *Bacillus thuringiensis* Cry34/35Ab1 insecticidal crystal protein and the genetic material for its production (plasmid insert PHP 17662) in event DAS-59122-7 corn. (2) File Symbol: 68467-L. Applicant: Mycogen Seeds c/o Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268. Product Name: Mycogen Brand *B.t.* Cry34/35Ab1 Construct 17662 Corn. Plant-incorporated protectant. Active Ingredient: *Bacillus thuringiensis* Cry34/35Ab1 insecticidal crystal protein and the genetic material for its production (plasmid insert PHP 17662) in event DAS-59122-7 corn. (69 FR 53434)
December 22, 2004	Federal Register Publication (Notice of Issuance)	Notice announcing extension/amendment of experimental use permits previously approved for Mycogen Seeds c/o Dow AgroSciences LLC (68467-EUP-7) and Pioneer Hi-Bred International, Incorporated (29964-EUP-5). (1) 68467-EUP-7: For use of 0.94 pounds of *Bacillus thuringiensis* Cry34/35Ab1 proteins and the genetic material necessary for their production (from the insert of plasmid PHP17662) in corn on 1,177 acres of field corn; Effective from April 29, 2004 to April 30, 2005. (2) 29964-EUP-5: For use of 3.97 pounds of *Bacillus thuringiensis* Cry34/35Ab1 proteins and the genetic material necessary for their production (from the insert of plasmid PHP17662) in corn on 4,690 acres of field corn; Effective from April 29, 2004 to April 30, 2005. (69 FR 76732)

Date	Action Type	Description
June 3, 2005	Federal Register Publication (Notice of Receipt)	Notice announcing receipt of the following applications for products containing new active ingredients: (1) File Symbol: 29964-L. Applicant: Pioneer Hi-Bred International, A DuPont Company, 7250 N.W. 62nd Ave., P.O. Box 552, Johnston, IA 50131-0552. Product Name: Herculex Xtra Insect Protection. Plant-incorporated protectant. Active Ingredient: *Bacillus thuringiensis* Cry34/35Ab1 insecticidal crystal protein and the genetic material for its production (plasmid insert PHP 17662) in event DAS-59122-7 corn and *Bacillus thuringiensis* Cry1F protein and the genetic material for its production (plasmid insert PHI 8999) in corn. (2) File Symbol: 68467-A. Applicant: Mycogen Seeds c/o Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268. Product Name: Mycogen Seeds Herculex Xtra Insect Protection. Plant-incorporated protectant. Active Ingredient: *Bacillus thuringiensis* Cry34/35Ab1 insecticidal crystal protein and the genetic material for its production (plasmid insert PHP 17662) in event DAS-59122-7 corn and *Bacillus thuringiensis* Cry1F protein and the genetic material for its production (plasmid insert PHI 8999) in corn. (70 FR 32610)
August 10, 2005	Federal Register Publication (Notice of Issuance)	Notice announcing extension/amendment of experimental use permits previously approved for Mycogen Seeds c/o Dow AgroSciences LLC (68467-EUP-7) and Pioneer Hi-Bred International, Incorporated (29964-EUP-5). (1) 68467-EUP-7: For use of 2,734.85 grams Cry34Ab1 and 10.88 grams Cry35Ab1 of the insecticides Cry34/35Ab1 proteins and the genetic material necessary for their production (from the insert of plasmid PHP17662) in corn on 3,096 acres of field corn; Effective from January 21, 2005 to April 30, 2006. (2) 29964-EUP-5: For use of 1,813.6 grams Cry34Ab1 and 47.2 grams Cry35Ab1 of the insecticides Cry34/35Ab1 proteins and the genetic material necessary for their production (from the insert of plasmid PHP17662) in corn on 5,115 acres of field corn; Effective from January 21, 2005 to April 30, 2006. (70 FR 46510)

Date	Action Type	Description
August 31, 2005	Registration	The Agency issued time-limited, conditional registration notices (under FIFRA section 3(c)(7)(C)) for event DAS-59122-7 corn to Mycogen Seeds c/o Dow AgroSciences LLC (EPA Reg. No. 68467-5) and Pioneer Hi-Bred International, Incorporated (EPA Reg. No. 29964-4). *Expiration Date: September 30, 2010
September 21, 2005	Federal Register Publication (Final Rule)	The following permanent exemption from the requirement of a tolerance was established under 40 CFR § 174.457: "*Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production in corn are exempted from the requirement of a tolerance when used as plant-incorporated protectants in the food and feed commodities of corn; corn, field; corn, sweet; and corn, pop." (70 FR 55254)
October 27, 2005	Registration	The Agency issued time-limited, conditional registration notices (under FIFRA section 3(c)(7)(A)) for Herculex® Xtra Insect Protection to Mycogen Seeds c/o Dow AgroSciences LLC (EPA Reg. No. 68467-6) and Pioneer Hi-Bred International, Incorporated (EPA Reg. No. 29964-5). *Expiration Date: October 15, 2008
April 25, 2007	Federal Register Publication (Direct Final Rule)	The tolerance exemption for Cry34/35Ab1 proteins was redesignated from 40 CFR § 174.457 to 40 CFR § 174.506 and changed to the following: "Residues of *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins in corn are exempted from the requirement of a tolerance when used as plant-incorporated protectants in the food and feed commodities of corn; corn, field; corn, sweet; and corn, pop." (72 FR 20431)
August 15, 2007	Federal Register Publication (Notice of Issuance)	Notice announcing conditional approval of two products (Herculex® Rootworm Insect Protection, EPA Reg. No. 29964-4; Herculex® RW Insect Protection, EPA Reg. No. 68467-5) containing *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (plasmid insert PHP17662) in event DAS-59122-7 corn, an active ingredient not included in any previously registered product. (72 FR 45807)

Date	Action Type	Description
October 8, 2008	Amendment	The Herculex® Xtra Insect Protection registrations (EPA Reg. Nos. 29964-5 and 68467-6) were amended by the Agency to extend the expiration date set forth in the original registration notices. *New Expiration Date: September 30, 2010
July 2009–April 2010	Registration	The Agency registered several combination PIP products expressing Cry34/35Ab1 and another protein(s), as well as two seed blends. See Appendix A for the complete list.
September 2010	Amendment	The Herculex® Xtra Insect Protection (EPA Reg. Nos. 29964-5 and 68467-6), Herculex® Rootworm Insect Protection (EPA Reg. No. 29964-4), and Herculex® RW Insect Protection (EPA Reg. No. 68467-5) registrations were amended by the Agency to extend the expiration date in accordance with the scheme explained in section III(C) of this Biopesticides Registration Action Document (BRAD). *New Expiration Date: September 30, 2015

II. SCIENCE ASSESSMENT

The classifications that are found for each data submission are assigned by Environmental Protection Agency (EPA) science reviewers and are an indication of the usefulness of the information contained in the documents for risk assessment. A rating of "ACCEPTABLE" indicates the study is scientifically sound and is useful for risk assessment. A "SUPPLEMENTAL" rating indicates the data provide some information that can be useful for risk assessment. The studies may have certain aspects determined not to be scientifically acceptable ("SUPPLEMENTAL: UPGRADABLE"). If a study is rated as "SUPPLEMENTAL: UPGRADABLE," EPA always provides an indication of what is lacking or what can be provided to change the rating to "ACCEPTABLE." If there is simply a "SUPPLEMENTAL" rating, the reviewer will often state that the study is not required by the current 40 Code of Federal Regulations (CFR) Part 158. Both "ACCEPTABLE" and "SUPPLEMENTAL" studies may be used in the risk assessment process as appropriate. An "UNACCEPTABLE" rating indicates that the study is not useful for risk assessment and cannot be upgraded.

A. Product Characterization

1. Event DAS-59122-7 Corn (Organization for Economic Cooperation and Development (OECD) Unique Identifier: DAS-59122-7) Expressing Cry34Ab1 and Cry35Ab1

a. Data Cited/Submitted for Initial Registrations of Event DAS-59122-7 Corn (Prior to August 2005)

The Agency's detailed assessment of the product characterization for *Bacillus thuringiensis* (*Bt*) Cry34/35Ab1 corn is found in U.S. EPA (2004). Portions of the product characterization data were also reviewed in U.S. EPA (2001a, 2002, 2003a, 2003c, 2003d, and 2003e). *Bacillus thuringiensis* Cry34/35Ab1 corn was produced by *Agrobacterium tumefaciens*-mediated transformation of public corn line (Hi-II) with the transfer deoxyribonucleic acid (T-DNA) from plasmid PHP17662[a], which contains *cry*34Ab1, *cry*35Ab1, *phosphinothricin acetyltransferase* (*pat*), and regulatory sequences necessary for the expression of the genes. The *cry*34Ab1 and *cry*35Ab1 transgenes were optimized for expression in maize, but the amino acid sequence of the expressed proteins is identical to the native proteins from *Bt*. Characterization of the deoxyribonucleic acid (DNA) isolated from *Bt* Cry34/35Ab1 corn, using restriction enzyme digests and Southern blot analysis (Master Record Identification Numbers (MRID Nos.) 461239-08 and 461239-09; reviewed in U.S. EPA (2004)), indicated that the T-DNA from plasmid PHP17662 inserted as a single, intact copy into the corn genome. In addition, DNA analysis indicated stability and inheritance of the inserted DNA within and across several generations.

Protein characterization data demonstrated that the plant-produced proteins have characteristics and activities that are equivalent to those of the proteins produced in *Pseudomonas fluorescens* transformed to produce Cry34Ab1 and Cry35Ab1 (MRID Nos. 461239-05 and 461239-06; reviewed in U.S. EPA

[a] Early in the development of this product, other plasmids were used and other events tested. Event DAS-59122-7, where plasmid PHP17662 was used, has been chosen for commercialization.

(2004)). The following techniques were used to characterize and compare the plant-produced proteins and the microbially produced proteins: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), N-terminal amino acid sequencing, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), glycosylation analysis, enzyme-linked immunosorbent assays (ELISAs), and western blot analysis. Glycosylation analysis indicated that the Cry34Ab1 and Cry35Ab1 proteins are not glycosylated. Biological activity of the plant-produced and *P. fluorescens*-produced proteins was also assessed and compared; Cry34Ab1 and Cry35Ab1 from both sources displayed similar insecticidal activity to western corn rootworm larvae. These analyses justified the use of microbially produced proteins in toxicity studies.

Studies on the mode of action of Cry34Ab1 and Cry35Ab1 indicated that, similar to other *Bt* delta-endotoxins, Cry34Ab1 and Cry35Ab1 appear to target midgut epithelial cells in susceptible larvae (Master Record Identification Number (MRID No.) 457906-01; reviewed in U.S. EPA (2003c)). Cry34Ab1 appears to cause pore formation in phospholipid membranes, and addition of Cry35Ab1 resulted in pores remaining open longer and improved membrane permeability (Masson *et al*. 2004). Ribosomal inhibition activity was also investigated (MRID No. 461239-10; reviewed in U.S. EPA (2004)). The results demonstrated that the insecticidal activity of Cry34Ab1 and Cry35Ab1 is not associated with the inhibition of protein synthesis.

Submitted studies on heat stability of the Cry34Ab1 and Cry35Ab1 proteins demonstrated that these proteins are inactivated at 90°C and 60°C, respectively (MRID Nos. 453584-01, 455845-01, 458086-01, and 458602-01; reviewed in U.S. EPA (2001a, 2002, and 2003a)).

Data on expression levels of the proteins in transgenic corn tissues were also provided for both inbred and hybrid lines from several different events (MRID No. 461239-04; reviewed in U.S. EPA (2004)). The protein expression levels were comparable between hybrid and inbred lines. Data on expression levels in a hybrid line are summarized in Table 1.

Table 1. Mean Expression Levels of Cry34Ab1 and Cry35Ab1 in Plant Tissues from a Hybrid Line Containing Event DAS-59122-7.

Tissue	Cry34Ab1 (ng/mg tissue dry weight ± standard deviation)*	Cry35Ab1 (ng/mg tissue dry weight ± standard deviation)*
Leaf	50 ± 8 – 220 ± 38	41 ± 7 – 85 ± 19
Root	37 ± 9 – 50 ± 20	3 ± 2 – 8 ± 3
Whole Plant	32 ± 16 – 77 ± 10	7 ± 2 – 14 ± 2
Pollen	74 ± 7	0.02 ± 0.04
Stalk	33 ± 4	10 ± 2
Forage	53 ± 19	12 ± 3
Grain	50 ± 16	1 ± 0.3

* Ranges reflect the range of means at different growth stages.

The product characterization studies that were submitted in support of event DAS-59122-7 corn are summarized in Table 2. Some of the studies were conducted on events other than DAS-59122-7, and

some of the studies refer to the Cry34Ab1 and Cry35Ab1 proteins as PS149B1 13.6- or 14-kiloDalton (kDa) and 43.8- or 44-kDa insecticidal crystal proteins. The term, PS149B1 proteins, was used prior to the proteins receiving their *Bt* toxin nomenclature designations (Crickmore *et al*. 2010). The PS149B1 13.6-kDa or 14-kDa proteins and 43.8-kDa or 44-kDa proteins are the same as the Cry34Ab1 and Cry35Ab1 proteins, respectively.

Table 2. Product Characterization Data for Event DAS-59122-7 Corn (Reviewed in U.S. EPA (2004) Unless Otherwise Noted).

Study Title	Summary	MRID No.
Product Characterization Data for *Bacillus thuringiensis* PS149B1 13.6 kDa and 43.8 kDa Insecticidal Crystal Proteins Expressed in Transgenic Maize Plants[b]	Maize immature embryo tissues were transformed using a linearized fragment from plasmid PHP12560 encoding two insecticidal crystal protein (ICP) genes, *8v6* (13.6 kDa) and *7v7* (43.8 kDa), each driven by the maize ubiquitin promoter, and the *pat* gene, driven by the CaMV 35S promoter. The two ICP genes from *Bt* PS149B1 are modified in coding sequence to optimize for plant expression. Amino acid sequences are, however, identical to the bacterial source proteins. Microprojectile bombardment of tissues, followed by selection on glufosinate, produced plants expressing the two insecticidal crystal proteins (ICPs) of PS149B1 and PAT. Maize plants expressing these genes were also produced by *Agrobacterium* - mediated transformation of immature embryos. Western corn rootworm (WCRW) bioassays were used to assess the bioactivity of the expressed ICPs. Within 48 hours of feeding on transgenic maize plants, WCRW larvae had midgut symptoms consistent with feeding on delta-endotoxins (e.g., swelling, lysis, blebbing, or vacuolization). WCRW feeding on control plants did not show pathological changes in midgut epithelium. The two ICPs were also tested for adenosine diphosphate (ADP)-ribosylating or glucosylating activities as well as other enzymic activities, but none were detected. **Classification: Acceptable**	452422-01
Characterization of Inserted DNA of Event 5639 Transgenic Corn Plants – Interim Report[b]	Based upon the Southern blot data presented, the most likely conclusion is that there are three insertions of the *8v6*, *7v7*, and *pat* genes into event 5639, although only one may be fully intact. The complexity of the hybridization patterns does not allow one to say with certainty that three insertions are present at this time. A more detailed analysis of gene insertion will be required as this product develops to fully ascertain the nature of the integration events. Data indicated that event 5639 does not contain a *kan*^{r} sequence. **Classification: Acceptable**	452422-02

[b] Reviewed in U.S. EPA (2001a)

Study Title	Summary	MRID No.
Equivalency of Microbial- and Maize-Expressed PS149B1 proteins[c]	Biochemical, molecular, and serological data presented indicate that the proteins produced in the *P. fluorescens* system are equivalent to the proteins produced in transgenic maize plants. Relative molecular mass and serological reactivity to polyclonal antibodies were the same for the 14-kDa proteins produced in either system. SDS-PAGE and western blots indicated identity between the two production systems. This was also true for the 44-kDa protein and its proteolytic cleavage product, the 42-kDa protein. The 44-kDa protein predominated in the fresh plant material and in the *P. fluorescens* production system. Storage of leaf material led to the production of the 42-kDa protein as the predominant form due to proteolytic processing. No glycosylation was detected on either protein from the maize system. Tryptic digestion of PS149B1 proteins and subsequent analysis by MALDI-TOF-MS, N-terminal sequencing, and electrospray ionization tandem mass spectrometry (ESI MS/MS) spectra all indicated that PS149B1 proteins produced by *P. fluorescens* and the transgenic maize are equivalent for toxicological testing to be performed with proteins from either source. In insect bioassays, proteins produced in the transgenic maize system and the microbial system both showed activity against the southern corn rootworm (SCRW) and WCRW larvae. While the transgenic maize assay indicated no reduction in damage from European corn borer (ECB) feeding, the diet-based overlay with the microbially produced protein indicated some slight inhibition of growth for ECB larvae. Additionally, the PS149B1 maize plants sustained greater leaf damage from ECB, based upon visual estimates, than the control plants. It is not clear if this difference was statistically significant since there was no analysis provided. **Classification: Acceptable**	452422-03
Microbial PS149B1 Binary Delta-Endotoxin: Maize-Insect-Pest Susceptibility Study[c]	The relative sensitivity of the test insects in the feeding bioassays suggests that larvae of coleopteran species are far more sensitive to the 14-kDa and 44-kDa proteins of PS149B1 than the lepidopteran or homopteran larval insects evaluated. Adult WCRW were not sensitive to the delta-endotoxins, however. Rootworms (northern corn rootworm (NCRW), WCRW, and SCRW) were the most susceptible to the PS149B1 proteins. European corn borer and corn earworm (CEW) demonstrated some inhibition of growth at higher concentrations of the test substance than the rootworm larvae. Black cutworm (BCW) was the least sensitive of the lepidopteran species tested. No activity was seen against the corn leaf aphid (CLA), a homopteran. Relative susceptibilities of the insect species to PS149B1, based upon the more sensitive measure of 50% growth inhibition (GI_{50}), were as follows: (most susceptible) WCRW, NCRW, SCRW > ECB, CEW >> WCRW adult, CLA, BCW (least susceptible). **Classification: Acceptable**	452422-04

[c] Reviewed in U.S. EPA (2001a)

Study Title	Summary	MRID No.
Quantitative ELISA Analysis of PS149B1 Protein Expression Levels in Hybrid and Inbred Lines of Maize Event TC5639[d]	Leaf, pollen, root, and senescent whole plant tissue samples were taken from field-grown plants cultured in the U.S. corn belt. Both test hybrid and inbred lines of event 5639 and control hybrid and inbred lines were compared for expression of the 14-kDa and 44-kDa PS149B1 proteins. Leaf, pollen, root, and senescent whole plant tissues expressed the 14-kDa and 44-kDa proteins at measurable levels in the modified maize lines. Hybrid and inbred lines had comparable levels of expression of these proteins when analyzed on a dry weight basis. On a total extractable protein basis, though, root tissues had the highest expression and pollen had the lowest expression. Control tissues were negative for the presence of the 14-kDa or the 44-kDa proteins. Coefficients of variation (CVs) were lacking for data presented and need to be included in order to adequately assess the results of the ELISAs. The use of samples spiked with known quantities of the two proteins separately is also needed to assess the possible influence of maize tissue extracts on the enzyme-linked immunosorbent assay (ELISA) values observed. These data and an evaluation of possible cross-reactivity of the primary antibody with other Cry proteins will be needed for Section 3 registration. **Classification: Acceptable**	452422-13
Lateral Flow Test Kit for the Detection of the PS149B1 Protein in Maize Grain[e]	The double antibody sandwich format of the lateral flow test strip from Strategic Diagnostics was capable of correctly detecting 48 positive aliquots of 24 extracts from 24 single kernel samples of PS149B1 maize. Conversely, 48 aliquots of 24 extracts from 24 kernels of a non-PS149B1 inbred line all tested negative, as expected. Ratios of positive (PS149B1) and negative (control) kernels were mixed and extracted. When 0.5% or greater PS149B1 kernels were present in the mixture, they were correctly detected as containing Cry34Ab1 protein. No ratios of positive to negative below 0.5% were evaluated in this study. **Classification: Acceptable**	453834-01

[d] Reviewed in U.S. EPA (2001a)
[e] Reviewed in U.S. EPA (2003b)

Study Title	Summary	MRID No.
Characterization of Cry34Ab1 and Cry35Ab1 from Recombinant *Pseudomonas fluorescens* and Transgenic Maize[f]	Cry34Ab1 and Cry35Ab1 proteins were separated from maize leaf extracts and from microbial production sources on SDS-PAGE gels and then blotted to nitrocellulose for serological detection. Both proteins were detected on the western blots by polyclonal antibody preparations and appeared at the appropriate M_r for both proteins on individual blots (i.e., 14 kDa - Cry34Ab1; 44 kDa - Cry35Ab1). A second protein band reacted with the Cry35Ab1-specific antibody in the microbially produced protein preparation at approximately 40 kDa, which is presumably a degradation product from protease activity. This band or signal was noted in the western blot of 3 of 6 maize leaf extracts representing the 6 events but at reduced amounts relative to the microbial preparation. Both Cry proteins from the maize extracts migrated very similarly to the Cry proteins produced in the microbial production system and, by this criterion (i.e., mobility in SDS-PAGE), are considered as indistinguishable. **Classification: Acceptable**	457904-01

[f] Reviewed in U.S. EPA (2003c)

Study Title	Summary	MRID No.
Characterization of DNA Inserted into Transgenic Corn Events E4497.42.1.34, E4497.45.2.16, E4497.59.1.10, E4497.66.1.27, E4497.71.1.29, and E4497.71.1.33[g]	Southern blots were used to discern the number of insertions from the plasmid constructs, PHP17662 and PHP17658, used in the transformation of maize. An analysis of integration sites in events E4497.42.1.34, E4497.45.2.16, and E4497.59.1.10 indicated a single integration of the T-DNA sequence into the maize genome. Each reactive band present in the blot resulted from the *XhoI* cleavage of the incorporated T-DNA region (containing *cry34Ab1*, *cry35Ab1*, and *pat* from PHP17622 in a single fragment) and a second *XhoI* restriction site in the adjacent maize genome. Hence, the bands represented individual, unique integrations of T-DNA in the maize genome. The size of the reactive bands cannot be determined *a priori* since the size of the flanking or border fragment is not known as it is determined by the distance from the single *XhoI* site within the T-DNA region. Events E4497.42.1.34 and E4497.45.2.16 each had single reactive hybridization events (bands) on the blot, indicating the presence of a single T-DNA insertion. The commonality of band sizes (i.e., 9.4 or >22 kilobase (kb)) suggests that the T-DNA insertion is the result of a single insertional event. A 9.4 kb signal in E4497.59.1.10 also indicates the integration of one full T-DNA containing *cry34Ab1*, *cry35Ab1*, and *pat*. The presence of a second hybridizing signal (7.4 kb) in the blot of genomic DNA from E4497.59.1.10 probed with *cry35Ab1* suggests that a partial construct insertion occurred containing only the *cry35Ab1* portion and not *cry34Ab1* or *pat*. *StuI* cuts once in PHP17658 and was used to define integration sites in events E4497.66.1.27, E4497.71.1.29, and E4497.71.1.33. Events E4497.71.1.29 and E4497.71.1.33 appear to both contain a single T-DNA insertion based on the reactive band signals being present at 11 and 8.3 kb for all three probes. Additionally, there was a reactive band at 14 kb and 2.7 kb for the *pat*-probed blots of E4497.71.1.29 and E4497.71.1.33, respectively. The second reactive band in the *pat*- probed blots was a consequence of the position of the cleavage site for the *StuI* enzyme being within the *pat* open reading frame (ORF). Genomic DNA from control plants (non-transgenic) was spiked with restricted plasmid DNA of PHP17661 and PHP17657 at a concentration of one copy per genome equivalent to serve as positive controls. **Classification: Acceptable**	457904-02

[g] Reviewed in U.S. EPA (2003c)

Study Title	Summary	MRID No.
Product Characterization Data for *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 Proteins Expressed in Transgenic Maize Plants (PHP17662)[h]	Genes encoding the PAT, Cry34Ab1, and Cry35Ab1 proteins were transferred into the T-DNA region of the disarmed *A. tumefaciens* strain LBA4404. This Ti-plasmid was then referred to as PHP17662 and is approximately 50 kb in size. This strain carrying PHP17662 was used to transform immature maize embryo tissues by co-cultivation for 6 days. Coding sequences for the Cry34Ab1 (14-kDa) and Cry35Ab1 (44-kDa) proteins were synthesized from the native sequence to optimize expression in maize. The PAT protein produced from a synthetic gene is not altered in its amino acid sequence as compared to the native protein. The gene and protein are considered as inert ingredients in this plant-incorporated protectant. Gut cells of the control maize-fed insects and the starved insects appeared normal, although the latter group showed some collapse of the gut lumen. After 48 hours of feeding on the transgenic maize plants, WCRW larvae had evidence of cell lysis, blebbing, and vacuolization, as would be expected from intoxication with delta-endotoxins in a susceptible insect. Subsequently, the gut tissues showed further collapse and more fragmentation. Enzyme assays were evaluated to determine if either Cry34Ab1 or Cry35Ab1 proteins were capable of activity: glucosylating or ADP-ribosylating of midgut proteins, protease at acid and neutral pH, neuraminidase (sialidase), acid and alkaline phosphatase activity. Protein synthesis inhibition of an *in vitro* system was also tested. All assays were negative for their corresponding enzyme activities. Amino acid sequences of the three proteins and micrographs of histopathological midgut responses were presented. **Classification: Acceptable**	457906-01

[h] Reviewed in U.S. EPA (2003c)

Study Title	Summary	MRID No.
Quantitative ELISA Analysis of Cry34Ab1 and Cry35Ab1 Proteins Expressed in Maize Plants Transformed with the Vector PHP17662[i]	ELISA was used to determine the quantity of the two ICPs present in leaf, pollen, root, and seed of transgenic maize from three separate transformation events (E4497.42.1.34, E4497.45.2.16, and E4497.59.1.10). Direct double antibody sandwich ELISA was used to quantify the Cry34Ab1 and Cry35Ab1 present in plant samples. The primary antibodies raised against Cry34Ab1 and Cry35Ab1 were used to capture the proteins (individually in separate reactions) in the microplate well, and a second specific antibody was used to detect the protein that was bound. This second antibody was conjugated to biotin, which was detected using streptavidin linked to alkaline phosphatase (AP). The colorimetric reaction catalyzed by the AP provides for a means of measuring optical density or absorbance and correlating it to the amount of target protein present based upon comparison to a standard curve generated from reference standards. The values obtained from the ELISA readings are converted to amounts of Cry34Ab1 or Cry35Ab1 protein and expressed as nanograms Cry34Ab1 protein per milligram total extractable protein (TEP). Since data for water content exists for the tissue types sampled, the conversion to nanograms Cry34Ab1 protein per milligram dry tissue is possible with conversion factors. Reference standards used to generate the standard curve were run in triplicate within each 96-well microplate. For Cry34Ab1, the magnitude of expression for tissue types was as follows in descending order: leaf, pollen, kernel, and root. All control tissues were negative for the expression of Cry34Ab1. Due to the Mendelian segregation of the transgenes, approximately 50% of the seeds were found to be expressing Cry34Ab1. For Cry35Ab1, the magnitude of expression for tissue types was as follows in descending order: leaf, root, and kernel. All control tissues were negative for the expression of Cry35Ab1. Cry35Ab1 protein was not detected in pollen. Due to the Mendelian segregation of the transgenes, approximately 50% of the seeds were found to be expressing Cry35Ab1. Seeds that were observed to contain no Cry proteins by ELISA were not included in the summary statistics since they represent segregants from the heterozygous populations of the three events. Mean values are presented in tabular format for all three events for each tissue type. **Classification: Acceptable**	458332-01

[i] Reviewed in U.S. EPA (2003c)

Study Title	Summary	MRID No.
Probe MOA Studies to Assess Potential for Protein Synthesis Inhibition by *Bacillus thuringiensis* PS149B1 Cry34Ab1/Cry35Ab1 Proteins in a Rabbit Reticulocyte Assay: Re-Examination of Lab Notebook Data[j]	Two experiments were performed in order to assess the potential for ribosomal inhibition activity by either Cry34Ab1 or Cry35Ab1. The first experiment concluded, based upon a sensitive luciferase (chemiluminescence) assay, that the Cry 34Ab1 and Cry35Ab1 proteins did not show the properties of ribosomal inhibition proteins (RIPs). This was largely based upon comparison to a bovine serum albumin (BSA) control and phosphate buffered saline (PBS) buffer control. A second experiment, with a different treatment regime and altered protocol, concluded that there was some ribosomal inhibition protein (RIP) activity associated with the PS149B1 proteins. Further, digesting the 14-kDa protein with trypsin may have also reduced translation a little. The two experiments used different amounts of BSA (i.e., 1x and 2x), and this added variable further confounded the interpretation of results. The lack of proper controls and different treatment regimes in the experiments made any assessment of the potential RIP activity dubious. The experiments would also have benefitted by including a greater number of samples per treatment (at least triplicate) and appropriate controls. A positive control (i.e., a protein with known RIP activity) is crucial to the interpretation of results. Note that the source (lot) of the proteins used for the two experiments was different as well. It is also probable that confounding stabilization reagents (e.g., glycerol, azide) were present in the reaction during the second experiment, further complicating any analysis of results. It is not possible, given the present data set and variance in methodologies, to assess the results accurately. The RIP assay has not been considered as a typical part of the submission process for plant-incorporated protectants. There is no *a priori* reasoning or similarity between the delta-endotoxins of *Bt* or *Bacillus sphaericus* insecticidal toxins and proteins with known RIP activity to expect Cry34Ab1 or Cry35Ab1 to exhibit such action. Proteins with this activity also require a means of intracellular vectoring in order to reach the ribosome target. **Classification: Supplemental**	459428-01
Thermolability of PS149B1 Binary Delta-Endotoxin[k]	Results indicate that the PS149B1 proteins, or at least one of them, are inactivated after exposure to 60°C for 30 minutes, based upon activity against the southern corn rootworm. It is possible that shorter times or lower temperatures may have also denatured the protein(s), but these were not tested. With the current protocol design, it is not possible to determine if one or both proteins are inactivated following heat treatment. To address this issue, the applicant should consider repeating the study under a method that would allow this distinction to be made as part of the submission for Section 3 registration. **Classification: Acceptable**	453584-01

[j] Reviewed in U.S. EPA (2003d)
[k] Reviewed in U.S. EPA (2001a)

Study Title	Summary	MRID No.
Heat Lability of Individual Proteins of the PS149B1 Binary ICP[1]	The percent growth inhibition of larvae, as observed in a southern corn rootworm feeding assay, was used as a measure or indicator that the Cry34Ab1 and Cry35Ab1 proteins were degraded to a point they have lost insecticidal activity. The decrease in growth inhibition activity following heating of the 14-kDa and 44-kDa mixture suggests that heat treatment at 60°C for 30 minutes is sufficient to denature at least one of the necessary components of the ICP. No mortality effect was seen with the Cry protein treatments, as expected. The author suggested that the degree of inhibition (30 to 42% larval growth inhibition) seen following heat treatment of the mixture is typical of an inactive protein preparation as observed in previous assays. This difference between the larval weights observed with the ICP-temperature treatments and the buffer control was a non-specific effect attributable to the presence of proteins in the diet irrespective of the amino acid sequence and conformation of the ICPs. The 44-kDa protein in the ICP was denatured by treatment at 60°C and higher temperatures. The decrease in insect growth inhibition, seen in the 44-kDa spike of the heated ICP as temperature of incubation was increased, indicates that the 14-kDa protein was more heat stable than the 44-kDa protein. The concomitant decrease in insect activity (i.e., growth inhibition) with temperature increase shows the relative stability of this protein at 60°C and 75°C but its denaturation to background levels (i.e., 36% growth inhibition compared to buffer control) at 90°C. **Classification: Supplemental**	455845-01

[1] Reviewed in U.S. EPA (2002)

Study Title	Summary	MRID No.
Summary of Heat Lability Studies with Cry34Ab1/Cry35Ab1[m] Slide Presentation Summarizing Cry34Ab1/Cry35Ab1 Heat Inactivation Studies[m]	Using an insect bioassay as the detection method for PS149B1 proteins (Cry34Ab1, Cry35Ab1) provides for a qualitative assessment of the protein(s) remaining after treatment but does not allow for quantitation. This assay utilized the decrease in growth of larval SCRW, measured as mass, as a means of assessing activity remaining in the Cry protein(s) but did not give a direct correlation with protein concentration. Nonetheless, the assay provided for a determination as to the conformational changes (denaturation) that occur following heat treatment. The two studies previously submitted (MRID Nos. 455845-01 and 453584-01), regarding heat stability of the Cry34Ab1 and Cry35Ab1 proteins, demonstrated that these proteins are inactivated at ≤90°C and ≤60°C, respectively. The two current submissions served to clarify the lability of the proteins to heat. However, differences in the execution of the two studies complicated the interpretation and comparison of the results. The feeding bioassays, using the southern corn rootworm, indicated that the proteins are no longer active following their respective heat treatments. This is taken as evidence of protein denaturation. The SCRW assay was based upon growth inhibition of the larvae and not mortality. A different diet formulation was used in each study with a greater than 10-fold increase in larval growth occurring in control insects with the artificial diet in the second study (MRID No. 455845-01). Additionally, the presence of salts and buffer impurities from the purification process used for the synthesis of the test substance (Cry34Ab1 and Cry35Ab1 proteins from a bacterial production system) resulted in experimental variance not expected or attributable to the insecticidal proteins being tested. Both Cry proteins evaluated herein are susceptible to heat denaturation under the conditions tested, albeit with different denaturation profiles. **Classification: Acceptable**	458086-01 458602-01
Independent Laboratory Validation of Pioneer Hi-Bred International, Incorporated ELISA Method for the Quantification of Cry34Ab1 Protein from Transgenic Plants	This study was conducted to provide independent laboratory validation (ILV) data for the determination of Cry34Ab1 insecticidal crystal protein in corn matrices and to support the stated limit of quantitation (LOQ) of 0.072 nanograms per milligram (ng/mg). The reported data satisfied the requirements of U.S. EPA Guideline Office of Chemical Safety and Pollution Prevention (OCSPP) 860.1340(c)(6) and the study protocol. Individual recovery values for each fortified control sample were within the range of 70 – 120% and averaged 78% and 98% at the LOQ and 2x the LOQ, respectively. The relative standard deviation (RSD) of replicate recovery measurements did not exceed 20% at or above the LOQ, and interferences were minor. **Classification: Acceptable**	461239-01

[m] Reviewed in U.S. EPA (2003a)

Study Title	Summary	MRID No.
Independent Laboratory Validation of Dow AgroSciences Method GRM 03.13, "Determination of Cry35Ab1 Insecticidal Protein in Maize Tissue by Enzyme-Linked Immunosorbent Assay"	This study was conducted to provide ILV data for the determination of Cry35Ab1 insecticidal crystal protein in corn matrices and to support the stated LOQ of 0.06 ng/mg. The reported data satisfy the requirements of U.S. EPA Guideline OCSPP 860.1340(c)(6) and the study protocol. Individual recovery values for each fortified control sample were within the range of 70 – 120% and averaged 93 and 101% at the LOQ and 2x the LOQ, respectively. The RSD of replicate recovery measurements did not exceed 20% at or above the LOQ, and interferences were minor. **Classification: Acceptable**	461239-02
Cry34/Cry35 Protein Distribution and Familiarity	DNA hybridization and polymerase chain reaction (PCR) analysis of total genomic DNA from collections of strains of *Bt* showed that homologs of *cry*34/35 genes were present in strains from North and South America and from Australasia taken from a variety of environments. The overall rate of occurrence of *cry*34/35 genes in *Bt* strains was 1.2%; this rate of occurrence is comparable to that of the *cry*3Aa gene, which encodes a previously registered insect control protein (EPA Registration Number 524-474). Sequence comparisons demonstrated that the Cry35 protein has some homology (26 to 29%, respectively) to the 42- and 51-kDa mosquitocidal proteins from *B. sphaericus* strain 2362 (*B. sphaericus* H5a5b strain 2362 is a registered biopesticide). **Classification: Acceptable**	461239-03
Agronomic Characteristics, Quantitative ELISA and Nutrient Composition Analysis of Hybrid Maize Lines Containing the *cry*34Ab1, *cry*35Ab1, and *pat* Genes: Chile Locations	The data from this study indicated that control and transgenic lines (containing events DAS-45216-6, DAS-59122-7, and DAS-45214-4) are similar with respect to agronomic characteristics and nutrient composition. Expression levels of Cry34Ab1, Cry35Ab1, and PAT proteins were analyzed by ELISA for various plant tissues at different growth stages for transgenic hybrid and inbred lines and compared with control lines for both herbicide-sprayed plants and non-sprayed plants. On a dry weight basis, the concentrations found for Cry34Ab1 ranged from a low of 31 ng/mg in R1 stalks to a high of 500 ng/mg in R4 leaves; concentrations for Cry35Ab1 ranged from a low of 0.01 ng/mg in pollen to a high of 120 ng/mg in R4 leaves; and concentrations for PAT ranged from 0 ng/mg in pollen to 36 ng/mg in R1 leaves. Transgenic protein levels were independent of spraying status, comparable between hybrid and inbred lines, and the proteins were not detected in any control samples. **Classification: Acceptable**	461239-04

Study Title	Summary	MRID No.
Biological Equivalency of Cry34Ab1/Cry35Ab1 Insecticidal Crystal Protein in Transgenic Plants and Derived from Transgenic *Pseudomonas fluorescens*	Three transgenic corn lines (DAS 45214-4, 45216-6, and 59122-7), carrying the *cry*34Ab1 and *cry*35Ab1 genes that produce a binary insecticidal protein, and control (non-transgenic) corn were field-tested for their resistance to damage from western corn rootworm, northern corn rootworm, black cutworm, and European corn borer. The transgenic corn lines were all shown to have a statistically significant effect against western and northern corn rootworm but were not effective against European corn borer and black cutworm. These results are consistent with the efficacy profiles observed in previous studies on other corn events expressing the same insecticidal protein (DAS Report 000367) and with the efficacy profiles obtained with purified binary insecticidal protein isolated from *P. fluorescens* (DAS Report 000366). **Classification: Acceptable**	461239-05
Characterization of Cry34Ab1 and Cry35Ab1 Proteins Derived from Transgenic Maize Event E4497.59.1.22 (DAS-59122-7)	Selected biochemical properties of Cry34Ab1 and Cry35Ab1 proteins, isolated from leaf tissue from *Bt* transgenic corn event E4497.59.1.22 (DAS-59122-7) [59.1.22], were evaluated and compared to biochemical properties of the proteins obtained from a *P. fluorescens* bacterial expression system containing the two *Bt* transgenes. The results were also compared with results from a previously submitted study on Cry34Ab1 and Cry35Ab1 isolated from *Bt* transgenic corn event 5638. SDS-PAGE and western blot analysis indicated the relative molecular weights of Cry34Ab1 and Cry35Ab1 from all sources were ~14 kDa and ~44 kDa, respectively. In addition, the Cry35Ab1 protein from both transgenic corn (event 5638) and *P. fluorescens* has been shown previously to be proteolytically degraded to a 40-kDa form (with an intact N–terminus), and the Cry35Ab1 protein isolated from corn event 59.1.22 also showed a 40-kDa degradation product. Glycosylation analysis indicated that Cry34Ab1 and Cry35Ab1 derived from event 59.1.22 are not glycosylated; this result was consistent with the earlier studies of these two proteins derived from corn event 5638 and from *P. fluorescens*. MALDI-TOF-MS and N–terminal sequencing provided additional evidence that Cry34Ab1 and Cry35Ab1 proteins derived from corn event 59.1.22 are identical to those produced by the *P. fluorescens* bacterial expression system and by corn event 5638. **Classification: Acceptable**	461239-06

Study Title	Summary	MRID No.
Characterization of Phosphinothricin Acetyltransferase (PAT) Derived from Transgenic Maize Event E4497.59.1.22	SDS-PAGE, western blot analysis, and ELISA were used to compare PAT protein derived from transgenic maize plant event E4497.59.1.22 with PAT protein from a recombinant *Escherichia coli* expression system. SDS-PAGE demonstrated that the *E. coli*-produced PAT protein had the expected molecular weight. Leaf extract samples from event E4497.59.1.22 showed an immunoreactive band in the western blot analysis that corresponded to the expected molecular weight for the PAT protein and comigrated with the *E. coli*-produced PAT. ELISA results demonstrated similar expression of the PAT protein in all of the transgenic leaf samples tested. The control plants did not contain immunoreactive proteins. Results were consistent with earlier studies of PAT protein derived from *E. coli* and from transgenic maize event 5638, which used MALDI-TOF-MS peptide mass fingerprinting (Korjagin 2000). The present study further supports the use of microbe-derived PAT protein as an appropriate surrogate for the PAT protein produced in maize plants. **Classification: Acceptable**	461239-07
Characterization of DNA Inserted into Transgenic Corn Events DAS-45216-6 and DAS-59122-7	Southern blot analysis was used to characterize the DNA inserted in transgenic corn events DAS-45216-6 and DAS-59122-7. The restriction enzyme fragments observed in the Southern blots matched the predicted fragments, indicating that a single, intact T-DNA from plasmid PHP17662 was integrated into the corn genome at a single locus. The data also demonstrated the absence of the tetracycline and spectinomycin resistance genes, the *vir*G gene, and vector backbone DNA regions immediately outside of the left and the right T-DNA border, indicating that only DNA contained within the T-DNA borders was integrated into the transgenic corn. Identical fragment sizes were observed for all samples (two distinct generations, four plants each), indicating stability of inheritance across and within generations. **Classification: Acceptable**	461239-08
Detailed Characterization of DNA Inserted into Transgenic Corn Events DAS-45216-6 and DAS-59122-7	Southern blot analysis was used to analyze the inserted DNA in events DAS-45216-6 and DAS-59122-7 and to develop detailed restriction enzyme maps of the insertions. Fragments of the expected sizes were observed, indicating that a single, intact T-DNA inserted into the corn genome at one locus without any rearrangements of the inserted DNA. **Classification: Acceptable**	461239-09

Study Title	Summary	MRID No.
Evaluation of Microbe-Derived Cry34Ab1 and Cry35Ab1 Proteins for Protein Synthesis Inhibition Activity	The rabbit reticulocyte assay was used to determine if Cry34Ab1 and Cry35Ab1 proteins inhibit protein synthesis. Ricin, used as a positive control, produced a 50% inhibition in protein synthesis with a concentration of 0.182 nanomolar (nM). In contrast, Cry34Ab1 and Cry35Ab1, as well as BSA used as a negative control, showed no inhibition of protein synthesis at concentrations up to 2,880 nM. The results suggest that the insecticidal activity of Cry34Ab1 and Cry35Ab1 is not associated with the inhibition of protein synthesis. **Classification: Acceptable**	461239-10

b. Terms and Conditions of the Event DAS-59122-7 Corn Registrations (August 2005 – September 2010)

When event DAS-59122-7 corn was initially registered on August 31, 2005, the Agency issued registration notices to both Mycogen Seeds c/o Dow AgroSciences LLC (EPA Reg. No. 68467-5) and Pioneer Hi-Bred International, Incorporated (EPA Reg. No. 29964-4) that contained the following requirement for further product characterization information:

> "Provide to the EPA laboratory (Ft. Meade, MD) methodology and/or reagents necessary for validation of a Cry34/35Ab1 analytical method within 6 months of the date of registration."

More recently, the Agency has decided to allow this requirement to be satisfied by the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA) performance verification of a qualitative rapid test kit for detecting the presence of the biotechnology event in grains and oilseeds (USDA 2004). In the case of Cry34/35Ab1, the Agency has confirmed that a test kit has been verified by GIPSA and, therefore, the aforementioned requirement has been satisfied for both registrations.

2. Herculex® XTRA Insect Protection Corn (OECD Unique Identifier: DAS-Ø15Ø7-1 x DAS-59122-7) Expressing Cry1F, Cry34Ab1, and Cry35Ab1

a. Data Cited/Submitted for Initial Registrations of Herculex® XTRA Insect Protection Corn (Prior to October 2005)

Herculex® XTRA Insect Protection Corn was developed through conventional breeding of a corn line containing event DAS-01507-1 (also known as event TC1507 and Herculex® I), which expresses the Cry1F (truncated) and PAT proteins, and a corn line containing event DAS-59122-7, which expresses the Cry34Ab1, Cry35Ab1, and PAT proteins. This product is considered a stacked plant-incorporated protectant because it targets both lepidopteran and coleopteran pests and contains two separate active

ingredients. DAS-01507-1 is briefly summarized in the paragraph that follows, while additional information on DAS-59122-7, specifically related to product characterization, can be found in section II(A)(1) of this Biopesticides Registration Action Document (BRAD).

DAS-01507-1 produces the *Bacillus thuringiensis* var. *aizawai* Cry1F protein to selectively control larvae of European corn borer (*Ostrinia nubilalis*) and other lepidopteran pests. In addition, DAS-01507-1 also produces the PAT protein from *Streptomyces viridochromogenes*, which confers tolerance to glufosinate-ammonium herbicides. On May 18, 2001, the Agency issued Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) section 3 registrations to Mycogen Seeds c/o Dow AgroSciences LLC (EPA Reg. No. 68467-2) and Pioneer Hi-Bred International, Incorporated (EPA Reg. No. 29964-3) for Herculex® I, expressing *Bacillus thuringiensis* Cry1F protein and the genetic material necessary (plasmid insert PHI8999A) for its production in corn event TC1507 (OECD Unique Identifier: DAS-Ø15Ø7-1). The product characterization data supporting the registration of Herculex® I, including the submitted study titles, conclusions, and their MRID Numbers, can be found in the 2001 *Bt* Crops Reassessment, August 2005 Cry1F BRAD, and the September 2010 Cry1Ab and Cry1F BRAD (U.S. EPA 2001b, 2005a, and 2010b).

The data submitted for Herculex® XTRA Insect Protection Corn, which include DNA analysis of the inserts in and protein expression data for the stacked corn product, are summarized in Table 3.

Table 3. Product Characterization Data for Herculex® XTRA Insect Protection Corn (Reviewed in U.S. EPA (2005b)).

Study Title	Summary	MRID No.
Characterization of Stacked Hybrid of Transgenic Corn Lines DAS-01507-1 and DAS-59122-7 by Comparison to the Individual Lines	Southern blot analysis was conducted on restriction enzyme digests of DNA from the stacked hybrid of transgenic corn lines DAS-01507-1 and DAS-59122-7 as well as the individual lines. Hybridization of *Hind* III and *Sac* I digests with probes for the *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* genes gave the expected bands on Southern blots, based on restriction enzyme maps of both of the inserts in the individual lines. The results indicate that the inserts in the 1507 x 59122 stacked hybrid are equivalent to the individual inserts in the inbred lines. **Classification: Acceptable**	462971-02

Study Title	Summary	MRID No.
Agronomic Characteristics, Quantitative ELISA, and Nutrient Composition Analysis of Hybrid Maize Lines Containing the *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* Genes: U.S. and Canada Locations	Agronomic characteristics, nutrient composition, and protein expression levels, for transgenic corn line DAS-01507-1 x DAS-59122-7 grown at five locations in the U.S. and Canada, were analyzed and compared with results from a non-transgenic control. The presence of the transgenes for the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins had no notable effect on agronomic parameters, such as early and final population, time to silking and to pollen shed, and plant and ear height. Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins were detected by ELISA in all plant tissues except that PAT was not detected in pollen. On a tissue dry weight basis, mean expression levels had the following ranges: Cry34Ab1 – a low of 43 ng/mg in grain to a high of 340 ng/mg in leaf tissue at the R4 growth stage; Cry35Ab1 – a low of 0.14 ng/mg in pollen to a high of 104 ng/mg in leaf tissue at the R4 growth stage; Cry1F – a low of 0.14 ng/mg in pollen to a high of 25 ng/mg in leaf tissue at the R4 growth stage; and PAT – a low of <LOQ in pollen to a high of 13 ng/mg in leaf tissue at the R4 and R1 growth stages and whole plant samples from the V9 growth stage. Herbicide spraying did not affect protein expression levels. **Classification: Acceptable**	463438-01

b. Terms and Conditions of the Herculex® XTRA Insect Protection Corn Registrations (October 2005 – September 2010)

When Herculex® XTRA Insect Protection Corn was initially registered on October 27, 2005, the Agency issued registration notices to both Mycogen Seeds c/o Dow AgroSciences LLC (EPA Reg. No. 68467-6) and Pioneer Hi-Bred International, Incorporated (EPA Reg. No. 29964-5) that contained the following requirement:

> "Submit all data required to support the individual plant-incorporated protectants in event TC1507 (Herculex I) and event DAS-59122-7 (Herculex Rootworm) corn…."

All requirements for additional product characterization information for events TC1507 (see U.S. EPA (2010b)) and DAS-59122-7 (see section II(A)(1)(b) of this BRAD) have been satisfied for both registrations.

3. 1507 x 59122 x MON 810 (OECD Unique Identifier: DAS-Ø15Ø7-1 x DAS-59122-7 x MON-ØØ81Ø-6) Expressing Cry1F, Cry34Ab1, Cry35Ab1, and Cry1Ab and 59122 x MON 810 (OECD Unique Identifier: DAS-59122-7 x MON-ØØ81Ø-6) Expressing Cry34Ab1, Cry35Ab1, and Cry1Ab

a. Data Cited/Submitted for Initial Registrations of 1507 x 59122 x MON 810 and 59122 x MON 810 (Prior to February 2010)

The stacked and pyramided product, 1507 x 59122 x MON 810, and the stacked product, 59122 x MON 810, are hybrids created by crossing the individuals events, TC1507 (Cry1F), DAS-59122-7 (Cry34/35Ab1), and MON 810 (Cry1Ab), via traditional breeding methods. Product characterization background on DAS-59122-7 and TC1507 was discussed or referenced in sections II(A)(1) and II(A)(2) of this BRAD, respectively; MON 810 is briefly summarized in the paragraph that follows.

MON 810 produces *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 Cry1Ab protein to selectively control larvae of European corn borer (*O*. *nubilalis*) and other lepidopteran pests. On December 20, 1996, the Agency issued a FIFRA section 3 registration to Monsanto Company for MON 810 (EPA Reg. No. 524-489), expressing *Bacillus thuringiensis* Cry1Ab protein and the genetic material necessary (PV-ZMCT01) for its production in corn event MON 810 (OECD Unique Identifier: MON-ØØ81Ø-6). The product characterization data supporting the registration of MON 810, including the submitted study titles, conclusions, and their MRID Numbers, can be found in both the 2001 *Bt* Crops Reassessment and the September 2010 Cry1Ab and Cry1F BRAD (U.S. EPA 2001b, 2010b).

The data submitted for 1507 x 59122 x MON 810 and 59122 x MON 810, which include molecular characterization data, protein expression analyses, and a synergism study, are summarized in Table 4.

Table 4. Product Characterization Data for 1507 x 59122 x MON 810 and 59122 x MON 810 (Reviewed in U.S. EPA (2010a)).

Study Title	Summary	MRID No.
Molecular Characterization of Maize Combined Trait Product DAS-01507-1 x DAS-59122-7 x MON-00810-6 Using Southern Blot Analysis and Event-Specific Polymerase Chain Reaction (Product Characterization)	Molecular analyses (restriction enzyme digests and Southern blots) were performed to compare the integrity of the transgenic inserts in events TC1507, DAS-59122-7, and MON 810 corn with the transgenic DNA inserts in the combination plant-incorporated protectant (PIP) 1507 x 59122 x MON 810 corn product, produced by conventional plant breeding. The Southern blot data showed the predicted DNA hybridization patterns of the *cry*1F gene from TC1507 corn, the *cry*34Ab1 and *cry*35Ab1 genes from DAS-59122-7 corn, and the *cry*1Ab gene from MON 810 corn. These data demonstrated equivalence among events because the transgenic inserts were stably integrated and retained when the parental lines are crossed to create 1507 x 59122 x MON 810 corn. In addition, event-specific PCR methods detected and confirmed the presence of each expected transgenic DNA insert in the combination PIP corn event. **Classification: Acceptable**	476778-01
Additional Addendum to Expressed Trait Protein Concentration of Maize Lines Containing Events TC1507, DAS-59122-7, MON 810, and Combination PIPs 1507 x MON 810, 59122 x MON 810, and 1507 x 59122 x MON 810 Corn Products (Protein Expression Levels)	A field study was conducted using quantitative ELISA methods to statistically compare the level of expression for the Cry1F, Cry34Ab1, Cry35Ab1, Cry1Ab, and PAT proteins expressed in 1507 x 59122 x MON 810, 1507 x MON 810, and 59122 x MON 810 combination PIP corn events to the individual parental events. No statistically significant differences were found for Cry1F, Cry34Ab1, Cry35Ab1, and Cry1Ab protein expression between 1507 x 59122 x MON 810 and parental corn events 1507, 59122, or MON 810. No statistical differences were observed in any plant tissue for Cry1F, PAT, and Cry1Ab protein expression when TC1507 x MON 810 was compared to events 1507 and MON 810 or when event 59122 was compared to event MON 810. Based on the results, the protein concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and Cry1Ab expressed in combination PIP events 1507 x 59122 x MON 810, 1507 x MON 810, and 59122 x MON 810 are comparable to the respective protein concentrations of their respective single parental events. **Classification: Acceptable** **Note: This study supersedes MRID Nos. 475109-04 and 478654-01.**	478800-01

Study Title	Summary	MRID No.
Laboratory Characterization of Key Lepidopteran Pest Response to Pyramided Events (Synergism Study)	A seven-day laboratory sensitive insect bioassay was conducted to determine if the combination PIP product, 1507 x 59122 x MON 810, has a synergistic effect in comparison to the individual parental events, TC1507 (expressing Cry1F protein) and MON 810 (expressing Cry1Ab protein), on target lepidopteran pests. The pests used in the bioassay were European corn borer (ECB, *Ostrinia nubilalis*), southwestern corn borer (SWCB, *Diatraea grandiosella*), fall armyworm (FAW, *Spodoptera frugiperda*), and corn earworm (CEW, *Helicoverpa zea*). The observed and expected larval mortality in the 1507 x 59122 x MON 810 group were similar and mean larval weight of the survivors exposed to 1507 x 59122 x MON 810 leaf tissue was not significantly different from the single parental events or the negative control of non-*Bt* maize. These results indicate that the Cry1F and Cry1Ab proteins act independently and do not have a synergistic or antagonistic effect on the target pests, other than an additive effect. Quantitative ELISA results also confirmed that the expression of each of the proteins in the combination PIP was not affected by the presence of the other protein. **Classification: Acceptable**	476778-02

b. Terms and Conditions of the 1507 x 59122 x MON 810 and 59122 x MON 810 Registrations (February 2010 – September 2010)

When 1507 x 59122 x MON 810 (EPA Reg. No. 29964-8) and 59122 x MON 810 (EPA Reg. No. 29964-9) were initially registered on February 24, 2010, the Agency issued registration notices to Pioneer Hi-Bred International, Incorporated that contained the following requirements:

- For 1507 x 59122 x MON 810 –

  "The data submitted by Pioneer are sufficient to support registration for the combination PIP product…provided that the registrant submits/cites any data required to support the PIP registrations of the individual parental events…as well as the combination PIP product TC1507 (DAS-Ø15Ø7-1) x DAS-59122-7…."

- For 59122 x MON 810 –

  "The data submitted by Pioneer are sufficient to support registration for the combination PIP product…provided that the registrant submits/cites any data required to support the PIP registrations of the individual parental events…."

All requirements for additional product characterization information for individual events TC1507 (see U.S. EPA (2010b)), MON 810 (see U.S. EPA (2010b)), and DAS-59122-7 (see section II(A)(1)(b) of

this BRAD), as well as for combination PIP product TC1507 x 59122-7 (see section II(A)(2)(b) of this BRAD), have been satisfied.

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B. Human Health Assessment

1. Human Health Assessment of Cry34Ab1 and Cry35Ab1

The detailed Agency human health assessment of Cry34Ab1/Cry35Ab1 corn is found in U.S. EPA (2005a and 2005d). Portions of the data used in the human health assessment are reviewed in U.S. EPA (2001a, 2002, 2003, and 2004a). A summary of the key findings is provided below.

a. Mammalian Toxicity and Allergenicity Assessment

Based upon the human health data provided, there is minimal risk of toxic and/or allergenic effects to humans or animals due to exposure to the Cry34Ab1 and Cry35Ab1 proteins. Based on review of the data, there is a reasonable certainty of no harm to humans and animals posed by the aggregate exposure to residues of these proteins.

i. Mammalian Toxicity

Acute oral toxicity data have been submitted demonstrating the lack of mammalian toxicity at high levels of exposure to the pure Cry34Ab1 and Cry35Ab1 proteins separately and combined. These data demonstrate the safety of the products at levels well above maximum possible exposure levels that are reasonably anticipated in the crops. 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 these plant-incorporated protectants were derived (see 40 CFR §§ 158.2130(d)(1)(i) and 158.2140(d)(7)). For microbial products, further toxicity testing and residue data are triggered by significant acute effects in studies, such as the mouse oral toxicity study, to verify and quantify the observed effects and clarify the source of these effects (Tiers II and III).

Three acute oral toxicity studies on Cry34Ab1 and Cry35Ab1 in mice were submitted, which indicated that these proteins are non-toxic to humans.

In an oral toxicity study of Cry34Ab1 alone (Master Record Identification Number (MRID No.) 452422-07; reviewed in U.S. EPA (2005a)), Cry34Ab1 produced from microbial culture was administered to five male mice (5,000 milligrams/kilogram (mg/kg) body weight) by oral gavage as a 20% mixture in a 0.5% aqueous methylcellulose vehicle. All animals survived the two-week study. No clinical signs were noted for any animals during the study. An initial weight loss was observed in 3 mice at test days 1 and 2, but they gained weight for the remainder of the study. The two other animals gained weight throughout the study. No treatment-related gross pathologic changes were observed during the study. Under the conditions of this study, the acute oral median lethal dose (LD_{50}) for the test substance in male CD-1 mice was greater than 5,000 mg/kg. Since the test substance contained Cry34Ab1 at 54% purity, the acute oral LD_{50} for the pure Cry34Ab1 protein was greater than 2,700 mg/kg.

In an oral toxicity study of Cry35Ab1 alone (MRID No. 452422-08; reviewed in U.S. EPA (2005a)), Cry35Ab1 produced from microbial culture was administered to five male mice (5,000 mg/kg body weight) by oral gavage as a 20% mixture in a 0.5% aqueous methylcellulose vehicle. All animals survived the two-week study. No clinical signs were noted for any animal during the study. An initial weight loss was observed in 2 mice at test days 1 and 2, but they gained weight for the remainder of the study. One animal had fluctuating body weight. The other two animals gained weight throughout the study. No treatment-related gross pathologic changes were observed during the study. Under the conditions of this study, the acute oral LD_{50} for the test substance in male CD-1 mice was greater than 5,000 mg/kg. Since the test substance contained Cry35Ab1 at 37% purity, the acute oral LD_{50} for the pure Cry35Ab1 protein was greater than 1,850 mg/kg.

Finally, in an oral toxicity study of Cry34Ab1 and Cry35Ab1 combined (MRID No. 452422-09; reviewed in U.S. EPA (2001a)), a mixture of the microbially produced Cry34Ab1 and Cry35Ab1 proteins (5,000 milligrams (mg) test material, containing 482 mg pure Cry34Ab1 and 1,520 mg pure Cry35Ab1 (corresponding to an equimolar ratio), per kilogram (kg) body weight) was administered by oral gavage to 5 female and 5 male mice as a 20% mixture in 0.5% aqueous methylcellulose. All animals survived the 2-week study. One female mouse exhibited protruding or bulging eyes on days 6 and 7, but this resolved thereafter. This observation was not attributed to the treatment as it was an isolated observation (i.e., no other animals exhibited this). No other clinical signs were noted for any animals during the study. An initial weight loss was observed in 2 mice at test days 1 and 2, but both gained weight for the remainder of the study. All other animals gained weight throughout the study. No treatment-related gross pathologic changes were noted. Under the conditions of the study, the acute oral LD_{50} of the test material in male and female CD-1 mice was greater than 5,000 mg/kg body weight, corresponding to 2,000 mg/kg of an equimolar ratio of the pure proteins.

In addition, a study was submitted where the amino acid sequences of the Cry34Ab1 and Cry35Ab1 proteins were compared with protein sequences in publicly available databases (GenPept dataset) to identify any potential similarities with known toxins (MRID No. 465847-01; reviewed in U.S. EPA (2005d)). No similarities were identified that would raise a safety concern.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad *et al*. 1992). Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry34Ab1 and Cry35Ab1 proteins are not considered toxic. Further, amino acid sequence comparisons showed no similarity between the Cry34Ab1 and Cry35Ab1 proteins and known toxic proteins available in public protein databases.

ii. Allergenicity Assessment

Since Cry34Ab1 and Cry35Ab1 are proteins, allergenic potential was also considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a weight-of-the-evidence approach where the following factors are considered: source of the trait; amino acid sequence similarity with known allergens; prevalence in food; and biochemical properties of the protein, including *in vitro* digestibility in simulated gastric fluid (SGF) and glycosylation. Current

scientific knowledge suggests that common food allergens tend to be resistant to degradation by acid and proteases, may be glycosylated, and can be present at high concentrations in the food. In the past, EPA has also considered heat stability in assessing allergenicity potential; however, at a March 1–2, 2005 meeting, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) stated that heat stability based on a bioactivity assay is of minimal to no value in predicting the allergenicity potential of novel proteins, and EPA agrees. Therefore, EPA did not consider heat stability of these proteins in its weight-of-the-evidence approach.

Source of the trait. *Bacillus thuringiensis* is not considered a source of allergenic proteins.

Amino acid sequence. A comparison of amino acid sequences of Cry34Ab1 and Cry35Ab1 with known allergens (MRID No. 452422-05; reviewed in U.S. EPA (2001a)) showed no overall sequence similarities or homology at the level of 8 contiguous amino acid residues.

Prevalence in food. Expression level analysis indicated that the proteins are present at relatively low levels in corn; on a dry weight basis, Cry34Ab1 is present at a concentration of approximately 50 nanograms per milligram (ng/mg) in grain from event 59122-7, and Cry35Ab1 is present at a concentration of approximately 1 ng/mg in grain from event 59122-7 (MRID No. 461239-04; reviewed in U.S. EPA (2004b)). Thus, expression of the Cry34Ab1 and Cry35Ab1 proteins in corn kernels has been shown to be in the parts per million range.

Digestibility. Two *in vitro* digestibility studies (MRID No. 452422-12; reviewed in U.S. EPA (2001a)) were conducted to determine the stability of the Cry34Ab1 and Cry35Ab1 proteins in simulated gastric fluid (i.e., an acid environment containing pepsin; SGF). In the first *in vitro* digestibility study, the proteins were incubated in SGF (pepsin concentration: 3.2 milligrams per milliliter (mg/mL); pH 1.2; 37°C) with a pepsin to protein substrate ratio of approximately 20:1, moles to moles (mol/mol) (equivalent to 60:1, weight-to-weight (w/w) for Cry34Ab1 and 17:1, w/w for Cry 35Ab1). Samples taken at 1, 5, 7, 15, 20, 30, and 60 minutes were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. Cry35Ab1 was no longer visible at the five-minute time point using both SDS-PAGE stained with Coomassie Brilliant Blue and western blot detection. Cry34Ab1 was visible on the stained gel for the 15-minute sample but not in later sample time points. In the western blot analysis, Cry34Ab1 was visible in the 20-minute sample but not in later sample time points. In conclusion, this first study showed that Cry34Ab1 was digested within 30 minutes, and Cry35Ab1 was digested within 5 minutes in SGF under the conditions of the study.

Because Cry34Ab1 appeared to be somewhat resistant to SGF in the study that used the time-to-disappearance endpoint (as described in the preceding paragraph), Dow AgroSciences LLC submitted a second study on the *in vitro* digestibility of Cry34Ab1 in SGF using a kinetic approach (MRID No. 455845-02; reviewed in U.S. EPA (2002)). The digestion was performed under the same conditions as the previous study, except that reaction mixtures were shaken during incubation and samples were analyzed at 1, 2, 3, 5, 7.5, 10, 15, and 20 minutes. The previous study on pepsin digestibility of Cry34Ab1 and Cry35Ab1, as well as other pepsin digestibility studies used in allergenicity assessments, focused on the time required for the protein to become undetectable and, therefore, the results are

dependent on the detection limit of the analytical method used. In this second study, Dow AgroSciences LLC determined the rate of pepsin digestion of Cry34Ab1 by measuring the relative amounts of Cry34Ab1 at each of the time points based on SDS-PAGE densitometry estimates. Under the conditions of the study, the rate of decay fit a first-order model (with respect to Cry34Ab1 concentration), and Dow AgroSciences LLC estimated the DT_{50} (half-life) and DT_{90} (time until 90% decay) to be 1.9 minutes and 6.2 minutes, respectively. In this experiment, Cry34Ab1 was visible on gels and blots in 15-minute time-point samples but not in 20-minute time-point samples.

Because the digestibility of Cry34Ab1 was assessed using a different method (i.e., the kinetic approach) rather than the typical end-point method that has been used previously, comparison studies, using the kinetic approach to assess the digestibility of known allergens and non-allergens, were submitted to validate the method and allow comparison of the digestibility of Cry34Ab1 with known allergens and non-allergens (Master Record Identification Numbers (MRID Nos.) 461239-20 and 463886-01). In the comparison study where the conditions used were the same as those used in the kinetic study on the digestibility of Cry34Ab1 (MRID No. 463886-01; reviewed in U.S. EPA (2005a)), two allergens and two non-allergens were shown to digest similarly to Cry34Ab1. From these studies and published studies, EPA concludes that Cry35Ab1 is rapidly digested, and Cry34Ab1 is digested at a moderate rate in SGF; Cry34Ab1 appears to digest slower than previously registered proteins and many other proteins that are not considered allergens but faster than most previously tested allergens.

On March 1–2, 2005, EPA held a FIFRA SAP meeting to address the scientific issues that arose during the human health safety assessment of Cry34Ab1 and Cry35Ab1. The SAP report (U.S. EPA 2005b) is summarized below and in U.S. EPA (2005c). EPA asked the SAP to comment on EPA's allergenicity assessment of Cry34Ab1. The SAP agreed with EPA's preliminary assessment that the allergenicity potential of Cry34Ab1 is low. However, the Panel based its conclusion, in part, on statements made by Dow AgroSciences LLC that Cry34Ab1 and Cry35Ab1 do not aggregate in solution. The Panel was concerned that if the proteins were to aggregate, protease binding sites could be masked, and the rate of digestion could be slower than was observed for the individual proteins. Therefore, EPA asked Dow AgroSciences LLC to submit data supporting the claim that Cry34Ab1 and Cry35Ab1 do not associate with one another in solution.

To support the digestibility studies on the individual proteins, Dow AgroSciences LLC submitted a study using size exclusion chromatography, which demonstrated that Cry34Ab1 and Cry35Ab1 do not associate with one another in solution under acidic conditions (MRID No. 465568-01; reviewed in U.S. EPA (2005d)).

Glycosylation. Cry34Ab1 and Cry35Ab1 expressed in corn were shown not to be glycosylated (MRID No. 461239-06; reviewed in U.S. EPA (2004b)).

Conclusion. Considering that (1) Cry34Ab1 and Cry35Ab1 originate from a non-allergenic source, (2) Cry34Ab1 and Cry35Ab1 have no overall sequence similarities or homology at the level of 8 contiguous amino acid residues with known allergens, (3) Cry34Ab1 and Cry35Ab1 will only be present at low levels in food, (4) Cry35Ab1 is rapidly digested in SGF, (5) Cry34Ab1 is digested at a moderate rate in

SGF, and (6) Cry34Ab1 and Cry35Ab1 are not glycosylated when expressed in maize, EPA has concluded that the potential for the Cry34Ab1 and Cry35Ab1 proteins to be food allergens is minimal. The FIFRA SAP that met on March 1–2, 2005 agreed with this conclusion regarding the allergenicity potential of Cry34Ab1. There were no triggers to raise concern about the allergenicity of Cry35Ab1, so the SAP was not asked to comment specifically on Cry35Ab1. As noted above, toxic proteins typically act as acute toxins with low dose levels. Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry34Ab1 and Cry35Ab1 proteins are not considered toxic.

SAP Opinion on the Kinetic Approach

In general, the Panel supported using a kinetic approach, rather than the disappearance endpoint, for assessing proteins that are not rapidly degraded because using several time points is "inherently more accurate than the single point assay." The Panel pointed out, however, that many of the statements and assumptions Dow AgroSciences LLC made about the kinetic model are incorrect. In particular, Dow AgroSciences LLC has stated that, as long as first-order conditions are met, first-order rate constants and half-lives are unaffected by changes in substrate and enzyme concentration (when enzyme is saturating) and, therefore, can be used to predict relative digestion efficiencies for proteins even if the concentrations are varied among experiments. The Panel stated that it was "impossible to comment" on these statements without knowing the affinity of the substrate for the enzyme and emphasized the need for standardized assay conditions. In addition, the Panel report stated the following:

> "A poor fit of early time points to Michaelis-Menten kinetics indicates a problem with the model. The Panel questioned the application of single substrate Michaelis-Menten kinetics to the registrant data. The registrant did not adequately address the problems of applying classical Michaelis-Menten kinetics to a situation where $[E_0] >> [S_0]$. They incorrectly applied the term V_{max}/K_M to this scenario."

In addition, "the Panel noted that at high enzyme to substrate ratios, the simple quasi-steady-state Michaelis-Menten equation cannot be applied."

Some Panel members expressed concern about omitting early time points to achieve a good fit to first-order decay but in general "agreed that modeling of the late first order decay was an adequate/conservative approach for a given simulated gastric fluid (SGF) degradation assay…provided that a significant fraction of the digestion takes place during this slow phase." The Panel report did not specify what a "significant fraction" is but pointed out that, in the case of Cry34Ab1, one-half of the substrate had been consumed by the first time point of one minute. The report stated that "one does not know whether or not the reaction follows first order kinetics during the initial 50% of the reaction," but in this case, "this may not be important since one is interested in the time course for total substrate hydrolysis." However, the Panel also pointed out that the late phase "may be hard to define in a practical setting and consequently impossible to standardize." In conclusion, although the Panel pointed out some

problems with the kinetic approach, it recommended using the approach for proteins that are not rapidly digested.

SAP Opinion on Assay Conditions

The Panel emphasized the need for standardized assay conditions throughout the report and gave some useful information on appropriate conditions. The Panel report clearly stated that a fixed amount of enzyme activity units should be used in digestion studies, and a standardized protocol should be used to determine enzyme activity.

In addition, the Panel indicated that the pepsin to substrate ratio and the concentrations of pepsin and substrate have a pronounced effect on the rate of hydrolysis, implying that both the ratio and concentrations should be standardized.

EPA asked if the concentration of test protein should be fixed on a weight basis (mg/mL) or a mole basis (mol/L). There was no consensus from the Panel on this issue: "Some Panel members recommended comparison of test proteins on a weight/mL, while others recommended a mole/mL."

One Panel member stated that a standardized pH should also be used for digestibility assays and indicated that there is no evidence that using more than one pH is necessary for predicting allergenicity. The Panel did not indicate one particular pH as being more appropriate than another.

The Panel report discussed the pros and cons of the different methods that can be used for monitoring digestion reactions (i.e., SDS-PAGE with staining, western blot analysis, and high-performance liquid chromatography (HPLC)). Typically, applicants use SDS-PAGE with staining and western blot analysis. The Panel did not provide any information that would suggest a change to the current methodology.

When asked about the pros and cons of using one digestion reaction and removing aliquots at various time points for monitoring or setting up separate reactions for each of the time points, the Panel responded that "there would be no difference…as long as there are no pipetting errors and the reactions are homogeneous." In addition, several Panel members indicated that using a single digestion reaction gives a "slightly better ability to control extraneous factors that could affect experimental variability." The report also pointed out that different statistical models would be required, depending on which approach is used.

SAP Opinion on Digestibility Studies in Allergenicity Assessments

When asked what weight *in vitro* digestibility studies should be given in the overall allergenicity assessment compared with other criteria, the majority of the Panel indicated that digestibility is of some value but is less important than the source of the protein, sequence homology, or a validated animal model. The Panel also stated that "the use of digestibility data also is only of value in the context of a total weight of evidence approach."

When asked about setting acceptable/unacceptable limits for digestibility in assessing the safety of a protein, the Panel stated that it is "difficult if not impossible given the lack of consistency between digestibility and allergenicity." The Panel also noted that the relative digestibility of proteins depends on the assay conditions used, and a database of digestibilities under standard conditions needs to be established.

When asked about the significance of the rate of digestion of protein fragments, the Panel recommended determining digestion rates for all fragments of a molecular mass >1500 Daltons (Da) (~10–15 amino acids) since a number of allergens have been shown to be peptides. The Panel recommended this size range because smaller peptides are not detectable using SDS-PAGE.

SAP Opinion on Cry34Ab1 Allergenicity Assessment

The Panel agreed with EPA's assessment that the weight-of-the-evidence indicates that Cry34Ab1 is unlikely to be a food allergen. The Panel reached this decision based on the following: (1) Cry34Ab1 is moderately digested in SGF, and it does not self-aggregate or aggregate with Cry35Ab1 in physiologic solutions; (2) Cry34Ab1 originates from a non-allergenic source; (3) Cry34Ab1 has no sequence similarity with known allergens "based on data presented on the lack of identity of 8 contiguous amino acids or more than 35% identity over 80 amino acids with known inhalant and ingested allergens"; and (4) Cry34Ab1 is not glycosylated when expressed in maize.

The majority of Panel members indicated that heat stability is of minimal to no value in predicting the allergenicity potential of Cry34Ab1. The Panel also stated that "there is no convincing evidence that *B. thuringiensis* is an allergen in that it has never conclusively been documented to provoke an allergic reaction." The Panel report also noted that "there are no data indicating that crystal proteins are allergens."

b. Aggregate Exposures

In examining aggregate exposure, section 408 of the Federal Food, Drug, and Cosmetic Act (FFDCA) directs EPA to consider available information concerning 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 the plant-incorporated protectants' chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Exposure via residential or lawn use to infants and children is also not expected because the use sites for the Cry34Ab1 and Cry35Ab1 proteins are all agricultural for control of insects. Oral exposure, at very low levels, may occur from ingestion of

processed corn products and, potentially, drinking water. Oral toxicity testing, however, did not show any adverse effects. Furthermore, the expression of the Cry34Ab1 and Cry35Ab1 proteins in corn kernels has been shown to be in the parts per million range, which makes the expected dietary exposure several orders of magnitude lower than the amounts of Cry34Ab1 and Cry35Ab1 proteins shown to have no toxicity. Therefore, even if negligible aggregate exposure should occur, the Agency concludes that such exposure would result in no harm due to the lack of mammalian toxicity and low potential for allergenicity demonstrated for the Cry34Ab1 and Cry35Ab1 proteins.

c. Cumulative Effects from Substances with a Common Mechanism of Toxicity

Section 408(b)(2)(D)(v) of FFDCA requires that, when considering whether to establish, modify, or revoke a tolerance, 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 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 resulting from the plant-incorporated protectants, the Agency concludes that there are no cumulative effects for the Cry34Ab1 and Cry35Ab1 proteins. For information regarding EPA's efforts to determine which chemicals have a common mechanism of toxicity and to evaluate the cumulative effects of such chemicals, see EPA's website at *http://www.epa.gov/pesticides/cumulative*.

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

i. Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry34Ab1 and Cry35Ab1 proteins include the characterization of the expressed Cry34Ab1 and Cry35Ab1 proteins in corn, as well as the acute oral toxicity and *in vitro* digestibility of the proteins. The results of these studies were determined applicable to evaluate human risk, and the validity, completeness, and reliability of the available data from the studies were considered.

Adequate information was submitted to show that the Cry34Ab1 and Cry35Ab1 protein test material derived from microbial cultures was biochemically and functionally similar to the protein produced by the plant-incorporated protectant ingredients in corn. Production of microbially produced protein was chosen in order to obtain sufficient material for testing.

The submitted acute oral toxicity data support the prediction that the Cry34Ab1 and Cry35Ab1 proteins would be non-toxic to humans. As mentioned in section II(B)(1)(a)(i) of this Biopesticides Registration Action Document (BRAD), when proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad *et al*. 1992). Since no effects were shown to be caused by the Cry34Ab1 and Cry35Ab1 proteins, even at relatively high dose levels, the Cry34Ab1 and Cry35Ab1 proteins are

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 *Bt* products from which these plant-incorporated protectants were derived (see 40 CFR §§ 158.2130(d)(1)(i) and 158.2140(d)(7)). For microbial products, further toxicity testing and residue data are triggered by significant acute effects in studies, such as the mouse oral toxicity study, to verify and quantify the observed effects and clarify the source of these effects (Tiers II and III).

Cry34Ab1 and Cry35Ab1 protein 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. Notwithstanding that, submitted data demonstrated low levels of the Cry34Ab1 and Cry35Ab1 proteins in corn tissues.

Since Cry34Ab1 and Cry35Ab1 are proteins, their potential allergenicity is also considered as part of the toxicity assessment. Considering that (1) Cry34Ab1 and Cry35Ab1 originate from a non-allergenic source, (2) Cry34Ab1 and Cry35Ab1 have no overall sequence similarities or homology at the level of 8 contiguous amino acid residues with known allergens, (3) Cry34Ab1 and Cry35Ab1 are not glycosylated when expressed in maize, (4) Cry34Ab1 and Cry35Ab1 will only be present at low levels in food, (5) Cry35Ab1 is rapidly digested in SGF, and (6) Cry34Ab1 is digested at a moderate rate in SGF, EPA has concluded that the potential for the Cry34Ab1 and Cry35Ab1 proteins to be food allergens is minimal. The FIFRA SAP that met on March 1–2, 2005 agreed with this conclusion regarding the allergenicity potential of Cry34Ab1. There were no triggers to raise concern about the allergenicity of Cry35Ab1, so the SAP was not asked to comment specifically on Cry35Ab1.

Neither available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children) nor safety factors that are generally recognized as appropriate for the use of animal experimentation data were evaluated. The lack of mammalian toxicity at high levels of exposure to the Cry34Ab1 and Cry35Ab1 proteins, 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 ingredients are the nucleic acids (deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)) that comprise genetic material encoding these proteins and their regulatory regions. The genetic material (DNA and RNA) necessary for the production of the Cry34Ab1 and Cry35Ab1 proteins have been exempted under the blanket exemption for all nucleic acids (40 CFR § 174.507).

ii. 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 data base 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 concludes that there is a finding of no toxicity for the Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production. Thus, there are no threshold effects of concern and, as a result, an additional margin of safety for infants and children is not necessary.

iii. 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 Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their 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 thus far in this chapter, no toxicity to mammals has been observed, nor has there been any indication of allergenicity potential for the plant-incorporated protectants.

e. Other Considerations

i. Analytical Enforcement Methodology

Validated enzyme-linked immunosorbent assays, for the detection and quantification of Cry34Ab1 and Cry35Ab1 in corn tissue, have been submitted and found acceptable by the Agency (MRID Nos. 461239-01 and 461239-02; reviewed in U.S. EPA (2004b)).

ii. International Residue Limits

In making its tolerance decisions, EPA seeks to harmonize U.S. tolerances with international standards whenever possible, consistent with U.S. food safety standards and agricultural practices. In this context, EPA considers the international maximum residue limits (MRLs) established by the Codex Alimentarius Commission (Codex), as required by FFDCA section 408(b)(4). The Codex Alimentarius is a joint United Nations Food and Agriculture Organization/World Health Organization food standards program, and it is recognized as an international food safety standards-setting organization in trade agreements to which the United States is a party. EPA may establish a tolerance that is different from a Codex maximum residue level (MRL); however, FFDCA section 408(b)(4) requires that EPA explain the reasons for departing from the Codex level.

The Codex has not established a MRL for the Cry34Ab1 and Cry35Ab1 proteins in corn.

f. Endocrine Disruptors

As required under FFDCA section 408(p), the Agency 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 the Agency 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, the Agency 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.

The Cry34Ab1 and Cry35Ab1 proteins are not among the group of 58 pesticide active ingredients on the initial list to be screened under the EDSP. Under FFDCA section 408(p), the Agency must screen all pesticide chemicals. Accordingly, the Agency anticipates issuing future EDSP orders/data call-ins for all pesticide active ingredients.

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/*.

g. Tolerance Exemption

Certain data submitted and reviewed for experimental use permits, as well as the FIFRA section 3 registrations of event DAS-59122-7 corn, also supported the petition for a tolerance exemption for residues of the *Bt* Cry34Ab1 and Cry35Ab1 proteins. Given the available data and information as summarized in Table 1, the Agency established a permanent exemption from the requirement of a tolerance for residues of the *Bt* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production in corn when used as plant-incorporated protectants in the food and feed commodities of corn; corn, field; corn, sweet; and corn, pop under 40 CFR § 174.457 (70 Federal Register (FR) 55254; September 21, 2005). Subsequently, administrative revisions were made to existing plant-incorporated protectant tolerance exemptions (e.g., some tolerance exemptions were moved from 40 CFR part 180 to 40 CFR part 174).

The original Cry34Ab1 and Cry35Ab1 tolerance exemption was subject to these revisions as it was redesignated from 40 CFR § 174.457 to 40 CFR § 174.506 and changed to the following (72 FR 20431; April 25, 2007):

> "**§ 174.506 *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins in corn; exemption from the requirement of a tolerance.**
>
> Residues of *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins in corn are exempted from the requirement of a tolerance when used as plant-incorporated protectants in the food and feed commodities of corn; corn, field; corn, sweet; and corn, pop."

Table 1. Human Health Assessment Data for Cry34Ab1 and Cry35Ab1.

Study Title	Summary	MRID No.
Comparison of the Amino Acid Sequence of the *Bacillus thuringiensis* strain PS149B1 13.6 kDa and 43.8 kDa Insecticidal Crystal Proteins to Known Protein Allergens[a]	Based upon the search requirements that at least 8 contiguous amino acids are identical to a known allergenic sequence, PS149B1 13.6-kiloDalton (kDa) and 43.8-kDa proteins are unrelated to any known allergens in that they do not share any linear epitopes with known protein allergens. **Classification: Acceptable**	452422-05
PS149B1 14 kDa and 44 kDa Proteins: Acute Oral Toxicity Study in CD-1 Mice[a]	All animals survived the two-week study. One female mouse exhibited protruding or bulging eyes on days 6 and 7, but this resolved thereafter. This observation was not attributed to the treatment as it was an isolated observation (i.e., no other animals exhibited this). No other clinical signs were noted for any animals during the study. An initial weight loss was observed in 2 mice at test days 1 and 2, but both gained weight for the remainder of the study. All other animals gained weight throughout the study. No gross treatment-related observations were recorded during the study as represented by gross pathologic observations. An acute oral LD_{50} was calculated for this study based upon a dosage of a 1:4.6 ratio mixture of PS149B1 proteins at greater than 5,000 mg/kg and greater than 2,000 mg/kg for an equimolar mixture (1:3) of the pure proteins. **Classification: Acceptable**	452422-09

[a] Reviewed in U.S. EPA (2001a)

Study Title	Summary	MRID No.
In vitro Digestibility of PS149B1 Proteins[b]	*Pseudomonas fluorescens* strains MR1253 and MR1256 were used to prepare the 14-kDa and 44-kDa PS149B1 proteins, respectively, as inclusion bodies. A simulated gastric fluid was produced to determine the lability of these proteins in an acid environment containing pepsin. The bovine serum albumin (BSA) positive control was rapidly digested (within 1 minute) and the b-lactoglobulin remained intact for 60 minutes, as expected, the duration of the experiment. The 14-kDa protein was visible on the SDS-PAGE gel at the 15-minute sample point but not afterwards. It was detected on the western blot, which has greater sensitivity, at the 20-minute time point but not in later sample points. A band was also noted on the western blot at approximately 30 kDa. This band was noted in an earlier study and considered the result of protein aggregation. For the 44-kDa protein western blot, bands were observed at approximately 42 kDa and 14 kDa. A single band was observed on the 44-kDa SDS-PAGE at approximately 15 to 16 kDa. These bands were only observed at the one-minute time point but not afterwards. It is concluded that both insecticidal crystal proteins (ICPs) are susceptible to degradation in the simulated gastric environment and differ in their lability to these conditions (i.e., the 44-kDa protein is more rapidly degraded). **Classification: Acceptable**	452422-12
In vitro Simulated Gastric Fluid Digestibility Study of Microbially Derived Cry34Ab1 Protein[c]	Bovine serum albumin, the positive control protein, was digested rapidly in the assay and could not be readily detected after 1 minute in the SGF. In contrast, the negative control protein, b-lactoglobulin, was detectable and appeared to change little during the assay time frame. When averaged across the 3 duplicate gels containing Cry34Ab1, the protein appeared to have approached full degradation by 7.5 minutes. Volumes remaining at the 10- and 15-minute time points were excluded from the calculations since they were below background levels. Using this first-order decay model, the DT_{50} and DT_{90} for this protein in the SGF were estimated to be 1.9 and 6.2 minutes, respectively. The Cry34Ab1 protein was rapidly degraded in the SGF using this assay and detection methodology. The conditions of the assay were biologically appropriate in temperature, pH, and chemical makeup of the digestive solution. The first-order decay rate kinetics accurately portrayed the digestion of Cry34Ab1. **Classification: Acceptable**	455845-02

[b] Reviewed in U.S. EPA (2001a)
[c] Reviewed in U.S. EPA (2002)

Study Title	Summary	MRID No.
SDS-PAGE Sensitivity Analysis for Cry35Ab1 in Support of the Simulated Gastric Fluid Digestion Study[d]	GelCode Blue staining of the SDS-PAGE gel used to electrophorese Cry35Ab1 samples indicated that the amount of protein remaining that was visualized by dye binding, 15.6 nanograms (ng), represented 2.6% of the total load (0.61 mg) in that lane. Therefore, 97% of the protein was digested by the simulated gastric digestion assay (pepsin treatment) at this 5-minute time point. The GelCode Staining Reagent does, however, have a sensitivity limitation, which may or may not allow for detection of Cry35Ab1 at levels of 8 ng or less. The sequential sample co-electrophoresed on the gel examined was a 7.8 ng Cry35Ab1 protein/lane, which was not detected by visual examination following staining and destaining. **Classification: Supplemental**	457904-08
Digestion of Allergenic and Non-Allergenic Proteins in Simulated Gastric Fluid[e]	The rate of digestion of allergenic and non-allergenic proteins in SGF was compared. Proteins were incubated in SGF (pH 1.2 and 2.0; pepsin:substrate ratio approximately 3:1, w/w; reaction not shaken), and samples analyzed by SDS-PAGE at various time points. The relative amount of protein remaining at each time point was estimated by densitometry, and the decay was analyzed using a first-order kinetic model. Under the conditions used in the study, the five allergenic proteins evaluated or a digestion fragment of the allergenic protein had half-lives in SGF of over 16 minutes, while the four non-allergenic proteins or a digestion fragment had half-lives of under 14 minutes (and 3 of those 4 had half-lives of less than 0.5 minutes). **Classification: Supplemental**	461239-20
Digestion Efficiency of Allergens and Non-Allergens in Simulated Gastric Fluid[f]	The digestion of seven allergens and eight non-allergens was assessed in SGF using a first-order kinetic model. Half-lives for the proteins tested ranged from <30 seconds to >60 minutes. Allergens tended to be more stable in SGF than non-allergens, but a strong correlation between digestion rate and allergenicity was not observed for this set of proteins. The data fit well to a first-order decay model, except for early time points, and half-lives calculated using initial substrate concentrations that differed by 5-fold were fairly consistent. **Classification: Acceptable**	463886-01

[d] Reviewed in U.S. EPA (2003)
[e] Reviewed in U.S. EPA (2004a)
[f] Reviewed in U.S. EPA (2005a)

Study Title	Summary	MRID No.
PS149B1 14 kDa Protein: Acute Oral Toxicity Study in CD-1 Mice[g]	PS149B1 14-kDa protein (Cry34Ab1; 54% pure) was evaluated for acute oral toxicity. Five male CD-1 mice received 5,000 mg of test material per kg body weight by oral gavage as a 20% mixture in a 0.5% aqueous methylcellulose vehicle. All animals survived the two-week study. No clinical signs were noted for any animals during the study. An initial weight loss was observed in 3 mice at test days 1 and 2, but they gained weight for the remainder of the study. The two other animals gained weight throughout the study. No treatment-related gross pathologic observations were observed during the study. Under the conditions of this study, the acute oral LD_{50} for the test substance in male CD-1 mice was greater than 5,000 mg/kg; since the test substance contained PS149B1 14-kDa protein (Cry34Ab1) at 54% purity, the acute LD_{50} for the pure protein was greater than 2,700 mg/kg. **Classification: Acceptable**	452422-07
PS149B1 44 kDa Protein: Acute Oral Toxicity Study in CD-1 Mice[g]	PS149B1 44-kDa protein (Cry35Ab1; 37% pure) was evaluated for acute oral toxicity. Five male CD-1 mice received 5,000 mg of test material per kg body weight by oral gavage as a 20% mixture in a 0.5% aqueous methylcellulose vehicle. All animals survived the two-week study. No clinical signs were noted for any animals during the study. An initial weight loss was observed in 2 mice at test days 1 and 2, but they gained weight for the remainder of the study, and one animal had fluctuating body weight. The other two animals gained weight throughout the study. No treatment-related gross pathologic observations were observed during the study. Under the conditions of this study, the acute oral LD_{50} for the test substance in male CD-1 mice was greater than 5,000 mg/kg; since the test substance contained PS149B1 44-kDa protein (Cry35Ab1) at 37% purity, the acute LD_{50} for the pure protein was greater than 1,850 mg/kg. **Classification: Acceptable**	452422-08
Lack of Cry34Ab1/Cry35Ab1 Co-Association in Solution[h]	Size exclusion chromatography was used to investigate whether Cry34Ab1 and Cry35Ab1 associate with one another in solution under acidic conditions. Two studies were conducted, one at pH 3.6 and one at pH 2.0. In both studies, Cry34Ab1 and Cry35Ab1 eluted as separate peaks, indicating that the proteins do not associate with one another in solution under these conditions. **Classification: Acceptable**	465568-01

[g] Reviewed in U.S. EPA (2005a)
[h] Reviewed in U.S. EPA (2005d)

Study Title	Summary	MRID No.
Evaluation of the Sequence Similarities of the Cry34Ab1, Cry35Ab1, and PAT Proteins with the Public Protein Sequence Datasets[i]	The sequences of the Cry34Ab1, Cry35Ab1, and PAT proteins were compared with protein sequences in publicly available databases (GenPept dataset) to identify any potential similarities with known toxins. The BlastP2.2.6 algorithm was used with a cutoff expectation (E) value of 1.0. The results of the Cry34Ab1 search identified 10 proteins, 5 of which are Cry proteins from *Bt* that are closely related or identical to the Cry34Ab1 protein. The other proteins represent putative microbial collagenases and hypothetical proteins from genome-sequencing projects with a low degree of similarity to the Cry34Ab1 protein. The Cry35Ab1 search identified 22 proteins, 7 of which are similar or identical Cry proteins from *Bt*. Other significant similarities were with proteins from a related species, *Bacillus sphaericus*, which has mosquitocidal activity. The PAT search identified 148 accessions. Only 18 of these represent actual GenPept accessions and are either phosphinothricin acetyltransferases or other acetyltransferases. The remaining 130 proteins are unidentified and/or hypothetical proteins from genome-sequencing data. The Cry34Ab1, Cry35Ab1, and PAT proteins do not appear to have similarities with any proteins that would raise safety concerns. **Classification: Acceptable**	465847-01

2. Human Health Assessment of PAT (Expressed in Event DAS-59122-7 Corn, Herculex® XTRA Insect Protection Corn, 59122 x MON 810, and 1507 x 59122 x MON 810), Cry1F (Expressed in Herculex® XTRA Insect Protection Corn and 1507 x 59122 x MON 810), and Cry1Ab (Expressed in 59122 x MON 810 and 1507 x 59122 x MON 810)

Based on previously completed Agency human health assessments, permanent tolerance exemptions have been established for the PAT, Cry1F, and Cry1Ab proteins:

> "**§ 174.522 Phosphinothricin Acetyltransferase (PAT); exemption from the requirement of a tolerance.**
>
> Residues of the Phosphinothricin Acetyltransferase (PAT) enzyme are exempt from the requirement of a tolerance when used as plant-incorporated protectant inert ingredients in all food commodities."

**original under 40 CFR § 180.1151 (62 FR 17717; April 11, 1997)
**revised under 40 CFR § 174.522 (72 FR 20431; April 25, 2007)

[i] Reviewed in U.S. EPA (2005d)

"**§ 174.520 *Bacillus thuringiensis* Cry1F protein in corn; exemption from the requirement of a tolerance.**

Residues of *Bacillus thuringiensis* Cry1F protein in corn are exempt from the requirement of a tolerance when used as plant-incorporated protectants in the food and feed commodities of corn; corn, field; corn, sweet; and corn, pop.

**original under 40 CFR § 180.1217 (66 FR 30321; June 6, 2001)
**revised under 40 CFR § 174.520 (72 FR 20431; April 25, 2007)

"**§ 174.511 *Bacillus thuringiensis* Cry1Ab protein in all plants; exemption from the requirement of a tolerance.**

Residues of *Bacillus thuringiensis* Cry1Ab protein in all plants are exempt from the requirement of a tolerance when used as plant-incorporated protectants in all food commodities."

**original under 40 CFR § 180.1173 (61 FR 40340; August 2, 1996)
**revised under 40 CFR § 174.511 (72 FR 20431; April 25, 2007)

The toxicological and allergenicity data supporting the establishment of these tolerance exemptions, as well as the associated registrations of MON 810 (expressing Cry1Ab protein) and Herculex® I (expressing Cry1F and PAT proteins), can be found in the 2001 *Bt* Crops Reassessment, the August 2005 Cry1F BRAD, and/or the September 2010 Cry1Ab and Cry1F BRAD (U.S. EPA 2001b, 2005e, and 2010b).

The individual data generated for the Cry1Ab, Cry1F, and PAT proteins (and Cry34Ab1 and Cry35Ab1) support their inclusion, as expressed in particular stacked and/or pyramided plant-incorporated protectants (i.e., Herculex® XTRA Insect Protection Corn, 59122 x MON 810, and 1507 x 59122 x MON 810), into the appropriate tolerance exemptions since the mode of action for the proteins does not suggest a synergistic activity in combination for mammalian species.

Human health assessment data, provided specifically in relation to the Herculex® Insect Protection Corn, 59122 x MON 810, and/or 1507 x 59122 x MON 810 registrations, are summarized in Table 2.

Table 2. Human Health Assessment Data for PAT, Cry1F, and Cry1Ab.

Study Title	Summary	MRID No.
Herculex® XTRA Insect Protection Corn Data		
Summary Information in Support of the Section 3 Registration of Herculex® Xtra Resistant Corn[j]	Summary information is presented in support of the Section 3 registration of Herculex® Xtra Insect Resistant Corn, which contains Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins. The information focuses on the individual traits (from Herculex® I and Herculex® RW) that comprise Herculex® Xtra and potential synergies of the proteins with respect to mammalian and environmental risk. Studies of the individual traits, as well as the stacked traits, indicate expected efficacy to certain lepidopteran and coleopteran pests in field corn and no synergistic effect from the combined proteins. Studies indicate no concerns for safety of humans, non-target organisms, or threatened and endangered species. Data waiver requests and rationale are presented in this submission. **Classification: Acceptable**	462971-01
Equimolar Ratio of Cry1F (tr) + Cry35Ab1 + Cry34Ab1: Acute Oral Toxicity Study in CD-1 Mice[k]	The acute oral toxicity of a mixture of microbially produced Cry1F (truncated), Cry34Ab1, and Cry35Ab1 proteins was assessed by administering an equimolar mixture of the proteins to five female mice and five male CD-1 mice. A dose of 525 mg pure Cry1F (truncated)/kg body weight (bw), 355 mg pure Cry35Ab1/kg bw, and 113 mg pure Cry34Ab1/kg bw was given as a suspension in 0.5% aqueous methylcellulose by two fractional gavage doses. All animals survived the two-week study and gained weight, no clinical signs were noted, and there were no gross pathologic changes observed at necropsy. The oral LD_{50} was greater than 993 mg/kg for the equimolar mixture of the pure Cry proteins. **Classification: Acceptable**	463843-01
59122 x MON 810 & 1507 x 59122 x MON 810 Data		
Evaluation of the Amino Acid Sequence Similarity of the Cry1Ab Protein from MON 810 to the NCBI Protein Sequence Datasets (Toxicity Study)[l]	The 818 amino acid sequence of event MON 810 corn expressing Cry1Ab protein was sequentially searched and compared for protein sequence homology to known toxins contained in the National Center for Biotechnology Information (NCBI) Protein Sequence datasets. The BLASTP search showed no significant amino acid sequence homologies, using the Cry1Ab protein sequence as a query, with any proteins known to be toxins. Therefore, the Cry1Ab protein does not share any amino acid sequence homology with any proteins known to be toxins. **Classification: Acceptable**	477864-06

[j] Reviewed in U.S. EPA (2005f)
[k] Reviewed in U.S. EPA (2005f)
[l] Reviewed in U.S. EPA (2010a)

Study Title	Summary	MRID No.
Evaluation of the Amino Acid Sequence Similarity of the Cry1F Protein from TC1507 to the NCBI Protein Sequence Datasets (Toxicity Study) [m]	The 605 amino acid sequence of event TC1507 corn expressing Cry1F protein was sequentially searched for comparisons to known toxins contained in the NCBI Protein Sequence datasets. The BLASTP search showed no significant amino acid sequence homologies, using the Cry1F protein sequence as a query, with any proteins known to be toxins. Therefore, the Cry1F protein does not share any amino acid sequence homology with any proteins known to be toxins. **Classification: Acceptable**	477864-05
Evaluation of the Amino Acid Sequence Similarity of the PAT Protein to the NCBI Protein Sequence Datasets (Toxicity Study)[m]	No significant amino acid sequence homologies were identified, using the PAT protein sequence as a query, with any proteins known to be toxins contained within the NCBI Protein Sequence datasets. Therefore, the updated amino acid homology search verifies no changes in the results and conclusions of the previously submitted toxin homology assessment for the PAT protein (see MRID No. 465847-01). **Classification: Acceptable**	477864-07
Evaluation of the Amino Acid Sequence Similarities of the Cry34Ab1 and Cry35Ab1 Proteins to the NCBI Protein Sequence Datasets (Toxicity Study)[m]	No significant amino acid sequence homologies were identified, using the Cry34Ab1 and Cry35Ab1 protein sequences as a query, with any proteins known to be toxins contained within the NCBI Protein Sequence datasets. Therefore, the updated amino acid homology search verifies no changes in the results and conclusions of the previously submitted toxin homology assessment for the Cry34Ab1 and Cry35Ab1 proteins expressed in event DAS-59122-7 corn (see MRID No. 465847-01). **Classification: Acceptable**	477864-08
Comparison of the Amino Acid Sequence Identity Between the Cry1F Protein and Known Protein Allergens (Allergenicity Study)[m]	No identity matches of ~35% over ~80 amino acid residues were observed for the Cry1F protein sequence against the protein sequences of known allergens. In addition, there were no 8 or greater contiguous identical amino acid matches observed with the Cry1F protein sequence. Therefore, the updated amino acid homology search verifies no changes in the results and conclusions of the previously submitted allergen homology assessment for the Cry1F protein expressed in event TC1507 corn (see MRID No. 449717-01). **Classification: Acceptable**	477864-01

[m] Reviewed in U.S. EPA (2010a)

Study Title	Summary	MRID No.
Comparison of the Amino Acid Sequence Identity Between the Cry34Ab1 and Cry35Ab1 Proteins and Known Protein Allergens (Allergenicity Study) [n]	No identity matches of ~35% over ~80 amino acid residues were observed for the Cry34Ab1 and Cry35Ab1 protein sequences against the protein sequences of known allergens. In addition, there were no 8 or greater contiguous identical amino acid matches observed with the Cry34Ab1 and Cry35Ab1 protein sequences. Therefore, the updated amino acid homology search verifies no changes in the results and conclusions of the previously submitted allergen homology assessment for the Cry34Ab1 and Cry35Ab1 proteins expressed in event DAS-59122-7 corn (see MRID No. 452422-05). **Classification: Acceptable**	477864-02
Comparison of the Amino Acid Sequence Identity Between the PAT Protein and Known Protein Allergens (Allergenicity Study)[n]	No identity matches of ~35% over ~80 amino acid residues were observed for PAT protein sequence against the protein sequences of known allergens. In addition, there were no 8 or greater contiguous identical amino acid matches observed with the PAT protein sequence. Therefore, the updated amino acid homology search verifies no changes in the results and conclusions of the previously submitted allergen homology assessment for the PAT protein (see MRID No. 449717-01). **Classification: Acceptable**	477864-03
Comparison of the Amino Acid Sequence Identity Between the Cry1Ab Protein from Event MON 810 and Known Protein Allergens (Allergenicity Study)[n]	No identity matches of ~35% over ~80 amino acid residues were observed for Cry1Ab protein sequence against the protein sequences of known allergens. In addition, there were no 8 or greater contiguous identical amino acid matches observed with the Cry1Ab protein sequence. Therefore, the updated amino acid homology search verifies no changes in the results and conclusions of the previously submitted allergen homology assessment for the Cry1Ab protein expressed in event MON 810 corn (see MRID No. 458789-02). **Classification: Acceptable**	477864-04

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U.S. EPA. 2010a. Review of Human Health and Product Characterization Data for Registration of *Bacillus thuringiensis* (*Bt*) Cry1F, Cry34Ab1, Cry35Ab1, and Cry1Ab Proteins and the Genetic Material Necessary for Their Production in the Combination Plant-Incorporated Protectant (PIP) Products: 1507 x MON 810 [EPA Reg. No. 29964-T], 59122 x MON 810 [EPA Reg. No. 29964-O], and 1507 x 59122 x MON 810 [EPA Reg. No. 29964-I]. Memorandum from A. Waggoner and J.L. Kough, Ph.D. to A. Sibold dated February 19, 2010.

U.S. EPA. 2010b. Biopesticides Registration Action Document – *Bacillus thuringiensis* Cry1Ab and Cry1F Corn (Updated September 2010). Available from: *http://www.regulations.gov* (see "Supporting & Related Materials" within Docket Number EPA-HQ-OPP-2010-0607).

C. Environmental Assessment

1. Tiered 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.[a]

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.

The Environmental Protection Agency (EPA) uses a tiered (Tiers I–IV) testing system to assess the toxicity of a plant-incorporated protectant (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 endpoint. 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, which utilize the tiered approach, were last published by EPA as Harmonized Office of Prevention, Pesticides, and Toxic Substances (OPPTS) Testing Guidelines (now Harmonized Office

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

of Chemical Safety and Pollution Prevention (OCSPP) Testing Guidelines), Series 850 and 885 (EPA 712-C-96-280, February 1996).[b] These guidelines 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. Therefore, plant-incorporated protectants (PIPs) containing microbial toxins are also covered by these testing guidelines.

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, or the Estimated Environmental Concentration (EEC).[c] Tier I tests serve to identify potential hazards and are conducted in the laboratory at high dose levels, which 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 thirty animals or three groups of ten test animals at the screening test concentration. The Guidelines further state that the duration of all Tier I tests should be approximately 30 days. 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%.

Failing the Tier I (10x EEC) screening at the MHD does not necessarily indicate the presence of an unacceptable risk in the field, but it triggers the need for additional testing.[d] A less than 50% mortality effect at the MHD is taken to indicate minimal risk. Greater than 50% mortality, however, 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.[e]

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[b] General OCSPP Harmonized Testing Guidelines available from: *http://www.epa.gov/ocspp/pubs/frs/home/guidelin.htm*.
Series 850 Testing Guidelines available from:
http://www.epa.gov/ocspp/pubs/frs/publications/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/Drafts/index.html.
Series 885 Testing Guidelines available from:
http://www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series885.htm.

[c] 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.

[d] Note that the 10x 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.

[e] The 1x EEC test dose is based on plant tissue content and is considered the highest dose in a worst-case scenario (sometimes referred to as the Highest Estimated Environmental Concentration or HEEC). This 1x EEC is still much greater

When screening tests indicate a need for additional data, the OCSPP Harmonized Guidelines call for testing at incrementally lower doses in order to establish a definitive LD_{50} (i.e., dose that will kill 50% of the test organisms within a designated period) 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 Adverse Effect Concentration (LOAEC), 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 LOAEC to the EEC; when the EEC is lower than the LOAEC, 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, and/or repetition are needed to enhance statistical power in these circumstances.

Data that shows less than 50% mortality at the maximum hazard dosage level (i.e., LC_{50}, ED_{50}, or LD_{50} >10x 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 (U.S. EPA 1998).[f]

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 Federal Insecticide, Fungicide, and Rodenticide Act (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 PIPs; 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 genetically modified (GM) crop fields should be conducted. Testing of *Bacillus thuringiensis* (*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 (U.S. EPA 2000, 2001a, 2002, and 2004b).

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. But, the 30-day test duration requirement does amount to subchronic

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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.

[f] The established peer and EPA Science Board reviewed guidance on screening test levels of concern is 50% mortality at 5x environmental concentration for terrestrial and 10x for aquatic species. 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.

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 either 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 I 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, however, then higher tier, longer term field testing will be required. As noted above, the October 2000 SAP and the National Academy of Sciences (NAS 2000) recommended testing non-target organisms directly in the field. This approach, with an emphasis on testing invertebrates found in corn fields, was also recommended by the August 2002 SAP and was supported by several public comments. Based on these recommendations and due to the lack of baseline data on the potential for long-term environmental effects from the cultivation of PIP-producing plants, the Agency has required long-term field studies on invertebrate populations/communities and Cry protein accumulation in soils as conditions of past PIP registrations.

Since the commercialization of *Bt* crops, the number of field studies published in scientific literature, in combination with the post-registration field studies submitted to the Agency, has accumulated to a level where empirical conclusions can be made. As a result, the issue of long-range effects of cultivation of these Cry proteins on the invertebrate community structure in *Bt* crop fields has since been adequately addressed. Specifically, a meta-analysis[g] of the data collected from 42 field studies indicated that non-target invertebrates are generally more abundant in *Bt* cotton and *Bt* maize fields than in non-transgenic fields managed with insecticides (Marvier *et al*. 2007). In addition, a comprehensive review of short- and long-term field studies on the effects of invertebrate populations in *Bt* corn and cotton fields indicated that no unreasonable adverse effects are taking place as a result of wide-scale *Bt* crop cultivation (Sanvido *et al*. 2007). Another review of field tests published to date concluded that the large-scale studies in commercial *Bt* cotton have not revealed any unexpected non-target effects other than subtle shifts in the arthropod community caused by the effective control of the target pests (Romeis *et al*. 2006). 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.

Overall, the Agency is in agreement with the conclusions of these studies and, collectively, these results provide extensive data to support that *Bt* crops have not caused long-term environmental effects, on a population level, to organisms not targeted by *Bt* proteins. Based on these considerations, regulatory

[g] This research was funded by EPA grant CR-832147-01. The *Bt* crop non-target effects database can be found on the National Center for Ecological Analysis and Synthesis (NCEAS) Web Site: *http://delphi.nceas.ucsb.edu/btcrops/*.

testing of the specialist predators and parasitoids of target pests may eventually be considered unnecessary.

2. Event DAS-59122-7 Corn (Organization for Economic Cooperation and Development (OECD) Unique Identifier: DAS-59122-7) Expressing Cry34Ab1 and Cry35Ab1

a. Data Cited/Submitted for Initial Registrations of Event DAS-59122-7 Corn (Prior to August 2005)

i. Background

In 2003, Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International, Incorporated requested registrations for *Bacillus thuringiensis* Cry34/35Ab1 binary protein and the genetic material necessary for its production in all corn lines and varieties. This protein controls the corn rootworm (CRW, *Diabrotica* spp.), a primary pest of corn in the United States. Corn rootworm larvae feed on corn roots, resulting in lodging and a reduction in a plant's ability to absorb water and nutrients from soil. In areas where the CRW is a pest (e.g., 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.

EPA conducted an environmental hazard assessment of the Cry34/35Ab1-producing corn lines. The general topics covered include gene flow to related wild plants, development of weediness, effects on wildlife, and fate of Cry34/35Ab1 protein in the environment. The assessment is based on data submitted to the Agency during the development of the corn lines; additional data submitted for registration; FIFRA SAP recommendations; consultations with scientific experts; and previous public comments on plant-incorporated protectant regulation.

ii. Non-Target Wildlife Hazard Assessment

Two separate SAP reports (October 2000 and August 2002) recommended that non-target testing should focus on species exposed to the crop being registered. Following SAP recommendations, the Agency determined that non-target organisms with the greatest exposure potential to 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 beneficials from several taxa was performed. The toxicity of the Cry34/35Ab1 protein has been evaluated on several species of invertebrates, including larval honey bees, a parasitic hymenoptera, green lacewings, lady beetles, collembola, and earthworms. Reproductive and developmental observations were also made on collembola, honey bee, and lady beetle larva.

The non-target organisms tested are chosen as representative indicators of the major groups of wildlife and on the potential for field exposure as deduced from data on Cry34/35Ab1 protein expression in the plant. Although *Bt* Cry proteins are very specific in their activity to only certain insect species, for Cry34/35Ab1 protein in corn, the Agency has examined the toxicity to birds, fish, honey bees, and

certain other beneficial insects even though the October 2000 SAP recommended against testing of non-target species not related to those susceptible to the specific activity of *Bt* Cry proteins. In order to comply with the Agency's published data requirements for registration of microbial toxins (40 CFR § 158.2150), data submissions or waiver justifications were submitted to address these requirements. Collembola and earthworm studies were also conducted and voluntarily submitted to the Agency by the applicants to ascertain effects of Cry34/35Ab1 on beneficial decomposer species because prolonged exposure to Cry34/35Ab1 protein in soil was a possibility. Honey bee effects on brood were also required as exposure of honey bee larvae to the Cry34/35Ab1 protein in pollen is expected.

The form of the test substances used in the studies for this assessment are plant material such as leaves, roots, pollen, or purified, bacterially produced Cry34/35Ab1 protein incorporated into the test species diet. The October 2000 SAP provided guidance to the Agency that, while actual plant material is the preferred test material, bacterially derived protein is also a valid test substance, particularly in testing where the test animals do not consume corn plant tissue and where large amounts of Cry protein are needed for maximum hazard dose testing. Consistent with the OCSPP Harmonized Testing Guidelines, the adult insect studies were generally of 30 days duration or until the negative control mortality reached 20%. Larval studies were carried out through pupation and adult emergence.

The results for each study on environmental effects for Cry34/35Ab1 are summarized in Table 1. Additionally, the results are presented in a more descriptive format in subsequent sections of this Environmental Assessment chapter. Complete reviews of each study can be found in the individual Data Evaluation Reports.

Table 1. Environmental Effects Data for Event DAS-59122-7 Corn (Reviewed in U.S. EPA (2005c) Unless Otherwise Noted).

Guideline Number	Study	Results	MRID Number
885.4050	Avian Dietary Testing	There were no treatment-related deaths or clinical signs observed in broilers fed a diet containing 59% (starter ration) or 63% (grower/finisher ration) Cry34/35 *Bt* corn for 42 days. Corn containing the test protein supported rapid growth of broiler chickens and was similar in performance to non-transgenic varieties. No adverse effects on avian wildlife are expected from consumption of Cry34/35Ab1 corn. **Classification: Acceptable**	461239-11

Guideline Number	Study	Results	MRID Number
885.4150	Wild Mammal Testing	Mammalian wildlife exposure to Cry34/35Ab1 protein is considered likely; however, the Cry34/35Ab1 toxicity data, as described in the Human Health Assessment (see section II(B)(1) of this Biopesticides Registration Action Document (BRAD)), indicate that there is no significant toxicity to rodents from testing at the maximum hazard dose. Therefore, no hazard to mammalian wildlife is anticipated, and data on wild mammal testing are not required.	N/A
850.1075	Freshwater Fish Testing[h]	There were no treatment-related deaths or clinical signs observed in rainbow trout fed 100 milligrams (mg) PS149B1 insecticidal crystal (IC) binary protein/kilogram (kg) diet for 8 days. Based on these observations, the 8-day LD_{50} for rainbow trout was $\geq$100 mg PS149B1 IC protein. No freshwater fish hazard is expected from commercial cultivation of Cry34/35Ab1 corn. **Classification: Supplemental**	457904-03
850.1010	Aquatic Invertebrate Acute Toxicity Test (*Daphnia magna*)[h]	A static-renewal, 48-hour limit test was performed on *Daphnia magna*. The test material was purified PS149B1 binary protein at a target concentration of 100 mg protein/Liter (L) water. No adverse effects were noted for the acute limit test. No hazards to daphnia are expected from incidental exposure to Cry34/35Ab1-containing corn pollen. **Classification: Supplemental** *Note for 2010: There is an update to this summary. See section II(C)(2)(b) ("Terms and Conditions of the Event DAS-59122-7 Corn Registrations (August 2005 – September 2010)") of this BRAD.	457904-04
885.4280	Estuarine and Marine Animal Testing	The estuarine and marine animal studies are not required for this product because Cry34/35Ab1 is not intended for direct application into the estuarine or marine environment and there is very low to no potential for exposure to Cry34/35Ab1 protein from field corn.	N/A
885.4300	Non-Target Plant Studies	The active ingredient is an insect toxin (*Bt* endotoxin) that is non-toxic to aquatic and terrestrial plants. Consequently, non-target plant studies have been waived for this product.	N/A
885.4380	Honey Bee Larva Testing[i]	The median lethal concentration (LC_{50}) for honey bee larvae and adult bee emergence was found to be >5.6 micrograms (µg) Cry34/35Ab1 protein (100x the concentration in pollen). Also, no behavioral or morphologic abnormalities were seen in bees exposed to 2 mg/larva Cry34/35Ab1 corn pollen. Therefore, no hazard to honey bee larvae and adult bee emergence is anticipated. **Classification: Acceptable**	453407-01

[h] Reviewed in U.S. EPA (2005a)
[i] Reviewed in U.S. EPA (2004a)

Guideline Number	Study	Results	MRID Number
885.4340	Parasitic Hymenoptera Larva Testing	The LC_{50} for parasitic Hymenoptera was determined to be >280 parts per million (ppm) Cry34/35Ab1 protein. No hazard to parasitic Hymenoptera is expected. Testing of a species more common to corn fields is recommended. **Classification: Acceptable**	457904-05
885.4340	Dietary Toxicity to Green Lacewing Larvae	The LC_{50} for green lacewing larvae was determined to be >280 ppm Cry34/35Ab1 protein (10x field exposure). No hazard to green lacewing is expected. It is questionable whether the test species ingests the test material in feeding trails. Consequently, another species (e.g., minute pirate bug or predatory carabid) with greater likelihood of exposure should be tested. **Classification: Acceptable** *Note for 2010: There is an update to this summary. See section II(C)(2)(b) ("Terms and Conditions of the Event DAS-59122-7 Corn Registrations (August 2005 – September 2010)") of this BRAD.	457904-07
885.4340	Lady Beetle Larval Feeding Study (*Coleomegilla maculata*)	Significant growth inhibition (80% growth reduction in 7-day study) was observed in *Coleomegilla maculata* larvae feeding on Cry34Ab1/Cry35Ab1 diet, compared to larvae feeding on control diets. However, since toxic effects were not seen at the field dose level when *Bt* corn pollen- or *Bt* corn plant-fed aphids were used as diet, there is reasonable certainty that Cry34/35Ab1 corn will not adversely affect *C. maculata.* **Classification:** **Acceptable (MRID Nos. 452422-10 and 452422-11)** **Supplemental (MRID No. 461239-12) because the study was terminated prior to pupation.**	452422-10[j] 452422-11[j] 461239-12
885.4340	Collembola Chronic Dietary Toxicity Study[k]	The LC_{50} for collembola was found to be >12.7 mg PS149B1 active ingredient/kg (10x field exposure). No adverse survival or reproductive effects were noted. No hazard to beneficial soil-inhabiting decomposers is expected. **Classification: Acceptable**	457904-06
850.6200	Earthworm Toxicity Study[l]	The LC_{50} for earthworm was found to be >76 mg Cry34/35Ab1 protein/kg dry soil (20x field exposure). No hazard to earthworms is expected. **Classification: Supplemental**	453602-01

[j] Reviewed in U.S. EPA (2001b)
[k] Reviewed in U.S. EPA (2005b)
[l] Reviewed in U.S. EPA (2004a)

Guideline Number	Study	Results	MRID Number
N/A	Insecticidal Activity Spectrum Study[m]	The relative sensitivity of the test insects in the feeding bioassays suggests that larvae of coleopteran species are far more sensitive to the 14-kiloDalton (kDa) and 44-kDa proteins of PS149B1 than the lepidopteran or homopteran larval insects evaluated. Adult western corn rootworm (WCRW) were not sensitive to the delta-endotoxins, however. Rootworms (northern corn rootworm (NCRW), WCRW, and southern corn rootworm (SCRW)) were the most susceptible to the PS149B1 proteins. European corn borer (ECB) and corn earworm (CEW) demonstrated some inhibition of growth at higher concentrations of the test substance than the rootworm larvae. Black cutworm (BCW) was the least sensitive of the lepidopteran species tested. No activity was seen against the corn leaf aphid (CLA), a homopteran. Relative susceptibilities of the insect species to PS149B1, based upon the more sensitive measure of 50% growth inhibition (GI_{50}), were as follows: (most susceptible) WCRW, NCRW, SCRW > ECB, CEW >> WCRW adult, CLA, BCW (least susceptible). **Classification: Acceptable**	452422-04
N/A	Non-Target Organism Field Scale Risk Assessment	Field monitoring results from two corn-growing seasons confirmed a lack of adverse effects on non-target invertebrates when exposed to Cry34/35Ab1 protein. These studies are supplemental to Tier I maximum hazard dose testing and are of inadequate statistical power for long-term effects determination. **Classification: Supplemental** *Note for 2010: There is an update to this summary. See section II(C)(2)(b) ("Terms and Conditions of the Event DAS-59122-7 Corn Registrations (August 2005 – September 2010)") of this BRAD.	461239-14
885.5200	Soil Fate	The GI_{50} value for SCRW fed a representative soil containing Cry34/35Ab1 protein was 3.2 days. This finding suggests that soil-incorporated binary insecticidal protein degrades over time. **Classification: Supplemental** *Note for 2010: There is an update to this summary. See section II(C)(2)(b) ("Terms and Conditions of the Event DAS-59122-7 Corn Registrations (August 2005 – September 2010)") of this BRAD.	452422-14

[m] Reviewed in U.S. EPA (2001c)

Guideline Number	Study	Results	MRID Number
N/A	Endangered Species Impact Assessment	Pioneer Hi-Bred International, Incorporated conducted a hazard assessment, exposure assessment, and risk characterization to demonstrate that Cry34/35Ab1 does not pose risk to endangered coleoptera. The Agency's independent assessment is in agreement with these conclusions. **Classification: Acceptable**	461239-17
N/A	Non-Target Invertebrate Ecological Risk Assessment	Submitted data indicate that Cry34/35Ab1 corn will not pose unreasonable adverse effects to corn field flora and fauna and that minimal short-term accumulation of Cry34/35Ab1 protein in agricultural soil is expected. Further, no adverse effect to endangered and threatened species listed by the U.S. Fish and Wildlife Service is expected from the proposed Cry34/35Ab1 CRW resistant corn registration. **Classification: Acceptable**	461239-13

I. Non-Target Wildlife Study Summaries

a. Mammalian Wildlife

Mammalian wildlife exposure to Cry34/35Ab1 protein is considered likely; however, the mammalian toxicology information gathered to date on *Bt* Cry proteins does not show a hazard to wild or domesticated mammals. The Cry34/35Ab1 toxicity data, as described in the Human Health Assessment (see section II(B)(1) of this BRAD), indicate that there is no significant toxicity to rodents from acute oral testing at the maximum hazard dose. Therefore, no hazard to mammalian wildlife is anticipated, and data on wild mammal testing is not required.

b. Avian Species

Published data and studies on file at EPA show that consumption of *Bt* corn has no measurable deleterious effects on avian species. Nonetheless, the following broiler study was submitted to the Agency in support of the Cry34/35Ab1 corn registrations.

i. Broiler Study (Master Record Identification Number (MRID No.) 461239-11)

Day-old commercial broiler chickens (Cobb x Cobb) were fed diets containing either transgenic corn line event TC15344 (Cry34Ab1 or Cry35Ab1), a non-transgenic isoline corn (near isoline of event TC15344), or commercial corn from one of two sources (A or B) for 42 days. A starter ration containing approximately 59% wet weight corn was fed *ad libitum* during days 1–20 (Phase I), and a grower/finisher ration containing approximately 63% wet weight corn was provided *ad libitum* during days 21–42 (Phase II) of the test period. No biologically significant differences in live weight, carcass

weight, or feed intake efficiency were seen among treatment groups. These data suggest that the hybrid maize line containing event TC15344 was nutritionally equivalent to the non-transgenic isoline and commercial maize sources and that long-term exposure to Cry34/35Ab1 corn is not expected to pose a hazard to avian wildlife.

c. Aquatic Species

There is no evidence for sensitivity of aquatic (including endangered) species to anti-coleopteran Cry proteins. Aquatic species toxicity studies with coleopteran-active Cry proteins have not revealed hazard for fish or invertebrates exposed to either corn pollen or to bacterially expressed Cry protein. Furthermore, aquatic exposure from *Bt* crops is extremely small.

i. Freshwater Fish (MRID No. 457904-03)

The Harmonized Testing Guidelines requirement for a static-renewal freshwater fish toxicity study is usually waived based on low to nonexistent exposure to Cry protein produced in corn. Exposure from corn pollen, if it does take place, will be of a very short duration and quantity and is not expected to have any detectable effect on freshwater fish. Nonetheless, an eight-day limit study was performed and submitted for review. This study is scientifically sound but was not performed according to OCSPP microbial testing guidelines.

This limit test was conducted at a target concentration of 100 mg PS149B1 IC binary protein/kg diet, fed at 10% approximate fish wet body weight per day for 8 days, in a static-renewal system with water and test chambers replaced every 24 hours after feeding. A control group was fed unamended diet only. A set of 30 fish (3 replicates, n = 10) were tested for each of the control (diet only) and treatment (diet with added PS149B1 IC binary protein) groups. Fish were observed for mortality and sublethal effects every 24 hours during the 8-day test period. Feed consumption ranged from 70–90% (as reported) during the 20-minute feeding period. No fish mortality or sublethal effects were reported in either the treatment or control groups. Based on these observations, the 8-day LD_{50} for rainbow trout was $\geq$100 mg PS149B1 IC protein/kg diet fed at 10% approximate fish wet body weight/day. No statistical test was performed because it was a single dose (limit) test. Biological loading exceeded guideline parameters of 0.5 grams (g) fish/L.

In view of the lack of demonstrated toxicity to rainbow trout and minimal aquatic exposure, no freshwater fish hazard is expected from commercial cultivation of Cry34/35Ab1 corn.

ii. Freshwater Aquatic Invertebrates (MRID No. 457904-04)

This study was conducted according to procedures specified in Series 72 of EPA's Registration Guidelines, Pesticide Assessment Guidelines, FIFRA Subdivision E, Hazard Evaluation for acute toxicity testing of pesticidal substances to freshwater aquatic invertebrates.

This static-renewal limit test was performed on *Daphnia magna*, a freshwater invertebrate. The test material consisted of purified *Bacillus thuringiensis* PS149B1 crystal binary protein added to water at a target concentration of 100 mg PS149B1 insecticidal crystal protein (ICP)/L. The study was procedurally sound, and no treatment mortality or behavior changes were reported between the dosed and control replicates during the 48-hour exposure period.

The October 2000 and August 2002 SAP reports recommended that non-target testing be focused on species exposed to the crop being registered (i.e., beneficial insects found in corn fields). Nevertheless, acute aquatic invertebrate species testing was performed, and no substantial aquatic exposure to Cry34/35Ab1 protein contained within corn plant tissue is expected. Therefore, no hazard from the registered uses of Cry34/35Ab1-containing corn is anticipated.

*Note for 2010: There is an update to this summary. See section II(C)(2)(b) ("Terms and Conditions of the Event DAS-59122-7 Corn Registrations (August 2005 – September 2010)") of this BRAD.

iii. Estuarine and Marine Animals

The estuarine and marine animal study was not required for this product because of no or very low potential for exposure to the Cry34/35Ab1 protein in these environments.

d. Terrestrial and Aquatic Plants

Plant toxicity studies were waived for this product because the active ingredient is an insect toxin (*Bt* endotoxin), and we are unaware of any demonstrated toxicity to plants.

e. Non-Target Insect Testing

i. Effects on Honey Bee Larvae (MRID No. 453407-01)

An acceptable study was conducted based on OCSPP Harmonized Test Guideline 885.4380 (Honey Bee Testing, Tier I). This study was conducted in accordance with Good Laboratory Practice Standards (40 CFR Part 160) with certain exceptions that did not affect the integrity of the test.

Testing was conducted with three- to five-day-old honey bee (*Apis mellifera*) larvae. Individual larvae were dosed with one of the following treatments: 2 mg pollen collected from non-genetically modified maize; 2 mg (0.056 µg PS149B1 ICP) pollen collected from corn plants expressing *Bt* strain PS149B1 ICP; 5.6 µg bacterially produced PS149B1 binary ICP (100x protein concentration present in 2 mg PS149B1 pollen); 3.2 µg 14-kDa protein; 2.4 µg 44-kDa protein; 20 µg potassium arsenate (positive control); or untreated cell (negative control). Larval survival was evaluated 6 and 12 days after treatment, and adult emergence was evaluated 26 days after treatment. There were no statistical differences ($p = 0.05$) in larval mortality between those fed PS149B1 pollen, unmodified pollen, or bacterially produced PS149B1 binary ICP. Based on results presented in this study, it can be concluded that honey bee development and survival are not affected by exposure to the Cry34/35Ab1 corn pollen.

There was 92.5% mortality among larvae treated with potassium arsenate, the positive control, which indicates that bees were adequately exposed to the treatments.

ii. Parasitic Hymenoptera Testing (MRID No. 457904-05)

The study was conducted in accordance with Good Laboratory Practice Standards (40 CFR Part 160) with certain exceptions that did not affect the integrity of the test. This study was conducted based on OCSPP Harmonized Test Guideline 885.4340 (Non-Target Insect Testing, Tier I).

A dietary toxicity study was conducted to evaluate the effect that *Bt* strain PS149B1 binary protein (purity equal to 37% 44-kDa protein and 54% 14-kDa protein) has on parasitic Hymenoptera (*Nasonia vitripennis*). The wasp test diet consisted of sugar water mixed with Cry protein at a rate of 280 ppm (combined total of 120 ppm 44-kDa protein and 160 ppm 14-kDa protein) PS149B1 protein, which is approximately equivalent to 10x the maximum protein concentration present in PS149B1 corn pollen.

Based on this test, the LC_{50} for adult parasitic Hymenoptera exposed to dietary Cry PS149B1 protein was determined to be >280 ppm. It should also be noted that parasitic Hymenoptera do not feed directly on corn plant tissues, including pollen; therefore, minimal exposure of parasitic Hymenoptera to Cry protein is expected. As a result of these findings, no hazard to *N. vitripennis* is expected from exposure to Cry34/35Ab1 corn.

The *N. vitripennis* study was found acceptable by the Agency. However, the August 27, 2002, SAP concluded that the parasitic Hymenoptera (*N. vitripennis*) is not an appropriate test species for coleopteran-active *Bt* corn because the dipteran parasitoid is not found in corn fields. A more appropriate parasitoid, that occurs in corn fields (e.g., *Tricogramma* spp. or *Macrocentrus grandii*), should be considered for future studies.

iii. Green Lacewing Larva Testing (MRID No. 457904-07)

An acceptable study was conducted in accordance with Good Laboratory Practice Standards (40 CFR Part 160) with certain exceptions that did not affect the integrity of the test. This study was conducted based on OCSPP Harmonized Test Guideline 885.4340 (Non-Target Insect Testing, Tier I).

Green lacewing larvae were fed PS149B1 insecticidal crystal protein in a moth egg (*Sitotroga* spp.) and water meal diet at a rate of 280 ppm, which is approximately equivalent to 10x the maximum protein concentration in plant tissue. There was 28% mortality in the negative control group on day 10. Compared to the negative control, at day 10, there was no significant increase in green lacewing larval mortality when fed 10x (280 ppm) the maximum PS149B1 protein concentration found in plant tissue. The data show that the LC_{50} for green lacewing larvae exposed to PS149B1 in diet was >280 ppm. Based on these results, it is not expected that the green lacewing will be adversely affected when exposed to Cry34/35Ab1 corn in the field.

The August 27, 2002, SAP concluded that green lacewing (*Chrysoperla carnea*) is not an appropriate test species for several reasons. Green lacewing are difficult to test in the laboratory because of a high rate of mortality. For example, in the study outlined above (MRID No. 457904-07), the test was terminated after 10 days because there was >28% mortality in the negative control. In addition, it is questionable whether green lacewings ingest the protein on coated moth eggs, since green lacewing have piercing-sucking mouthparts and do not consume the external surface of eggs. For these reasons, the applicants should conduct a laboratory insect toxicity test on an alternate organism, such as the minute pirate bug (*Orius insidiosus*). This egg predator, which feeds on pollen when prey is scarce, is typically found in corn fields. An appropriate evaluation would involve feeding *O. insidiosus* pollen, a natural food source, and purified protein in diet in two separate diet bioassays. The purified protein assay would be useful in determining toxicity at the maximum hazard dose, while the pollen assay would provide information on the potential effect of an actual exposure scenario. As noted in Table 2, the Agency has requested that the applicants submit this study as a condition of registration.

*Note for 2010: There is an update to this summary. See section II(C)(2)(b) ("Terms and Conditions of the Event DAS-59122-7 Corn Registrations (August 2005 – September 2010)") of this BRAD.

iv. Lady Beetle Testing

The Cry34/35Ab1 protein specifically targets coleopteran (beetle) insects. Consequently, particular attention should be paid to the potential effects that Cry34/35Ab1 protein may have on lady beetles, which are in the family Coccinellidae.

- **MRID No. 452422-10** – In a pure *Bt* binary crystal protein adult lady beetle feeding study, no mortality or abnormal clinical signs were observed in treatment or control groups on Day 0 at 3/4 and 1 3/4 hours after test initiation. At test termination (Day 11), there was 22% (33 of 150) mortality in the negative control group. Beetles in the control group appeared normal, except for five lethargic individuals. The 280 µg active ingredient/milliliter (mL) treatment group resulted in 13% (20 of 150) mortality at test termination. All beetles in the treatment group appeared normal except for two immobile beetles. There was a lower mortality rate and fewer clinical signs of abnormality in the 280µg active ingredient/mL treatment group then the control group. Mortality and abnormal behavior was not treatment related. The dietary LC_{50} value for ladybird beetles exposed to PS149B1 ICP was determined to be greater than 280 µg active ingredient/mL, which was the highest concentration tested.

- **MRID No. 452422-11** – A tri-trophic interaction study between field corn expressing PS149B1, corn leaf aphids (*Rhopalosiphum maidis*), and lady beetle (*C. maculata*), in larval and adult stages, was also submitted and reviewed. Field corn plants expressing the PS149B1 protein event TC5638 and non-transgenic isolines were planted in the greenhouse and infested with naturally occurring aphid populations. Aphid colonies were allowed to establish on each plant before being removed and fed to lady beetles. One to three aphids were fed to individual lady beetle adults and larvae in 7 mL scintillation vials daily. Mortality and time between molts was monitored daily until day 10. No significant difference in adult lady beetle mortality or weight

was found between lady beetles feeding on PS149B1-intoxicated aphid and aphids that fed on non-transgenic corn. Feeding on PS149B1-intoxicated aphids also did not affect the length of time between larval molts.

- **MRID No. 461239-12 –** Two diet assays were conducted: (1) a Tier I assay in which lady beetle larvae (*C. maculata* DeGeer) were fed purified Cry34/35Ab1 protein mixed with artificial diet, and (2) a Tier II assay in which larvae were exposed to a diet consisting of 50% inbred Cry34/35Ab1 pollen from events E4497.45.2.16, E4497.59.1.22 (renamed as DAS-59122-7, which is the event being registered), E4497.45.2.14, and E4497.71.1.33 corn pollen and 50% ground corn earworm eggs. These assays are described in more detail below.

  Tier I Assay – Lady beetle larvae (*C. maculata*) were fed an artificial diet containing purified Cry34/35Ab1 protein at a concentration equal to approximately 10 times the expected environmental exposure concentration through pollen. Cry34Ab1 protein was incorporated at 900 ppm and Cry35Ab1 protein at 1 ppm (ratio based on Cry34 and Cry35 expression in event DAS-59122-7 corn pollen). The purified proteins were suspended in deionized water and mixed with commercially available lady beetle diet. Neonate larvae were placed in individual bioassay wells and allowed to feed for 7 days. Larvae were then assessed for mortality and weight.

  There was no significant difference in mortality among *C. maculata* larvae fed for seven days on one of two control diets, which did not contain active protein, and the treatment containing purified Cry34/35Ab1 toxin. However, significant growth inhibition (80% growth reduction) was observed in *C. maculata* larvae feeding on the Cry34/35Ab1 diet compared to larvae feeding on the control diets.

  Tier II Assay – Corn pollen may comprise up to 50% of a lady beetle larva's diet during corn anthesis. Consequently, a study was conducted to evaluate the effect that event Cry34/35Ab1 corn pollen may have on lady beetle larvae. Pollen from four different events, which were tested as four different treatments, was fed to lady beetle larvae in a diet consisting of 50% Cry34/35Ab1 corn pollen and 50% ground corn earworm eggs. First neonate larvae were placed in individual bioassay wells and allowed to feed *ad libitum* throughout the 14-day larval growth period.

  There was no significant difference in mortality, development, or adult weight among *C. maculata* larvae fed a control diet, which did not contain active protein, and treatments containing Cry34/35Ab1 pollen. Among the four events tested, protein expression for Cry34Ab1 proteins ranged from 116 to 175 nanograms per milligram (ng/mg). Among reported events (values were not provided for two of the four events because they were not quantifiable or exceeded the upper limit of detection), Cry35Ab1 protein expression levels ranged from 75.5 to 83.5 ng/mg. For the registered event (DAS-59122-7), Cry34Ab1 protein expression was 117 ng/mg; Cry35Ab1 expression was not determined because one or more of the entries were not quantifiable (additional information on Cry34Ab1 and Cry35Ab1 protein expression levels may be found in section II(A)(1)(a) of this BRAD).

Conclusions – Submitted data show that Cry34/35Ab1 protein is toxic to *C. maculata* at dose levels that exceed field exposure (Tier I Assay). However, since toxic effects were not seen at the field dose level (Tier II Assay) when *C. maculata* larvae were fed natural prey and pollen, there is reasonable certainty that Cry34/35Ab1 corn will not adversely affect *C. maculata*.

Since the Cry34/35Ab1 protein is toxic to Coleopteran species, evaluations of other appropriate non-target beetle species should be submitted as a component of this risk assessment. The 2002 SAP suggested that Carabids (ground beetles) would be suitable for PIP Tier I testing because beetles found within this family play important ecological and economic roles within agroecosystems, including corn fields. As noted in Table 2, the Agency is requesting that the applicants submit this study as a condition of registration.

v. Collembola Feeding Study (MRID No. 457904-06)

This study was conducted in accordance with Good Laboratory Practice Standards (40 CFR Part 160) with certain exceptions that did not affect the integrity of the test. This study was conducted based on OCSPP Harmonized Test Guideline 885.4340 (Non-Target Insect Testing, Tier I).

Juvenile collembola (*Folsomia candida*) were fed diets consisting of purified PS149B1 protein (purity = 54% 14-kDa protein and 37% 44-kDa protein) mixed with dry granulated Brewer's yeast at a single treatment level of 12.7 mg PS149B1 active ingredient/kg. Fresh diet was provided to test organisms every third day. On days 0 and 28, mortality and observations of sublethal effects on surviving collembola were recorded.

Results of this study show no adverse effects on survival and reproduction of collembola exposed to PS149B1 insecticidal crystal protein at 10x concentrations found in transgenic corn plants. It is noted that the primary route of collembola exposure to Cry34/35Ab1 protein in the field is from decaying root tissue, which is expressed in corn roots at a range of 3–66 micrograms per gram (µg/g) and is significantly lower than the treatment level used in this test.

This study adequately addresses potential concerns for Cry34/35Ab1 protein to collembola (*F. candida*), a representative of beneficial soil insect species. The results of this study demonstrate that Cry34/35Ab1 protein found in transgenic corn poses no hazard to soil-inhabiting collembola species, and by inference, to other beneficial non-coleopteran soil insects. It is noted that the October 2000 Scientific Advisory Panel stated that it is unnecessary to conduct non-target testing on invertebrate orders that are not known to be affected by the Cry protein in question.

vi. Earthworm Toxicity Testing

Earthworm feeding studies submitted to the Agency demonstrate that Cry proteins are not toxic to earthworms at the worst-case environmental concentration. Although some public comments have

questioned whether earthworm test organisms actually ingested the soil incorporated *Bt* Cry proteins, recently published data show that earthworms do ingest and excrete soil-incorporated *Bt* Cry proteins.

- **MRID No. 453602-01** – This study complied with Good Laboratory Practice Standards (40 CFR Part 160) and OECD Principles of Good Laboratory Practice with certain exceptions that did not affect the integrity of the test. The testing was conducted based on OCSPP Harmonized Test Guideline 850.6200 (Earthworm Subchronic Toxicity Test) and OECD Guideline 207. This study meets current testing requirements for assessing risks to earthworms from plant-incorporated protectants derived from *Bt*.

  The 14-day LC_{50} for earthworms exposed to PS149B1 ICP in an artificial soil substrate was determined to be greater than 76 mg active ingredient/kg dry soil (the highest concentration tested), or greater than 20 times the expected field concentration. Earthworm mortality and changes in average body weights were not statistically different ($p > 0.05$) among the controls and protein-amended soils. The LC_{50} value for earthworms exposed to chloroacetamide, the positive control, was approximately 19.4 mg active ingredient/kg dry soil. This finding was consistent with historical results, and further confirmed the adequacy and consistency of the protocol used in the definitive test. The submitted data suggest that earthworms will not be adversely affected by Cry34/35Ab1 corn plants.

vii. Insecticidal Activity Spectrum Study (MRID No. 452422-04)

The relative sensitivity of the test insects in the feeding bioassays suggested that larvae of coleopteran species are far more sensitive to the 14-kDa and 44-kDa proteins of PS149B1 than the lepidopteran or homopteran larval insects evaluated. Adult WCRW were not sensitive to the delta-endotoxins, however. Rootworms (NCRW, WCRW and SCRW) were the most susceptible to the PS149B1 proteins. European corn borer and CEW demonstrated some inhibition of growth at higher concentrations of the test substance than the rootworm larvae. Black cutworm was the least sensitive of the lepidopteran species tested. No activity was seen against the CLA, a homopteran. Relative susceptibilities of the insect species to PS149B1, based upon the more sensitive measure of GI_{50}, were as follows: (most susceptible) WCRW, NCRW, SCRW > ECB, CEW >> WCRW adult, CLA, BCW (least susceptible).

viii. Field Evaluation of Cry34/35Ab1 Corn Exposure on Non-Target Invertebrates (MRID No. 461239-14)

This Tier IV field experiment was submitted as a supplement to the Tier I maximum hazard dose findings presented above.

Methods:
The field study was conducted over two growing seasons (2001–2002) at two locations in the central Corn Belt (York, Nebraska and Johnston, Iowa). The experimental design was a randomized complete block design with two replicates for each of five treatments. Treatments were the following: (1) Cry34/35Ab1 maize hybrid TC5639; (2) Cry34/35Ab1 maize hybrid TC15344; (3) non-transgenic

maize hybrid with a planting time application of tefluthrin (Force® 3G); (4) non-transgenic maize hybrid sprayed with an application of bifenthrin (Capture® 2EC) at the V10 and R2 corn growth stages to control first and second generation CRW adults; and (5) non-transgenic maize hybrid with no corn rootworm control measures taken. Treatment plots covered approximately 2,800 square feet (70 feet x 40 feet) and were separated by 18 feet of non-transgenic corn.

Arthropod abundance was evaluated in soil, on the soil surface, and at the crop canopy level. Methods used were the following: (1) visual observations; (2) soil sampling with Berlese-Tullgren funnels for invertebrate extraction; (3) soil surface collection using pitfall traps; and (4) canopy level sampling using yellow sticky traps. Samples were taken at specified intervals throughout the season, and four samples were collected within each plot for each collection method at each sampling interval.

Although data were collected on individual invertebrate families, some families were grouped for statistical analyses. At the community level, groupings were based on a family's abundance in corn fields, as well as their function within the agricultural ecosystem. Representatives from several functional groups (decomposers, herbivores, predators, parasitoids, and generalist feeders) served as indicator species.

Two analyses were applied. Principal response curves (Van den Brink and Ter Braak 1999) were used to investigate and describe treatment effects at the community level, while analysis of variance (ANOVA) was performed for each key indicator species and taxa group to detect invertebrate abundance among treatments.

Results:
Neither Cry34/35Ab1 event showed signs of adverse effects on non-target arthropods at the community level or in key taxa. The two primary effects observed were an overall abundance decrease at the 50% pollen shed sampling date in the foliar insecticide-treated plots compared to the untreated control plots, and an increase in collembola abundance at the R2 and R5 sampling stages of the foliar insecticide-treated plots compared to the untreated control plots. It is noted that the two Cry34/35Ab1 events presented in this study are not planned for commercial release; however, they represent equal or greater Cry34/35Ab1 expression levels when compared to commercial event DAS-59122-7.

Conclusions:
According to submitted data, Cry34/35Ab1 corn does not adversely impact the abundance of non-target invertebrates found in corn fields. The utility of these data, however, is limited because the plot size (70 feet × 40 feet) was too small, and the experiment included only two replicates at each of two locations. In addition, only four samples, for each collection method, were taken from each plot. The August 2002 SAP concluded that field experiments must be appropriately designed (larger fields, more replicates, more samples) to provide a measure of ecological impacts and, further, that a two-year field study would not be sufficient to determine if Cry34/35Ab1 corn will have long-term impact on non-target invertebrates. However, since the endpoint for field census studies has not been identified, it is difficult to determine the appropriate field size, number of replicates, and number of samples needed per plot.

The data provided in this report suggest that corn events containing the Cry34/35Ab1 protein did not adversely affect non-target arthropods. Supplemental studies, employing larger plot sizes, more replicates, and more samples per plot, are recommended for further verification of a long range no adverse effect finding. In response to these concerns, as requested by the Agency in Table 2, the applicants are preparing additional field census data that will be submitted to the Agency as a condition of registration.

Although the experimental design of the field assays needs improvement, the submitted field census data are useful for short-term hazard assessment and as supplementary information that supports the no-hazard trend seen in the maximum hazard dose single species laboratory testing described above. It is an accepted practice in the Office of Pesticide Programs to use the trends seen in several supplemental studies for hazard assessment when a perfect study is not available.

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II. Soil Fate (MRID No. 452422-14)

Soil organisms may be exposed to Cry34/35Ab1 protein through contact with corn plant roots (by direct feeding), corn plant root exudates, incorporation of aboveground plant tissues into soil following harvest, or by soil-deposited pollen. Some evidence suggests that acidic soils (pH 5.6), and those which are high in clays and humic acids, are more likely to bind Cry protein, and thus decrease the rate of protein degradation by soil microorganisms. It is noted, however, that the pH factor should not contribute to protein binding in corn fields, since maize is generally grown on neutral soils (above pH 5.6). 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 suggests that Cry proteins do not accumulate in soil to arthropod-toxic levels. Nonetheless, the Agency requires soil fate evaluations for each new insect-protected crop.

This study was conducted in accordance with Good Laboratory Practice Standards (40 CFR Part 160).

Methods:
An insect bioassay was conducted to measure the rate of purified PS149B1 delta-endotoxin degradation in a representative silt loam field soil. The southern corn rootworm (SCRW, *Diabrotica undecimpunctata* subsp. *howardi*) was selected as the test species for this bioassay due to its susceptibility to PS149B1 insecticidal proteins. An aqueous mixture of the test material, composed of 14-kDa and 44-kDa binary insecticidal proteins, was pipetted onto 1 gram (dry weight) soil at a concentration of 5 mg per protein per gram of soil. The soil-protein mixture was transferred to 50 mL perforated vials (to allow gas exchange) and incubated at approximately ~25˚C for 0, 1, 3, 7, 14, or 28 days. Four vials were prepared for each incubation period. Aliquots (50 microliters (µL)) of these dilutions were applied to the surface of small wells (1.5 square centimeters in diameter) containing 500 µL of artificial insect diet. Soil-agar and agar-only negative controls were also included in bioassay trays. Following treatment application, single neonate rootworm larvae were introduced into each well

and housed for six days at ~27˚C. To reach the artificial diet, larvae had to eat through the soil-protein surface layer and thus consume the insecticidal proteins. Fourteen to 16 larvae were tested at each dilution level, for a total of 94–96 larvae per incubation interval. Larvae mortality and weights were collected for all treatments.

Results:
Larval mortality was negligible and did not correlate with insecticidal protein content. Consequently, protein degradation at each incubation interval was defined as the amount of insecticidal protein required to reduce larval growth by 50% (GI_{50}). GI_{50}s were calculated from treatment averages for each soil-protein incubation interval, along with their 95% upper and lower confidence limits. Results indicate that larval growth inhibition was inversely correlated with soil-protein incubation periods, suggesting that protein degradation increased with time. Regression of the GI_{50}s with time (0 to 7 days) showed a degradation half-life of 3.2 days for the insecticidal protein. The 14- and 28-day GI_{50}s did not fit the first-order decay model and, consequently, were excluded from half-life calculations.

Conclusions:
Based on these results, it may be concluded that purified PS149B1 insecticidal proteins degrade rapidly in silt loam soil; however, persistence and accumulation at low levels is not addressed. Silt loam soil is just one of many soil classes used for corn production in the United States. A more useful study would evaluate protein degradation/accumulation in a range of soil types, including those with high clay and humic acid content, due to their known binding affinity for proteins.

In addition, this study utilized field soil spiked with purified insecticidal protein. This approach is useful because dose responses can be easily quantified. However, the degradation and accumulation of Cry proteins found within decaying plant tissue may behave differently than proteins in artificially spiked soil. Thus, the relevance of these study results is unclear other than to show that degradation in soil does take place.

To account for the above concerns, it is recommended that additional studies be conducted to evaluate insecticidal protein degradation, accumulation, and persistence in a variety of soil types, including those high in clay and humic acids into which all non-harvested corn plant material is incorporated. Sampling should be conducted at the end of three years in a field sown with continuous Cry34/35Ab1 corn. Soil should be monitored for a minimum of one growing season after harvest and continued until the Cry34/35Ab1 protein can no longer be detected. As noted in Table 2, the Agency has requested that the applicants submit these studies as a condition of registration.

*Note for 2010: There is an update to this summary. See section II(C)(2)(b) ("Terms and Conditions of the Event DAS-59122-7 Corn Registrations (August 2005 – September 2010)") of this BRAD.

III. 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.

IV. Horizontal Transfer of Transgenes from Bt Crops to Soil Organisms

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 Cry34/35Ab1 corn are derived from soil-inhabiting bacteria, EPA has concluded that there is a low probability of risk from HGT of transgenes found in Cry34/35Ab1-producing corn.

V. 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. Nonetheless, 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 obtain 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 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 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 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 incapable of survival in the wild as a result of breeding practices (i.e., selection) during domestication of the crop.

iii. Impacts on Endangered Species (MRID No. 461239-17)

The primary route of exposure to Cry34/35Ab1 protein 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 the Cry protein. Since Cry34/35Ab1 corn pollen has shown no toxicity at the estimated environmental concentration rates to mammals, birds, plants, aquatic species, insects, and other invertebrate species tested, 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 Cry34/35Ab1 protein for coleopteran species, endangered species concerns are mainly restricted to the order Coleoptera. Examination of an overlay map showing the county-level distribution of the 16 endangered/threatened coleopteran species (as 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 American burying beetle (*Nicrophorus americanus*). The American burying beetle is the largest carrion beetle in North America and is only found today in limited areas in Rhode Island and the portions of the Great Plains into Arkansas and Georgia. Adults are nocturnal and feed on carrion and sometimes prey on other arthropods. Larvae feed exclusively on buried carrion provided by their parents. The American burying beetle's habitat is variable and often includes deciduous forest, grassland, and agricultural areas. Considering that both larvae and adult insects feed exclusively on carrion with some limited adult predation, even if American burying beetles did occur in proximity to *Bt* corn fields, there would be little chance of exposure to *Bt* protein due to their feeding habits. After careful review of the available data, EPA determined that exposure of American burying beetle to harmful levels of Cry34/35Ab1 corn tissue is not expected. Likewise, a review of the preferred habitats of other coleopteran species, listed as endangered by the U.S. Fish and Wildlife Service, indicated that no exposure to harmful levels of Cry34/35Ab1 protein would take place. The main reasons for the lack of exposure are geographical and habitat limitations. These other species are located in non-corn production areas and/or their habitat does not encompass agricultural areas.

Likewise, other endangered/threatened insect species in the orders Diptera, Hemiptera, Lepidoptera, Odonata, and Orthoptera 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 show the relative insensitivity of a range of insects from non-Coleopteran orders to the Cry34/35Ab1 protein, indicating that the Cry34/35Ab1 corn hybrids will not likely cause detrimental effects to the non-Coleopteran insects on the endangered/threatened species list.

Likewise, several insect species listed are aquatic species and are unlikely to come in contact with Cry34/35Ab1 corn. Many of the endangered and threatened beetles occur in cave or aquatic habitats. Since movement of Cry34/35Ab1 in soil and into water bodies is expected to be negligible, pollen drift was considered the primary source of potential hazard to endangered aquatic Coleoptera. According to

estimates based on published studies, if 100% of the pollen grains leaving a corn field were deposited in a 1 hectare pond with 2 meters depth and located ≥1 meter from the edge of the corn field, <0.0001 µg Cry34/35Ab1/mL of water would be expected. This is a few orders below the toxic level to any insect.

Conclusions:
The reviewed non-target data confirm the expectation that Cry34/35Ab1 corn is not likely to jeopardize the continued existence of any endangered and/or threatened species listed by the U.S. Fish and Wildlife Service, including mammals, birds, or terrestrial and aquatic plants and invertebrate species. Therefore, no consultation with the U.S. Fish and Wildlife Service is required under the Endangered Species Act.

iv. Environmental Assessment Summary (MRID No. 461239-13)

The Agency is using a maximum hazard dose tiered system for biopesticide non-target wildlife hazard assessment. When no adverse effects are observed at the maximum hazard dose, the Agency concludes that there are no unreasonable adverse effects from the use of the pesticide. From all of the required and voluntarily developed indicator and host range species test data on Cry34/35Ab1 corn, the Agency concludes that the levels of Cry34/35Ab1 protein in corn will not pose unreasonable adverse effects to corn field flora and fauna. Available data also indicate that there should be minimal short-term accumulation of Cry34/35Ab1 protein in agricultural soil. In addition, no adverse effect on endangered and threatened species, as listed by the U.S. Fish and Wildlife Service, is expected from the proposed Cry34/35Ab1 CRW resistant corn registration.

At present, the Agency is aware of no identified significant adverse effects of Cry34/35Ab1 protein on the abundance of non-target beneficial organisms in any population in the field, whether they are pest parasites, pest predators, or pollinators. Field testing and field census data submitted to the Agency show minimal to undetectable changes in beneficial insect abundance or diversity. To date, available field test data show that, compared to crops treated with conventional chemical pesticides, the transgenic crops have no detrimental effect on the abundance of non-target insect populations.

The Agency believes that cultivation of Cry34/35Ab1 corn may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, Cry34/35Ab1 corn requires substantially fewer applications of chemical pesticides. This should result in fewer adverse impacts to non-target organisms because application of non-specific conventional chemical pesticides is known to have an adverse effect on non-target beneficial organisms found living in the complex environment of an agricultural field. Many of these beneficial organisms are important integrated pest management controls for secondary pests, such as aphids and leafhoppers. The overall result of Cry34/35Ab1 corn cultivation is that the number of chemical insecticide applications for non-target pest control is reduced for management of multiple pest problems.

The movement of transgenes from Cry34/35Ab1 host plants into weeds and other crops has also been considered. 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 territories. The fate of Cry34/35Ab1 protein in soils and indirect effects on soil biota have also been evaluated. Test

data show that most of the Cry protein deposited into soil is quickly degraded, although a residual amount may persist in biologically active form for a much longer period of time. It is also reported that the same degree of *Bt* Cry protein persistence takes place in soils that have been exposed to repeat *Bt* spray applications when compared to soil exposed to growing *Bt* crops. Limited data do not indicate that Cry proteins have any measurable effect on microbial populations in the soil. Horizontal transfer of genes from transgenic plants to soil bacteria has not been demonstrated. Published studies of *Bt* Cry protein in soil show no effect on bacteria, actinomyces, fungi, protozoa, algae, nematodes, springtails, or earthworms. In addition, new plants planted in *Bt* Cry protein-containing soil do not take up the *Bt* protein.

Conclusion:
At the present time and for the purposes of a time-limited registration, this risk assessment finds no hazard to the environment from cultivation of corn expressing the Cry34/35Ab1 protein.

v. Supplemental Studies Needed for Long-Term Cry34/35Ab1 Non-Target Hazard Assessment

The Agency has sufficient information to believe that there is no risk from the proposed uses of Cry34/35Ab1 corn to non-target wildlife, aquatic, and soil organisms. Nonetheless, in response to the August 2002 SAP recommendations, the Agency is requesting supplementary studies that will evaluate more appropriate non-target invertebrates (e.g., those found in corn fields) and facilitate identification of potential adverse effects that may result from long-term use of Cry34/35Ab1 corn. The Agency does not believe that these data requirements were reasonably foreseeable by Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International, Incorporated at the time of their respective applications.

Table 2. Supplemental Data Requirements.

Testing Category	Type of Data
Non-target insect more appropriate for corn fields	Conduct a maximum hazard dose laboratory toxicity test with *Orius insidiosus* (minute pirate bug).
Non-target insect more appropriate for corn fields	Conduct a maximum hazard dose laboratory toxicity test with a carabid (ground beetle).
Ecosystem effects	Additional long-range field studies should be conducted based on recommendations from the August 2002 SAP (as presented in summary form in the conclusions section of the MRID No. 461239-14 review).
Soil fate studies	Additional long-range soil degradation field studies should be conducted to include the parameters outlined by the August 2002 SAP (as presented in summary form in the conclusions section of the MRID No. 452422-14 review).

*Note for 2010: There is an update to this summary. See section II(C)(2)(b) ("Terms and Conditions of the Event DAS-59122-7 Corn Registrations (August 2005 – September 2010)") of this BRAD.

b. Terms and Conditions of the Event DAS-59122-7 Corn Registrations (August 2005 – September 2010)

When event DAS-59122-7 corn was first registered on August 31, 2005, the Agency issued registration notices to both Mycogen Seeds c/o Dow AgroSciences LLC (EPA Reg. No. 68467-5) and Pioneer Hi-Bred International, Incorporated (EPA Reg. No. 29964-4) that, given the conclusions set forth in the initial environmental risk assessment (see section II(C)(2)(a) of this BRAD), contained the following three requirements for further environmental effects data:

> "Submit field degradation studies evaluating accumulation and persistence of Cry34/35Ab1 in several different soils in various strata. Representative fields must have been planted with Cry34/35Ab1 corn and include both conventional tillage and no-till samples and be harvested under typical agronomic conditions. Sampling must continue until the limit of detection is reached. Studies should include soils with high levels of a variety of clays. Both ELISA and insect bioassays need to be conducted and compared to determine if Cry34/35Ab1 is accumulating or persisting in soil samples… A final report regarding data from fields that have had three continuous years of cultivation of event DAS-59122-7 corn is due by January 31, 2010."
>
> "Submit laboratory toxicity tests with *Orius insidiosus* (minute pirate bug) [and] carabid (ground beetle) within 24 months of the date of registration…."
>
> "Additional 3 year full-scale field or semi-field studies for evaluation of Cry34/35Ab1 event DAS-59122-7 corn exposure on non-target invertebrates must be conducted. Full-scale field experiments must be appropriately designed to provide a measure of ecological impacts (larger fields, more replicates, more samples per plot based on recommendations of the August 2002 SAP). The previously submitted two-year field study is not sufficient to determine if Cry34/35Ab1 corn will have long term impact on non-target invertebrates…A final report is due September 30, 2009."

For both registrations, the abovementioned requirements for additional environmental effects data have been satisfied by submission of appropriate studies; summaries of the studies are presented in Table 3.

Current environmental effects data, to include those conditional data referenced in Table 3, and EPA reviews of Cry34/35Ab1 protein support the Agency's original determination that adverse effects will not occur to non-target organisms. Due to a demonstrated lack of toxicity and/or exposure, no effects from Cry34/35Ab1 protein are anticipated for any non-target species, including federally listed threatened and endangered ("listed") lepidopteran and coleopteran species and their designated critical habitats. The Agency is therefore upholding its determination that the registered uses of Cry34/35Ab1 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 and the National Marine Fisheries Service.

When the docket for the expiring *Bt* corn registrations was opened for public comment on August 4, 2010 (Docket Number EPA-HQ-OPP-2010-0607), the Agency noted its awareness of a recently published laboratory study showing reduced growth in shredding caddis flies exposed to anti-lepidopteran Cry1A protein corn litter (Rosi-Marshall *et al*. 2007). Given the findings of this particular study, the Agency proposed requiring additional aquatic invertebrate data for the Cry34Ab1 and Cry35Ab1 proteins—either a 7- to 14-day *D. magna* study or a dietary study evaluating the effects of these proteins on an aquatic invertebrate that represents the functional group of a leaf shredder in headwater streams. Since the 2007 Rosi-Marshall *et al*. publication, numerous researchers have published peer-reviewed studies that identify issues with the scientific merit and relevance of the original caddis fly study (Swan *et al*. 2009, Jensen *et al*. 2010, summarized by Beachy *et al*. 2008, Parrott 2008, and Wolt and Peterson 2010). In response to comments received on the proposed terms and conditions for the extension of the 2010 expiring *Bt* corn registrations, the Agency conducted a literature review of these recently published studies. Criticisms of the Rosi-Marshall *et al*. study included several findings: (1) adverse effects were not caused by toxicity of Cry1A but, rather, by other differences between plant test substances (Jensen *et al*. 2010); (2) the abundance of Trichoptera in streams containing residues of Cry1A was not reduced (Chambers *et al*. 2007); and (3) while post-harvest crop residue was identified as the most likely route of exposure (Carstens *et al*. 2010), aquatic exposure to biotech crops has been shown to be limited temporally and spatially with low to negligible exposure concentrations of Cry proteins in post-harvest crop tissues (Swan *et al*. 2009, Chambers *et al*. 2010, Jensen *et al*. 2010, Wolt and Peterson 2010, Carstens *et al*. 2010). In light of these results, the Agency is not requiring additional aquatic invertebrate studies to assess hazard to aquatic shredder species for existing Cry protein PIP registrations.

Table 3. Environmental Effects Data Submitted in Response to Conditions of Registration for Event DAS-59122-7 Corn

Study Title	Summary	MRID No.
Three-Year Field Monitoring of Cry34/35Ab1 and Cry1F x Cry34/35Ab1 Maize Hybrids for Non-Target Arthropod Effects[n]	A multi-year field study evaluated the impact of event DAS-59122-7 maize (expressing Cry34Ab1 and Cry35Ab1 proteins) and its respective stacked maize hybrid, DAS-01507-1 x DAS-59122-7 (expressing Cry1F and Cry34/35Ab1 proteins), on non-target arthropods after continuous cropping at three locations in the Corn Belt (Iowa, Nebraska, and Indiana). Community-level analyses and key indicator species data were collected using visual observations, sticky traps, pitfall traps, and litter bag traps. Results showed similar dynamics on non-target arthropod populations between non-*Bt* isogenic maize hybrid fields and the *Bt* transgenic PIP corn events, DAS-59122-7 and DAS-59122-7 x TC1507. Therefore, there is negligible potential for long-term adverse effects on non-target arthropods on the community level and key taxon abundance after continuous cultivation of both Herculex® RW and Herculex® XTRA *Bt* corn events. **Classification: Acceptable**	478703-01

[n] Reviewed in U.S. EPA (2010c)

Study Title	Summary	MRID No.
Soil Accumulation of Cry34Ab1 and Cry35Ab1 Proteins after Three Years of Cropping with DAS-59122-7 Corn[o]	After 3 years of continuous cropping with DAS-59122-7 corn, the enzyme-linked immunosorbent assay (ELISA) analyses showed no detectable residues of Cry34Ab1 and Cry35Ab1 proteins in soil (bulk and rhizosphere) extracted from 3 different field sites (Illinois, Minnesota, and North Carolina) containing a range of soil properties. In addition, no biological activity was observed in insect bioassays of southern corn rootworm (*Diabrotica undecimpunctata howardi*) neonates after exposure to rhizosphere soil samples. Therefore, there is negligible potential for Cry34Ab1 and Cry35Ab1 proteins to persist or accumulate in agricultural soils in various soil matrices across cropping cycles of DAS-59122-7 corn. **Classification: Acceptable**	479595-01
Summary Report of Carabid Beetle Laboratory Toxicity Study Using Cry34Ab1 and Cry35Ab1[p]	In a laboratory bioassay, ground beetle (*Poecilus cupreus*) larvae were fed blowfly (*Calliphora vomitoria*) pupae containing target concentrations of a combination of 1,000 nanograms (ng) Cry34Ab1 + 333.3 ng Cry35Ab1/mg of pupa. A negative control group of larvae was fed pupae treated with deionized water, and a positive control group was fed pupae treated with teflubenzuron. Survival of the ground beetle larvae was monitored throughout pupation and adult emergence. There was no statistically significant difference in pre-imaginal mortality between the negative control and Cry34Ab1 + Cry35Ab1 groups (15% and 8%, respectively), while mortality in the positive control group was 83%. Mean development time and mean adult weight of the negative control and Cry34Ab1 + Cry35Ab1 groups were comparable. This study shows that when carabid beetle larvae are fed diet containing Cry34/35Ab1 protein, no negative adverse effect is observed. Therefore, at field concentrations, it is unlikely that Cry34/35Ab1 will have negative adverse effects on carabid beetles. **Classification: Acceptable**	467141-01

[o] Reviewed in U.S. EPA (2010c)
[p] Reviewed in U.S. EPA (2010b)

Study Title	Summary	MRID No.
Evaluation of Potential Dietary Effects of Cry34/35Ab1 Protein on Insidious Flower Bugs, *Orius insidiosus* [q]	Four separate 10- to 14-day tests were conducted from December 2005 to July 2007 to evaluate the dietary effects of combined Cry34Ab1 and Cry35Ab1 proteins on survival and development of insidious flower bug (*O. insidious*) nymphs. Each test included a group given a combination of 750 ng Cry34Ab1 + 85 ng Cry35Ab1/mg of diet (bee pollen), a positive control group given potassium arsenate (8 ng active ingredient/mg of diet volume by weight), and an assay control group given purified reagent grade water mixed with diet. In addition, one test included a group given 750 ng of heat-treated Cry34Ab1 + 85 ng of heat-treated Cry35Ab1/mg of diet. The control group mortality in each test exceeded the allowable rate specified in the test protocol of less than 20% control mortality. However, the survival of the test species in the control groups and test groups was similar in each test. It is concluded that, at field concentrations, it is unlikely that Cry34/35Ab1 will have negative adverse effects on the insidious flower bug. **Classification: Supplemental**	474367-01

3. Herculex® XTRA Insect Protection Corn (OECD Unique Identifier: DAS-Ø15Ø7-1 x DAS-59122-7) Expressing Cry1F, Cry34Ab1, and Cry35Ab1

a. Data Cited/Submitted for Initial Registrations of Herculex® XTRA Insect Protection Corn (Prior to October 2005)

i. Background

In 2004, Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International, Incorporated requested full and unrestricted FIFRA section 3 registrations for commercialization of Herculex® XTRA Insect Protection Corn, a stacked product containing the following registered active ingredients: Cry1F (EPA Reg. No. 68467-2 and EPA Reg. No. 29964-3; Herculex® I) and Cry34/35Ab1 (EPA Reg. No. 68467-5 and EPA Reg. No. 29964-4; Event DAS-59122-7 corn or Herculex® RW). Herculex® XTRA was developed by crossing inbred lines of Cry1F lepidopteran-active and Cry34/35Ab1 coleopteran-active corn to produce hybrid maize line 1507 x 59122. Southern blot analyses demonstrated that the insertions in the Herculex® XTRA hybrid are equivalent to those of the Herculex® I and Herculex® RW lines and indicated that the stacked hybrid was a successful cross of the two inbred lines, 1507 and 59122 (MRID No. 462971-02; reviewed in U.S. EPA (2005f)). In addition to the insecticidal genes, the *phosphinothricin acetyltransferase* gene, which encodes PAT protein, is also present in the stacked trait corn plants. The inclusion of the *pat* gene provides tolerance to glufosinate-ammonium herbicides.

The insecticidal proteins expressed in single-trait events DAS-01507-1 (Herculex® I) and DAS-59122-7 (Herculex® RW) have been previously evaluated for adverse impacts on non-target organisms and have

[q] Reviewed in U.S. EPA (2010b)

been shown to pose negligible risk. Thus, the evaluation of Herculex® XTRA is simplified, requiring only demonstration of no synergy in the biological activity of the combined traits from Herculex® I and Herculex® RW.

ii. Non-Target Wildlife Hazard Assessment

An environmental risk assessment was conducted to characterize the risk of adverse impacts of the traits expressed in Herculex® XTRA on non-target organisms, particularly invertebrates found in or near agro-ecosystems. Concerns over possible effects on endangered and threatened species, especially butterfly (Lepidoptera) and beetle (Coleoptera) species were also addressed.

Expression of the Cry1F, Cry34Ab1, and Cry35Ab1 proteins in Herculex® XTRA was determined in various tissue types at various stages of development utilizing ELISA kits specific for each protein (MRID No. 463438-01; reviewed in U.S. EPA (2005f)). These data indicate that protein levels are comparable between the Herculex® I, Herculex® RW, and Herculex® XTRA hybrids. Therefore, the environmental effects data submitted and reviewed in support of the Herculex® I and Herculex® RW registrations, including studies submitted as conditions of registration, may be bridged to support the Herculex® XTRA product registrations. The environmental risk assessments for the insecticidal proteins (Cry1F and Cry34/Cry35Ab1) expressed in the single-trait events have been evaluated for adverse impacts on non-target organisms and have been shown to pose negligible risk (see U.S. EPA 2001d, 2005d, and 2010d for Cry1F; see section II(C)(2) of this BRAD for Cry34/35Ab1).

Of unique concern to the environmental risk assessment of stacked PIPs, is the potential for synergistic effects that may result from the insertion of multiple active ingredients into a single crop plant. Therefore, the conceptual model for evaluation of the stacked Herculex® XTRA hybrid is focused on the demonstration of no synergy in the biological activity of the two traits, Herculex® I and Herculex® RW. To address this issue and in support of the Herculex® XTRA registrations, four new studies were submitted: three comparing the effects of the single and stacked proteins in the sensitive target insect pests and two non-target insect species and one evaluating the effects of the stacked proteins on broiler chickens. These studies were reviewed in U.S. EPA (2005f) and are summarized in the sections that follow.

I. Non-Target Wildlife Study Summaries

a. Potential Synergistic Interaction Between the Cry1F and Cry34/35Ab1 Proteins (MRID No. 463438-06)

Laboratory bioassays were conducted to determine if combining the Cry1F and Cry34/35Ab1 proteins increased their activity against southern corn rootworm or European corn borer larvae. No synergism or antagonism between Cry1F and Cry34/35Ab1 was seen in the tests. Therefore, the absence of additive or synergistic effects in sensitive organisms makes it extremely unlikely that the Cry1F x Cry34/35Ab1 proteins produced in Herculex® XTRA will have any adverse effect on non-target organisms different from what was seen in individual Cry protein testing.

b. Lady Beetle Study (MRID No. 463438-07)

In a 12-day laboratory feeding study, ladybird beetle (*C. maculata*) larvae were provided a prepared test diet consisting of 50% Cry1F x Cry34/35Ab1 maize pollen and 50% ground corn earworm eggs until adult emergence. Larvae fed the treated diet for 12 days did not show any developmental delays compared to those fed the control diet. There was no statistically significant difference in larval mortality between the treated and control groups (7% and 0%, respectively). The average weight of adults in the treated and control groups was comparable. These results demonstrated no additional adverse effect to *C. maculata*, at the expected field exposure dosage, from the mixture in Herculex® XTRA over that observed with the Cry1F and Cry34/35Ab1 proteins alone.

Since the Cry34/35Ab1 protein toxicity is specific to coleopteran species, evaluations of other appropriate non-target beetle species should be submitted in support of Cry34/35Ab1-containing corn lines. Studies submitted in support of Herculex® RW may be bridged to support the Herculex® XTRA registrations.

c. Monarch Butterfly Testing (MRID No. 463438-08)

In a 4-day laboratory feeding study, first-instar monarch butterfly (*Danaus plexippus*) larvae were exposed to transgenic (Cry1F x Cry34/35Ab1 stack) or non-transgenic pollen at densities of 50, 100, 200, 400, 800, or 1600 pollen grains/square centimeter on milkweed leaves. After 96 hours, the larvae were transferred to clean milkweed leaves for an additional 120 hours. Exposure to the transgenic pollen did not significantly reduce larval survival, weight gain, developmental life stage, or leaf consumption during the 216-hour observation period. These data indicate that subchronic effects were not seen when *D. plexippus* was exposed to the Cry1F and Cry34/35Ab1 proteins at the recommended field dosage for 4 days. Therefore, no hazard to monarch butterflies is expected from incidental exposure to Herculex® XTRA corn pollen.

d. Avian Study (MRID No. 463438-03)

Broiler chickens were fed diets containing transgenic maize grain (containing Cry1F x Cry34/35Ab1 and PAT proteins), a non-transgenic near-isoline maize grain, or non-transgenic commercial reference maize grains. Feeding began on the day of hatch and continued for 42 days. There were no statistically significant differences in broiler mortality, body weight, body weight gain, feed efficiency, or carcass/carcass part yields for any of the diets. These results indicate that hybrid maize line 1507 x 59122 (Cry1F x Cry34/35Ab1) was nutritionally equivalent to the near-isoline and commercially available non-transgenic hybrids and, like with the corn events producing the individual Cry proteins, there were no adverse effects noted on the growth and development of broiler chickens. Therefore, long-term exposure to Herculex® XTRA is not expected to pose a hazard to avian wildlife.

e. Other Non-Targets Organism Risk Assessments

Waiver rationales were also submitted for the additional non-target studies normally required for environmental risk assessments: Avian Oral (OCSPP Harmonized Guideline 885.4050); Avian Injection (now Avian Inhalation) (OCSPP Harmonized Guideline 885.4100); Freshwater Fish (OCSPP Harmonized Guideline 885.4200); Freshwater Invertebrate (OCSPP Harmonized Guideline 885.4240); Estuarine and Marine Animal (OCSPP Harmonized Guideline 885.4280); Non-Target Plant (OCSPP Harmonized Guideline 885.4300); Non-Target Insect (OCSPP Harmonized Guideline 885.4340); and Honey Bee (OCSPP Harmonized Guideline 885.4380). A poultry broiler study was submitted, so a standard avian oral study is not required. The rationale for the waiver from avian injection and freshwater fish is that the active ingredient is not an infectious agent and is expressed at very low levels in plants. The rationale for the waiver from estuarine and marine animal studies is that the active ingredient is not an infectious agent, is expressed at very low levels in plants, and there is very low to no potential for exposure to the protein from field corn. The rationale for waivers from freshwater invertebrate, non-target insect, and honey bee studies is that appropriate studies were completed for the single-trait products (Cry34/35Ab1 and Cry1F). The rationale for the waiver from non-target plant studies is that the active ingredient is an insect toxin (*Bt* endotoxin) that is non-toxic to aquatic and terrestrial plants. Lastly, toxicity data submitted for the Human Health Assessment (see section II(B)(2) of this BRAD) indicate that there is no significant toxicity to rodents from testing at the maximum hazard dose and, therefore, no hazard to mammalian wildlife is anticipated.

Additional support for a complete environmental risk assessment is gained from an overview of the assessments done for the individual Cry1F (Herculex® I) and Cry34/35Ab1 (Herculex® RW) registrations. A brief summary of the Cry1F environmental risk assessment is provided in the section that follows, while the comprehensive environmental risk assessment for Cry34/35Ab1 can be found in section II(C)(2) of this BRAD.

f. Herculex® I (Lepidopteran-Active Cry1F Protein) Environmental Risk Assessment

EPA has conducted an extensive review of effects of the Cry1F protein present in Herculex® I to non-target organisms in an environmental risk assessment as part of the registration process (U.S. EPA 2001d, 2005d, and 2010d). EPA reviewed studies conducted on representative non-target species with Cry1F protein and performed risk or impact assessments on plants, wild mammals, birds, fish, aquatic invertebrates, estuarine and marine animals, earthworms, terrestrial non-target insects (including honey bee adults and larvae, parasitic wasps, green lacewings, lady beetles, springtails (collembola toxicity/reproduction), and monarch butterflies), field invertebrate abundance studies, soil degradation/persistence studies, and endangered species with special emphasis on the Karner blue butterfly. In addition, weediness and gene flow assessments (via pollen and Cry protein deoxyribonucleic acid (DNA) uptake by plants and soil microorganisms) were also performed.

The Agency concluded that, considering all available information, the weight-of-evidence indicates no unreasonable adverse effects of Cry1F proteins in corn to non-target wildlife, plants, or beneficial invertebrates.

iii. Endangered Species Considerations

The reviewed non-target data confirm the expectation that Cry1F x Cry34/35Ab1 corn is not likely to jeopardize the continued existence of any endangered and/or threatened species listed by the U.S. Fish and Wildlife Service, including mammals, birds, or terrestrial and aquatic plants and invertebrate species. Herculex® XTRA is not likely to adversely affect Karner blue butterflies because exposure to harmful levels of Cry1F corn pollen is not expected (U.S. EPA 2001d, 2005d, and 2010d). Likewise, a review of the preferred habitats of other lepidopteran species listed as endangered by the U.S. Fish and Wildlife Service, including the endangered Mitchell satyr butterfly, indicates that no exposure to harmful levels of Cry1F protein-containing pollen will take place. Furthermore, there is also no overlap of endangered beetle species habitat with corn production. Therefore, EPA believes that this action will have "No Effect" on listed species. This finding was triggered by the reviewed Cry protein toxicity and exposure data on terrestrial and aquatic species. As a result, no endangered species labeling is required for the registration of Herculex® XTRA corn.

iv. Environmental Assessment Summary

From all of the required and voluntarily developed indicator and host range species test data on Cry1F, Cry34/35Ab1, and Cry1F x Cry34/35Ab1 corn, the Agency concludes that the levels of Cry1F x Cry34/35Ab1 protein in Herculex® XTRA corn will not pose unreasonable adverse effects to corn field flora and fauna. Available data also indicate that there should be minimal short-term accumulation of Cry1F x Cry34/35Ab1 protein in agricultural soil. No evidence of synergy between the lepidopteran-active and coleopteran-active insecticidal proteins expressed by Herculex® XTRA corn was found in laboratory studies of target and non-target insects evaluated for these registrations.
Endangered/threatened species assessments conducted in support of individual trait (Herculex® I and Herculex® RW) registrations were sufficient to demonstrate a "No Effect" finding for the stacked product. In conclusion, Herculex® XTRA is projected to control certain lepidopteran and coleopteran pests in field corn without concerns of risk to non-target organisms.

Conclusion:
At the present time and for the purposes of a time-limited registration, this risk assessment finds no hazard to the environment from cultivation of corn expressing the Cry1F and Cry34/35Ab1 proteins.

v. Supplemental Studies Needed for Long-Term Cry1F x Cry34/35Ab1 Non-Target Hazard Assessment

The Agency has sufficient information to believe that there is no evidence of risk from the proposed uses of Herculex® XTRA corn to non-target wildlife, aquatic, and soil organisms. However, after consultation with the Scientific Advisory Panel in August 2002, and based upon several public comments, the Agency is requesting more data. The supplementary studies would provide additional weight to support the Agency's conclusions. The Agency does not believe that these data requirements

were reasonably foreseeable by Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International, Incorporated at the time of their respective applications.

The following data (Table 4) may be required to ascertain any possible adverse environmental effects from long-term use of Herculex® XTRA corn.

Table 4. Supplemental Data Requirements.

Testing Category	Type of Data
Toxicity data on non-target insects more appropriate for corn fields, ecosystem effects, and soil persistence determination	Laboratory toxicity testing with *Orius insidiosus* (minute pirate bug) and a carabid (ground beetle) may be required, as well as long range effects on invertebrate populations in the field and soil persistence studies with Cry1F x Cry34/35Ab1 corn as per the parameters outlined by the August 2002 FIFRA SAP. In the event that Cry34/35Ab1 (Herculex® RW) studies sufficiently demonstrate a lack of long range adverse effects, no additional data with Herculex® XTRA will be required.

*Note for 2010: There is an update to this summary. See section II(C)(3)(b) ("Terms and Conditions of the Herculex® XTRA Insect Protection Corn Registrations (October 2005 – September 2010)") of this BRAD.

b. Terms and Conditions of the Herculex® XTRA Insect Protection Corn Registrations (October 2005 – September 2010)

When Herculex® XTRA Insect Protection Corn was initially registered on October 27, 2005, the Agency issued registration notices to both Mycogen Seeds c/o Dow AgroSciences LLC (EPA Reg. No. 68467-6) and Pioneer Hi-Bred International, Incorporated (EPA Reg. No. 29964-5) that contained the following requirement in relation to further environmental effects data:

> "Submit all data required to support the individual plant-incorporated protectants in event TC1507 (Herculex I) and event DAS-59122-7 (Herculex Rootworm) corn…In the event that the Agency concludes Cry34/35Ab1 (Herculex Rootworm) studies do not sufficiently demonstrate a lack of long range adverse effects, additional data with Herculex Xtra must be submitted. This data may include a) laboratory toxicity testing with *Orius insidiosus* (minute pirate bug), b) laboratory toxicity testing with a carabid (ground beetle), c) long range effects testing on invertebrate populations in the field, and d) long range soil persistence testing."

All requirements for additional environmental effects data for events TC1507 (see U.S. EPA (2010d)) and DAS-59122-7 (see section II(C)(2)(b) of this BRAD), as set forth in the October 27, 2005 Herculex® XTRA Insect Protection Corn registration notices, have been satisfied for both registrations.

Moreover, after evaluating the conditional data submitted to support the registrations of event DAS-59122-7 corn, the Agency has concluded that these data do not demonstrate the potential for long-range adverse effects to the environment as a result of the cultivation of Cry34/35Ab1 corn. It should be noted that Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International, Incorporated, although not specifically requested by the Agency, did include Herculex® XTRA Insect Protection Corn in the three-year non-target arthropod field study (MRID No. 478703-01; reviewed in U.S. EPA 2010(b)).

4. 1507 x 59122 x MON 810 (OECD Unique Identifier: DAS-Ø15Ø7-1 x DAS-59122-7 x MON-ØØ81Ø-6) Expressing Cry1F, Cry34Ab1, Cry35Ab1, and Cry1Ab and 59122 x MON 810 (OECD Unique Identifier: DAS-59122-7 x MON-ØØ81Ø-6) Expressing Cry34Ab1, Cry35Ab1, and Cry1Ab

a. Data Cited/Submitted for Initial Registrations of 1507 x 59122 x MON 810 and 59122 x MON 810 (Prior to February 2010)

The stacked and pyramided product, 1507 x 59122 x MON 810, and the stacked product, 59122 x MON 810, are hybrids created by crossing the individuals events, TC1507 (Cry1F), DAS-59122-7 (Cry34/35Ab1), and MON 810 (Cry1Ab), via traditional breeding methods. Although background information on individual events DAS-59122-7, TC1507, and MON 810 is provided throughout this BRAD, the most specific information on each of these events is discussed or referenced in the Product Characterization chapter (see sections II(A)(1)–II(A)(3) of this BRAD).

To support their registration applications for 1507 x 59122 x MON 810 and 59122 x MON 810, Pioneer Hi-Bred International, Incorporated submitted molecular characterization data, protein expression analyses, and a synergism study. Collectively, these data (as summarized in the paragraphs that follow) justified bridging the existing findings and conclusions from the environmental assessments conducted for the single PIP events (TC1507, DAS-59122-7, and MON 810) to the combination PIP products (1507 x 59122 x MON 810 and 59122 x MON 810) (MRID Nos. 476778-01, 476778-02, and 478800-01; reviewed in U.S. EPA (2010a)).

The molecular characterization of *Bt* Cry1F, Cry34Ab1, Cry35Ab1, and/or Cry1Ab insect control proteins and the genetic material necessary for their production in 59122 x MON 810 and 1507 x 59122 x MON 810 combination PIP corn products demonstrated the stability and integrity of the intended transgenic DNA inserts of Cry1F protein expressed in TC1507, Cry34Ab1 and Cry35Ab1 proteins expressed in DAS-59122-7, and/or Cry1Ab protein expressed in MON 810 corn events, when combined through traditional plant breeding. Protein expression analyses also demonstrated similar levels of Cry1F, Cry34Ab1, Cry35Ab1, and/or Cry1Ab protein concentrations of each respective parental event when compared to 59122 x MON 810 and 1507 x 59122 x MON 810 combination PIP corn events.

Laboratory assays, using sensitive target pest species, found no antagonistic or synergistic effects among the Cry proteins expressed in each combination PIP product. This determination demonstrates functional equivalence among the single parental events and the combination PIP products, and the results and

conclusions of the non-target organism studies (conducted for each single parental event) can be bridged (see U.S. EPA 2001d, 2005d, and 2010d for Cry1F; see section II(C)(2) of this BRAD for Cry34/35Ab1; and see U.S. EPA 2001d and 2010d for Cry1Ab). Therefore, no unreasonable adverse effects on non-target organisms and the environment are expected from 59122 x MON 810 and 1507 x 59122 x MON 810.

b. Terms and Conditions of the 1507 x 59122 x MON 810 and 59122 x MON 810 Registrations (February 2010 – September 2010)

When 1507 x 59122 x MON 810 (EPA Reg. No. 29964-8) and 59122 x MON 810 (EPA Reg. No. 29964-9) were initially registered on February 24, 2010, the Agency issued registration notices to Pioneer Hi-Bred International, Incorporated that contained the following requirements:

- For 1507 x 59122 x MON 810 –

  "The data submitted by Pioneer are sufficient to support registration for the combination PIP product…provided that the registrant submits/cites any data required to support the PIP registrations of the individual parental events…as well as the combination PIP product TC1507 (DAS-Ø15Ø7-1) x DAS-59122-7…."

- For 59122 x MON 810 –

  "The data submitted by Pioneer are sufficient to support registration for the combination PIP product…provided that the registrant submits/cites any data required to support the PIP registrations of the individual parental events…."

All requirements for additional environmental effects data for individual events TC1507 (see U.S. EPA (2010d)), MON 810 (see U.S. EPA (2010d)), and DAS-59122-7 (see section II(C)(2)(b) of this BRAD), as well as for combination PIP product TC1507 x 59122-7 (see section II(C)(3)(b) of this BRAD), as set forth in the February 24, 2010 1507 x 59122 x MON 810 and 59122 x MON 810 registration notices, have been satisfied.

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D. Insect Resistance Management (IRM)

1. Supporting Information for Development of the Cry34/35 Event DAS-59122-7 Corn Durability Plan (2005 Registration)

a. Pest Biology

A clear understanding of pest biology is essential to the development of a sound IRM plan. Cry34/35 *Bacillus thuringiensis* (*Bt*) corn was developed to control three target pest species: western corn rootworm (*Diabrotica virgifera virgifera* LeConte, WCRW), northern corn rootworm (*Diabrotica barberi* Smith and Lawrence, NCRW) and Mexican corn rootworm (*Diabrotica virgifera zeae* Krysan and Smith, MCRW). Key factors believed to influence corn rootworm (CRW) adaptation to Cry34/35 corn include distribution, univoltinism, adult dispersal among fields, adult dispersal within fields, larval dispersal across rows, larval mortality due to density-dependent processes, insecticide use, egg mortality, fecundity, and adult and larval population density. Some of these factors are discussed below.

Distribution

WCRW is the most prevalent target pest in the United States and throughout most of the Corn Belt[1]. NCRW, also found throughout the Corn Belt, is considered the second most prevalent rootworm pest in the United States and is the primary target pest of the north-central region[2]. MCRW, the third target pest, is limited to Texas, is considered a relatively minor pest in the United States, and is generally excluded from discussion in the data package. Southern corn rootworm (SCRW), also a minor pest of corn in the U.S., is not identified as a target pest of Cry34/35-protected corn.

Life Cycle

The traditional life cycle of CRW is as follows: adult female rootworms deposit eggs in corn fields during late summer; eggs overwinter and hatch in late-spring, generally between late May and mid-June; larvae feed on corn roots for three to four weeks, during which time they complete three instar stages (most significant damage to corn plants caused during this period); the third instar transforms into a pupa, which develops for one to two weeks; pupae mature to become adult beetles, which emerge from the soil in mid-July to feed on corn foliage, pollen, and silks; adults remain active for 10 to 12 weeks, during which time they feed, mate, and deposit eggs.

Recently, deviations from the traditional life-cycle have emerged. A biotype of WCRW is depositing eggs in soybean fields, while a NCRW biotype has broken from the traditional univoltine life cycle by utilizing an extended diapause. In affected regions, these adaptations diminish the pest control benefits

[1] WCRW is the primary rootworm pest in Colorado, Kansas, Nebraska, Ohio, Indiana, Illinois, Iowa, Missouri, and Michigan.

[2] NCRW is the primary rootworm pest in Wisconsin, Minnesota, South Dakota, and northern Iowa.

associated with corn-soybean/corn-alternate crop rotations, a management practice that has long been recognized as the most effective means of controlling CRW.

Dispersal/Movement

Much remains to be learned about CRW larval and adult dispersal patterns; however, some preliminary research has been completed. In their study of larval movement between corn rows, Hibbard *et al*. (2003) reported that 0.75% and 6% of larvae moved across rows. Larval movement was largely dependent on the density of plants within a row, food availability, soil porosity, and level of soil compaction.

In their discussion of adult dispersal factors (reference to model discussion), the applicants referenced Nowatzki *et al*.'s research (Nowatzki *et al*. 2003), which described a study of male and female WCRW dispersal in a commodity grain field. Results of this research indicate that males moved at least 184 meters (m) in 4 days, or 45 m per day, while females took around 14 days to move the same distance, traveling up to 13 m per day.

The applicants also referenced Hill and Mayo's (1980) research on interfield dispersal, which shows that NCRW was three times more prevalent than WCRW in first-year corn, yet that NCRW populations were half of WCRW in second-year corn. Lance *et al*. (1988) later reported that NCRW adults are more likely than WCRW to disperse from mature corn and, further, that NCRW has a tendency to disperse from corn to feed on flowers and pollen of other plants (Naranjo 1994).

Biopesticides and Pollution Prevention Division (BPPD)'s Analysis of Pest Biology

Sufficient information has been provided on the pest biology of western corn rootworm, northern corn rootworm, and Mexican corn rootworm to develop an IRM strategy. Because there are still information gaps, additional information on WCRW, NCRW, and MCRW biology and ecology could improve understanding of resistance risks and lead to improvements in the IRM strategies. Specific areas to be examined include the following: dispersal behavior (categorized by sex and developmental stage); reproductive biology, including mating habits and frequency, ovipositional patterns, role of density-dependent mortality, use of non-corn hosts, and fecundity; level of Cry34/35 expression in corn roots relative to developmental stage of CRW pests; determination of whether IRM strategies designed for WCRW and NCRW are appropriate for MCRW; and the effect that new rotation-resistant WCRW and NCRW biotypes may have on the development of appropriate IRM strategies. These research efforts are recommended to be continued and bolstered. New information published since the trait durability plan was submitted to the Environmental Protection Agency (EPA) regarding CRW larval dispersal in the field and use of non-maize host as unstructured refuge should be considered, as appropriate, in the model (Hibbard *et al*., 2003; Hibbard *et al*., 2004; Moeser and Hibbard 2005; Oyediran *et al*. 2004; Clark and Hibbard 2004).

b. Mode of Action and Cross-Resistance

Cry34Ab1 (14-kiloDalton (kDa)) and Cry35Ab1 (44-kDa) proteins are both required for high activity against WCRW larvae (Ellis *et al*. 2002). The mode of action appears to be similar to other *Bt* proteins. Experimental feeding studies conducted by Moellenbeck *et al*. (2001) with WCRW neonates and later (2^{nd} and 3^{rd}) instar indicated that these proteins bind to midgut receptors, followed by membrane disruption. As a result, high levels of resistance to Cry34/35Ab1 will likely result from loss of binding or loss of activity following bindings, as reported for other Cry proteins (Ferré and Van Rie 2002).

Amino acid sequence comparisons did not show any significant similarity between the 14- and 44-kDa proteins (Cry34Ab1 and Cry35Ab1, respectively) and other registered *Bt* plant-incorporated protectants, including the Cry3 family (Ellis *et al*. 2002). These proteins also share no sequence homology with the *Bt* vegetative insecticidal proteins (VIPs). Because of this lack of sequence homology with known *Bt* plant-incorporated protectants (PIPs) and VIPs, Storer and Lefko (2003, Master Record Identification Number (MRID No.) 461239-18) assume that high levels of cross-resistance development is unlikely with any other rootworm-targeted *Bt* proteins. Field trials conducted in areas where methyl-parathion resistance is present and absent showed no associated difference in adult emergence (Storer *et al*. 2003, MRID No. 461239-15). Similarly, field trials in areas where rotational-adapted populations (behavioral resistance) of NCRW and WCRW are present and absent showed no associated differences in adult emergence (Storer *et al*. 2003, MRID No. 461239-15). Agricultural systems in which Cry34/35 corn is deployed will likely reduce the selection pressure for resistance to alternative corn rootworm control tactics, including other PIPs. Simulation models have shown that the use of Cry34/35 corn can reverse selection for rotation resistance in WCRW (Storer 2003b, MRID No. 461239-19).

The 14-kDa protein (Cry34Ab1) alone shows some insecticidal activity against southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber), but was enhanced by the 44-kDa protein (Cry35Ab1), indicating that both proteins are necessary for maximal insecticidal activity (Herman *et al*. 2002). A recent study by Masson *et al*. (2004) provided evidence that Cry34Ab1/Cry35Ab1 causes pore formation in the midgut, which results in membrane permeability. This study provides evidence that the mode of action is similar to that previously identified for other *B*. *thuringiensis* and *Bacillus sphaericus* toxins.

Receptor binding sites for Cry34Ab1 and Cry35Ab1 have not been identified and characterized. Ellis *et al*. (2002) indicate that there is little sequence homology between the Cry34Ab1 and Cry35Ab1 proteins and other registered Cry proteins and VIPs. Lack of sequence similarity would suggest that high levels of cross-resistance conferred by Cry34Ab1 and Cry35Ab1 would be unlikely. There is some evolutionary relatedness between the larger Cry35Ab1 protein (44-kDa) and the 42-kDa and 51-kDa dipteran-active toxins from *B. sphaericus* (Baumann *et al*. 1988). However, since *B*. *sphaericus* biopesticide products act against dipterans rather than coleopterans, cross-resistance with these toxins should not be an issue. Field trials (Storer *et al*. 2003, MRID No. 461239-15) suggest that there is little possibility of cross-resistance between Cry34Ab1/Cry35Ab1 and any other rootworm-control tool. The use of Cry34Ab1/Cry35Ab1 corn will likely reduce the selection pressure for resistance to other corn rootworm control tactics, including other PIPs. Simulation models predict that use of

Cry34Ab1/Cry35Ab1 corn can reverse selection for rotation resistance in WCRW (Storer 2003b, MRID No. 461239-19).

BPPD's Analysis of Mode of Action and Cross-Resistance

To confirm that high levels of Cry34Ab1 or Cry35Ab1 cross-resistance with other *Bt* proteins is unlikely, it is recommended that research efforts should focus on determining the likelihood of cross-resistance. Midgut binding receptors for Cry34Ab1 and Cry35Ab1 are recommended to be identified and characterized. Resistant colonies might be used to study the cross-resistance potential. Models could be run with scenarios that include the possibility for cross-resistance to see how the selection for resistance is impacted. Resistance may evolve through a detoxification or binding modification mechanism, for example, and/or through a behavioral modification mechanism. Multiple mechanisms should be considered as one evaluates the cross-resistance potential. This information would provide further evidence that cross-resistance is unlikely.

There is also a potential "repellent" behavior that should be further investigated. It is required that additional research efforts focus on characterizing and understanding the implications of this mode of action on selection for WCRW and NCRW resistance.

c. Genetics and Mechanisms of Resistance

The resistance mechanism of the greatest concern is that which could occur through a single gene mutation (i.e., monogenic) through loss of effective receptor binding. In nearly all known *Bt* resistance cases that have conferred high levels of resistance, this is the single most common mechanism (Ferré and Van Rie 2002). Other forms of resistance, such as metabolic resistance or increased midgut protease activity, do not confer high levels of resistance because multiple mutations in individual insects would be required to have high fitness on the transgenic plants. The IRM plan is based on the worst-case assumption that resistance will be monogenic, with two alleles, one conferring resistance (r-allele) and the other conferring susceptibility (S-allele). In addition, there is another worst-case assumption that homozygous resistant insects will be completely resistant to Cry34Ab1/Cry35Ab1 corn.

Tabashnik *et al*. (2003) reports that most resistance (laboratory-selection) to Cry proteins is incomplete meaning that less than 100% of target pests survive on a *Bt* crop. Incomplete resistance will evolve more slowly than complete resistance since the relative fitness of the r-allele compared with the S-alleles will be lower. The IRM plan for Cry34Ab1/Cry35Ab1 corn has made the worst-case assumption that resistance will be complete and that homozygous resistant individuals would have the same fitness on transgenic and non-transgenic plants (i.e., complete resistance).

The fitness costs of rootworm resistance to Cry34Ab1/Cry35Ab1 are unknown. Resistance to other *Bt* proteins has generally been shown to have high fitness costs (Ferré and Van Rie 2002). Despite this evidence, the IRM plan assumes the worst-case assumption that there will be no loss of fitness associated with resistance.

Receptor modifications are the most likely cause of resistance. Resistance will be incompletely to completely recessive, and heterozygotes will retain partially functioning Cry protein receptors. Resistance associated with receptor modifications usually results in low resistance ratios for heterozygotes. IRM plans developed for lepidopteran-protected *Bt* corn products have assumed that heterozygous insects will be 25-fold resistant to the associated Cry proteins, an assumption that was deemed sufficiently conservative for PIPs (U.S. EPA 1998 and 2001a). The IRM plan for Cry34Ab1/Cry35Ab1 corn assumes that heterozygous insects will be 25-fold resistant to Cry34Ab1/Cry35Ab1. This assumption enables the estimation of the functional dominance of the r-alleles and, therefore, heterozygote survival used in the Storer model (Storer 2003b, MRID No. 461239-19). Based on the dose information, the functional dominance is predicted to be around 0.08 in WCRW and between 0.15 and 0.37 in NCRW (Storer 2003b, MRID No. 461239-19).

Given the limited availability of information on the genetics of resistance to Cry34/35, the IRM plan utilizes "worst-case" assumptions of resistance potential in the modeling simulations of target pest adaption to Cry34Ab1/Cry35Ab1 corn (Storer 2003b, MRID No. 461239-19). These assumptions include the following: (1) all resistance to Cry34Ab1/Cry35Ab1 is monogenic; (2) all resistance is complete; (3) resistance does not confer a fitness cost; and (4) heterozygotes will be 25-fold resistant to Cry34Ab1/Cry35Ab1. BPPD agrees with this approach.

BPPD's Analysis of Genetics and Mechanism of Resistance

When resistance does occur, little is known about how that resistance would affect rootworm fitness. Most studies of target pest resistance to *Bt* products and Cry proteins have shown that resistance is unstable, most likely due to fitness costs associated with resistance genes or with other closely linked loci (Ferré and Van Rie 2002). These findings suggest that the affected midgut receptors may perform alternate functions. Despite insights provided by these preliminary investigations, the trait durability plan acknowledges that "the fitness costs of rootworm resistance to Cry34/35Ab1 are currently unknown" and that further research on fitness costs is needed.

Given that functional dominance and/or recessiveness of resistance to Cry34/35 is presently unknown and will remain unknown until field resistance occurs, resistant (homozygous or heterozygous) colonies would be useful to study functional dominance. It is recommended that the applicants establish Cry34/35 resistant CRW laboratory colonies to study possible mechanisms, genetics, and inheritance of resistance. While useful information is likely from the study of resistant colonies, it is important to note that conclusions made from laboratory studies may not necessarily be extended to situations in the field. However, even without this confirmatory information, the IRM durability plan is sufficiently conservative and assumes the worst case that there will be no fitness costs associated with resistance.

Laboratory assays submitted by the applicants also suggest a "repellent" behavior. While larvae feeding on control plants tended to feed in one location, CRW larvae failed to become established on Cry34/35-protected corn roots. It follows that inconsistent feeding patterns could contribute to poor larval development and decreased fitness. A combination of both behavioral and binding modification resistance mechanisms would also slow the rate of resistance evolution. It is recommended that the

applicants further study the "repellent" or behavioral mode of action and its implications in the selection of resistance.

d. Dose

The term "high dose" is not well characterized for the CRW complex, and the previous dose definition developed for lepidopteran pests ("a level of toxin 25 times greater than is needed to kill all susceptible insects") may not apply to CRW species. Dose evaluation for CRW-targeted PIPs is also complicated by the fact that (1) CRW larvae and adults both feed on corn tissues and each life stage must be evaluated separately for dose; (2) CRW larvae feed underground, making direct observations of mortality difficult; and (3) CRW are notoriously difficult to rear in the laboratory.

In terms of adult susceptibility and dose, Dow AgroSciences LLC/Pioneer Hi-Bred International, Incorporated (Dow/Pioneer) conducted adult feeding studies with WCRW and Cry34/35 corn tissue. These studies indicated that adult WCRW feeding (solely) on Cry34/35Ab1 aboveground corn tissue does not reduce adult fitness as characterized by longevity, fecundity, or egg viability. Therefore, dose considerations for Cry34/35 corn can be relegated to the larval life stage.

To investigate larval dose, Dow/Pioneer conducted a series of single-season field trials that measured adult emergence while correcting for density-dependent mortality to provide an indirect estimate of larval mortality. These trials indicated that WCRW larval mortality ranged between 99.82% and 99.98%, while NCRW larval mortality ranged between 92.77% and 99.14%. Laboratory feeding studies were also conducted with neonate, as well as 2nd and 3rd instar, WCRW and NCRW. The lab studies documented evidence of repellent or avoidance behavior but did not provide estimates of mortality or dose measures.

The mortality estimates obtained from the field indicate that it is likely that Cry34/35 corn expresses slightly less than a high dose for CRW (perhaps a "borderline" high dose) when evaluated relative to the previously used "25x" high dose definition. These data for dose were directly applied to CRW models to evaluate the durability of the IRM plan.

BPPD's Analysis of Dose

The studies (both larval and adult dose) are acceptable for the purposes of registration and the development of an IRM plan for Cry34/35 corn. Nonetheless, there are still significant uncertainties regarding the CRW dose expressed in Cry34/35 corn. At best, the results are an educated estimate of the true effects of Cry34/35 corn on CRW. The exact toxicity (i.e., mode of action) of the Cry34/35 proteins to CRW is unclear: the field studies indicated high levels of mortality, while the laboratory studies suggested less toxic mechanisms such as growth inhibition or feeding deterrence. Given these uncertainties, it is recommended that Dow/Pioneer continue to research the dose issue, including the concept of dose for CRW, specific assays to evaluate dose for rootworm-protected corn hybrids, and the role of density dependence in dose determinations.

e. Refuge (General Considerations)

Unstructured refuge

Several non-maize grassy weeds are known to support CRW species. Earlier work characterizing non-maize hosts of WCRW and NCRW (Branson and Ortman 1970; Branson and Ortman 1971; Siegfried and Mullin 1990) has been augmented by the work of Oyediran *et al*. (2004) and Clark and Hibbard (2004) on prairie and forage grasses, and weedy species. The results of these new studies indicate that WCRW can readily develop to adulthood by feeding on non-maize hosts and, in the case of pubescent wheatgrass, the number of reared adults was not significantly different from that produced on maize (Oyediran *et al*. 2004).

When alternate hosts are present in close proximity to Cry34/35 corn fields, it is assumed that some percentage of target pests develop on alternate plant hosts. As a result, alternate hosts may be considered unstructured refuge for target pests. Although much remains to be learned about the role of alternate hosts as refuge, the Storer CRW model (Storer 2003a; Storer 2003b, MRID No. 461239-19) makes the assumption that 0.5% of larvae attacking Cry34/35 maize have developed on non-maize hosts.

Technology adoption is expected to be incomplete (and will likely never reach 100%). Therefore, not all fields will be planted to Cry34/35 corn, and these will serve as essentially unstructured refuge. This will reduce the selection pressure and provide susceptible adults that can mate with any resistant survivors from Cry34/35 corn fields. It is important to note, however, that the trait durability plan proposed by Dow/Pioneer does not rely on this source of unstructured refuge.

Alternative hosts, such as common grassy weeds, should not be considered as refuge for CRW-protected transgenic corn until more information is available regarding those plants' ability to produce susceptible CRW individuals. Potential areas of investigation could include:

- Expanded listing of potential alternate hosts, including identification of most promising hosts (e.g., most attractive to target pests, most prevalent near Cry34/35 production regions, etc.);
- CRW movement between maize and non-maize plants at different points in target pest life cycle;
- Impact that movement between Cry34/35 maize and non-maize plants could have on resistance/susceptibility of target pests to Cry proteins.

Even though 100% adoption of Cry34/35 corn is unlikely, unstructured refuge consisting of corn fields not planted in Cry34/35 corn is not sufficiently reliable in all production areas.

Structured refuge

A structured refuge of non-Cry34/35 corn will function to delay monogenic[3] resistance evolution by (1) reducing population-wide selection pressure for resistance and (2) providing a source of non-selected (*SS* genotype) adults to mate with any partially or fully-resistant (genotypes *rr* or *rS*) adults that may survive in the Cry34/35 corn fields. The higher the dose, the more effective the refuge, and thus a smaller refuge is required for a given durability.

Refuge location is important, especially for higher dose traits. Susceptible insects must be produced in sufficiently close proximity so that any resistant survivors in the *Bt* fields are likely to mate with susceptible insects rather than amongst themselves. CRW biology potentially favors this inter-mating. Males emerge 3–5 days before females, and they actively disperse to seek females. Females, by contrast, tend to mate while they are teneral and cannot fly and, therefore, are in close proximity to their emergence site. Although CRW long-range dispersal remains poorly understood, a preliminary investigation of WCRW movement in a commodity grain field showed that male and female adults moved 45 m and 13 m per day, respectively (Nowatzki *et al*. 2003). Another study, which imposed high target pest infestation rates on experimental fields, documented between-row CRW larval movement of 0.76 m in susceptible corn and between-plant larval movement equal to "three plants down the row," or approximately 0.54 centimeter (cm)(Hibbard *et al*. 2003, 2004). This degree of larval dispersal is density dependent (increasing movement with increasing density) and is associated with significant host plant damage (less food results in greater movement). These studies support the assumption that CRW larvae and adults are poorly mobile. Because long-distance movement by WCRW and NCRW prior to mating is still poorly understood, refuge areas should be close to or within the Cry34/35 corn fields to be most effective.

Another important factor in refuge management is the use of insecticides in Cry34/35 fields and accompanying refuge to control CRW and non-CRW pests. An effective refuge must produce an abundance of susceptible CRW adults that are available to breed with resistant adults from the Cry34/35 field. To encourage technology adoption and refuge deployment, it is important for growers to have the option of treating the refuge (protecting it against rootworm larval feeding), but this must be balanced against maintaining the refuge as a source of large numbers of susceptible Cry34/35 insects. Pest management should be carefully considered. Consequently, insecticide applications that function to kill refuge-produced adult CRW must also be applied to the Cry34/35 field to nullify any negative impact on the value of the refuge.

Adulticide treatments can be very effective at reducing CRW adult numbers if applied at the right time (i.e., appearance of gravid females) and thus management through adult control should be discouraged in the refuge

[3] Monogenic resistance is assumed given what is presently known about the genetics of resistance.

Use of seed and granular insecticide treatments to control CRW larvae could be allowed on refuge acres, even if not applied to the Cry34/35 field, since these treatments are shown to be non-high dose controls (Meinke *et al*. 1998). Data have been collected that support the premise that banded insecticides and seed treatments will allow considerable survival of rootworms to adult (Sutter *et al*. 1991; Cormier and Martel 1997). There is ongoing research as to the impact of clothianidin and Cruiser® seed treatments on rootworm fitness, but preliminary data suggest that there is a minimal impact of CRW exposed to these seed treatments.

Crop rotation will have a high impact on larval survival. If a refuge is planted on rotated ground, it will encourage adaptation to Cry34/35 unless the Cry34/35 corn is also planted on rotated ground.

A structured refuge is necessary to reduce the population-wide selection pressure for resistance and providing a source of non-selected (*SS* genotype) adults to mate with any partially or fully resistant (genotypes *rr* or *rS*) adults that may survive in the Cry34/35 corn fields. Four basic aspects should be examined: refuge size, refuge deployment, refuge management, and grower feasibility. The higher the dose, the smaller the refuge needed. Because of the uncertainties associated with CRW long-range dispersal, the refuge should be placed adjacent to or within the same field as Cry34/35 corn. Refuge management should consider the roles of chemical insecticides, crop rotation, and other practices in the context of an integrated pest management program. For a detailed discussion of structured refuge specific for Cry34/35 corn, see the "Analysis of Dow/Pioneer's Proposed Cry34/35 Event 59122-7 Corn Durability Plan (2005 Assessment)" section later in this chapter.

f. Computer Simulation Models

Simulation models were used to predict the impact of various factors on the rate of adaptation to Cry34/35 (event DAS-59122-7) corn by western corn rootworm and northern corn rootworm. In designing the Cry34/35 durability plan, Dow and Pioneer used simulation models. Simulation modeling, leading to the development of the proposed trait durability plan, is summarized and documented in Storer (2003b). The product durability plan based on the simulated modeling is discussed in Storer and Lefko (2003). These materials and published CRW resistance management models (Onstad *et al*. 2001; Storer 2003a) were also considered.

Overview of the Storer CRW model

The CRW model used in the submission (Storer 2003b, MRID No. 461239-19) is described in detail in Storer (2003a). A brief overview is presented in this section. The model is stochastic. Mortality, dispersal, development, and crop development are probabilistic. The model describes an agricultural ecosystem in a spatially explicit manner, with 25-hectare (ha) square fields arranged in a grid of size 10 x 10 or 12 x 12 fields. The fields can be planted in either corn or soybean. Corn fields can be planted to conventional or rootworm-resistant corn. Also, corn fields can be subdivided into blocks or strips so that a rootworm-resistant field can have an internal refuge of conventional corn. External refuge fields can also be simulated, including adjacent refuge fields or randomly distributed refuge fields. The model can simulate continuous corn cultivation, such as practiced in the western Corn Belt, or crop rotation with

soybean, as practiced in much of the central and eastern Corn Belt. The model can examine the rate of adaptation of two types of rootworm-protected corn planted simultaneously as a mosaic in the environment. The following processes were modeled: crop development; larval development, survival, and movement; adult emergence, development, and survival; mating and oviposition; and adult dispersal.

Input Parameters

Dow has determined that Cry34/35 corn expresses a dose that kills at least 99.75% of susceptible WCRW larvae (using the lower end of the range of field studies to estimate dose; see previous discussion in section II(D)(1)(d) of this Biopesticides Registration Action Document (BRAD)). For NCRW, there was greater uncertainty in the intensity of density-dependent mortality, and two conservative estimates of dose were derived: 95.0% and 99.0% (midpoint of the range of the lower dose estimate and higher dose estimate, respectively; see previous discussion in section II(D)(1)(d) of this BRAD). The expected mortality of the heterozygotes was calculated by assuming that the dose-response curves were parallel and that heterozygotes are 25 times more tolerant of the proteins than are homozygous susceptibles (Storer 2003a). Although it is not possible to calculate a dose-mortality relationship from a limited set of data points, the relationships for both WCRW and NCRW species are consistent with a default assumption of a slope of 1.0. Based on the dose estimates, assumptions of dose-response slopes, and the assumption that heterozygotes are 25x resistant, the functional dominance of resistance for WCRW is estimated as 0.08 and, for NCRW, between 0.34 and 0.15. Previous research submitted by Dow (Storer and Lang 2003, MRID 461239-16) showed that Cry34/35 corn has no effect on adults that feed on it, so the dose against adults was 0 for all genotypes.

The default model parameters and sensitivity analysis of the parameters for the runs with wild-type WCRW are summarized in the Dow modeling submission (Storer 2003b, MRID No. 461239-19). Additional discussion is provided on the following parameters.

1. *Larval movement*. A default value of 2.5% of larvae moving one row was used. Hibbard *et al*. (2003) showed that between 0.75% and 6% of larvae moved across rows. This represents a relatively high-end estimate of the number of larvae that cross rows. Homozygous susceptible (*SS*) larvae that crossed from transgenic to refuge were assumed to suffer 100% mortality after feeding on transgenic plants, while those crossing in reverse were assumed to suffer 90% mortality. Partially adapted heterozygotes (*rS*) were assumed to suffer 50% of the mortality after crossing from transgenic to refuge plants, while those crossing in reverse were assume to suffer 30% of the mortality. Adapted homozygous resistant larvae did not suffer any mortality. Field data showing the level of survival of *SS* and *rS* larvae when crossing between transgenic Cry34/35 and refuge rows when collected can be incorporated into the model.

2. *Adult in-field movement*. Dow cited to a study by Nowatzki *et al*. (2003) that described male and female WCRW intrafield dispersal using rubidium as a marker. Based on this study, females were shown to move about one third the distances of males in irrigated corn fields. By the time females reached the same distance as males, they were assumed to be mated. On average, females mated

within 2 rows of their emergence row. For block refuge scenarios, the destination of dispersing adults is always the other block (i.e., transgenic to refuge or vice-versa). For strip refuge scenarios, the probability that those leaving each strip end up in the opposite kind of strip (i.e., transgenic strip to refuge strip or vice-versa) is equal to the proportion of the field that is in the opposite kind of strip.

3. *Soybean biotype WCRW biology parameters*. The soybean-biotype WCRW lays eggs in crops other than corn. Only the dispersal parameters differ from that of the wild-type WCRW (see Table 1 in Storer (2003b)). Investigations on durability of Cry34/35 corn in areas where soybean-adapted populations are prevalent were initialized with an allele frequency of 0.5.

4. *Wild-type NCRW biology parameters*. The parameter values for NCRW are assumed to be the same as for wild-type WCRW, except for adult survival, daily fecundity, adult between-field dispersal, pre-oviposition period, and density dependence. The survival probability decreases from 0.99 at the first day of adult emergence, rather than from 0.999 for WCRW. A value of 16 eggs per female per day is used to ensure the population was sustainable. The longevity of NCRW is shorter than WCRW, the fecundity is lower, and the equilibrium population is around 6-fold lower. Between-field dispersal is three times higher for NCRW than for WCRW. The pre-oviposition period of 10 to 14 days was the same for both NCRW and WCRW. Less information is available regarding density-dependent mortality in NCRW than in WCRW, and the WCRW parameter for density-dependent survival was modified.

Model Output

The model output is the predicted rate of resistance evolution expressed relative to a benchmark rate. The same benchmark used in Storer (2003a) was used in the model submission (Storer 2003b, MRID No. 461239-19). The benchmark rate is for runs with:

- A product that kills 100% of the susceptible rootworm larvae, 90% of larvae heterozygous for resistance, and 0% of resistant larvae;
- A product planted on 100% of the acreage with the exception of the refuge;
- 20% refuge in external fields whose location is randomized each year;
- Default values for all model parameters as described in Storer (2003a).

The relative rate of adaptation (RRA) is calculated by the following equation: $RRA = (1/Y \ln q_y/q_0)/0.327$; where, the q_y equals the r-allele frequency after Y years and 0.327 is the adaptation rate (yr^{-1}) for the baseline run (Storer 2003a). The relative rate of adaptation is calculated when the r-allele frequency first exceeds 0.075, when the rootworm egg population in the autumn falls below 20,000 per field, or after 10 years, whichever is soonest. The relative rate of adaptation for the benchmark setting is 1.0. A relative rate of 2.0 implies resistance would evolve twice as fast as the benchmark, while a relative rate

of 0.5 implies resistance would evolve twice as slowly. From the equation described above, a relative rate of adaptation of 1 implies that the modeled r-allele frequency would increase 100-fold (e.g., from 0.001 to 0.1) in approximately 14 model years.

Runs or Scenarios Conducted

Several scenarios were modeled to understand how various refuge options may affect the rate at which WCRW and NCRW adapt to Cry34/35 corn. One set of simulations was designed to investigate the effects of refuge size, refuge placement, and refuge strip width on WCRW adaptation. Other model runs were designed to look at the durability obtained with Cry34/35 against WCRW compared with a lower dose event. Another set of simulations was designed to examine the effects of incomplete adoption of the technology, incomplete deployment of the refuge, and a competitive mosaic with an alternate rootworm plant-incorporated (PIP) expressed at a lower dose. Simulations also included, adaptation in a crop-rotation system, where 50% of the WCRW population is rotation resistant (soybean biotype). Adaptation by NCRW was compared with wild-type and extended diapause biotype. Each run was conducted at two refuge sizes (10% and 20%) repeated for a total of five runs at each setting to investigate the effects of stochasticity.

Analysis of Variance

Output from the runs was subjected to analysis of variance for a completely randomized factorial design to identify the parameters that had a significant effect on RRA. All factors were treated as fixed. Means separation was conducted using Tukey's method to identify significant differences among refuge placement options.

Sensitivity Analysis

Conservative estimates of the mean values of biological and genetic parameters in the models were used for the simulations, based on empirical data. The uncertainty in these values, as they affect the confidence of product durability, was studied using a Monte Carlo analysis (the WCRW model was run 2,000 times). Twenty-one parameters were analyzed and a 10% in-field refuge was used. The relative importance of each of the parameters was analyzed through rank correlation between parameter value and adaptation rate (Storer 2003b, MRID No. 461239-19). The parameters with the largest effects on the relative rate of adaptation were the functional dominance of the r-allele (explaining 58.5% of the variation) and the dose (explaining 33.5% of the variation). The next most important parameters were the fitness cost of resistance (3.1% of the variation), winter survival (2.9%), and fecundity (1.1%). Winter survival and fecundity were correlated. All other parameters accounted for less than 1% of the variation.

Model Results

Western Corn Rootworm

1. *How does dose affect durability?*
An in-field block refuge design was used, and the position of the refuge within the field was not held constant from year to year. Refuge sizes were 10% and 20%. The simulations predict that higher doses result in longer durability (i.e., lower relative rate of adaptation), and refuge size was most important at higher doses.

2. *How does refuge size affect durability of Cry34/35 corn?*
With a 20% refuge, the mean RRA of the moderate dose (one that kills 90% of susceptibles with a functional dominance of 0.5) line was 3.17 (standard error = 0.01), while for the Cry34/35 event DAS-59122-7 line, the mean RRA was 0.64 (standard error = 0.002). These results indicate that the adaptation would take five times longer for the Cry34/35 corn than for a moderate dose rootworm corn. With a 10% refuge, the mean RRA for Cry34/35 event DAS-59122-7 was 1.24 (standard error = 0.009) or approximately half the durability as predicted with the 20% refuge. This means that a 4% refuge used with Cry34/35 event DAS-59122-7 corn lines would have the same predicted durability as a 20% refuge used with a moderate dose event corn line.

3. *How does refuge placement affect durability?*
The effect of different refuge designs, using the conservative dose estimate of 99.75% (WCRW) for the Cry34/35 corn line, was simulated. The following deployment strategies were compared: in-field single block with an adjacent field and a random pattern of Cry34/35 and refuge fields. A 20% refuge was used because this allowed the model to place every Cry34/35 field directly adjacent to one refuge field. The model predicts that the RRA was lower (approximately half the relative rate of adaptation) for all three scenarios where the refuge was in the same location each year (i.e., a fixed refuge) than for refuge locations randomized each year. As discussed in Storer (2003a), the fixed refuge allows a higher population of susceptible insects from year to year. For randomized locations, the RRA was higher the greater the distance between the refuge and the Cry34/35 fields, but the differences among these three scenarios was relatively small.

4. *How does refuge strip width affect durability?*
The effect of different strip widths for the refuge on the durability of Cry34/35 was simulated. These simulations assume that the strips are not in the same place each year, so that the distribution of hatching eggs across the field is independent of the position of the refuge and the Cry34/35 strips. The survival estimates were based on the assumption that the heterozygous individuals the move between the refuge and the Cry34/35 plants have a higher probability of surviving than either the heterozygous individuals that remain on the Cry34/35 plants or homozygous susceptible individuals that move between the refuge and Cry34/35 plants. The overall conclusion is that strip width is not likely to greatly affect predictions of durability with either a 10% or 20% refuge. That is, there is very little difference in the relative rate of adaptation between a two-row strip and a ten-row strip, although there are some minor benefits. The model predicts that blocks are more durable than strips. This is because there are more interfaces

between Cry34/35 and refuge strips, allowing greater larval movement and thus greater survival differential between *SS* and *rS* genotypes, and the adults that disperse from Cry34/35 strips are more likely to end up in other Cry34/35 strips rather than refuge strips because there are more Cry34/35 strips relative to the number of refuge strips. Based on these simulations, as strip width increases, the durability will increase up to the point of a single block, but the effect will be small due to the practical range of planting wider strips.

5. *How does crop rotation affect durability?*
Farmers may use Cry34/35 corn (or other CRW-protected *Bt* corn) to help manage rotation-resistant WCRW (soybean biotype) or NCRW (extended diapause biotype). The model was used to investigate the possible effects crop rotation (i.e., corn-soybean rotations vs. corn-corn) may have on durability of Cry34/35 corn under two refuge deployment structures: in-field block and external fixed block. Both a 10% and 20% refuge were considered. Based on the model predictions, crop rotation reduced the relative rate of adaptation for both in-field and external refuge options. There was a significant interaction between refuge size and rotation in the in-field refuge scenarios. For external refuges, there was a main effect of crop rotation, but no interaction with refuge size.

6. *How does rotation-resistant WCRW affect durability?*
In certain parts of the eastern range of the WCRW (i.e., Illinois, Indiana, part of Michigan), WCRW has adapted to crop rotation by laying eggs in fields that are not planted in corn, most notably in soybean fields ("soybean biotype"). The soybean biotype lays its eggs in soybean fields and they hatch when it is rotated to corn. The model was used to examine the effect of this behavior on durability of Cry34/35 corn. Three refuge scenarios were run: fixed external refuge (continuous corn), with Cry34/35 corn rotated with soybean; in-field refuge, with all fields in corn-soybean rotation; and in-field refuge, with all fields planted to continuous corn. All scenarios were run with both 10% and 20% refuge size. Rotation resistance is assumed to be controlled by a single gene. Based on the modeling simulations, rotation resistance did not significantly affect the durability of any refuge scheme. That is, the soybean biotype adapted to Cry34/35 at the same rate as wild-type WCRW. However, there were differences in effects on the rate of relative adaptation caused by different deployment schemes. The external, fixed refuge served as a refuge for both Cry34/35 susceptibility and rotation susceptibility and, therefore, reduced the incidence of rotation resistance. These results suggest that Cry34/35 corn has the potential to decrease the incidence of WCRW rotation resistance if used in rotation with soybean while an external refuge is planted to continuous non-rootworm protected corn.

7. *How do refuge implementation rates and technology adoption rates affect durability?*
Adoption of Cry34/35 corn will not be 100% in all locations from the time of commercialization and will vary from area to area. The rate of technology adoption is affected by grower attitudes, market acceptance, perceived need for the technology, and effectiveness of the technology. The model was used to investigate the impact of Cry34/35 corn adoption and refuge implementation on Cry34/35 corn durability. A grid of 12 x 12 fields planted to continuous corn was divided into 16 farms, each consisting of 3 x 3 fields with each farm designated as either an adopter or non-adopter of Cry34/35 corn. Adopting farms were further designated by those either deploying the required refuge block within each field or not deploying a refuge at all (i.e., 100% Cry34/35 corn). Adoption rates were between 25% and 100%

and refuge deployments were between 50% and 100%. Based on the modeling simulations, rootworm adaptation rate is least when adoption is lowest (i.e., 25%). When adoption was 25%, refuge implementation had no effect on the overall r-allele frequencies. Refuge size (whether 10% or 20%) had a significant effect on the relative rate of adaptation only when there was 100% adoption of the technology. At 50% and 75% levels of technology adoption, refuge implementation had virtually no impact on the relative rate of adaptation.

Modeling simulations indicated that the spatial patterns of population size and *r*-allele frequencies are the inverse of one another. The overall *r*-allele frequency was dominated by populations in the farms with the refuge and non-adopting farms. Sufficient dispersal of the adults from these farms to those without a refuge would prevent the *r*-allele frequencies from becoming locally elevated.

8. *How does a mosaic with an alternative rootworm PIP affect durability against WCRW?*
Dow modeled the effect of a spatial mosaic of two rootworm PIP products on durability. It is expected that multiple rootworm PIP products will be grown as a spatial mosaic across the landscape. Currently, there are two registered rootworm PIPs, YieldGard® Rootworm Corn (expresses the Cry3Bb1 protein, Event MON 863, EPA Reg. No. 524-528) and YieldGard® Plus Corn (expresses both the Cry3Bb1 and Cry1Ab proteins, EPA Reg. No. 524-545). The 2002 Scientific Advisory Panel (SAP) concluded that MON 863 expressed a moderate dose to control rootworm (U.S. EPA 2002). For the simulations, MON 863 was assumed to kill 90% of the insects susceptible to it, and the functional dominance was assumed to be 0.5. It was assumed there is no cross-resistance between Cry3Bb1 and Cry34/35 proteins. Simulations were run with 0, 25, 50, 75, and 100% of the rootworm-protected corn expressing Cry34/35, and corresponding 100, 75, 50, 25, and 0% expressing Cry3Bb1. All corn fields had an in-field refuge. EPA has required that both YieldGard® Rootworm Corn and YieldGard® Plus Corn have a 20% structured refuge (U.S. EPA 2007a, 2010d). For Cry34/35 corn, the refuge size was assumed to be 10%.

The modeling simulations indicate that the relative rate of adaptation for Cry34/35 corn was lower at comparable market share levels than Cry3Bb1 corn. For example, at equal deployment of 50% market share for Cry3Bb1 corn and 50% market share for Cry34/35 corn, the model predicts that adaptation to Cry34/35 would take four times longer (RRA = 0.33, standard error = 0.005) than at 100% Cry34/45 market share (RRA = 1.25, standard error = 0.016). There is a more dramatic reduction in the rate of adaptation to Cry34/35 corn as its market share increases than the rate of adaptation to Cry3Bb1 corn as its market share increases. This is because the Cry34/35 fields produce very few Cry3Bb1-susceptible insects. However, these simulations suggest that in a competitive spatial mosaic, both products would benefit in terms of durability from the presence of the other.

Northern Corn Rootworm

1. *How is the durability prediction altered for Northern Corn Rootworm?*
The model was run to compare differences in predictions of durability between NCRW and WCRW. A range of doses and refuge sizes were run. The output was compared with WCRW. Dose and refuge size significantly affected the relative rate of adaptation. The predicted values for NCRW showed a very similar relationship to dose and refuge as for WCRW, although for any given dose and refuge size, the

relative rate of adaptation for NCRW is lower than that for WCRW. It is thought that a lower estimate for dose of Cry34/35 corn would be 95% and a higher estimate for dose of Cry34/35 corn would be around 99% for control of NCRW. Based on the simulations, a 20% refuge had lower comparable relative rate of adaptation values than a 10% refuge at both the lower and higher estimates of dose. For example, at the lower dose estimate, the 20% refuge increased durability by about 50% compared with the 10% refuge, while for the higher dose estimate, the 20% refuge increased durability two-fold compared with the 10% refuge.

2. *How do crop rotation and rotation-resistant NCRW affect durability?*
The model was run to examine how crop rotation and rotation-resistant NCRW affected durability of Cry34/35 corn. The model predicted that crop rotation had the biggest advantage when practiced only on the Cry34/35 corn fields and when the refuge fields were planted in the same location each year. Just as for WCRW, there are two modes of action operating in the rotated Cry34/35 corn fields: the direct effect of Cry34/35 and the effect on the progeny of insects laying their eggs in corn fields that could not survive since they hatched in soybean fields.

Rotation-resistant NCRW spend two winters as eggs in the soil; therefore, they act as a temporal refuge from selection for refuge. The model predicted that for in-field refuge options, rotation resistance approximately doubled the durability of Cry34/35 corn when the corn/soybean rotation was maintained and increased the durability 60% to 80% when corn was grown continuously. On the other hand, if the refuge was external in fixed locations, while Cry34/35 corn was rotated with soybean, the rotation resistance accelerated resistance evolution to Cry34/35. This is in contrast to the relative rates of adaptation predicted in WCRW. The NCRW rotation-resistant genotype was only exposed to Cry34/35 every second year, while the wild type was exposed every year. Each year, Cry34/35 corn would kill half of the proportion of rotation-resistant biotype as compared to the wild type and, thus, the frequency of the rotation-resistant biotype was increased from its initial value of 0.5. The rate of increase is affected by how Cry34/35 corn and the refuge were deployed. When the refuge was planted in the same location each year (continuous corn) and the Cry34/35 corn was rotated with soybean, the rate of increase was lowest.

Storer Modeling Summary

Using the lower end of the range of field studies to estimate dose, Cry34/35 event DAS-59122-7 corn expresses a dose that kills at least 99.75% of susceptible WCRW larvae. The model predicts that the durability is likely to be adequate with a 10% refuge, planted in-field or in an adjacent field. Durability is extended even further when there is market competition with other transgenic and non-transgenic rootworm management options. Cry34/35 corn has the potential to decrease the incidence of WCRW rotation resistance if used in rotation with soybean while an external refuge is planted to continuous non-rootworm protected corn. Both the Onstad *et al*. (2001) model and the Storer model (Storer 2003a; Storer 2003b, MRID No. 461239-19) predict that a 10% refuge would provide acceptable durability against WCRW resistance.

In contrast, Cry34/35 event DAS-59122-7 expresses a dose (midpoint of the range of the lower dose estimate and higher dose estimate, respectively) that kills at least 95 to 99% of susceptible NCRW larvae. The model predicts that the durability of a 20% refuge for wild-type NCRW at the higher dose estimate results in comparable durability of a 10% refuge for wild-type WCRW, and less durability at the lower dose estimate. Rotation resistance through extended diapause results in more than 50% longer durability.

Onstad and Guse WCRW model

Dow also discussed the WCRW simulation model of Onstad and his colleagues (Onstad *et al*. 2001). The Onstad *et al*. (2001) model of WCRW adaptation differs from the Storer model in that it is deterministic and non-spatial (the Storer models are stochastic and spatial). Two refuge structures were considered: external blocks and in-field strips. It is assumed that the block refuge is fixed (same location annually for maintaining large, non-selected populations) and the strip refuge moves (planted randomly each year). The difference between fixed and moving refuge drives the model. The most important parameter in the Onstad *et al*. (2001) model affecting durability of a rootworm-protected PIP was the functional dominance of the resistance allele (expressed as "allele expression").

If the allele was fully recessive, resistance did not occur in this model within 99 years. If the allele was fully dominant, resistance always occurred within 2–5 years for refuge sizes between 5% and 30%. For incompletely recessive alleles, the time to resistance depended on both dose and refuge size. For the 95%, 90%, and 80% doses, the functional dominance was calculated to be 0.5. The model predicted that the time to resistance in these runs was 3 to 9 model years with a 20% refuge, depending on whether the refuge was fixed (block) or moved from year to year (row strips). For a dose of 99.9% (i.e., a "practical high dose"), the functional dominance was calculated to be 0.009. In this instance, the time to resistance with the moving 20% refuge (strips) was 29 model years and with a moving 10% refuge was 22 model years. At the theoretical high dose, 100%, the functional dominance was 0, and resistance did not evolve within 99 model years with a 20% moving refuge and evolved in 52 model years with a 5% moving refuge. For a fixed 5% refuge, resistance did not evolve within 99 model years at 99.9% or 100% dose.

Using a low-end estimate, Cry34/35 event DAS-59122-7 corn expresses a dose of at least 99.75% against WCRW, which is similar to Onstad *et al*.'s (2001) designation of a "practical high dose." At this dose, the model predicts that a 5% refuge would give more than four times the durability of a 80%, 90%, or 95% dose PIP with a 20% refuge. A 10% refuge would give more than 20 model years of durability if the refuge is moved around each year (strips), and greater than 99 model years of durability if the refuge is fixed (blocks).

BPPD's Analysis of Proposed Computer Models

BPPD agrees with Dow and Pioneer's assessment of CRW adaptation using the Storer simulation models (Storer 2003a; Storer 2003b, MRID No. 461239-19) and Onstad *et al*.'s (2001) simulation model. Using the lower end of the range of field studies to estimate dose, Cry34/35 event DAS-59122-7 corn expresses a dose that kills at least 99.75% of susceptible WCRW larvae. The Storer simulation

model (Storer 2003b, MRID No. 461239-19) predicts that the durability is likely to be adequate with a 10% refuge, planted in-field or in an adjacent field. Durability is extended even further when there is market competition with other transgenic and non-transgenic rootworm management options. Cry34/35 corn has the potential to decrease the incidence of WCRW rotation resistance if used in rotation with soybean while an external refuge is planted to continuous non-rootworm protected corn. Both the Onstad *et al*. (2001) model and the Storer models (Storer 2003a; Storer 2003b, MRID No. 461239-19) predict that a 10% refuge would provide acceptable durability against WCRW resistance.

In contrast, Cry34/35 event DAS-59122-7 expresses a dose (midpoint of the range of the lower dose estimate and higher dose estimate, respectively) that kills at least 95% to 99% of susceptible NCRW larvae. The Storer model (Storer 2003b, MRID No. 461239-19) predicts that the durability of a 20% refuge for wild-type NCRW at the higher dose estimate results in comparable durability of a 10% refuge for wild-type WCRW, and less durability at the lower dose estimate. Rotation resistance through extended diapause results in more than 50% longer durability.

g. Technology Adoption

Product durability is affected by market penetration. Modeling efforts (Storer 2003b, MRID No. 461239-19) make the conservative assumption of complete adoption (i.e., all corn acres are planted in Cry34Ab1/35Ab1 corn) because the applicants do not wish the durability of the product to depend upon the presence of competing products (Storer and Lefko 2003, MRID No. 461239-18). The applicants stated, however, that actual adoption is likely to be significantly less than 100% and governed by multiple parameters (availability of alternate controls, economic and market factors, etc.)(Storer and Lefko 2003, MRID No. 461239-18). Implementation of a variety of controls, the most likely scenario, discourages over-reliance on a single control method and, consequently, helps to delay resistance development to control options (Gray 2001; Ostlie 2001; Carrière *et al*. 2004).

The assumption of 100% adoption, while not likely, is a reasonable one on which to base a conservative IRM plan to manage the Cry34Ab1/Cry35Ab1 corn durability.

2. Analysis of Dow/Pioneer's Proposed Cry34/35 Event 59122-7 Corn Durability Plan (2005 Assessment)

Dow and Pioneer proposed a Cry34/35 event 59122-7 corn durability plan that has the following elements: (1) structured refuge, (2) resistance monitoring, (3) remedial action plan, and (4) compliance and education. Simulation models were used to assist in evaluating and comparing structured refuge options (discussed previously). Using the model predictions, Dow and Pioneer were able to propose specific refuge guidelines. Given the uncertainties in understanding the dose, genetics and mechanism(s) of resistance, functional dominance, pest biology and ecology, and level of adoption of the technology, Dow and Pioneer have proposed a conservative insect resistance management (durability) plan. The proposed plan is "acceptable," with one exception—that of the size of the in-field strips (see discussion below). Additional data are needed to support independent treatment of the refuge for other pests, other than corn rootworm when corn rootworm may be present, but not targeted and its impact on corn

rootworm resistance management. Additional research should be conducted to further evaluate the sustainability of the proposed durability plan and confirm the assumptions made in the simulation models. Each aspect of Dow and Pioneer's proposed durability plan will be discussed below.

a. Structured Refuge

The guidelines for structured refuge address the following: refuge size, refuge deployment (proximity of the refuge to the Cry34/35 corn fields), and refuge management (acceptable chemical management of target pests in the transgenic fields and refuge and agronomic management). In developing the refuge guidelines, Dow and Pioneer stated that a goal was to ensure that the structured refuge would remain productive for the grower, while serving as an effective reservoir of susceptible target pests. The applicants' plan is designed around NCRW for which there is greater uncertainty surrounding the estimation of the Cry34/35 dose and, thus, the extent of functional dominance of resistance alleles. Based on the modeling simulations by Storer (2003b), this plan would then be more conservative for WCRW. Since WCRW is related to MCRW (Clark *et al*. 2001), the plan should also be applicable (and conservative) for MCRW. Additionally, the simulations by Storer (2003b) indicated that rotation resistance in the WCRW soybean biotype did not affect durability of Cry34/35 corn, while rotation resistance in NCRW populations (extended diapause) increased the durability of Cry34/35 corn. These simulations indicated the trait durability plan could reverse the spread of the soybean biotype WCRW.

Several assumptions make this plan conservative. The Monte Carlo simulations (see the earlier simulation models section) indicate that the choice of default parameter settings lie within the 5-percent worse-case settings. The assumptions of the genetics of resistance are conservative because it is unlikely that there will be incomplete recessiveness of the resistance allele, rather the more realistic expectation is for complete or near-complete functional recessiveness. Finally, the trait durability plan is based on the unrealistic expectation that 100% of the corn in a given area will be planted to Cry34/35 corn. Storer's simulations (Storer 2003b, MRID No. 461239-19) indicate that the plan would be effective at managing region-wide resistance evolution if only 75% of farms correctly implement the refuge.

Dow and Pioneer's Proposed Structured Refuge Guidelines

1. *Refuge size*. The use of Cry34/35 corn from event DAS-59122-7 would require an accompanying 20% refuge. The refuge size is governed by the greater uncertainties in the dose determination of NCRW to Cry34/35. Based on the modeling simulations for WCRW and NCRW (Storer 2003b, MRID No. 461239-19), the refuge size is considered to be conservative for WCRW (and MCRW).

2. *Refuge location*. Given the uncertainties regarding rootworm long-range dispersal, the rootworm refuge would be required to be planted within or adjacent (e.g., across the road) to the Cry34/35 corn field.

3. *Refuge management options*. The rootworm refuge must be managed in such a way that there is little or no yield loss to rootworms, but it is sufficiently productive of susceptible rootworm adults. Dow and Pioneer propose the following refuge management guidelines.

- The in-field refuge options may be planted as a single block or as a series of strips measuring at least two crop rows wide. (BPPD does not agree with this portion of the proposed plan as discussed later; at least four crop rows wide should be required.) Modeling (Storer 2003b, MRID No. 461239-19) showed that the strip width had a very small effect on durability. Single-row strips could be too narrow and allow too much larval movement across rows to sufficiently maintain low functional recessiveness.

- Seed mixtures of Cry34/35 and refuge corn are not permitted. Larval movement within rows would compromise the value of the effective dose of the Cry34/35 corn and allow enhanced heterozygote survival.

- If the refuge is planted on rotated ground, then Cry34/35 corn must also be planted on rotated ground. Refuge on rotated ground is likely to produce few individuals. Therefore, adult production would need to be mirrored in the Cry34/35 corn field to nullify the effect on resistance evolution.

- If the refuge is planted in continuous corn, the Cry34/35 field may be planted on either continuous or rotated land (option encouraged where WCRW rotation-resistant biotype may be present). The Storer simulations (Storer 2003b, MRID No. 461239-19) predicted that planting the refuge on continuous corn ground and rotating the Cry34/35 corn with a second crop can lead to a reduction in the prevalence of rotation-resistant WCRW.

- Banded application of soil insecticide is permitted in the refuge. As discussed previously, there are several studies that have shown that this control option permits very high survival of rootworm and, therefore, will not affect refuge performance but will protect the yield in the refuge making this option attractive to growers. Simulations by Storer (2003a and 2003b) were based on the assumption that the refuge will be managed by soil-insecticides in banded applications when egg populations warrant protection.

- Seed treatment is permitted in the refuge, either at a high rate for rootworm protection or at a low rate for controlling secondary soil pests. As discussed previously, insecticide seed treatments have been shown to have little impact on the number or timing of adult emergence and, therefore, are acceptable for use in a Cry34/35 corn structured refuge.

- If aerial insecticides are applied to the refuge for control of CRW adults, the same treatment must also be applied to Cry34/35 corn. It is important that aerial applications be properly timed and that egg population reductions are not reduced more in the refuge than the Cry34/35 corn fields. Application of adult-targeted aerial insecticides, applied at the same time to Cry34/35 corn areas and the refuge, will nullify any negative effects on the value of the refuge.

- The refuge and Cry34/35 corn may be treated with independent insecticide applications that comply with local integrated pest management guidelines, to control pests other than adult rootworms. Foliar sprays targeted at other pests, other than rootworm adults, will have a much lower effect on the rootworm egg populations. The timing of the applications is also important to avoid any significant impact on future adult emergence. (BPPD does not agree with this portion of the proposed plan at this time. Additional data are needed to address independent treatment of the refuge for other pests (not corn rootworm) and its impact on corn rootworm resistance management.)

- The rootworm refuge can be planted to any corn hybrid that does not express PIPs for rootworm control (e.g., lepidopteran-protected *Bt* corn, herbicide-tolerant corn, or conventional corn).

- The refuge and Cry34/35 corn should be sown on the same date, or with the shortest window possible between planting dates, to ensure that corn root development is similar among varieties.

- Based on simulations by Storer (2003a and 2003b) and Onstad *et al*. (2001), it may be best for growers to plant the rootworm refuge in the same location each year, as it allows the rootworm population to remain high and the durability of the trait is extended. This refuge can be protected with banded application of soil insecticides or with seed treatments. This option may be preferable to growers who wish to only think of their refuge design once and for growers who grow continuous corn. However, for those growers who need to employ crop rotation, a fixed refuge would be impractical.

BPPD's Analysis of Dow and Pioneer's Proposed Structured Refuge Guidelines

Given the uncertainties in understanding the dose, genetics and mechanism(s) of resistance, functional dominance, pest biology and ecology, and level of adoption of the technology, Dow and Pioneer have proposed a conservative insect resistance management (trait durability) plan for Cry34/35 corn. The trait durability plan, while based on the best available scientific information regarding rootworm biology and ecology, has also strongly considered grower feasibility and practicality. If the grower does not implement the structured refuge and manage it correctly, then the value of the trait durability plan is minimal. The refuge guidelines are designed around NCRW to which there is greater uncertainty surrounding the estimation of the Cry34/35 dose and, thus, the extent of functional dominance of resistance alleles. Based on the modeling simulations by Storer (2003a and 2003b), this plan would be more conservative for WCRW. Since WCRW is related to MCRW, the plan should also be applicable to and conservative for MCRW. Based on BPPD's analysis, the proposed plan is acceptable with two modifications: one to the proposed size of the in-field strips and the other to the agronomic treatment of the refuge.

1) BPPD disagrees with Dow and Pioneer's recommendation that the in-field strip width be ≥2 row strips.

Recent larval movement data, published by Hibbard *et al*. (2003), showed that between 0.75% and 6%

of larvae moved across rows. This represents a relatively high-end estimate of the number of larvae that could cross rows. This means that much narrower in-field strips should be sufficient to provide adequate protection from sublethal selection caused by CRW larval movement across rows and maintain low functional recessiveness. Any increase in sublethal selection would be offset by a greater probability that potentially resistant adults emerging from the *Bt* corn rows would mate with susceptible adults from the refuge row. Simulations by Storer (2003b) incorporated the Hibbard *et al*. (2003) larval movement data to compare how strip width can affect the durability. These simulations predicted that narrower in-field strips, between 2 and 10 rows, did not affect trait durability. Single-row strips could be too narrow and allow too much larval movement across rows to sufficiently maintain low functional recessiveness. While the Storer simulations (Storer 2003b, MRID No. 461239-19) indicate that trait durability is virtually unaffected by strip width, in-field strips of ≥4 rows would provide some marginal, additional protection (see Figure 4 of MRID No. 461239-19) and also provide the advantage of being more compatible with the current in-field strip width requirement, ≥4 row strips (≥6 row strips preferred) for lepidopteran-protected *Bt* corn hybrids. In-field strips of ≥4 rows would also be practical and flexible for the grower, just as a ≥2 row strips. Because Cry34/35 will likely be stacked with Cry1F, for example, a recommendation of ≥4 row strips will provide the grower a more easily understandable and consistent message regarding the width of in-field strips and reduce confusion associated with in-field row strips for lepidopteran-protected *Bt* corn hybrids, rootworm-protected *Bt* corn hybrids, and those *Bt* corn hybrids that have both traits. Overall, BPPD believes that a requirement of ≥4 row in-field strips will simplify refuge deployment and potentially increase compliance with refuge requirements.

Note: Herculex® RW (Cry34/35 corn) was registered with an in-field strip width refuge requirement of at least 4 rows.

2) BPPD disagrees with Dow and Pioneer's recommendation for independent treatment of the refuge for other pests (not corn rootworm).

Properly timed aerial sprays are very effective at reducing the corn rootworm egg population in the field, and it is important that the refuge productivity is not reduced more than the Cry34/35Ab1 corn field productivity. Sprays targeted at adults in the current year's refuge may significantly reduce the number of adults available for mating with Cry34/35-resistant survivors in Cry34/35 corn fields. Sprays targeted at adults in the area to be used as refuge the next year may significantly reduce the number of adults emerging from that area the following year, reducing refuge effectiveness. BPPD agrees with Dow and Pioneer's conclusion that insecticide applications that function to kill refuge-produced adult CRW must also be applied to the Cry34/35 corn field to nullify any negative impact on the value of the refuge.

Foliar sprays targeted at pests other than rootworm adults are thought to have a much lower effect on the rootworm egg populations. If the sprays are applied before peak adult emergence, then residual activity may be too low to have a significant impact on future adult emergence. Similarly, if the sprays are applied after peak egg-laying, they are likely to have a reduced impact on egg numbers. Therefore, if the treatment windows for other pests do not overlap with the critical period in which corn rootworms would be treated, growers could be permitted to use aerial sprays on any fields (refuge or not) if they are needed for management of other pests besides adult corn rootworm. Dow and Pioneer proposed

allowing independent treatment of the refuge and *Bt* fields if pests other than corn rootworm are targeted. While this would prevent unnecessary spraying of the *Bt* corn fields, additional data are needed to determine if the treatment windows for other insect pests, e.g., European corn borer (2nd generation), Southwestern corn borer, spider mites and corn rootworm overlap and what impact treatment for other pests would have on the effectiveness of the corn rootworm refuge. At this time, pests other than adult corn rootworms can only be treated with CRW-labeled insecticide on the refuge acres without treating the Cry34/35 acres if treatment occurs when corn rootworms are not present. Pests on the Cry34/35 acres can be treated as needed without having to treat the refuge.

BPPD agrees with Dow and Pioneer that the use of seed and granular insecticide treatments to control CRW larvae should be allowed on refuge acres, even if not applied to the Cry34/35 corn field, since these treatments are shown to be non-high dose controls (Meinke *et al*. 1998). Data have been collected that support the premise that banded insecticides and seed treatments will allow considerable survival of rootworms to adult (Sutter *et al*. 1991; Cormier and Martel 1997). There is ongoing research that suggests that clothianidin and Cruiser® seed treatments have a minimal impact on CRW fitness.

Crop rotation will have a high impact on larval survival, e.g., corn-soybean rotations. If a refuge is planted on rotated ground, it will encourage adaptation to Cry34/35 unless the Cry34/35 corn is also planted on rotated ground. This is because susceptible corn rootworm production is lower on rotated ground (i.e., soybean is not a host of corn rootworms). BPPD agrees with Dow and Pioneer that if the refuge is planted on rotated ground, then Cry34/35 corn must also be planted on rotated ground.

Based on simulation models (Storer 2003a; Onstad *et al*. 2001), it may be best for growers to plant the rootworm refuge in the same location each year, as it allows the rootworm population to remain high and the durability of the rootworm-protection trait to be extended. This refuge can be protected with banded application of soil insecticides or with seed treatments. For those growers who need to employ crop rotation, a fixed refuge would be impractical.

Based on BPPD's analysis, a 20% structured non-lepidopteran *Bt* corn refuge planted adjacent to, or as ≥4 row strips within the Cry34/35 corn field (rather than ≥2 row strips), is sufficiently conservative to mitigate CRW resistance to the Cry34Ab1 and Cry35Ab1 proteins expressed in event DAS-59122-7 corn hybrids. BPPD agrees with all of Dow and Pioneer's proposed agronomic management recommendations except for one. Additional data are needed to address independent treatment of the refuge for other pests (not corn rootworm) and its impact on corn rootworm resistance management. Additional research on corn rootworm pest biology and ecology, genetics and mechanisms of resistance, functional dominance, fitness costs, cross-resistance potential, dose, and mode of action will also be useful to evaluate the proposed durability plan and confirm the assumptions made in the simulation models.

Note: Herculex® RW (Cry34/35 corn) was registered with the following insecticide use restrictions to address the concerns regarding refuge treatment discussed above:

- If aerial insecticides are applied to the refuge for control of CRW adults, the same treatment must also be applied in the same time-frame to Cry34/35 corn.

- Pests other than adult corn rootworms can only be treated with CRW-labeled insecticide on the refuge acres without treating the Cry34/35 acres if treatment occurs when adult corn rootworms are not present. Pests on the Cry34/35 acres can be treated as needed without having to treat the refuge.

- Application of soil insecticide is permitted in the refuge.

- Seed treatment is permitted in the refuge, either for rootworm protection or for controlling secondary soil pests.

b. Resistance Monitoring

The need for proactive resistance detection and monitoring is critical to the survival of *Bt* technology. Consequently, the Agency mandates that a resistance monitoring plan must be implemented for all registered *Bt* corn products. Resistance can evolve regionally or as a local increase in resistance (*r*-) allele frequency. The resistance monitoring plan designed for Cry34/35, an adaptation of the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) program developed for lepidopteran-protected corn, will attempt to detect either local or regional resistance early enough to initiate effective remedial action (see below). Resistance monitoring will be implemented through a two-pronged approach, field reports of unexpected damage and population testing and sampling.

Field Reports of Unexpected Damage

Field monitoring is intended to detect localized resistance. The applicants are working to accumulate a data set of Cry34/35 field efficacy under a wide range of pest pressures, soil types, and environmental conditions. This information will be used to develop efficacy guidelines that applicants will use to determine whether the level of rootworm damage in their fields is considered "normal" or outside the range of normal.

Once field guidelines have been set, reports of unexpected CRW damage will be evaluated to determine if crop damage is due to failure of Cry34/35 or to some alternative factor. Specifically, the applicant will confirm that plants under question were expressing Cry34/35Ab1 proteins, that the Cry34Ab1 and Cry35Ab1 proteins were expressed at expected levels in corn plant roots, and that damage was caused by target CRW pests. If, after having completed the confirmatory procedures described above, the damage report still points to Cry34/35 failure, the case will be considered "suspected resistance" and remedial action will be implemented. Eggs or adults will also be collected for the "Population Testing and Sampling" program.

Population Testing and Sampling

Population sampling and testing will be used to identify area-wide increases in *r*-allele frequency before widespread field failure occurs. *R*-allele detection programs will employ dose-response and discriminating dose bioassays to detect non-recessive and fully recessive *r*-alleles in the homozygous state (ABSTC 2003).

CRW species are challenging to rear in the laboratory. Of the identified target pest species, scientists have had the most success rearing WCRW and have had little success with NCRW. Consequently, monitoring through population sampling will focus on WCRW and include NCRW populations when available.

A bioassay that "accurately and reliably" determines dose-response relationships for WCRW is being used by a third-party laboratory to establish baseline sensitivity. Efforts to establish baseline sensitivity will continue through the early years of commercial Cry34/35 corn deployment, before significant selection pressure is placed on rootworm populations. Dow and Pioneer are also working to develop a "high-throughput diagnostic screen," which uses Cry34/35 seedlings to determine discriminating dose. The applicants state that this screen will be able to identify potential field-resistant insects, may be able to detect heterozygotes for *r*-alleles, and will provide estimates of phenotype and genotype frequencies.

European and southwestern corn borer are multivoltine insects that produce one to three generations per year. Monitoring programs developed for these pest species evaluate every second or third generation for resistance. In contrast, rootworm species are univoltine, meaning that they produce a single generation of insects per year. Since corn borer is evaluated every second or third generation (two or three generations produced per year), the applicants argue that a similar level of monitoring intensity would be achieved by testing four or five CRW populations per year.

Rootworm populations will be collected from targeted areas where resistance is most likely to develop. Market penetration of Cry34/35 is considered the most important factor in determining the risk for resistance. Consequently, county sales data will be used to identify areas with the greatest Cry34/35 adoption rates.

BPPD's Analysis of the Proposed Resistance Monitoring Plan

BPPD agrees with Dow and Pioneer's approach to resistance monitoring. Detecting shifts in the frequency of resistance genes (i.e., susceptibility changes) through resistance monitoring can be an aggressive method for detecting the onset of resistance prior to widespread crop failure. As such, the utilization of sensitive and effective resistance monitoring techniques is critical to the success of an IRM plan.

Dow and Pioneer state that a third-party laboratory is using a bioassay that "accurately and reliably" determines dose-response relationships for WCRW to establish baseline sensitivity. While BPPD agrees

that a sensitive and reliable bioassay should be developed, these efforts are currently under development. Once this work is completed, the applicants need to develop guidelines as to what level of root damage will be expected under various conditions, and what level of rootworm control is normally achieved. Applicants will investigate grower reports of reduced product performance to determine whether damage is unexpected. However, without guidelines as to what is "acceptable" rootworm damage, then it will not be possible to determine what the "unexpected" rootworm damage is. Once these guidelines are established, it will be possible to define what is "suspected resistance" as described under the "Remedial Action Plans" to mitigate the spread of putative resistant populations. Because of the importance of these guidelines, it should be required that the applicants develop interim rootworm damage guidelines by 2008 and final guidelines by 2010 and submit these to the Agency for review.

Dow and Pioneer also mention that they are working to develop a "high-throughput diagnostic screen," which uses Cry34/35 seedlings to determine discriminating dose. The premise is that this screen will be able to identify potential field-resistant insects and provide estimates of phenotype and genotype frequencies. The applicants state that the screen should also be able to detect heterozygotes for *r*-alleles. While this type of screen would be highly valuable, the submission does not provide enough information about this diagnostic test. It is required that the applicants provide BPPD with a detailed explanation and validation (steps for) of the "high-throughput diagnostic screen" if it is to be considered an acceptable addition to present monitoring strategies.

Finally, it is recommended that Cry34/35 corn be given the following resistance monitoring requirements:

1. The applicants should monitor for resistance and/or trends in increased tolerance for corn rootworm. Sampling should be focused in those areas in which there is the highest risk of resistance development. The applicants should submit to EPA an appropriate sampling protocol as part of its monitoring plan.
2. The applicants should provide EPA a description of its resistance monitoring plan by January 31, 2006. The description would include the following: sampling (number of locations and samples per locations), sampling methodology, bioassay methodology, standardization procedures (including quality assurance/quality control provisions), detection technique and sensitivity, and the statistical analysis of the probability of detecting resistance. A final resistance monitoring plan is required by January 31, 2008.
3. The applicants should develop an appropriate discriminating or diagnostic dose assay by January 31, 2008.
4. The applicants should follow-up on grower, extension specialist, or consultant reports of unexpected damage or control failures for corn rootworm.
5. The applicants should provide EPA with an annual resistance monitoring report.

Note: A discussion of the ongoing resistance monitoring program for Cry34/35, including the conditions listed above and results from the annual testing, is contained in the "Conditionally Required Data for Cry34/35 Corn (2010 Update)" section of this chapter.

c. Remedial Action

The remedial action plan is designed as a tiered approach for mitigating WCRW, NCRW, and MCRW resistance development to the Cry34/35Ab1 proteins. The following program summary describes, in order of events, the steps that will be taken to implement a remedial action plan if resistance to target pests is confirmed.

1. **Definition of Suspected Resistance**: Resistance will be suspected if investigations of unexpected damage reports show that:

a. Implicated corn plant roots were expressing Cry34/35Ab1 proteins at the expected levels;
b. Alternative causes of damage or lodging, such as non-target pest insect species, weather, physical damage, larval movement from alternate hosts, planting errors, and other reasonable causes for the observations, have been ruled out;
c. The level of damage exceeds guidelines for expected damage.

If resistance is "suspected," the applicants will instruct affected growers to use alternate pest control measures such as adulticide treatment, crop rotation the following year, or soil or seed insecticides the following year. These measures are intended to reduce the possibility of potentially resistant insects contributing to the following year's pest population.

2. **Confirmation of Resistance**: Resistance will be confirmed if all of the following criteria are met by progeny from the target pest species sampled from the area of "suspected resistance":

a. The proportion of larvae that can feed and survive on Cry34/35 roots from neonate to adult is significantly higher than the baseline proportion (currently being established);
b. The median lethal concentration (LC_{50}) of the test population exceeds the upper limit of the 95% confidence interval for the LC_{50} of a standard unselected population and/or survival in the diagnostic assay is significantly greater than that of a standard unselected population, as established by the ongoing baseline monitoring program;
c. The ability to survive is heritable;
d. Cry34/35 plant assays determine that damage caused by surviving insects would exceed economic thresholds;
e. The identified frequency of field resistance could lead to widespread product failure if subsequent collections in the affected field area(s) demonstrated similar bioassay results.

3. **Response to Confirmed Resistance**: When resistance is "confirmed," the following steps will be taken:

a. EPA will receive notification within 30 days of confirming resistance;
b. Affected customers and extension agents will be notified about confirmed resistance;

c. Affected customers and extension agents will be encouraged to employ alternative CRW control measures;
d. Sale and distribution of Cry34/35 corn in the affected area will cease immediately;
e. A long-term resistance management action plan will be devised according to the characteristics of the resistance event and local agronomic needs.

BPPD's Analysis of Proposed Remedial Action Plan

BPPD agrees with the general framework for the Remedial Action Plan; however, because the "baseline sensitivity" has not been calibrated, this plan cannot be implemented. The submission states that mitigation measures will be initiated when unexpected levels of CRW damage occur. However, confirmation of insect resistance cannot be done until levels of baseline sensitivity are determined (see previous discussion on "Resistance Monitoring"). Consequently, it should be required that baseline sensitivity be established within two years of product commercialization. Unexpected damage guidelines and confirmation of target pest resistance (using baseline sensitivity information) will be used to initiate a remedial action plan when needed.

Note: Baseline susceptibility data through the 2008 growing season are discussed in the "Conditionally Required Data for Cry34/35 Corn (2010 Update)" section of this chapter. However, a credible diagnostic assay may need to be developed for resistance confirmation (e.g., Sublethal Seedling Assay; Nowatzki *et al*. 2008) so that a functional remedial action plan can be enacted.

d. Compliance

Compliance programs are important in that they encourage growers to comply with IRM requirements, while providing mechanisms by which registrants can be held accountable for noncompliant growers. The compliance program presented in this submission mirrors those developed for existing *Bt* corn registrations. Program components include the following: grower and sales force education/training programs that include workshops and educational pamphlets/brochures; contractual technology agreements that require grower adherence to IRM requirements; public relation activities, including media interviews, press releases, and conferences; and an annual affirmation program, which includes use of seed bag tags and tag language.

Grower Education

The applicants state that "grower education is the single most important element of any strategy for promoting compliance with the IRM requirements." The grower education program described in this submission is similar to that developed for other registered insect-protected corn products, so growers are familiar with the proposed concepts.

BPPD's Analysis of Grower Education and Compliance Assurance Program

Grower education and compliance are critical to the success of the trait durability plan. The applicants' grower education plans are acceptable. However, the applicants have not presented any additional information as to their compliance assurance program analogous to what has previously been required for all *Bt* corn PIP products. It is recommended that a compliance assurance program be required and submitted to the Agency that is in concert with existing *Bt* corn PIP products. Computer simulations by Storer (2003b) have shown that the level of refuge deployment needed for the trait durability plan to be fully effective depends on the level of technology adoption (see previous discussion under "Computer Simulation Models"). The proposed trait durability plan is based on 100% adoption, a very conservative assumption. However, as a worst-case scenario, refuge implementation by 75% of farmers would be needed for the proposed plan to be fully effective. At lower levels of adoption such as 75%, only refuge implementation by 50% of farmers would be effective in managing region-wide resistance. To ensure refuge implementation is high, a compliance assurance program should be required.

Note: A discussion of the ongoing compliance program for Cry34/35 corn is contained in the "Conditionally Required Data for Cry34/35 Corn (2010 Update)" section of this chapter.

e. Annual Reports

It is required that the applicants provide BPPD with the following annual reports: resistance monitoring, compliance assurance program, and sales data at the state level (county level data to be available if requested). Data from the resistance monitoring and compliance reports are discussed in the next section.

3. Conditionally Required Data for Cry34/35 Corn (2010 Update)

As part of the terms and conditions of the Herculex® RW (Cry34/35 corn) registrations, the registrants were required to submit programs for resistance monitoring and refuge compliance. In addition, the registrants were required to submit annual reports summarizing the data collected under these requirements. The following elements of resistance monitoring and compliance were required:

- Resistance Monitoring
    - Description of the steps to be taken to establish corn rootworm baseline sensitivity and damage guidelines (due January 31, 2006);
    - Submission of plan. Description of the program including baseline sensitivity, sampling (number of locations and samples per locations), sampling methodology, bioassay methodology, standardization procedures, detection technique, sensitivity, and the statistical analysis of the probability of detecting resistance, and an interim description of rootworm damage guidelines (due January 31, 2008);
    - Submission of rootworm damage guidelines (for unexpected pest damage) (due January 31, 2010);

- Development of diagnostic dose assay/high throughput screen (due January 31, 2008);
- Annual report of the insect resistance monitoring program. Results of monitoring and investigations of damage reports (due August 31st each year, beginning in 2008).

- Compliance

  - Written description of Compliance Assurance Program (due January 31, 2006);
  - Grower agreements. Proposed system to assure growers sign grower agreements and affirmation system to assure annual affirmation by growers of their IRM obligations (due January 31, 2006);
  - Annual report on Compliance Assurance Program (CAP) activities and results. Third-party grower survey, on-farm visitation program, phased-compliance report, tips and complaints, and grower education programs (due January 31st each year, beginning in 2007);
  - Grower education program for Cry34/35 corn. Subsequent changes to the grower education program must be included in the annual compliance assurance program report (due January 31, 2007).

Dow and Pioneer have submitted all of the required reports as mandated under the terms of registration. A discussion of the reports and data for the resistance monitoring and compliance programs for Cry34/35 corn follows in the next sections.

a. Resistance Monitoring

Monitoring Plan for Cry34/35 Corn (2008)

A preliminary resistance monitoring program was submitted to EPA in 2006 (reviewed in U.S. EPA 2006a). This submission described work to establish baseline susceptibility data for Cry34/35, develop a diagnostic bioassay (sublethal seedling assay), and develop unexpected pest damage guidelines.

Dow provided a revised monitoring plan in 2008 (MRID No. 473342-01), which is based on the framework of the ABSTC document titled "Updated Monitoring Plan for *Bt* Corn." Specifically, Dow addressed the following elements of monitoring: initial baseline sensitivity, sampling plan, bioassay methodologies and analyses, probability of detecting resistance, and an interim description of process for developing rootworm damage guidelines.

Monitoring for CRW resistance to the *Bt* crystal protein Cry34/35Ab1 relies on two approaches: population screening (random sampling, F_1 screen with diet bioassays, and possible Sublethal Seedling Assay) and field observations of product performance by growers, consultants, and extension agents. To understand the biological relevance of any future variation in target pest response to Cry34/35Ab1 and whether changes in susceptibility are due to selection to the protein, the population variation in sensitivity needs to be understood.

Sampling for CRW resistance will focus on WCRW (one of three *Diabrotica* target pests in the US) because this species represents the worst-case scenario (wide geographic distribution, greater abundance, and hence more likely to evolve resistance to *Bt* protein first) and is coordinated by the IRM technical subcommittee of ABSTC. Collection efforts will focus on regions where WCRW pest pressure is greatest, and CRW-protected *Bt* corn adoption is greatest. Such areas of focus are Illinois, Iowa, and Nebraska; however, Indiana, Missouri, Wisconsin, Minnesota, and Kansas are also potential candidates for sampling collections. ABSTC's core sampling area is divided into three regions: Region 1 consists of eastern Illinois and Indiana (rotation-resistant variant); Region 2 consists of western Illinois, Iowa, and Missouri (wild-type variant); and Region 3 consists of Nebraska and Kansas (organophosphate-resistant variant). Sampling locations will change on a yearly basis, and sampling intensity will reflect adoption of CRW-protected *Bt* corn. ABSTC will obtain coordinates of sampling locations so that re-sampling in subsequent years can occur when needed. Fifteen or 16 population subsamples from different counties across the sampling region will be collected, and colonies reared and maintained in the lab. Each collection should consist of 2,000 adult beetles to ensure a minimum of 5,000 eggs from at least eight of the total samples collected.

The *diet bioassay tool* is available to monitor for changes in WCRW susceptibility to Cry34/35Ab1 and the *Sublethal Seedling Assay* (SSA) (Nowatzki *et al*. 2008) may be available as well. It is Dow's intention to eventually select for use a single method once the advantages and validity of each approach have been verified. A final description of how the SSA can be used in resistance monitoring should be available in 2010.

BPPD's Analysis of Cry34/35 Resistance Monitoring Plan

BPPD has reviewed Dow's revised monitoring plan for DAS-59122-7 and has found it adequate for Cry34/35 (see review in U.S.EPA 2009c). BPPD recommends that Dow/Pioneer consider the use of the Sublethal Seedling Assay (Nowatzki *et al*. 2008) for detecting shifts in susceptibility rather than the diet bioassay approach (EC_{50}/LC_{50} data). Nowatzki *et al*. (2008) were able to show that the SSA was more sensitive in detecting shifts in susceptibility than the bioassay because it used the increased sensitivity of a sublethal measure.

BPPD also recommends that Dow/Pioneer address monitoring for resistance with northern corn rootworm. Baseline susceptibility data have been developed in the past by the Siegfried lab at the University of Nebraska, which now can serve as a reference. Past difficulties with rearing of NCRW and arguments of limited geographic distribution should not be the driving factors for failure to monitor for resistance in this corn pest. Efforts to develop reliable mass rearing and bioassay methods for NCRW should be continued.

Unexpected Pest Damage Guidelines (2010)

The few follow-ups to unexpected pest damage reports showed that most crop damage was due to weather-related causes rather than trait failure. The "*interim unexpected pest damage guidelines*" (submitted in 2008) encompassed the variability seen in registrant-generated field trial data from 2005–

2007. The Iowa State 0–3 Node Injury Scale (NIS) was used to assess root damage and assign ratings. To create worst-case guidelines, only results from locations where the average damage to the untreated isoline control exceeded a rating of 1.0 were used. Since environmental and ecological factors affect both trait performance and manifestation of rootworm damage and can cause outliers from the expected distribution, the interim guidelines for unexpected pest damage call for assessment of reports of root damage to be considered on a case-by-case basis.

Final unexpected pest damage guidelines were submitted in 2010 (MRID No. 480455-01). Dow reported that corn rootworm damage on Cry34/35-protected *Bt* corn was a function of the non-high dose effects of the crystal toxin and environmental conditions. Because of the multitude of factors playing a potential role in root-feeding damage, stalk and root lodging, Dow stated that it was impossible to derive a threshold damage level (i.e., NIS score) that would qualify as "unexpected" for all possible scenarios. The following are factors that affect the root damage levels:

- Rootworm population pressure
- Rootworm egg distribution across fields
- Field history (previous crop)
- Soil conditions in the previous year (affecting egg distribution)
- Soil structure and type
- Use of insecticides
- Use of fertilizers
- Presence of grassy weeds as alternate larval plant hosts
- Presence of off-type plants not expressing the toxin
- Presence of volunteer corn
- Soil moisture at rootworm egg hatch
- Timing of rootworm egg hatch and corn root growth
- Duration of rootworm egg hatch
- Size of root mass at the time of rootworm feeding
- Re-growth of corn roots following feeding
- Root damage due to other agents other than corn rootworm
- CRW species mix
- Soil chemical environment
- Soil fertility
- Winter conditions (e.g., temperature, snow cover, freeze-thaw cycles)
- Weather events before and during the period of rootworm feeding (storms, drought, etc.)
- Presence of CRW pathogens and predators
- Density-dependent CRW larval competition for feeding sites
- Phenotypic variation in expression of the proteins
- Temporal and spatial pattern of Cry34/35 concentration in roots
- Rootworm ability to find suitable host tissue
- Chronic effects of Cry34/35 on larvae
- Environmental impacts on corn growth and development

Both Dow and Pioneer have conducted field trials with their corn rootworm products expressing DAS-59122-7 and have found that, under extreme conditions and in the absence of resistance, Cry34/35 may not provide economic protection in part due to its non-high dose nature.

Dow and Pioneer have therefore set up their own protocol for investigating reports of unexpected CRW damage on DAS-59122-7 corn. A field visit will occur as soon as reasonably possible following a grower complaint. Lodging, stunting, and other symptoms will be investigated through the entire affected area on DAS-59122-7 expressing *Bt* and non-*Bt* corn plants, and the cause of damage will be determined (CRW or other pest damage, adverse weather, or other factors). If causes other than CRW or planting error do not explain the damage, then plants will be sampled from the affected field and root injury scored and plant samples will be tested to verify the presence of the trait. If the trait was expressed in the injured plants, then Dow proposed to collect more root samples from the affected field(s) to identify whether inadvertent seed mixing occurred. If the trait was expressed in these additional samples and non-expressing plants were not present in significant numbers, then Dow would escalate the incident for further investigation.

Further investigation involves collecting adult CRW and root tissue for expression analysis. If the expression analysis verified the expected expression level in roots and other factors did not explain the damage levels observed, then the collected CRW samples would be submitted to a third-party laboratory for bioassay analysis, which would determine whether the population samples are less sensitive to Cry34/35 than normal.

Dow expects this process to last up to a year. However, when damage reports come in too late to collect adults, then the investigation would start in the next growing season. The field and surrounding area would be assessed and, if damage cannot be explained by any other factors, then adults would be collected for rearing and consequent bioassay purposes.

Dow proposes that resistance would be confirmed if two years of unexplained damage and significantly lower than normal sensitivity to Cry34/35 are observed in offspring of collected adults. Dow's response to confirmed resistance was outlined in the registration letters and triggers the long-term resistance management remedial action plan according to the characteristics of the *Bt* trait and local agronomic needs.

BPPD's Analysis of Unexpected Damage Guidelines

Dow proposed that a damage threshold was no longer feasible to use as an indicator for potential CRW field resistance because other non-toxin related factors could act together in such a manner as to mimic field resistance. BPPD does not concur with Dow's proposition nor with their arguments to completely abandon the "damage threshold level" as an indicator for CRW field resistance and proposes a revised approach instead (see review in U.S. EPA 2010c). At the time, the 2002 SAP proposed a Node Injury Score threshold level of 0.5 for Cry3Bb1 as an indicator of "damage across the economic threshold level." BPPD notes that this value cannot be transferred to other single toxins, or even pyramided toxins, and that each report of unexpected CRW damage needs to be dealt with on an individual basis. The "damage threshold level" as expressed by node injury scores is an important tool for alerting the stakeholder community and EPA that some unusual event in *Bt* corn has occurred; however, this tool should not be used alone and out of context.

The following points should be considered when following up with a reported incident of unexpected pest damage:

1) The inherent dose of the toxin to control CRW (high dose vs. non-high dose control);
2) Prior use and crop history in the *Bt* field where excessive damage was observed;
3) Damage on non-*Bt* plants in the same field or immediately adjacent to the *Bt* plants;
4) Insect pressure during that corn-growing season (low vs. moderate vs. high); and
5) Weather pattern during the corn-growing season and possible effects on *Bt* protein expression and pest population dynamics.

When greater node injuries are observed on the refuge corn than on the *Bt* corn, then this could be an indication that damage is not due to resistance but some other factors. Likewise, if *Bt* plants have less damage than refuge plants that were treated with insecticides, then the cause of damage is less likely due to resistance. However, if damage levels on *Bt* plants exceed the level observed on non-*Bt* plants, then field resistance could be a concern. A NIS damage threshold level of 1.0 may be suitable for single toxin, non-high dose CRW products such as DAS-59122-7. When CRW pressure is exceedingly high, however, then both *Bt* and refuge corn will incur greater damage. Under these circumstances, a threshold level of 1.5 may be appropriate to use by the registrants.

BPPD concluded that Dow/Pioneer's response strategy for dealing with unexpected damage reports in DAS-59122-7 corn is protracted and could be improved (U.S. EPA 2010c). For example, Dow/Pioneer stated that they would first survey the area with unexpectedly high CRW damage, then collect root tissues and conduct tissue tests to verify appropriate *Bt* expression. If the *Bt* toxin was expressed, Dow/Pioneer proposed to collect more tissue samples to rule out planting error and then, if unable to rule out CRW resistance, begin collecting adults. With this sequence of steps, BPPD noted that Dow/Pioneer could lose valuable time to obtain adult CRW collections. BPPD recommended that Dow/Pioneer collect adult CRW immediately after first being alerted to unusually high root damage to DAS-59122-7 corn. These adults could be held until all the other proposed causative agents have been ruled out. The benefit to BPPD's approach could be that if field resistance is the cause of product failure and the unexpected damage reports, then Dow/Pioneer would already have a population sample available and could start the rearing process, bioassays, and have results to report possibly within one year rather than Dow/Pioneer's worst-case scenario of two years. Subsequent to BPPD's review, Dow/Pioneer provided clarification that most of the sequential steps outlined to test for resistance do in fact occur during the initial visit (Dow and Pioneer 2010). This includes testing for the presence of Cry34/35 and starting insect collections (except when reports are received too late in the season and CRW are not available to sample). If this is the case, the registrants will have addressed BPPD's primary concern regarding delayed insect collection.

When CRW adults are collected in response to an unexpected damage report, BPPD recommends that samples originate from the *Bt* field of concern and the adjacent refuge corn. However, studies by Naranjo (1990) and Coats *et al*. (1986) suggest that a significant fraction of the female WCRW population engages in longer distance and pre-ovipositional dispersal. Hence, collections of putative

resistant individuals should also be obtained past the ½ mile radius from the affected field should resistance be detected.

Resistance Monitoring Results (Through 2008)

In 2008 (report detailed in MRID No. 479008-01), nine populations of western corn rootworm were collected across the Corn Belt where the species was most abundant and where all three biotypes occurred (see below). At each collection site, 2,000 individuals were collected in one day and maintained for oviposition purposes by Custom Bio-Products in Maxwell, Iowa—an independent laboratory conducting bioassays for Dow since 2006.

ABSTC Region 1, rotation-resistant variant
- McLean County, Illinois
- Piatt County, Indiana
- Henry County, Illinois

ABSTC Region 2, wild-type variant
- Scott County, Iowa
- Palo Alto County, Iowa
- Clinton County, Iowa

ABSTC Region 3, organophosphate-resistant variant
- York County, Nebraska
- Polk County, Nebraska
- Seward County, Nebraska

Concentration response bioassays were conducted using neonates from each of the nine collected 2008 populations; the same protocol was followed as for the baseline susceptibility studies described in Dow's report to the Agency (MRID No. 473342-01). The protein used in the assays consisted of microbially produced, partially purified powders mixed in a ratio of 3:1 by active ingredient weight (Cry34 to Cry35). Concentrations tested included 0, 0.56, 1.67, 5.0, 15.0, and 45.0 micrograms per square centimeter ($\mu g/cm^2$). Growth inhibition (EC_{50}) and mortality (LC_{50}) were measured from neonates of the collected insects and compared to the established baseline susceptibility. In addition, the variation in sensitivity was measured using a ratio of the greatest to lowest LC_{50} and ratio of greatest to lowest EC_{50} and compared to prior results.

Data from 2008 were reported in MRID No. 479008-01 (reviewed in U.S. EPA 2010c). Estimated EC_{50} and LC_{50} values for the nine geographically distinct 2008 WCRW populations ranged from 2.0 $\mu g/cm^2$ to 7.9 $\mu g/cm^2$ and 6.2 $\mu g/cm^2$ to 15.4 $\mu g/cm^2$, respectively. For EC_{99} and LC_{90} measures, the estimates ranged from 83.8 $\mu g/cm^2$ to 596.6 $\mu g/cm^2$ (EC_{99}) and 58.4 $\mu g/cm^2$ to 613.6 $\mu g/cm^2$ (LC_{90}). The 2008 results are summarized in Table 1 below, along with data from susceptibility assays in previous seasons

(2004–2008). Dow concluded that the sensitivity of WCRW, as measured by the ratio of highest to lowest LC_{50} and EC_{50}, was similar to those in previous years (see Figure 1).

Table 1. Cumulative WCRW Cry34/35 Susceptibility Data (2004–2008).

Year	EC_{50} (µg/cm^2)	EC_{99} (µg/cm^2)	LC_{50} (µg/cm^2)	LC_{90} (µg/cm^2)
2004–2005	0.8–1.1		2.0–2.4	
2006	0.8–2.3	21.0–56.6	1.2–7.3	9.2–78.1
2007	1.0–3.7	51.9–213.6	3.0–11.5	23.9–463.5
2008	2.0–7.9	83.8–596.6	6.2–15.4	58.4–613.6

BPPD generated table from data submitted by Dow.

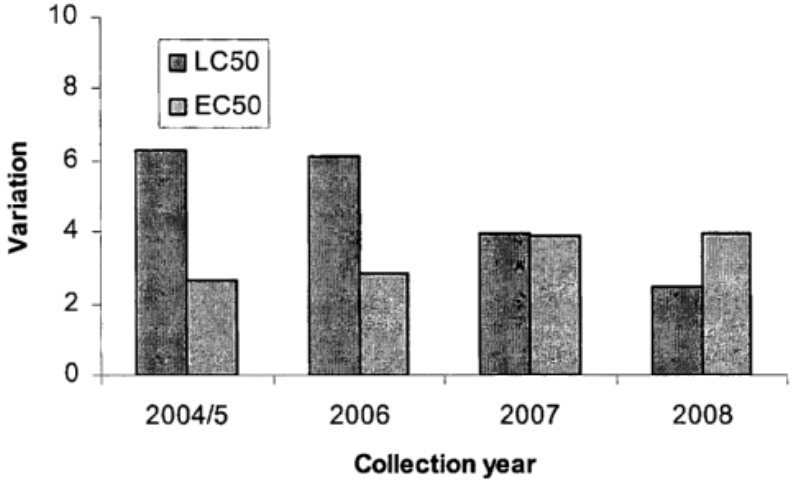

Figure 1. Variation in response to Cry34/35 (as measured by the ratio of high:low LC_{50} and EC_{50}) among WCRW field populations collected between 2004 and 2008 (Dow figure extracted from MRID No. 479008-01).

Overall, there has been a trend of decreasing susceptibility in WCRW to Cry34/35 when the data are evaluated on a qualitative basis. All measured and extrapolated mean EC and LC values were higher in 2008 than in previous years (see ranges for EC_{99} and LC_{90} values in Table 1). From 2006 to 2008, the range of mean EC_{99} and LC_{90} values obtained in bioassay tests or from extrapolations increased from 21.0–56.6 µg/cm^2 to 83.8–596.6 µg/cm^2 and 9.2–78.1 µg/cm^2 to 58.4–613.6 µg/cm^2, respectively. Dow and Pioneer have indicated, however, that the year-to-year differences in EC_{99} and LC_{90} are not statistically relevant (Dow and Pioneer 2010). The EC_{99} and LC_{90} values are extrapolated beyond the concentrations used in the bioassays and have overlapping confidence intervals. Further, the registrants

recommended focusing on the LC_{50} ratio of the least sensitive CRW population to the most sensitive population for comparison between seasons. An increase in this ratio could result if a population shows reduced susceptibility to the toxin. Using this measure, CRW susceptibility to Cry34/35 has not significantly decreased since monitoring was initiated in 2004 (Figure 1).

As an additional point of analysis, two counties that were previously sampled in 2006 and 2007 were also sampled in 2008, and a more direct data comparison could be made for these two locations (see Table 2). U.S. EPA (2010c) focused on EC data, since these showed less variation than LC data. When the mean EC_{50} and EC_{99} values were qualitatively compared across three consecutive years in Henry County (Illinois) and Scott County (Iowa), it was apparent that the extrapolated EC_{99} values had increased. The mean EC_{99} values for Henry and Scott County ranged from 21.0 $\mu g/cm^2$ to 388.3 $\mu g/cm^2$ and 48.7 $\mu g/cm^2$ to 95.8 $\mu g/cm^2$, respectively. BPPD concluded that susceptibility to DAS-59122-7 may have decreased in these areas (Henry County, Illinois and Scott County, Iowa) and recommended that additional sampling occur in these counties during future corn-growing seasons to assess whether these susceptibility trends continue. Dow and Pioneer noted that the apparent decrease in susceptibility may in fact be due to a change in test methodology. The timing of the exposure period in the bioassays was reduced from 4–7 days to 4 days, which could have resulted in lower mortality and growth inhibition (Dow and Pioneer 2010).

Table 2. Cry34/35 Susceptibility Data for WCRW populations in Henry County (IL) and Scott County (IA) (2006–2008) (BPPD generated table from data submitted by Dow/Pioneer).

Location/Year	EC_{50} ($\mu g/cm^2$)	EC_{99} ($\mu g/cm^2$)
Henry Co (2006)	1.3	21.0
Henry Co (2007)	2.1	213.6
Henry Co (2008)	4.0	388.3
Scott Co (2006)	0.8	48.7
Scott Co (2007)	1.8	63.2
Scott Co (2008)	2.9	95.8

b. Compliance

Dow/Pioneer's compliance reports, including grower agreements, grower affirmation procedures, and CAP, were reviewed in U.S. EPA (2006b). The compliance program for DAS-59122-7 (Cry34/35 corn) is aligned with that developed by ABSTC (in consultation with the EPA) for corn-borer protected *Bt* corn products. Specific elements of the program and additional reports with annual survey data are discussed below.

DAS-59122-7 Compliance Assurance Program

As a term of the Herculex RW (DAS-59122-7; Cry34/35Ab1) registration, Dow and Pioneer were required to develop and submit to EPA a CAP to ensure grower adherence to IRM requirements. The terms of registration mandated a number of components for the compliance program including:

- Grower Agreements: Contractual arrangement between the registrant and grower to obligate adherence to IRM requirements.
- Annual IRM Survey: A survey (conducted anonymously by an independent research firm) intended to provide a statistically representative sample of growers from various corn-growing regions in the U.S. Results from the survey should assess levels of grower compliance with refuges, as well as grower motivations, attitudes, and reasons for non-compliance.
- On-Farm Assessments: The registrant is required to develop an on-site assessment program in which trained personnel from each company make visits to farms growing *Bt* corn. During these visits, compliance with refuge requirements is assessed and growers not in compliance are identified for corrective action under the Phased Compliance Approach.
- Tips and Complaints: The registrant must establish a means for the reporting and investigation of incidences of refuge non-compliance.
- Phased Compliance Approach (PCA): A consistent set of procedures (for all *Bt* corn registrants) to be employed to address non-compliance among growers and seed dealers.

ABSTC, a consortium of *Bt* corn registrants, previously developed and submitted a CAP for lepidopteran *Bt* corn PIPs in 2002. Subsequently, ABSTC submitted revised versions of the CAP in 2004 and 2005 in response to EPA reviews of annual growing season reports (see discussion in U.S. EPA 2010e). Herculex® RW (and other registered products with Cry34/35) have been included as part of this existing program, though data are tabulated separately for lepidopteran, rootworm, and stacked (lepidopteran + rootworm) *Bt* corn PIPs. EPA reviews of compliance data for rootworm-protected PIPs (some of which predates the registration of Herculex® RW) can be found in U.S. EPA (2004, 2007b, and 2009a).

Annual Grower Surveys

As a condition of each individual *Bt* corn registration, the registrant must perform an annual third-party survey of a statistically representative sample of *Bt* corn growers. The grower survey functions to measure compliance adherence to refuge size and distance requirements at a regional level and to identify educational opportunities to increase grower compliance with IRM requirements. More than 500 growers from four separate regions are anonymously surveyed annually. The methodology for conducting the grower survey has remained virtually unchanged since it was first conducted by Marketing Horizons, Incorporated in 2000 for the lepidopteran *Bt* corn registrations. Starting in 2007, however, due to an increasing complexity of growers' *Bt* corn planting practices and a need to standardize the grower survey across insect-protected traits, Marketing Horizons, Incorporated utilized an internet-based survey approach.

Surveys for the corn rootworm PIPs encompass all growers planting rootworm-protected traits (Cry3Bb1, mCry3A, and Cry34/35Ab1). Cumulative results of the surveys are summarized in Table 3 below. Results from the stacked (lepidopteran + rootworm) *Bt* corn surveys are tabulated separately (Table 4) and also include all registered rootworm PIP traits.

Table 3. Summary of Telephone (2005–2006) and Online (2007–2008) Survey Results for Rootworm-Protected *Bt* Corn Growers.

Survey Question	2005[1] % Respondents	2006[2] % Respondents	2007 % Respondents	2008 % Respondents
Adherence to Refuge[3] Size	93	89	80	74
Adherence to Distance Requirements[3]	87	82	79	63
Awareness of IRM Requirements	97	93	97	96
Unaided Recall of Refuge Size	51	57	63	72
Unaided Recall of Refuge Distance	58	55	33	34

[1] Includes YieldGard® RW and YieldGard® Plus corn growers
[2] Includes YieldGard® RW, YieldGard® Plus, Herculex® RW, and Herculex® XTRA corn growers
[3] Weighted averages across all four regions surveyed

Table 4. Summary of Telephone and Online Survey Results for Stacked *Bt* Corn Growers (2006–2008).

Survey Question	2006 % Respondents	2007 % Respondents	2008 % Respondents
Adherence to Refuge Size[1]	78	70	72[4]
Adherence to Distance Requirements[1]	92	66	66[3]
Awareness of IRM Requirements	95	96	97
Unaided Recall of Refuge Size	59	62 and 55[2]	81
Unaided Recall of Refuge Distance CRW	48	39	36
Unaided Recall of Refuge Distance European Corn Borer (ECB)	32	77	86

[1] Weighted average across all four regions surveyed
[2] First number listed is for ECB and the second number for CRW refuge compliance.
[3] On a per field basis, adherence was 76%.
[4] On a per field basis, adherence was 73%.

Overall compliance (per grower) with refuge requirements for both single-trait and stacked rootworm-protected PIPs has declined from 2005 to 2008. Grower adherence to the necessary refuge size fell to below 75% in 2008 for single-trait and stacked rootworm PIPs. Compliance with refuge proximity was lower; in 2008, ≤66% of rootworm PIP growers deployed refuges within the required distance to the *Bt* field. The percent of growers who were able to recall the correct refuge distance requirements (unaided) for rootworm PIPs drastically declined to below 40% in 2008. Refuge distance requirements for rootworm-protected *Bt* corn products may be more challenging for growers because the refuge must be deployed either within or immediately adjacent to the *Bt* field. Stacked products present additional challenges due to the need to plan either two refuges (for lepidoptera and rootworm) or a combined refuge for both pest complexes. Grower awareness of the distance requirements has been poor and likely explains much of the reported non-compliance.

On-Farm Assessments

The on-farm assessment program is the portion of the CAP that identifies individual non-compliant growers (regardless of farm size) for remedial IRM education, follow-up reassessments, and other activities as part of the PCA. It can also serve as a tool to enhance the registrant's understanding of the obstacles growers face in implementing IRM requirements. The mandatory on-farm assessment program was fully implemented for the first time in 2003 (for lepidopteran registrations) and has typically encompassed more than 2,000 growers per season (for all types of *Bt* corn). On-farm assessments for rootworm-protected PIPs (including Cry34/35 products) began in 2006.

Data from the on-farm assessments (2006 through 2008) of rootworm-protected *Bt* corn PIPs are summarized in Table 5 below. These on-farm assessments encompass all growers planting rootworm-protected PIPs, including varieties expressing the Cry3Bb1, mCry3A, and Cry34/35Ab1 toxins. Results for the on-farm assessments of stacked (lepidopteran + rootworm) PIPs are detailed in Table 6. The assessments do not have the statistical power associated with the consistently stratified and randomized telephone/on-line surveys and are not used to measure representative rates of non-compliance. In 2007 and 2008, no information was provided regarding specific non-compliance with refuge size and distance. This information should be provided in future reports to be consistent with previously collected data and to illustrate how growers are out of compliance (U.S. EPA 2009a).

Table 5. Cumulative Results for the On-Farm Assessments of Coleopteran-Protected *Bt* Corn Growers (2006–2008)[1].

	2006	2007	2008
Growers Assessed	395	247	134
Refuge Distance Deviations[2]	13	N/A	N/A
Refuge Size Deviations	7	N/A	N/A
Significant Deviations	11 (2.8%)	16 (6.5%)	12 (9.0%)
Insignificant Deviations	10 (4.0%)	8 (3.2%)	7 (5.2%)
Compliant Growers	374 (94.7%)	223 (90.3%)	115 (85.8%)
Non-Compliant Growers	21 (5.3%)	24 (9.7%)	19 (14.2%)

[1]Table adapted from page 12 of MRID No. 470444-01.
[2] Some growers had compliance deviations other than refuge size or distance; thus, the total of refuge distance and size deviations does not equal the number of non-compliant growers.

Table 6. Cumulative Results of the On-Farm Assessments of Stacked *Bt* Corn (Rootworm + Corn Borer) Growers (2006–2008)[1].

	2006	2007	2008
Growers Assessed	600	1,069	1,799
Refuge Distance Deviations	51[2]	N/A	N/A
Refuge Size Deviations	8	N/A	N/A
Significant Deviations	45 (7.5%)	77 (7.2%)	86[3]
Insignificant Deviations	16	33 (3.1%)	36[3]
Compliant Growers	539 (89.8%)	959 (89.7%)	1546 (85.9%)
Non-Compliant Growers	61 (10.2%)	110 (10.3%)	253 (14.1%)

[1] Table adapted from page 12 of MRID No. 470444-01.
[2] Some growers had compliance deviations other than refuge size or distance; thus, the total of refuge distance and size deviations does not equal the number of non-compliant growers.
[3] The numbers of deviations do not add up to the 253 non-compliant cases reported.

Tips and Complaints

As required by the terms of registration, *Bt* corn registrants must have a "tips and complaints" system as a mechanism for individuals (e.g., growers, sales representatives, etc.) to report alleged instances of IRM non-compliance. The number of tips and complaints (summarized for all *Bt* corn registrations, including lepidopteran and rootworm varieties) received through 2008 is summarized in Table 7 below. Each of these growers identified through the tips and complaints mechanism were visited as part of the on-farm assessment program. However, it is not possible to determine whether any of the non-compliant growers, identified via the tips and complaints route, were subject to the Phased Compliance Approach.

Table 7. Anonymous Tips and Complaints about Non-Compliance with IRM Requirements (Data from ABSTC Annual Reports 2003 through 2008).

Year	Number of Tips and Complaints
2003	0
2004	0
2005	5
2006	3
2007	14
2008	5

Phased Compliance Approach

ABSTC's CAP for lepidopteran and rootworm-protected PIPs includes a standard set of procedures (shown in Table 8), known as the Phased Compliance Approach (PCA), which is to be used by registrants when responding to instances of grower non-compliance with the IRM requirements. The PCA also established a tiered approach for non-compliance with "significant" deviations and "other" deviations. For a 20% CRW refuge requirement (Corn Belt), a significant size deviation is defined as a *Bt* grower planting less than 15% non-*Bt* corn refuge. This definition is also applicable to "combined" refuges planted for both lepidoptera and CRW for stacked *Bt* corn PIPs. On the other hand, a significant deviation based on refuge proximity has not been clearly defined for CRW refuges and, as of the 2008 CAP report, it is unclear what standards are being used by ABSTC. For lepidopteran *Bt* corn, a significant deviation is triggered if fewer than 2/3 of the *Bt* corn fields are planted within ½ mile of a non-*Bt* corn refuge. However, this definition is not compatible with CRW refuge because the distance requirement mandates that refuges be placed adjacent to or within the *Bt* corn field. A reasonable extension of the lepidopteran definition for CRW could be "less than 2/3 of the non-*Bt* refuge is deployed adjacent to or within the *Bt* field" and "fewer than two-thirds (2/3) of the in-field strips are at least four rows wide" (U.S. EPA 2006b). This formula would also be applicable to combined refuges for stacked PIPs.

Table 8. Phased Compliance Approach (PCA) – Standards for *Bt* Corn Refuge Non-Compliance (Submitted with the ABSTC 2002 CAP).

	Mandatory Responses	Additional Responses
Significant Deviations	• IRM education • Warning letter • Compliance assistance contact prior to planting • Compliance assessment contact for the following growing season • Deny access to the *Bt* corn product for any significant deviation two years in a row	• Invoice monitoring • Technical assistance • Grower IRM training • Reaffirmation of IRM obligations • Deny access to the *Bt* corn product for other deviations that are repeated over a period of years
Other Deviations	• IRM education • Letter and/or compliance assistance contact prior to planting • Compliance assessment contact in the following growing season	

Under the PCA, sales are to be suspended to individual growers for one year after two years of significant deviations. Following the one-year suspension, growers will need to be requalified to purchase seeds. Grower identified as non-compliant (significant or other deviations) are required to receive a "compliance assessment contact" the following year under the PCA. Non-compliant growers are typically identified through the on-farm assessment program (see previous discussion in the "On-Farm Assessments" section). Table 9 summarizes the numbers of non-compliant growers reassessed under the PCA, and the growers still found to be out of compliance. As of the 2008 growing season, one grower was denied access to *Bt* corn technology due to a refusal to be reassessed in the following season after significant non-compliance. Compliance data, including results of on-farm assessments and PCA activities, are detailed in U.S. EPA (2007b and 2009a).

Table 9. Reassessment of Rootworm-Protected and Stacked *Bt* Corn Growers Under the Phased Compliance Approach (Taken from ABSTC Annual CAP Reports)[1].

Year	Reassessments [2]	Significant Deviations [3]	Loss of Access to Technology
2006	62	0	1 [4]
2007	82	0	0
2008	134	0	0

[1] The data in this table includes both growers planting single-trait rootworm PIPs and stacked (lepidopteran + rootworm) PIPs. The data in the table has been summed for both groups.
[2] Reassessments of growers identified with deviations (significant and other) to refuge requirements the previous growing season.
[3] Significant deviations recorded the following season. Two successive years of significant deviations results in loss of access to *Bt* corn technology.
[4] One grower refused to be reassessed in 2006 and was denied access to *Bt* corn

4. Stacked *Bt* Corn PIPs Containing Cry34/35

Several "stacked" *Bt* corn PIPs have been registered that contain Cry34/35. Stacked products express two or more toxins targeted at separate pest complexes. Cry34/35 (rootworm control) has been paired with lepidopteran-active proteins for control of European corn borer and other pests. The stacked Cry34/35 products (registered as of 2010) are described below. A separate product has been registered with a "pyramid" (two or more toxins targeted at the same pest complex) of Cry34/35 and Cry3Bb1 for rootworm control. This product (SmartStax™) is discussed in a separate Fact Sheet (U.S. EPA 2009b). Another product containing a seed blend mix of Cry34/35 corn (Optimum® AcreMax™ 1) has also been registered and is discussed in a separate Biopesticides Registration Action Document (U.S. EPA 2010b and 2010f).

a. Herculex® Xtra (DAS 59122-7 x TC1507)

Herculex Xtra (EPA Reg. Nos. 68467-6 and 29964-5) is a stacked product that is the result of a conventional breeding cross between Herculex® I (Event DAS 01507-1; EPA Reg. Nos. 68467-2 and

29964-3) and Herculex® Rootworm (Event DAS-59122-7; EPA Reg. Nos. 68467-5, 29964-4). Herculex® I (first registered in 2001) expresses the Cry1F *Bt* toxin and is targeted against stalk-boring lepidopteran pests, while Herculex® Rootworm corn (registered August 31, 2005) expresses the Cry34Ab1/35Ab1 binary toxin for control of CRW. The complete list of targeted pests is as follows: European corn borer (ECB, *Ostrinia nubilalis*), southwestern corn borer (SWCB, *Diatraea grandiosella*), corn earworm (CEW, *Helicoverpa zea*), fall armyworm (FAW, *Spodoptera frugiperda*), black cutworm (BCW, *Agrotis ipsilon*), western bean cutworm (WBCW, *Richia albicosta*), western corn rootworm (WCRW, *Diabrotica virgifera virgifera*), northern corn rootworm (NCRW, *Diabrotica barberi*), and Mexican corn rootworm (MCRW, *Diabrotica virgifera zeae*).

When evaluating a stacked PIP created from previously registered single-trait PIPs, the dose profiles for each of the toxins in the stack will have been previously established for the isolines. Therefore, the dose of the stacked PIP can be verified relatively easily through confirmatory studies. Two sets of data are typically analyzed: (1) plant expression data to verify that the toxin expression in the stacked product is at least as high as in the single-gene isolines; and (2) efficacy data to confirm that the level of control against the target pests in the stacked product is at least as high as the isolines. Dow/Pioneer submitted efficacy studies (Master Record Identification Numbers (MRID Nos.) 463438-04 and 463438-05) that were designed largely to confirm efficacy in the newly created stacked product, Herculex® Xtra. The data revealed that Herculex® Xtra offered comparable levels of protection against ECB, FAW, and WCRW as the single-gene hybrids (Herculex® I and Herculex® Rootworm) used to create the stacked product. Other labeled pests (e.g., BCW, SWCB, and NCRW) were not evaluated in these studies. However, since previously submitted data have demonstrated efficacy against all the labeled pests in the single-gene hybrids (Herculex® I and Herculex® Rootworm), it is expected that Herculex® Xtra will offer comparable protection. The results are also supported by toxin expression data (MRID No. 463438-04), which showed that the stacked hybrid expresses both Cry1F and Cry34/35 at comparable (or higher levels) than the single-gene hybrids (U.S. EPA 2005c).

IRM must be addressed for lepidopteran and corn rootworm by deploying either separate 20% non-*Bt* corn refuges (for each pest complex) or a combined 20% refuge that addresses both pest complexes. A combination refuge must address the proximity restrictions for CRW (i.e., the refuge must be placed adjacent to or within the *Bt* field and in-field strips must be at least 4 rows wide).

Other aspects of the IRM program for Herculex® Xtra (resistance monitoring, remedial action, and compliance) were incorporated into the existing programs for the single-toxin PIPs (Cry34/35 and Cry1F). The IRM program for Cry34/35 is discussed in sections II(D)(2) and II(D)(3) of this BRAD; Cry1F is discussed in the August 2005 Cry1F BRAD and the September 2010 Cry1Ab and Cry1F BRAD (U.S. EPA 2005b and 2010d).

b. 59122 x MON 810 and 1507 x 59122 x MON 810

Both 59122 x MON 810 (EPA Reg. No. 29964-9) and 1507 x 59122 x MON 810 (EPA Reg. No. 29964-8) are stacked products registered by Pioneer for control of rootworm and corn borer pests. The first, 59122 x MON 810, expresses Cry34/35 and Cry1Ab, while 1507 x 59122 x MON 810 expresses Cry1F,

Cry34/35, and Cry1Ab. Like other stacked *Bt* corn PIPs, these two products were supported by efficacy and expression data to confirm the dose profile against the major target pests.

Protein expression data were generated for each of the stacked products (59122 x MON 810 and 1507 x 59122 x MON 810) and the single-toxin constituent PIPs (MRID No. 475109-04). Studies were conducted in greenhouses, and plant tissues were analyzed by enzyme-linked immunosorbent assay (ELISA) for protein expression. Pioneer sampled leaf, stalk, root, whole plant, and grain tissues for the analyses (pollen tissue was also collected but was often below the limit of detection). Corn tissues were sampled during the R1 corn growth stage, except for grain which was taken from the R6 stage. The results from the ELISA assays showed that Cry1F, Cry1Ab, Cry34Ab1, and Cry35Ab1 protein were expressed at comparable levels in the stacked products to the single-toxin constituent products. Expression was consistent throughout all tissues—only small differences in protein levels, which were not statistically significant, were noted between the stacked and single-toxin products.

Pioneer conducted field studies to investigate the efficacy of their stacked products against ECB, SWCB, FAW, and CRW. The company asserted that testing was not necessary for other labeled pests (i.e., secondary lepidopteran corn pests) given that the constituent PIPs (1507, 59122, and MON 810) have well established efficacy profiles. These studies were described in MRID No. 476778-03. The submitted field efficacy data were sufficient to demonstrate that the *Bt* toxins in the three proposed stacked products have comparable dose expression profiles as their single-trait constituent products (U.S. EPA 2010a). Both MON 810 (Cry1Ab) and 1507 (Cry1F) have been considered "high dose" for ECB and likely SWCB but not for CEW. With CRW, 59122 (Cry34/35) is known not to have high dose expression. Given the confirmatory protein expression and efficacy studies, the stacked products can also be considered high dose for ECB and SWCB and non-high dose for CEW and CRW.

Similar to other stacked products, refuge must be addressed by planting separate (corn borer and rootworm) 20% refuges or a combination 20% refuge for both pests. Other aspects of the IRM program (resistance monitoring, remedial action, and compliance) are largely the same as the existing programs for the single-toxin PIPs (Cry34/35, Cry1Ab, and Cry1F). The IRM program for Cry34/35 is discussed in sections II(D)(2) and II(D)(3) of this BRAD; Cry1Ab and Cry1F are discussed in the 2001 *Bt* Crops Reassessment, August 2005 Cry1F BRAD, and/or the September 2010 Cry1Ab and Cry1F BRAD (U.S. EPA 2001b, 2005b, and 2010e).

c. Refuges for Stacked *Bt* Corn Products

Table 10 below describes the refuge requirements for stacked *Bt* corn PIPs targeting lepidopteran (e.g., ECB) and rootworm pests. Growers must either plant two separate refuges for each pest complex or a combined refuge that addresses both insect groups.

Table 10. Comparison of Lepidopteran (e.g., TC 1507, MON 810) and Rootworm (e.g., DAS-59122-7) IRM Requirements for Stacked *Bt* Corn PIPs (e.g., Herculex® Xtra)

Requirements	(Separate Lepidopteran Refuge)	(Separate Rootworm Refuge)	(Combined Refuge)
Refuge Size	≥ 20%	≥ 20%	≥ 20%
Refuge Placement	≤ ½ mile	Adjacent or within field	Adjacent or within field
Refuge Configuration	Block, in-field strips (≥ 4 rows), or edges	Block or strips (≥ 4 rows)	Block or strips (≥ 4 rows)
Refuge Management	Any corn rotation meeting placement and configuration requirements	Same corn rotation as the rootworm-protected *Bt* fields (e.g., first year corn or corn followed by corn)	Same corn rotation as the *Bt* fields (e.g., first year corn or corn followed by corn)
	Insecticides can be used in refuge to control ECB/SWCB when above economic thresholds.	Conventional insecticides or seed treatments can be used in refuge to control CRW larvae and other soil pests. If the refuge is treated with a foliar insecticide labeled for CRW control when CRW adults are present, then the *Bt* fields also must be treated.	Conventional insecticides or seed treatments can be used in refuge to control CRW larvae and other soil pests. If the refuge is treated with a foliar insecticide labeled for CRW control when CRW adults are present, then the *Bt* fields also must be treated.
	Microbial *Bt* insecticides are not allowed.	(Not applicable)	Microbial *Bt* insecticides are not allowed.
Refuge Corn Types	Conventional (Non-*Bt*)	Conventional (Non-*Bt*) Corn borer-protected *Bt* corn (a corn borer refuge planted ≤½ mile also will be required) Herbicide-tolerant corn (Non-*Bt*)	Conventional (Non-*Bt*) Corn borer-protected *Bt* corn (an additional refuge for corn borer will be required) Herbicide-tolerant corn (Non-*Bt*)

5. IRM Terms and Conditions of Registration

The terms and conditions for each of the Cry34/35Ab1 registrations contain a complete description of the IRM requirements. Details are provided on the requirements for refuge (size and structure), resistance monitoring, remedial action, compliance assurance, grower education, and annual IRM reports. For more information, please refer to the document, in Docket Number EPA-HQ-OPP-2010-0607, presenting the registration terms and conditions established with the 2010 amendments.

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E. Benefits and Public Interest Finding for Initial Registrations of Event DAS-59122-7 Corn

1. Overview

The Environmental Protection Agency (EPA) has reviewed the public interest document for event DAS-59122-7 corn (Master Record Identification Numbers (MRID Nos.) 461227-01 and 461239-21; reviewed in U.S. EPA (2005)) and concludes that, under section 3(c)(7)(C) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), conditional registration of event DAS-59122-7 corn is in the public interest. Cry34/35Ab1 corn rootworm (CRW)-protected corn hybrids (event DAS-59122-7 corn) will extend the benefits of insecticide use reduction that have been established for the Cry3Bb1 (event MON 863) CRW-protected corn products. Cry34/35Ab1 CRW-protected corn is comparatively less risky to human health and the environment than currently registered chemical pesticides. It also provides a different mode of action for CRW control, comparable or better efficacy and yield compared to other CRW-control products, expanded product choices for growers, and indirect benefits (e.g., energy savings resulting from reduced chemical insecticide use).

2. Public Interest Finding

The criteria for determining whether registration of a pesticide chemical is in the public interest are set forth in a Federal Register Notice dated March 5, 1986 (51 Federal Register (FR) 7628). There is a presumption that registration of a pesticide chemical is in the public interest if one of the following criteria is met: (1) the use is for a minor crop; (2) the use is a replacement for another pesticide that is of continuing concern to the Agency; (3) the use is one for which an emergency exemption under FIFRA section 18 has been granted (i.e., the basis for the exemption was lack of a registered alternative product); or (4) the use is against a pest of public health significance. Further, EPA may determine that such a registration is in the public interest on the basis of the following criteria: (1) there is a need for the new chemical that is not being met by currently registered pesticides; (2) the new pesticide is comparatively less risky to health or the environment than currently registered pesticides; or (3) 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.

Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International, Incorporated (hereafter referred to as Dow and Pioneer, respectively) have provided data to support their claim that Cry34/35Ab1 CRW-protected corn is in the public interest. EPA's analysis supports the following conclusions:

1. Cry34/35Ab1-protected corn provides effective control of key rootworm pests of field corn and may prove more efficacious than chemical insecticides presently registered for this purpose.

2. Economic models suggest that, under conditions of high rootworm pressure, use of Cry34/35Ab1-protected corn will provide greater net returns to farmers. Cost benefits include reduced expenditures on insecticides, application equipment, and personnel, complemented by greater potential corn yields. Under high rootworm pressure, these benefits are expected to outweigh the higher cost of seed.

3. Registration of Cry34/35Ab1-protected corn is expected to result in further reduction of chemical insecticide use by growers. This is of special importance since many pesticides registered for CRW-control are highly toxic to humans and the environment, while Cry34/35Ab1-expressing corn poses no foreseeable human health or environmental risks.

4. If Cry34/35Ab1 corn is registered, it will be the second CRW-protected *Bacillus thuringiensis* (*Bt*) corn product on the market (the first was Cry3Bb1). The availability of multiple CRW-protected corn products will increase grower choice and price competition, likely resulting in lower seed prices for consumers and higher adoption rates.

5. The Cry34/35Ab1 CRW-protected corn will provide a different mode of action and extend the durability of other CRW control measures, including other *Bt* CRW-protected corn hybrids.

a. Background

Corn is the most widely cultivated U.S. crop, in terms of acreage planted and net value. In 2004, U.S. corn acreage totaled 80.9 million, yielding 11.8 billion bushels. Corn rootworm (CRW, *Diabrotica* spp.), one of the most damaging pests of field corn, can cause yield losses in the range of 8 to 16 percent if left uncontrolled.

Prior to the advent of insect-protected field corn, CRW was controlled through the use of crop rotation and insecticides. Although crop rotation is regarded as an effective CRW-control tool (Levine and Oloumi-Sadeghi 1991), behavior changes in northern corn rootworm (extended diapause) and western corn rootworm (soybean rotation) have reduced the effectiveness of this management option in some corn-growing regions. Insecticidal control, a pest management alternative to crop rotation, employs chemicals that are highly toxic to fish, birds, and other wildlife species. In addition, resistance to some CRW insecticides, such as methyl parathion and carbaryl (Meinke *et al*. 1998; Scharf *et al*. 1999; Zhu *et al*. 2001), may result in increased chemical use.

Since 2003, transgenic CRW-protected *Bt* corn, a third method of CRW control, has been available to farmers. The first *Bt* field corn product registered for CRW control was Monsanto's event MON 863 (expresses the Cry3Bb1 protein). This review concerns the second CRW-protected *Bt* corn product submitted for registration, Dow's and Pioneer's DAS-59122-7, which produces the Cry34Ab1 and Cry35Ab1 insecticidal proteins (originally isolated from *Bt* strain PS149B1). Similar to event MON 863 corn hybrids, event DAS-59122-7 *Bt* corn is targeted against the western corn rootworm (WCRW, *Diabrotica virgifera virgifera* LeConte*),* northern corn rootworm (NCRW, *Diabrotica barberi* Smith and Lawrence), and Mexican corn rootworm (MCRW, *Diabrotica virgifera zeae* Krysan and Smith).

b. Efficacy

i. Summary of Submitted Information

In 2003, Dow and Pioneer conducted field trials at multiple locations to evaluate the performance of Cry34/35Ab1 CRW-protected corn against coleopteran insect pests. Several efficacy trials were designed to compare Cry34/35Ab1 hybrids to their non-*Bt* isolines across multiple genetic backgrounds following exposure to natural and/or artificial CRW infestations. Results suggest that DAS-59122-7 offers excellent control of WCRW, NCRW, and MCRW. Further details on studies of individual pest species are provided below.

WCRW: Field trials were conducted to evaluate the efficacy of WCRW control by Cry34/35Ab1 rootworm-protected corn, compared to non-*Bt* isolines and chemical control measures. Each applicant conducted separate efficacy trials, and Pioneer completed two independent evaluations (Dow completed one evaluation). All trials were replicated in four locations, using a randomized complete block design with three blocks per location. Fields used in these trials had been planted to corn in previous years and contained natural infestations of western and northern corn rootworm. In addition, corn plants were artificially infested with WCRW eggs (1,000 eggs per plant) at plant growth stage V3. Roots were harvested and scored when larvae reached pupation, which was at approximately plant growth stage R1. Event DAS-59122-7 corn roots consistently showed less WCRW damage compared to non-*Bt* isolines with no chemical control (Tables 1, 2, and 3). However, results suggest that efficacy of Cry34/35Ab1 corn is similar, or slightly better, than that of non-*Bt* isolines treated with chemical insecticides.

Table 1. Efficacy of Cry34/35Ab1 Rootworm-Protected Corn for Control of WCRW in 2003 (1st Pioneer Study).

Pioneer Experiment #1					
Corn Line	**York, NE**	**Johnston, IA**	**Janesville, WI**	**Windfall, IN**	**Average**
Non-*Bt* Hybrid	2.46[1] a[2]	1.06 a	0.59 a	0.12 a	1.22 a
Force 3G	0.57 b	0.11 b	0.16 b	0.05 a	0.31 b
DAS-59122-7	0.06 b	0.10 b	0.06 b	0.03 a	0.12 c

[1] Iowa State University Node Injury Root Rating Scale (Oleson 1998): 0.00 = no feeding damage, 1.00 = one node eaten back to within two inches of stalk, 2.00 = two nodes eaten, and 3 = three or more nodes eaten.
[2] Within each column, means that are followed by the same letter are not significantly different.

Table 2. Efficacy of Cry34/35Ab1 Rootworm-Protected Corn for Control of WCRW in 2003 (2nd Pioneer Study).

Pioneer Experiment #2					
Corn Line	**York, NE**	**Johnston, IA**	**Janesville, WI**	**Rochelle, IL**	**Average**
Non-*Bt* Hybrid	0.99[1] a[2]	0.79 ab	1.96 a	0.78 a	1.03 a
Force 3G	0.32 bc	0.45 bc	1.20 b	0.11 b	0.44 b
Counter 15G	0.25 bc	0.04 c	0.52 c	0.13 b	0.19 c
DAS-59122-7 (hybrid 1)	0.02 c	0.04 c	0.44 c	0.03 b	0.11 c
DAS-59122-7 (hybrid 2)	0.07 c	0.04 c	0.06 c	0.02 b	0.04 c
DAS-59122-7 (hybrid 3)	0.02 c	0.17 c	0.30 c	0.04 b	0.11 c
DAS-59122-7 (hybrid 4)	0.01 c	0.05 c	0.24 c	0.02 b	0.07 c

[1] Iowa State University Node Injury Root Rating Scale (Oleson 1998): 0.00 = no feeding damage, 1.00 = one node eaten back to within two inches of stalk, 2.00 = two nodes eaten, and 3 = three or more nodes eaten.
[2] Within each column, means that are followed by the same letter are not significantly different.

Table 3. Efficacy of Cry34/35Ab1 Rootworm-Protected Corn for Control of WCRW in 2003 (Dow Study).

Dow Experiment #1					
Corn Line	**Huxley, IA**	**York, NE**	**Arlington, WI**	**Fowler, IN**	**Average**
Non-*Bt* Hybrid	1.94 a[2]	0.93 a	1.03 a	2.43 a	1.58 a
Force 3G	0.15 d	0.11 b	0.19 c	0.57 b	0.25 b
Counter 15G	0.02 d	0.10 b	0.09 c	0.27 b	0.12 b
DAS-59122-7	0.03 d	0.04 b	0.05 c	0.09 b	0.05 b

[1] Iowa State University Node Injury Root Rating Scale (Oleson 1998): 0.00 = no feeding damage, 1.00 = one node eaten back to within two inches of stalk, 2.00 = two nodes eaten, and 3 = three or more nodes eaten.
[2] Within each column, means that are followed by the same letter are not significantly different.

NCRW: In 2003, each applicant completed a NCRW trial that compared the efficacy of Cry34/35Ab1 rootworm-protected corn to a non-*Bt* isoline. Each trial was conducted in a single location, using a randomized complete block design with three replicates.

Pioneer's evaluation of NCRW (natural infestations) was conducted in conjunction with their WCRW study (artificial infestations) at Janesville, WI. Beetle emergence traps showed that the ratio of naturally emerging NCRW and WCRW was 1:1, but quantification of natural population pressure was difficult due to the artificial infestation of WCRW (1,000 WCRW/plant). Root ratings (Tables 1 and 2) show that

Event DAS-59122-7 is more efficacious against CRW than non-*Bt* hybrids with no insecticide treatment and comparable to non-*Bt* hybrids treated with conventional insecticides.

Dow's NCRW efficacy trial, conducted at Lamberton, MN, utilized a randomized complete block design with three replicates. Fields used in this trial had been planted to corn in previous years and contained natural infestations of WCRW and NCRW; however, the ratio of WCRW and NCRW was not determined. Thus, results indicate product efficacy against an undetermined combination of NCRW and WCRW. Roots were harvested and scored when larvae reached pupation, at approximately growth stage R1. Results suggest that event DAS-59122-7 corn had less CRW damage compared to the non-*Bt* hybrids (Table 4).

Table 4. Efficacy of Cry34/35Ab1 Rootworm-Protected Corn for Control of NCRW and WCRW at Lamberton, MN in 2003 (Dow Study).

Corn Line	Average Root Rating (1–6 scale)[1]
Non-*Bt* Hybrid	4.38 a[2]
DAS-59122-7	2.54 b

[1] Modified root rating scale: 1 = no visible damage, 2 = slight feeding damage on one or more roots, 3 = 1–5 roots pruned to within 1.5 inches of stalk, 4 = one whorl of roots pruned, 5 = two whorls of roots pruned, and 6 = three whorls of roots pruned.
[2] Means that are followed by the same letter are not significantly different.

MCRW: Pioneer conducted a field trial to evaluate the efficacy of MCRW control by event DAS-59122-7 corn, compared to a non-*Bt* isoline and a non-*Bt* isoline treated with a chemical control. The trial was conducted in Sugarek, TX using a randomized complete block design with four replicates. Fields used in this trial had been planted to corn in previous years and contained natural infestations of MCRW. Results show that event DAS-59122-7 corn had less MCRW damage compared to non-*Bt* corn with and without chemical treatment (Table 5).

Table 5. Efficacy of Cry34/35Ab1 Rootworm-Protected Corn for Control of Mexican Corn Rootworm at Sugarek, TX in 2003 (Pioneer Study).

Corn Line	Average Root Rating
Non-Bt Hybrid	0.80[1] a[2]
Non-Bt Hybrid + Aztec	0.21 a
DAS-59122-7	0.05 b

[1] Iowa State University Node Injury Root Rating Scale (Oleson 1998).
[2] Within each column, means that are followed by the same letter are not significantly different.

ii. Biopesticides and Pollution Prevention Division (BPPD) Review

Results indicated that event DAS-59122-7 corn had less CRW damage when compared to non-*Bt* corn and comparable efficacy to non-*Bt* corn treated with conventional insecticides. Field plots used for WCRW and NCRW efficacy studies contained natural infestations of both pest species and discrimination between the species was not determined at all sites. Despite the presence of WCRW and NCRW species, efficacy against WCRW is likely to have been adequately measured because WCRW densities were boosted through artificial infestations (1,000 eggs per plant). However, efficacy results for NCRW evaluations could not be clearly determined. At the Janesville, WI location, NCRW populations were dwarfed by artificial WCRW infestations and, at the Lamberton, MN location, the ratio of NCRW to WCRW was not determined. Consequently, one may conclude that low root ratings associated with CRW-protected corn suggests that NCRW was controlled at some undetermined level.

Mexican corn rootworm efficacy was measured at one location, Sugarek, TX, under natural infestation pressure. Results indicate that event DAS-59122-7 corn was more efficacious against MCRW than non-*Bt* hybrids with and without chemical insecticide treatment. Additional efficacy data, collected from multiple locations and years, would be necessary to determine whether lower root ratings were due to high product efficacy, low initial pest populations, or a combination of both.

c. Yield and Agronomic Performance

i. Summary of Submitted Information

Several yield and agronomic performance studies were completed by the applicants. Each evaluation is described below.

In 2003, Pioneer conducted a yield trial (in conjunction with the first WCRW efficacy trial described above) to evaluate the benefit of event DAS-59122-7 hybrids in terms of yield performance, when compared to a non-*Bt* isoline hybrid treated with and without a chemical insecticide (Force 3G, applied at 5 ounces/1000 row feet). The following agronomic traits were evaluated: yield, grain density, percent moisture at harvest, accumulated growing degree-days to 50% silk, accumulated growing degree-days to 50% shed, percent stalk lodging, root lodging, plant height, ear height, and staygreen; these traits were evaluated to determine the overall health and harvestability of a hybrid. Trials were conducted in four locations (see WCRW efficacy trial description above), using a randomized complete block design with three replicates per location, twelve treatments per replicate, and four rows per treatment. At plant growth stage V2, plants were artificially infested with WCRW eggs at a rate of 1,000 eggs per plant.

Results show that the DAS-59122-7 corn hybrid provided similar agronomic performance against CRW when compared to the isoline hybrid with and without applied insecticide. Yields were not statistically different among treatments, despite relatively high rootworm pressure (1,000 eggs per plant).

Dow and Pioneer conducted additional agronomic performance trials in 2003. Performance of Cry34/35Ab1 CRW-protected hybrids (two expressing the single event DAS-59122-7 and two

expressing DAS-59122-7 x event TC1507 (Cry1F)) were compared to that of near-isogenic hybrids containing event TC1507 and near-isogenic hybrids containing no transgenes. An elite hybrid was also included in the trials. The Pioneer trials were planted at 7 locations, and the Dow evaluations at 12 and 13 locations (Dow completed 2 different experiments). Trials took place throughout the U.S. Corn Belt and fields were managed according to standard agronomic practices. Trials utilized randomized complete block design, with three replicates per location and two rows per plot. It is not clear if fields had been planted to corn prior to experiment initiation and/or whether rootworm pressure was severe in field trial locations.

Results indicate that hybrids with and without event DAS-59122-7 performed similarly for most agronomic traits; however, expression of the Cry34/35Ab1 proteins in event DAS-59122-7 CRW-protected corn was shown to confer advantages in yield and root lodging in some locations with high rootworm pressure. Agronomic performance may have been affected by base genetics of different hybrids and genetic variability in Cry34/35Ab1 corn hybrid lines.

ii. BPPD Review

Agronomic performance of Cry34/35Ab1 CRW-protected hybrids, two containing event DAS-59122-7 stacked with event TC1507 expressing Cry1F (Herculex® I) and two containing only event DAS-59122-7, was compared to that of near-isogenic hybrids containing event TC1507 and near-isogenic hybrids containing no transgenes. An elite hybrid was also included in the trials. Based on the review of all of the agronomic trials conducted in 2003, the following conclusions can be made. With low CRW pressure, event DAS-59122-7 (Cry34/35Ab1 CRW-protected corn) performed similarly to conventional corn hybrids treated with insecticides. There was no yield drag associated with event DAS-59122-7. Under high CRW pressure, however, some event DAS-59122-7 corn hybrids were shown to confer advantages, in some locations, in both yield and root lodging over that of non-transgenic corn treated with granular insecticides. There were no significant differences between stacked Cry34/35Ab1/Cry1F corn hybrids and Cry34/35Ab1 corn hybrids. The overall range of values for the measured agronomic parameters are all within the range of values obtained for traditional corn hybrids and do not indicate increased weediness.

d. Economic Benefits

i. Summary of Submitted Information

Economic benefit analyses were conducted to evaluate the impact of Cry34/35Ab1 CRW-protected corn hybrids (event DAS-59122-7) on grower profitability, compared to various insect management alternatives. The following paragraphs describe the considerations/assumptions used to develop the benefits analyses.

Several key assumptions were developed for economic modeling based on data collected by universities on insecticide performance under various corn rootworm densities. Published information was used to predict relationships between root damage in untreated corn and expected root damage in insecticide-

treated corn for granular, liquid, and seed treatments. Separate equations were developed using the 0–3 nodal injury scale (25 locations, 1999–2002) and the 1–6 Hills & Peters scale (45 locations, 1997–2001). The relationship between event DAS-59122-7 and control root ratings was determined from 12 Dow and Pioneer trials conducted in 2003. Based on slopes of the regression equations, the most effective to least effective product classes in reducing root damage are as follows: (1) event DAS-59122-7 hybrids, (2) granular insecticides, (3) liquid insecticides, and (4) seed treatments.

The relationship between root ratings and grain yield has been extensively studied under both artificial infestations and natural populations. The economic thresholds for insecticide use typically are exceeded at a root rating of 3 or higher (1–6 scale) but can be higher or lower depending upon environmental conditions. There is also data that conventional breeding programs and planting rates influence the yield response to corn rootworm damage. To predict the relationship between root damage and yield, data from 10 corn rootworm trials conducted by Iowa State University (Oleson *et al*. 2000, 2001, 2002, and 2003) were evaluated. The predicted difference in yield between insecticide-treated and untreated plots increases linearly with level of damage in untreated check plots over a range from 0 to 37 bushels per acre. Regression analysis from 37 university yield trials from Nebraska, Minnesota, Indiana, Pennsylvania, Illinois, and Iowa provide a similar equation from the 1–6 rating scale with predicted yield losses ranging from 0.6 to 39.5 bushels per acre.

Another key concern of growers is that rootworm feeding increases the risk of lodging. Lodged cornfields yield less both due to direct physiological losses and to increased harvest losses. Two additional harvest costs associated with lodging are labor costs due to reduced harvest speed and increased fuel usage if the field must be harvested in one direction. For this economics assessment, the relationship between level of root damage and lodging was determined using insecticide treatments and untreated check plots (1–6 scale: 13 locations, Figure 6; 0–3 scale, 18 University trials and 49 Pioneer strip trials). Although several other factors (e.g., soil moisture level, wind speed, and root re-growth) influence the severity of lodging, root ratings were fairly good at predicting level of root lodging with R^2 values of 0.3 and 0.6.

Cropping Scenarios

Three different farming operations were evaluated, using partial budget analysis, to illustrate the potential value that Cry34/35Ab1 CRW-protected corn hybrids may bring to the grower. Three farming situations were analyzed: (1) moderate pressure rootworm in continuous corn, (2) high pressure rootworm in rotated corn, and (3) moderate pressure rootworm in rotated corn. Variable costs for corn following soybeans and continuous corn include fertilizers, herbicides, insecticide, crop insurance, tillage, planting, harvest, and drying. These variable costs were based on values reported by Duffy and Smith (2003) for 150 bushels per acre and a corn price of $2.10 was used in all situations. Note: Tables 6–8 were revised by the applicant, per the Agency's request, to more accurately reflect variable costs.

Example 1: Moderate corn rootworm pressure, corn following corn

Traditionally, corn planted in fields where corn was planted in prior years is considered to be at moderate risk for rootworm infestations throughout the central Corn Belt, including Nebraska and parts of Iowa, Illinois, and Kansas. A high proportion of corn growers treat their continuous

corn acres with a planting-time application of granular or liquid insecticides applied at the full, labeled rate. Adult beetle management, which involves scouting and typically two aerial applications of insecticide, is also implemented in parts of Nebraska, Colorado, Kansas, and South Dakota (Meinke 1996). Recently, high rate seed treatment options have become available and are included in this analysis.

If left untreated, moderate rootworm pressure is expected to reduce harvestable yield 15 bushes per acre. Based on modeling assumptions, neither a two-application adult control program nor use of high rate seed treatments increased variable returns over the control despite improved standability and yield (Table 6). In contrast, use of event DAS-59122-7 or full, labeled rates of granular insecticides are expected to return from $10 to $14 per acre more than untreated fields. Use of the full, labeled rate of liquid insecticides returned slightly less than event DAS-59122-7 or granular insecticides ($9–$12 per acre over untreated fields).

Example 2: High corn rootworm pressure, corn following soybeans
In east central Illinois and northwestern Indiana, fields of corn following soybeans are at high risk for root damage due to an eastern variant of the WCRW. If fields in this region have not been scouted, growers are advised to apply a planting-time insecticide at full, labeled rate. It is not unusual to have untreated 1st year cornfields average two or more nodes of roots destroyed, resulting in high levels of lodging. In a situation where first-year corn is infested with high populations of corn rootworms, harvestable yield is predicted to be reduced by 37 bushes per acre if no treatment is applied. Liquid insecticides and high rate seed treatments are not as effective in reducing losses and lodging as granular insecticides. High, consistent efficacy against even high rootworm pressure gives event DAS-59122-7 a significant economic advantage over granular ($6–$13/acre), liquid ($16–$23/acre), and seed treatment options ($37–$40/acre)(Table 7).

Example 3: Moderate corn rootworm pressure, corn following soybeans
The eastern variant of the WCRW is considered a moderate risk to corn in northeastern Indiana and northern Illinois. This moderate risk area is expanding into southern Wisconsin, southwestern Michigan, and western Ohio. In addition, extended diapause by the northern corn rootworm is placing first-year corn in southern Minnesota, northern Iowa, and southwestern South Dakota at significant risk to damage. Both granular and liquid planting-time applications are being used in fields, particularly in fields with a history of root lodging. Frequently, growers elect to use a reduced rate of insecticide if rootworm pressure in the first-year corn is moderate.

If first-year corn is infested with moderate populations of corn rootworm, use of either hybrids containing event DAS-59122-7, a reduced rate of granular insecticides, or reduced rates of liquid insecticides is expected to increase variable returns $10 to $17 per acre over the untreated field (Table 8). Use of reduced rate of granular insecticide is slightly more profitable ($2 to $7) than either event DAS-59122-7 or a reduced rate of liquid insecticides.

Table 6. Economic Analysis of Moderate Pressure Corn Rootworm in Corn Following Corn.

	Untreated	DAS-59122-7	Granular Insecticide	Liquid Insecticide	High rate IST	Adult Control
CRW Damage						
Root rating (0-3)[1]	1.0	0.08	0.20	0.36	0.67	0.20
% Lodged[2]	23%	2%	5%	8%	15%	5%
Variable Cost	----------**$/acre**----------					
Seed @ 30,000/acre[3]	$37.50	$37.50	$37.50	$37.50	$37.50	$37.50
CRW premium	0	$13.13–16.88	0	0	0	0
Low rate IST	$4.50	$4.50	0	0	0	$4.50
Scouting	0	0	0	0	0	$7.00
Insecticide	0	0	$12.00–15.00	$12.00–15.00	$17.25	$12.80–16.00
Insecticide equipment & application	0	0	$2.00	$2.00	0	$6.00
Other variable costs[4]	$155.00	$155.00	$155.00	$155.00	$155.00	$155.00
Fuel adjustment[5]	0	0	0	0	0	0
Labor adjustment[6]	$0.55	0	0	0	$0.55	0
Harvest adjustment[7]	-$2.44	-$0.15	-$0.40	-$0.70	-$1.80	-$0.70
SUBTOTAL	$195.11	$209.98–213.73	$206.10–209.10	$205.80–208.80	$208.50	$222.10–225.30
Yield	----------**bushels/acre**----------					
Maximum yield	150	150	150	150	150	150
Harvest loss from lodging[8]	-3	0	0	0	-3	0
CRW loss[9]	-12	-1	-3	-4	-8	-4
SUBTOTAL	135	149	147	146	139	146
	----------**$/acre**----------					
Gross Returns @ $2.10/bushel	$283.50	$312.90	$308.70	$306.60	$291.90	$306.60
Returns over Variable Cost	$88.39	$99.17–102.92	$99.60–102.60	$97.80–100.80	$83.40	$81.30–84.50

[1] Moderate pressure defined as root damage rating (RDR) of 1 on 0–3 rating scale in untreated field. Expected RDR in DAS-59122-7. Expected RDR in insecticide & IST treatments.
[2] % lodging = 0.4175 + 22.083*RDR.
[3] Planting rate is 30,000 kernels per acre; seed cost for 80,000 kernels/unit is $100.00.
[4] Includes costs for fertilizer, lime, herbicide, drying, storage, machinery, harvest, insurance, and interest (Duffy and Smith 2003) for 150 bushels/acre production.
[5] Fuel adjustment: 50% of normal combine cost @ $7.87/acre (Duffy and Smith 2003) if lodging is >40%.
[6] Labor adjustment for harvesting lodged corn: $9.00/hour; normal combine speed 4.5 mph; for lodging ≥10% but <40%, combine speed reduced by 25%; for lodging ≥40%, combine speed reduced by 50%.
[7] Variable costs for harvest (hauling, drying, and handling) reduced $0.16/bushel for yields below 150 bushels/acre.
[8] Assumes 2% loss for lodging ≥10% but <40% and 5% loss for lodging ≥40%.
[9] CRW loss: Change in yield = 12.254*RDR .

Table 7. Economic Analysis of High Pressure Corn Rootworm in Corn Following Soybean.

	Untreated	DAS-59122-7	Granular Insecticide	Liquid Insecticide	High rate IST
CRW Damage					
Root rating (0-3)[1]	2.5	0.08	0.20	0.36	1.70
% Lodged[2]	56%	5%	12%	20%	38%
Variable Cost	----------**$/acre**----------				
Seed @ 30,000/acre[3]	$37.50	$37.50	$37.50	$37.50	$37.50
CRW premium	0	$13.13–16.88	0	0	0
Low rate IST	$4.50	$4.50	0	0	0
Insecticide	0	0	$12.00–15.00	$12.00–15.00	0
Insecticide equipment & application	0	0	$2.00	$2.00	0
Other variable costs[4]	$139.00	$139.00	$139.00	$139.00	$139.00
Fuel adjustment[5]	$3.93	0	0	0	0
Labor adjustment[6]	$1.65	0	$0.55	$0.55	$0.55
Harvest adjustment[7]	-$6.10	-$0.37	-$1.48	-$2.24	-$3.77
SUBTOTAL	$180.48	$193.76–197.51	$189.57–192.57	$188.81–191.81	$190.53
Yield	----------**bushels/acre**----------				
Maximum yield	150	150	150	150	150
Harvest loss from lodging[8]	-7	0	-3	-3	-3
CRW loss[9]	-31	-2	-6	-11	-21
SUBTOTAL	112	148	141	136	126
	----------**$/acre**----------				
Gross Returns @ $2.10/bushel	$234.90	$310.17	$295.54	$285.62	$265.49
Returns over Variable Cost	$54.42	$112.66–115.66	$102.97–105.97	$93.81–96.81	$74.96

[1] High pressure defined as RDR of 2.5 on 0–3 rating scale in untreated field. Expected RDR in DAS-59122-7. Expected RDR in insecticides & IST treatments.
[2] % lodging = 0.4175 + 22.083*RDR.
[3] Planting rate is 30,000 kernels per acre; seed cost for 80,000 kernels/unit is $100.00.
[4] Includes costs for fertilizer, lime, herbicide, drying, storage, machinery, harvest, insurance, and interest (Duffy and Smith 2003) for 150 bushels/acre production.
[5] Fuel adjustment: 50% of normal combine cost @ $7.87/acre (Duffy and Smith 2003) if lodging is >40%.
[6] Labor adjustment for harvesting lodged corn: $9.00/hour; normal combine speed 4.5 mph; for lodging ≥10% but <40%, combine speed reduced by 25%; for lodging ≥40%, combine speed reduced by 50%.
[7] Variable costs for harvest (hauling, drying, and handling) reduced $0.16/bushel for yields below 150 bushels/acre.
[8] Assumes 2% loss for lodging ≥10% but <40% and 5% loss for lodging ≥40%.
[9] CRW loss: Change in yield = 12.254*RDR .

Table 8. Economic Analysis of Moderate Pressure Corn Rootworm in Corn Following Soybean.

	Untreated	DAS-59122-7	Granular Insecticide	Liquid Insecticide	High rate IST
CRW Damage					
Root rating (0-3)[1]	1.0	0.08	0.2	0.4	0.7
% Lodged[2]	23	2	5	8	15
Variable Cost	----------**$/acre**----------				
Seed @ 30,000/acre[3]	$37.50	$37.50	$37.50	$37.50	$37.50
CRW premium	0	$13.13–16.88	0	0	0
Low rate IST	$4.50	$4.50	0	0	0
Insecticide	0	0	$9.00–11.25	$9.00–11.25	$17.25
Insecticide equipment & application	0	0	$2.00	$2.00	0
Other variable costs[4]	$139.00	$139.00	$139.00	$139.00	$139.00
Fuel adjustment[5]	0	0	0	0	0
Labor adjustment[6]	$0.55	0	0	0	$0.55
Harvest adjustment[7]	-$2.44	-$0.15	-$0.40	-$0.70	-$1.80
SUBTOTAL	$174.61	$193.98–197.73	$187.10–189.35	$186.80–189.05	$192.50
Yield	----------**bushels/acre**----------				
Maximum yield	150	150	150	150	150
Harvest loss from lodging[8]	-3	0	0	0	-3
CRW loss[9]	-12	1	3	4	8
SUBTOTAL	135	149	147	146	139
	----------**$/acre**----------				
Gross Returns @ $2.10/bushel	$282.97	$313.07	$309.74	$305.77	$291.41
Returns over Variable Cost	$108.36	$115.34–119.09	$120.39–122.64	$116.72–118.97	$98.91

[1] Moderate pressure defined as RDR of 1 on 0–3 rating scale in untreated field. Expected RDR in DAS-59122-7. Expected RDR in insecticide & IST treatments .
[2] % lodging = 0.4175 + 22.083*RDR.
[3] Planting rate is 30,000 kernels per acre; seed cost for 80,000 kernels/unit is $100.00.
[4] Includes costs for fertilizer, lime, herbicide, drying, storage, machinery, harvest, insurance, and interest (Duffy and Smith 2003) for 150 bushels/acre production.
[5] Fuel adjustment: 50% of normal combine cost @ $7.87/acre (Duffy and Smith 2003) if lodging is >40%.
[6] Labor adjustment for harvesting lodged corn: $9.00/hour; normal combine speed 4.5 mph; for lodging ≥10% but <40%, combine speed reduced by 25%; for lodging ≥40%, combine speed reduced by 50%.
[7] Variable costs for harvest (hauling, drying, and handling) reduced $0.16/bushel for yields below 150 bushels/acre.
[8] Harvest loss: assumes 2% loss for lodging ≥10% but <40% and 5% loss for lodging ≥40%.
[9] CRW loss: Change in yield = 12.254*RDR .

ii. BPPD Review

Dow and Pioneer analyzed three different farming operations to illustrate the economic benefits that event DAS-59122-7 (Cry34/35Ab1) corn hybrids may provide to growers. Per the Agency's request, Dow and Pioneer revised the original tables (Tables 19–21; Master Record Identification Number (MRID No.) 461239-21) to include variable cost adjustments, based on predicted yield, for hauling, drying, and handling. By using a static value for variable costs (original tables), economic models were slightly biased in favor of higher yielding treatments (Cry34/35Ab1 corn hybrids) and against lower yielding treatments because static values do not consider the effect that yield has on operating costs. The revised tables showed increased returns over variable costs for the untreated corn hybrids and high-rate insecticide treatments but had minimal impact on other treatments (Tables 6–8).

BPPD agrees with Dow and Pioneer's analysis of the economic benefits that event DAS-59122-7 corn hybrids may provide to growers. Potential benefits include higher corn yields, reduced expenditures on insecticides and insecticide application, and the potential for lower harvest costs due to reduced lodging. Additional costs associated with event DAS-59122-7 include higher seed prices and use of a refuge. Net benefits depend on the severity of the corn rootworm problem and on the price of event DAS-59122-7 seed. Models suggest that, in continuous corn fields with moderate CRW pressure, hybrids containing event DAS-59122-7 and insecticide-treated non-transgenic varieties should provide similar returns (Table 6). In contrast, slightly higher returns are predicted in corn-soybean rotations with high rootworm pressure (Table 7). Additional benefits, which are not quantified in these models, include the potential for more predictable yields and reduced exposure to insecticides. Future models should also consider the cost-benefit of resistance management programs.

e. Practical Benefits

i. Summary of Submitted Information

Cry34/35Ab1 (event DAS-59122-7) corn offers many practical advantages to corn growers over other CRW control measures**.** First, longer season varieties can be used, because later planting dates, which have traditionally helped to mitigate CRW damage, are unnecessary. Second, compared to use of chemical insecticides, growers should be able to plant more quickly because they won't have to stop and refill the insecticide boxes. Third, insecticidal seed treatments may be applied to Cry34/35Ab1 seeds to permit control of associated pests such as wireworm, grub, maggots, and cutworms, providing protection against multiple pests. Fourth, planting Cry34/35Ab1 corn is expected to save the grower money in application, insecticide, labor, fuel, equipment, storage, and packaging disposal costs (no pesticide containers). Fifth, Cry34/35Ab1 corn is labeled for general use and thus, provides an alternative to restricted use products. Sixth, Cry34/35Ab1 corn is expected to provide the grower and other occupational workers greater safety, as well as fewer adverse environmental impacts, compared to use of chemical insecticides.

ii. BPPD Review

BPPD agrees that Cry34/35Ab1 corn offers many practical advantages to corn growers over conventional insecticides, as stated above.

f. Market Adoption and Pesticide Use Reduction

i. Summary of Submitted Information

It is expected that growers who plant Cry34/35Ab1 CRW-protected corn will significantly reduce use of chemical insecticides for rootworm control. The addition of commercially available Cry34/35Ab1 corn will benefit growers for the following reasons: (1) corn varieties containing the Cry34/35Ab1 CRW-protected corn will compete with Cry3Bb1 corn varieties, creating price competition among transgenic CRW control technologies; and (2) corn varieties containing the Cry34/35Ab1 offer alternatives to current Cry3Bb1 CRW-protected corn varieties in terms of plant genetics and performance attributes.

ii. BPPD Review

Rootworm damage may cost U.S. corn growers $1 billion annually in control costs and crop losses (Gianessi *et al*. 2002). In the year 2000, almost 8 million pounds of CRW insecticide, costing $172 million, were applied to 14 million acres or 17% of U.S. corn acreage ($12.29 per acre). CRW-infested acreage is projected to increase from approximately 31.8 million acres in 2005 to 39 million acres by 2013 (Table 9).

Grower demand for CRW control technologies is influenced by the level of CRW infestation (acreage and degree of infestation), comparative cost-benefit of competing CRW control technologies, U.S. and global market acceptance and approval of a technology, and other regulatory constraints (e.g., refuge requirements). BPPD anticipates, through evaluation of National Agricultural Statistics Service (NASS) (of the United States Department of Agriculture) and Doane® Agricultural Services databases on CRW damage and control costs, that the market for transgenic in-plant CRW protection will increase by 2.6% per year, reaching 18 to 19 million acres by the year 2013 (Table 9).

Anticipated economic and pesticide use reduction benefits of Cry34/35Ab1 corn largely depend on market penetration. Introduction of commercially available Cry34/35Ab1 corn hybrids is expected to create price competition among commercially available transgenic CRW-protected corn products and will offer a greater variety of plant genetics and performance attributes to growers.

Increased adoption of transgenic CRW-protected corn products is expected to result in reduced use of many of the chemical insecticides registered for CRW control, which are highly toxic to humans and the environment, are in Special Review, and have restricted use labels. The chemical insecticides (larvicides and adulticides) subject to the greatest use reduction following adoption of transgenic CRW-protected hybrids are the following: organophosphates (chlorpyrifos, tebupirimphos, terbufos, methyl parathion, and chlorethoxyfos), pyrethroids (tefluthrin, cyfluthrin, bifenthrin, and lambda cyhalothrin), carbamates

(carbofuran), and pyrazoles (fipronil) (Table 10). Adoption of transgenic CRW-protected corn products is not expected to result in reduced use of seed treatments (nicotinoids); however, seed treatment products are generally less toxic than at-plant and post-plant products.

Table 9. Projected Acreage with Corn Rootworm Infestation and Breakdown of Associated CRW Control Practices for the Years 2000 to 2013 (Note: Information Presented for 2000 and 2002 Reflect Actual Infestation and Insect Control Tactics).

Year	Acres Infested	Acres Treated	CRW-Protected *Bt* Corn Acreage	Conventional Treatments
	----------acreage x 10^6----------			
2000	28.0	14.0	0.0	14.0
2001[1]	-	-	-	-
2002	29.5	14.7	0.0	14.7
2003	30.2	15.1	1.0	14.1
2004	31.0	15.5	2.5	13.0
2005	31.8	15.9	4.0	11.9
2006	32.6	16.3	6.0	10.3
2007	33.5	16.7	7.2	9.5
2008	34.3	17.2	8.6	8.5
2009	35.2	17.6	10.4	7.2
2010	36.1	18.1	11.9	6.1
2011	37.1	18.5	13.7	4.8
2012	38.0	19.0	15.8	3.2
2013	39.0	19.5	16.8	2.7
Annual Growth Rate	2.58%	2.58%	16.8%	-14.36%

[1] The Agency does not have data for the 2001 growing season.

Table 10. Use of Chlorpyrifos, Phorate, Tefluthrin, and Terbufos Insecticides for Rootworm Control in Corn.

	Chlorpyrifos			Phorate			Tefluthrin			Terbufos		
Source of data[1]	G	U-97	U-03	G	U-97	U-03	G	U-97	U-03	G	U-97	U-03
State	. ----------1,000 pounds active ingredient ----------											
Colorado	7		- 2	12			1		-	218		125
Illinois	810	2,105	747	120	–		68	57	205	466	1,117	–
Indiana	299	267	621	62			36	31	93	558	–	473
Iowa	1,044	1,248	366	114	–		71	35	–	366	922	–
Kansas	91		–				19		–	226		
Maryland	45						5			11		
Michigan	135	153	146	47			15		–	155	–	–
Minnesota	265	-	214	129		–	21	–	–	206	–	–
Missouri	232	315	106	25	–		1	–	–	23		
Nebraska	294	382	-	134			68	270	76	879	748	246
New York	216		80				147		11	24		–
North Dakota				48			1			107		–
Ohio	363	429	67	36	–		46	–	–		–	–
Oklahoma	1						2			14		
Pennsylvania	132		151	15		–	15		21	31		–
South Dakota	480	–	–	226			10	7	–	502	–	
Texas	50		44			–			5	512		228
Wisconsin	286	254	96	70	–		32	32	43	385	–	–
Total - sum of rows	4,750	5,153	2,638	1,037	–	–	558	432	454	4,681	2,787	1,072
Total - as reported in USDA		5,341	3,024					522	523		3,200	1,660

[1] G = Gianessi *et al*. (2002), data from 1997
U-97 = USDA/NASS (1998), data for 1997
U-03 = USDA/NASS (2004), data for 2003

[2] A - indicates that usage data not published for this active ingredient

g. Direct and Indirect Economic Benefits

i. Summary of Submitted Information

Shifting from conventional CRW control technologies to Cry34/35Ab1 CRW-protected corn is expected to provide direct economic benefits to farmers, by lessening the costs associated with pest control; transgenic corn seed is generally less expensive than chemical pesticide control programs. Indirect benefits are also expected to result from adoption of Cry34/35Ab1 CRW-protected corn. Benefits may include reduced energy consumption, resulting from a decline in the manufacture, transport, and application of chemical insecticides. Waste products, arising from pesticide manufacturing and residual chemical stocks, may also be reduced pending a decline in chemical pesticide manufacture and sales.

Dow and Pioneer calculated indirect economic benefits based on reduced chemical insecticide use following adoption of Cry34/35Ab1 technology. Chemical insecticides consume roughly 2.25×10^8 British thermal units (BTU) per ton for manufacture and distribution, and approximately 94,000 BTU per acre are used for a single pesticide application. Estimated energy savings are approximately 660,000 barrels of crude oil (3.83×10^{12} BTU), assuming a targeted adoption of 23.4×10^6 acres Cry34/35Ab1 *Bt* corn by 2014. These calculations are based on 2.25×10^6 BTU/ton of [active ingredient] pesticide for manufacturing and distribution, and 94,000 BTU/acre for application (one application per acre). Using Gianessi *et al*. (2002)'s 14.5 million pound (7,250 tons) estimate of applied CRW insecticide (annually), estimated annual energy savings would be: [(7,250 tons)(2.25×10^8 BTU/ton) + (23.4×10^6 acres)(94,000 BTU/acre) = 3.83×10^{12} BTU, at 5.8×10^6 BTU/barrel of oil = 660,000 barrels of oil per year.

ii. BPPD Review

BPPD agrees with Dow and Pioneer's conclusion that indirect economic benefits will be obtained from adoption of Cry34/35Ab1 CRW-protected corn. However, the applicants' estimates of energy savings may be inflated. There are four assumptions to check: (1) energy for pesticide manufacturing and distribution; (2) amount of pesticide applied; (3) energy for application; and (4) number of acres. BPPD agrees with the applicants' conclusions on assumptions 1, 2, and 4. However, assumption 3 ("energy for application") may be over estimated[a]. A value within the range of 15,730 to 25,220 BTU/acre may be more accurate than the applicants' estimate of 94,000 BTU/acre.

[a] From Bhat *et al*. (1994), energy to manufacture insecticides is estimated to be 210.7×10^6 BTU/ton and for formulation, packaging, and transportation ranges from 11.2 to 33.5 x 10^6 BTU/ton of active ingredient. An estimate of 2.25×10^8 BTU/ton of [active ingredient] pesticide for manufacturing and distribution is valid. From a number of crop budgets, application on corn is typically assumed to be done using a 50-foot boom sprayer pulled by a 60-horsepower (hp) tractor that covers 25.6 acres/hour. A 60-hp tractor consumes 2.64 gallons of fuel/hour based on the American Society of Agricultural Engineers (ASAE 2001) standards for calculating fuel consumption (0.044 gallons of diesel/horsepower-hour). The American Agricultural Economics Association (2000) methodology for estimating costs of farming incorporates the ASAE standards and also assumes that powered equipment, such as tractors, operate 10% longer than is needed for the actual field operation. Thus, the 60-hp tractor would consume 2.904 gallons of diesel/machine hour in the field (402,760 BTU/machine hour based on 138,690 BTU/gallon of diesel). To calculate the area covered by the sprayer, assume it travels 6.5 miles per hour (mph) with a field efficiency of 0.65 (i.e., it does productive work 65% of the time) (ASAE 2001) [6.5 mph * 5280 ft/mi * 50 ft/(43,560 ft^2/acre) = 25.6 acres/hour]. Based on 25.6 acres/hour, this is equivalent to 15,730 BTU/acre.

h. Human Health Benefits

i. Summary of Submitted Information

Cry34/35Ab1 CRW-protected corn is expected to be safer for handlers, applicators, farmers, and the public than chemical pesticides in current use. Twenty-five of the 39 registered conventional insecticides used to control CRW are classified as "Restricted Use," 12 have the "Danger" label classification, and several are in Agency Special Review (e.g., dimethoate, phorate, and terbufos). Further, each year there are confirmed reports of human illness associated with these registered chemical insecticides.

Adoption of Cry34/35Ab1 corn hybrids has the potential to reduce occupational, farmer, and public health risks associated with the manufacture, transportation, storage, handling, application, and disposal of conventional insecticides, by providing a safer alternative for CRW control.

ii. BPPD Review

BPPD has concluded that the Cry34Ab1 and Cry35Ab1 proteins are unlikely to be allergens and that there is reasonable certainty of no harm from aggregate exposure to the proteins as expressed in corn. (see the Human Health Assessment and Product Characterization chapters of this Biopesticides Registration Action Document (BRAD) for more information on potential human health effects.)

i. Environmental Benefits

i. Summary of Submitted Information

All of the major chemicals used for CRW control can cause major adverse environmental effects under conditions of normal use. Further, these chemicals can spread via spray drift and runoff, contaminating both land and water bodies and impacting non-target organisms. Fifteen of these products are labeled as "toxic," 6 as "highly toxic," 1 as "very highly toxic," and 14 as "extremely toxic" to birds, fish, and other wildlife. Several of the synthetic insecticides, in particular organophosphates and synthetic pyrethroids, exhibit moderate to high toxicity to terrestrial non-target species. Of special concern are methyl parathion and carbofuran, both of which are implicated in bird kills. The three top CRW insecticides (Table 11)—terbufos, chlorpyrifos, and tefluthrin—account for the majority of the acres treated (63%) for CRW control. These three CRW insecticides pose greater ecological risk than do Cry34/35Ab1 CRW-protected corn hybrids.

In contrast, the Cry34/35Ab1 protein is essentially non-toxic to non-target species, including endangered and beneficial species. The Cry34Ab1 and Cry35Ab1 proteins rapidly degrade soil, off-target exposure to non-target species through pollen or soil residues will be insignificant, and there is a minimum potential for runoff; exposure to Cry34Ab1 and Cry35Ab1 is minimal or non-existent. Reduced runoff reduces environmental pollution. The ecological risk assessment and characterization of the Cry34Ab1

and Cry35Ab1 proteins, as expressed in corn, suggests that these proteins pose no significant ecological risk to non-target species, including endangered and beneficial species.

ii. BPPD Review

See BPPD's Cry34/35Ab1 CRW-protected corn hybrid Environmental Assessment chapter in this BRAD for more information on the potential for ecological effects.

Table 11. Comparison of Ecological Risks Associated with Terbufos, Chlorpyrifos, and Tefluthrin.

Endpoint	Terbufos	Chlorpyrifos	Tefluthrin
Mammalian Acute RQ	50	1	0.008
Avian Acute RQ	0.27	0.55	0.0001
Fish acute RQ	11	2	0.77
Freshwater invertebrate RQ	50	20	0.77
Marine/Estuarine Invertebrate RQ	53	162	0.87

[a] Risk is defined as the risk quotient (RQ) >level of concern (LOC). RQ = Toxicity/Exposure. LOC = 1

j. Insect Resistance Management Benefits

i. Summary of Submitted Information

There is concern about the ability of target pests to evolve resistance to CRW control mechanisms, including crop rotation, chemical insecticides, and CRW-protected *Bt*-corn products. Currently, the favored management practice for CRW control is crop rotation, specifically corn-soybean rotations, complimented by application of chemical insecticides (e.g., pyrethroids, organophosphates, carbamates, and pyrazoles) and, more recently, neonicotinoids as seed treatments. Due to continuous use of a small number of active ingredients, CRW has developed resistance to a number of these chemical control products. The recent development of the NCRW extended diapause and WCRW soybean resistant variants have further reduced the efficacy of crop rotation and chemical control options. Since Cry34/35Ab1 CRW-protected corn presents a new mode of action and has good efficacy against CRW, introduction of this corn product is expected to extend the durability of existing rootworm control measures, including other commercially available CRW-protected *Bt*-corn products (e.g., MON 863). To ensure the long-term efficacy of Cry34/35Ab1 CRW-protected corn hybrids, an insect resistance management plan should be implemented.

ii. BPPD Review

See BPPD's Cry34/35Ab1 Insect Resistance Management chapter of this BRAD for the analysis of the dose, mode of action, durability modeling, and the proposed insect resistance management plan for Cry34/35Ab1 CRW-protected corn hybrids.

3. References

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III. REGULATORY POSITION FOR EVENT DAS-59122-7 CORN

A. Initial Registration (August 31, 2005)

Pursuant to section 3(c)(7)(C) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Environmental Protection Agency (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), as mentioned above, has been met because insufficient time has elapsed since the imposition of the requirements for the following data:

1. Independent laboratory analytical method validation.
2. Field degradation studies evaluating accumulation and persistence of the Cry34Ab1 and Cry35Ab1 proteins in several different soils in various strata.
3. Laboratory toxicity test with *Orius insidiosus* (minute pirate bug).
4. Laboratory toxicity test with carabid (ground beetle).
5. Multi-year non-target organism field studies.

The applicants submitted and/or cited data sufficient for EPA to determine that conditional registration of the *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (PHP17662 T-DNA) in event DAS-59122-7 corn (Organization for Economic Cooperation and Development (OECD) Unique Identifier: DAS-59122-7) under FIFRA section 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 and non-target organism effects data are considered sufficient for the period of the conditional registration. These data demonstrate that no foreseeable human health hazards or environmental effects are likely to arise from the use of the products and, furthermore, that the risk of resistance developing to the Cry34Ab1 and Cry35Ab1 proteins during the conditional registrations is not expected to be significant.

Registration of the *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (PHP17662 T-DNA) in event DAS-59122-7 corn (OECD Unique Identifier: DAS-59122-7) is in the public interest for the following reasons:

1. Cry34/35Ab1-protected corn provides effective control of key rootworm pests of field corn and may prove more efficacious than chemical insecticides presently registered for this purpose.

2. Economic models suggest that, under conditions of high rootworm pressure, use of Cry34/35Ab1-protected corn will provide greater net returns to farmers. Cost benefits include reduced expenditures on insecticides, application equipment, and personnel, complimented by greater potential corn yields. Under high rootworm pressure, these benefits are expected to outweigh the higher cost of seed.

3. Registration of Cry34/35Ab1-protected corn is expected to result in further reduction of chemical insecticide use by growers. This is of special importance since many pesticides registered for corn rootworm (CRW) control are highly toxic to humans and the environment, while Cry34/35Ab1-expressing corn poses no foreseeable human health or environmental risks.

4. Cry34/35Ab1 corn will be the second CRW-protected corn trait on the market (the first was Cry3Bb1). The availability of multiple CRW-protected corn products will increase grower choice and price competition, resulting in lower seed prices for consumers and higher adoption rates.

5. The Cry34/35Ab1 CRW-protected corn will provide a different mode of action and extend the durability of other CRW control measures, including other *Bacillus thuringiensis* CRW-protected corn hybrids.

In view of these minimal risks and the clear benefits related to the *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (PHP17662 T-DNA) in event DAS-59122-7 corn (OECD Unique Identifier: DAS-59122-7), EPA believes that the use of the products during the limited period of the conditional registrations 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 section 3(c)(5). Additional data are necessary to evaluate the risk posed by the continued use of these products. Consequently, EPA is imposing the data requirements specified earlier in this chapter.

EPA has determined, as explained in section II(E) of this Biopesticides Registration Action Document (BRAD), that the third criterion for a FIFRA section 3(c)(7)(C) conditional registration has been fulfilled because use of the *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (PHP17662 T-DNA) in event DAS-59122-7 corn (OECD Unique Identifier: DAS-59122-7) under these registrations is in the public interest.

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

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

B. 2010 Update

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 Cry34/35Ab1 corn product registrations, set to expire in September 2010 and described in-depth throughout this BRAD, meet both criteria (1) and (2):

(1) Event DAS-59122-7 Corn (EPA Reg. Nos. 68467-5 and 29964-4)
(2) Herculex® XTRA Insect Protection Corn (EPA Reg. Nos. 68467-6 and 29964-5)

All of these Cry34/35Ab1 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 Cry34/35Ab1-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 and summarized in section II(C)(2)(b) of this BRAD, 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(E) of this BRAD to support the 2005 registration of Event DAS-59122-7 corn (e.g., reduction in use of conventional insecticides that are highly toxic to both humans and the environment).

In conclusion, as the expiring Cry34/35Ab1 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[a] as follows:

Product Name (EPA Reg. No.)	Expiration Date
Herculex® RW Insect Protection (68467-5)	September 30, 2015
Herculex® Rootworm Insect Protection (29964-4)	September 30, 2015
Herculex® XTRA Insect Protection (68467-6)	September 30, 2015
Herculex® XTRA Insect Protection (29964-5)	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 will remain as terms or conditions for the purposes of the amendments.

[a] See section III(C) of this BRAD for an explanation describing how the expiration dates were determined.

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 Cry1Ab or Cry1F 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 plant-incorporated protectant (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 Agricultural Biotechnology Stewardship Technical Committee (ABSTC), in conjunction with the National Corn Growers Association, proposed uniform insect resistance management (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.

APPENDIX A

GLOSSARY OF ACRONYMS AND ABBREVIATIONS

AAEA	American Agricultural Economics Association
ABSTC	Agricultural Biotechnology Stewardship Technical Committee
ADP	adenosine diphosphate
AIBS	American Institute of Biological Sciences
ANOVA	analysis of variance
AP	alkaline phosphatase
APHIS	Animal and Plant Health Inspection Service (of the United States Department of Agriculture)
ARS	Agricultural Research Service (of the United States Department of Agriculture)
ASAE	American Society of Agricultural Engineers
BCW	black cutworm
BLAST	Basic Local Alignment Search Tool
BLASTP	BLAST search that compares an amino acid query sequence with others stored in protein sequence databases
BPPD	Biopesticides and Pollution Prevention Division
BRAD/BRADs	Biopesticides Registration Action Document/Biopesticides Registration Action Documents
BSA	bovine serum albumin
Bt/*B.t.*	*Bacillus thuringiensis*
BTU	British thermal units
bw	body weight
°C	Celsius (degrees)
CAP/CAPs	Compliance Assurance Program/Compliance Assurance Programs
CEW	corn earworm
CLA	corn leaf aphid
CFR	Code of Federal Regulations
cm	centimeter/centimeters
Codex	Codex Alimentarius Commission
CRW	corn rootworm
CVs	coefficients of variation
Da	Dalton/Daltons
DNA	deoxyribonucleic acid
DT_{50}	half-life
DT_{90}	time until 90% decay
EC_{50}	growth inhibition
ECB	European corn borer
ED_{50}	median effective dose (produces desired effect in 50% of population)
EDSP	Endocrine Disruptor Screening Program
EEC	Estimated Environmental Concentration
ELISA/ELISAs	enzyme-linked immunosorbent assay/enzyme-linked immunosorbent assays
EPA	Environmental Protection Agency (the Agency)
EPA Reg. No.	Environmental Protection Agency Registration Number
EPA Reg. Nos.	Environmental Protection Agency Registration Numbers
ESI MS/MS	electrospray ionization tandem mass spectrometry
FAW	fall armyworm
FFDCA	Federal Food, Drug, and Cosmetic Act

GLOSSARY OF ACRONYMS AND ABBREVIATIONS, CONTINUED

FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FR	Federal Register
ft	foot/feet
ft/mi	feet per mile
ft^2/acre	square feet per acre
g	gram/grams
GI_{50}	50% growth inhibition
GIPSA	Grain Inspection, Packers and Stockyards Administration (of the United States Department of Agriculture)
GM	genetically modified
ha	hectare/hectares
HEEC	Highest Estimated Environmental Concentration
HGT	horizontal gene transfer
hp	horsepower
HPLC	high-performance liquid chromatography
IA	Iowa
IC	insecticidal crystal
ICP/ICPs	insecticidal crystal protein/insecticidal crystal proteins
IL	Illinois
ILSI-CERA	International Life Sciences Institute Research Foundation – Center for Environmental Risk Assessment
ILV	independent laboratory validation
IN	Indiana
IRM	insect resistance management
ISBN	International Standard Book Number
K_M	Michaelis-Menten constant
kb	kilobase
kDa	kiloDalton
kg	kilogram/kilograms
L	liter/liters
LC_{50}	median lethal concentration. A statistically derived concentration of a substance that can be expected to cause death in 50% of test animals. It is usually expressed as the weight of substance per weight or volume of water, air, or feed (e.g., mg/L, mg/kg, or ppm).
LD_{50}	median lethal dose. A statistically derived single dose that can be expected to cause death in 50% of the test animals when administered by the route indicated (oral, dermal, inhalation). It is expressed as a weight of substance per unit weight of animal (e.g., mg/kg).
LLC	limited liability company
LOAEC	Lowest Observed Adverse Effect Concentration
LOC	Level of Concern
LOQ	limit of quantitation
m	meter/meters
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MCRW	Mexican corn rootworm
µg	microgram/micrograms
µg/cm^2	micrograms per square centimeter

GLOSSARY OF ACRONYMS AND ABBREVIATIONS, CONTINUED

µg/g	micrograms per gram
µL	microliter/microliters
mg	milligram/milligrams
mg/kg	milligrams per kilogram
mg/mL	milligrams per milliliter
MHD	maximum hazard dose
mL	milliliter/milliliters
MN	Minnesota
MO	Missouri
MOA	mode of action
mol/L	moles per liter
mol/mol	moles to moles
mph	miles per hour
MRID No./MRID Nos.	Master Record Identification Number/Master Record Identification Numbers
MRL/MRLs	maximum residue level/maximum residue levels
N/A	not applicable
NAS	National Academy of Sciences
NASS	National Agricultural Statistics Service (of the United States Department of Agriculture)
NCBI	National Center for Biotechnology Information
NCEAS	National Center for Ecological Analysis and Synthesis
NCRW	northern corn rootworm
NE	Nebraska
ng	nanograms/nanograms
ng/mg	nanograms per milligram
NIS	Node Injury Scale
nM	nanomolar
NOAEL	No Observed Adverse Effect Level
OCSPP	Office of Chemical Safety and Pollution Prevention
OECD	Organization for Economic Cooperation and Development
OPP	Office of Pesticide Programs
OPPTS	Office of Prevention, Pesticides, and Toxic Substances
ORF	open reading frame
PAT/*pat*	phosphinothricin acetyltransferase
PBS	phosphate buffered saline
PC Code	Pesticide Chemical Code
PCA	Phased Compliance Approach
PCR	polymerase chain reaction
PIP/PIPs	plant-incorporated protectant/plant-incorporated protectants
ppm	parts per million
RDR	root damage rating
RIP/RIPs	ribosomal inhibition protein/ribosomal inhibition proteins
RNA	ribonucleic acid
RQ	risk quotient
RRA	relative rate of adaptation
RSD	relative standard deviation
SAP	Scientific Advisory Panel (the Panel)
SCRW	southern corn rootworm

GLOSSARY OF ACRONYMS AND ABBREVIATIONS, CONTINUED

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SSA	Sublethal Seedling Assay
SWCB	southwestern corn borer
T-DNA	transfer deoxyribonucleic acid
TEP	total extractable protein
TGAI	technical grade of the active ingredient
TX	Texas
tr	truncated
U.S.	United States
USDA	United States Department of Agriculture
V_{max}	maximum velocity of the reaction
VIPs	vegetative insecticidal proteins
WBCW	western bean cutworm
WCRW	western corn rootworm
WI	Wisconsin
w/w	weight-to-weight

Table 1. Currently Registered PIPs Expressing Cry34Ab1 and Cry35Ab1 Proteins.

EPA Registration Number	Registration Name	Company and Address	Active Ingredient(s)	Initial Date of Registration
524-581	MON 89034 x TC1507 x MON 88017 x DAS-59122-7 (or SmartStax™)	Monsanto Company 800 North Lindbergh Boulevard St. Louis, MO 63167	• Cry1A.105 • Cry2Ab2 • Cry1F • Cry3Bb1 • Cry34Ab1 • Cry35Ab1	July 20, 2009
524-584	MON 89034 x DAS-59122-7 Note: This registration is for breeding purposes, agronomic testing, increasing inbred seed stocks, and producing hybrid seed only.	Monsanto Company 800 North Lindbergh Boulevard St. Louis, MO 63167	• Cry1A.105 • Cry2Ab2 • Cry34Ab1 • Cry35Ab1	December 14, 2009
524-586	MON 88017 x DAS-59122-7 Note: This registration is for breeding purposes, agronomic testing, increasing inbred seed stocks, and producing hybrid seed only.	Monsanto Company 800 North Lindbergh Boulevard St. Louis, MO 63167	• Cry3Bb1 • Cry34Ab1 • Cry35Ab1	December 14, 2009
524-588	MON 89034 x TC1507 x DAS-59122-7 Note: This registration is for breeding purposes, agronomic testing, increasing inbred seed stocks, and producing hybrid seed only.	Monsanto Company 800 North Lindbergh Boulevard St. Louis, MO 63167	• Cry1A.105 • Cry2Ab2 • Cry1F • Cry34Ab1 • Cry35Ab1	December 14, 2009
524-589	MON 89034 x MON 88017 x DAS-59122-7 Note: This registration is for breeding purposes, agronomic testing, increasing inbred seed stocks, and producing hybrid seed only.	Monsanto Company 800 North Lindbergh Boulevard St. Louis, MO 63167	• Cry1A.105 • Cry2Ab2 • Cry3Bb1 • Cry34Ab1 • Cry35Ab1	December 14, 2009
524-590	TC1507 x MON 88017 x DAS-59122-7 Note: This registration is for breeding purposes, agronomic testing, increasing inbred seed stocks, and producing hybrid seed only.	Monsanto Company 800 North Lindbergh Boulevard St. Louis, MO 63167	• Cry1F • Cry3Bb1 • Cry34Ab1 • Cry35Ab1	December 14, 2009
29964-4	Herculex® Rootworm Insect Protection	Pioneer Hi-Bred International, Incorporated 7100 N.W. 62nd Avenue P.O. Box 1000 Johnston, IA 50131-1000	• Cry34Ab1 • Cry35Ab1	August 31, 2005
29964-5	Herculex® XTRA Insect Protection	Pioneer Hi-Bred International, Incorporated 7100 N.W. 62nd Avenue P.O. Box 1000 Johnston, IA 50131-1000	• Cry1F • Cry34Ab1 • Cry35Ab1	October 27, 2005

EPA Registration Number	Registration Name	Company and Address	Active Ingredient(s)	Initial Date of Registration
29964-6	Optimum® AcreMax™ 1 Note: This registration is a seed blend.	Pioneer Hi-Bred International, Incorporated 7100 N.W. 62nd Avenue P.O. Box 1000 Johnston, IA 50131-1000	• Cry1F • Cry34Ab1 • Cry35Ab1	April 30, 2010
29964-8*	1507 x 59122 x MON 810	Pioneer Hi-Bred International, Incorporated 7100 N.W. 62nd Avenue P.O. Box 1000 Johnston, IA 50131-1000	• Cry1F • Cry34Ab1 • Cry35Ab1 • Cry1Ab	February 24, 2010
29964-9*	59122 x MON 810	Pioneer Hi-Bred International, Incorporated 7100 N.W. 62nd Avenue P.O. Box 1000 Johnston, IA 50131-1000	• Cry34Ab1 • Cry35Ab1 • Cry1Ab	February 24, 2010
29964-10	Optimum® AcreMax™ RW Note: This registration is a seed blend.	Pioneer Hi-Bred International, Incorporated 7100 N.W. 62nd Avenue P.O. Box 1000 Johnston, IA 50131-1000	• Cry34Ab1 • Cry35Ab1	April 30, 2010
68467-5	Herculex® RW Insect Protection	Mycogen Seeds c/o Dow AgroSciences LLC 9330 Zionsville Road Indianapolis, IN 46268-1054	• Cry34Ab1 • Cry35Ab1	August 31, 2005
68467-6	Herculex® XTRA Insect Protection	Mycogen Seeds c/o Dow AgroSciences LLC 9330 Zionsville Road Indianapolis, IN 46268-1054	• Cry1F • Cry34Ab1 • Cry35Ab1	October 27, 2005
68467-7	MON 89034 x TC1507 x MON 88017 x DAS-59122-7 (or SmartStax™)	Mycogen Seeds c/o Dow AgroSciences LLC 9330 Zionsville Road Indianapolis, IN 46268-1054	• Cry1A.105 • Cry2Ab2 • Cry1F • Cry3Bb1 • Cry34Ab1 • Cry35Ab1	July 20, 2009
68467-9	MON 89034 x TC1507 x DAS-59122-7 Note: This registration is for breeding purposes, agronomic testing, increasing inbred seed stocks, and producing hybrid seed only.	Mycogen Seeds c/o Dow AgroSciences LLC 9330 Zionsville Road Indianapolis, IN 46268-1054	• Cry1A.105 • Cry2Ab2 • Cry1F • Cry34Ab1 • Cry35Ab1	December 14, 2009
68467-10	MON 89034 x MON 88017 x DAS-59122-7 Note: This registration is for breeding purposes, agronomic testing, increasing inbred seed stocks, and producing hybrid seed only.	Mycogen Seeds c/o Dow AgroSciences LLC 9330 Zionsville Road Indianapolis, IN 46268-1054	• Cry1A.105 • Cry2Ab2 • Cry3Bb1 • Cry34Ab1 • Cry35Ab1	December 14, 2009
68467-11	MON 89034 x DAS-59122-7 Note: This registration is for breeding purposes, agronomic testing, increasing inbred seed stocks, and producing hybrid seed only.	Mycogen Seeds c/o Dow AgroSciences LLC 9330 Zionsville Road Indianapolis, IN 46268-1054	• Cry1A.105 • Cry2Ab2 • Cry34Ab1 • Cry35Ab1	December 14, 2009

EPA Registration Number	Registration Name	Company and Address	Active Ingredient(s)	Initial Date of Registration
68467-13	MON 88017 x DAS-59122-7 Note: This registration is for breeding purposes, agronomic testing, increasing inbred seed stocks, and producing hybrid seed only.	Mycogen Seeds c/o Dow AgroSciences LLC 9330 Zionsville Road Indianapolis, IN 46268-1054	• Cry3Bb1 • Cry34Ab1 • Cry35Ab1	December 14, 2009
68467-15	TC1507 x MON 88017 x DAS-59122-7 Note: This registration is for breeding purposes, agronomic testing, increasing inbred seed stocks, and producing hybrid seed only.	Mycogen Seeds c/o Dow AgroSciences LLC 9330 Zionsville Road Indianapolis, IN 46268-1054	• Cry1F • Cry3Bb1 • Cry34Ab1 • Cry35Ab1	December 14, 2009

*Registration expires October 31, 2010.