US Environmental Protection Agency
Office of Pesticide Programs

BIOPESTICIDE REGISTRATION ACTION DOCUMENT

Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 Proteins and the Genetic Material Necessary for Their Production in Event DAS-59122-7 Corn

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U.S. Environmental Protection Agency
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Biopesticides and Pollution Prevention Division

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I. Overview

A. Executive Summary

EPA has conditionally registered Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International's new active ingredient, Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (plasmid insert PHP 17662) in Event DAS-59122-7 corn. The Agency has determined that the use of this pesticide is in the public interest and that it will not cause any unreasonable adverse effects on the environment during the time of conditional registration.

The new products are the second PIP to offer protection against corn rootworm (CRW), and they are expected to result in a further reduction of chemical insecticide use by growers. The reduced chemical pesticide use will benefit the environment directly and can mean less exposure to people who apply chemical pesticides to corn. The availability of multiple CRW-protected corn products will also increase grower choice and price competition, resulting in lower seed prices for growers and higher adoption rates.

The new corn plant-incorporated protectant, Event DAS-59122-7 Corn, produces its own insecticide within the corn plant derived from *Bacillus thuringiensis* (Bt), a naturally occurring soil bacterium. The Bt proteins used in this product, called Cry34Ab1 and Cry35Ab1 (Cry 34/35), control corn rootworm, a highly destructive pest responsible for the single largest use of conventional insecticides in the United States.

In order to reduce the possibility of corn rootworm developing resistance to Bt, EPA is requiring Mycogen and Pioneer to ensure that 20 percent of the planted acreage of this product be set aside where non-CRW-protected Bt corn will be grown to serve as a "refuge." These refuge areas will support populations of corn rootworm not exposed to the Bt corn. The insect populations in the refuges will help prevent resistance development when they cross-breed with insects in the Bt fields. This resistance management strategy was developed as a condition of the registration, and EPA will require routine monitoring and documentation that these measures are followed. The submitted insect resistance management data support a 5-year registration until 2010.

A tolerance exemption under 40 CFR Part 174.457 has also been approved for *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production in corn.

<u>Product Characterization</u>

Cry34Ab1 and Cry35Ab1 proteins are from *Bacillus thuringiensis* PS149B1 and have activity against certain beetles.

B.t. Cry34/35Ab1 corn was produced by *Agrobacterium tumefaciens*-mediated transformation of public corn line (Hi-II) with the T-DNA from plasmid PHP17662, which contains *cry*34Ab1, *cry*35Ab1, *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 *B.t*. Characterization of the DNA isolated from *B.t.* Cry34/35Ab1 corn using restriction enzyme digests and Southern blot analysis 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 demonstrate 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.

Studies on the mode of action of Cry34Ab1 and Cry35Ab1 indicate that similar to other *B.t*. delta-endotoxins, Cry34Ab1 and Cry35Ab1 appear to target midgut epithelial cells in susceptible larvae. 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 Biochem. 43. 12349-12357). Ribosomal inhibition activity was also investigated. The results demonstrated that the insecticidal activity of Cry34Ab1 and Cry35Ab1 is not associated with the inhibition of protein synthesis.

Human Health Assessment

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

Three acute oral toxicity studies on Cry34Ab1 and Cry35Ab1 in mice were submitted, which indicated that these proteins are non-toxic to humans. 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 sequence databases to identify any potential similarities with known toxins. No similarities were identified that would raise a safety concern. 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.

Regarding allergenicity potential, 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 simulated gastric fluid, and

Cry34Ab1 is digested at a moderate rate in simulated gastric fluid; and 5) Cry34Ab1 and Cry35Ab1 are not glycoslyated when expressed in maize. EPA has concluded that the potential for the Cry34Ab1 and Cry35Ab1 proteins to be food allergens is minimal.

A tolerance exemption exists under 40 CFR Part 174.457 has also been approved for *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production in corn that includes Event DAS-59122-7 corn.

Environmental Assessment

The Agency is using a Maximum Hazard Dose Tiered system for biopesticide non-target wildlife hazard assessment. When no adverse effects at the maximum hazard dose are observed, 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 Event DAS-59122-7 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 listed by the US Fish and Wildlife Service is expected from the Event DAS-59122-7 corn registration.

At present, the Agency is aware of no identified significant adverse effects of Cry34/35Ab1 proteins 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 the 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 Event DAS-59122-7 corn may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, Event DAS-59122-7 corn requires substantially fewer applications of chemical pesticides. This should result in fewer adverse impacts to non-target organisms because application of nonspecific 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 (IPM) for secondary pests such as aphids and leafhoppers. The overall result of cultivation of corn expressing Cry34/35Ab1 proteins 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, 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 crop. 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.

The Agency finds no hazard to the environment at the present time from cultivation of Event DAS-59122-7 corn for a time-limited registration.

Insect Resistance Management

Dow and Pioneer proposed a Cry34/35 Event 59122-7 corn durability plan 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. In order to reduce the possibility of corn rootworm developing resistance to Bt, EPA is requiring Mycogen and Pioneer to ensure that 20 percent of the planted acreage of this product be set aside where non-CRW-protected Bt corn will be grown to serve as a "refuge." These refuge areas will support populations of corn rootworm not exposed to the Bt proteins. The insect populations in the refuges will help prevent resistance development when they mate with any potentially resistant adult rootworm beetles emerging from the Bt fields. This resistance management strategy was developed as a condition of the registration, and EPA will require routine monitoring and documentation that these measures are followed. The submitted insect resistance management data support a 5-year registration until 2010.

Benefits

In assessing the potential benefits from Event DAS-59122-7 corn, EPA compared the efficacy of Event DAS-59122-7 corn to other chemical controls for CRW, evaluated the human health and environmental benefits compared to registered alternatives, estimated the grower benefits, and estimated the chemical pesticide use reduction from adoption of Event DAS-59122-7 corn. EPA made a determination that the registration of Event DAS-59122-7 corn was in the public interest and that the benefits outweigh the risks.

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.

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.

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.

Cry34/35Ab1 corn is the second CRW-protected corn PIP to be registered (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 growers and higher adoption rates.

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.

B. Use Profile

- **Pesticide Name:** *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (plasmid insert PHP 17662) in Event DAS-59122-7 corn

- **Trade and Other Names:** Event DAS-59122-7 corn, Herculex Rootworm, Herculex RW, Herculex RW Rootworm Protection

- **OPP Chemical Code: 006490**

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

  Pioneer Hi-Bred International, A Dupont Company
  7250 N.W. 62nd Ave., P.O. Box 552, Johnston, IA

- **Type of Pesticide: Plant-Incorporated Protectant**

- **Uses: Field Corn**

- **Target Pest(s): Corn Rootworm.**

II. Science Assessment

The classifications that are found for each data submission are assigned by the EPA science reviewer and are an indication of the usefulness of the information contained in the documents and if the data meet the intent of the test guidelines. A rating of "ACCEPTABLE" indicates the study is scientifically valid and has been satisfactorily performed according to accepted EPA guidelines or other justified criteria. A "SUPPLEMENTAL" rating indicates the data provide some information that can be useful for risk assessment. However, the studies may either have certain aspects not determined to be scientifically acceptable (SUPPLEMENTAL. UPGRADABLE) or that the studies have not been done to fulfill a specific EPA guideline requirement. 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 current EPA guidelines or does not need to be reclassified as "ACCEPTABLE." Both ACCEPTABLE and SUPPLEMENTAL studies may be used in the risk assessment process as appropriate.

II A. PRODUCT CHARACTERIZATION

The Agency's detailed assessment of the product characterization for *Bacillus thuringiensis* Cry34/35Ab1 is found in Edelstein (2004b). Portions of the product characterization data were also reviewed in Wozniak (2001), Wozniak (2002a), and Wozniak (2003a, c, d, and e). *B.t.* Cry34/35Ab1 corn was produced by *Agrobacterium tumefaciens*-mediated transformation of public corn line (Hi-II) with the T-DNA from plasmid PHP17662[a], which contains *cry*34Ab1, *cry*35Ab1, *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 *B.t*. Characterization of the DNA isolated from *B.t.* Cry34/35Ab1 corn using restriction enzyme digests and Southern blot analysis (MRIDs 461239-08 and 461239-09, reviewed in Edelstein, 2004b) 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 demonstrate 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 (MRIDs 461239-05 and 461239-06, reviewed in Edelstein, 2004b). The following techniques were used to characterize and compare the plant-

[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.

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. Glycoslyation analysis indicated that the Cry34Ab1 and Cry35Ab1 proteins are not glycoslyated. 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 indicate that similar to other *B.t.* delta-endotoxins, Cry34Ab1 and Cry35Ab1 appear to target midgut epithelial cells in susceptible larvae (MRID 457906-01, reviewed in Wozniak, 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 Biochem. 43. 12349-12357). Ribosomal inhibition activity was also investigated (461239-10, reviewed in Edelstein, 2004b). 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 demonstrate that these proteins are inactivated at 90 °C and 60 °C, respectively (MRIDs 453584-01, 455845-01, 458086-01, and 458602-01, reviewed in Wozniak (2001, 2002a, 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 461239-04, reviewed in Edelstein, 2004b). The protein expression levels are 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 this product registration are summarized below 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 kDa and 43.8 or 44 kDa insecticidal crystal protein. The term PS149B1 proteins was used prior to the proteins receiving their *B. thuringiensis* toxin nomenclature designations (Crickmore, et al., 2005). The PS149B1 13.6 kDa or 14 kDa and 43.8 or 44kDa proteins are the same as the Cry34Ab1 and Cry35Ab1 proteins, respectively.

Table 2. Submitted Product Characterization Data

Study Title	Summary	MRID #
Product characterization data for *Bacillus thuringiensis* PS149B1 13.6 kDa and 43.8 kDa insecticidal crystal protein expressed in transgenic maize plants	Maize immature embryo tissues were transformed using a linearized fragment from plasmid PHP12560 encoding two ICP genes, *8v6* (13.6 kDa) and *7v7* (43.8 kDa), each driven by the maize ubiquitin promoter, and the phosphinothricin acetyl transferase gene (*pat*), driven by the CaMV 35S promoter. The two ICP genes from *B. thuringiensis* 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 ICP of PS149B1 and PAT. Maize plants expressing these genes were also produced by *Agrobacterium* -mediated transformation of immature embryos. Western corn rootworm (WCR) bioassays were used to assess the bioactivity of the expressed ICP. Within 48 hours of feeding on transgenic maize plants, WCR larvae had midgut symptoms consistent with feeding on d-endotoxins (*e.g.*, swelling, lysis, blebbing, vacuolization). WCR feeding on control plants did not show pathological changes in midgut epithelium. The two ICP were also tested for 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	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 the event 5639 does not contain a *kan* ^{r} sequence. **Classification: Acceptable.**	452422-02
Equivalency of microbial- and maize- expressed PS149B1 proteins	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 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 (SCR) and WCR larvae. While the transgenic maize assay indicated no reduction in damage from	452422-03

	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 149B1 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.**	
Microbial PS149B1 binary delta-endotoxin: maize-insect pest susceptibility study	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 WCR were not sensitive to the d-endotoxins, however. Rootworms (NCR, WCR and SCR) were the most susceptible to the PS149B1 proteins. ECB and Corn ear worm (CEW) demonstrated some inhibition of growth at higher concentrations of the test substance than the rootworm larvae. Black cut worm (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 GI_{50}, were as follows: (most susceptible) WCR, NCR, SCR > ECB, CEW >> WCR adult, CLA, BCW (least susceptible). **Classification: Acceptable.**	452422-04
Quantitative ELISA analysis of PS149B1 protein expression levels in hybrid and inbred lines of maize event TC5639	Leaf, pollen, root and senescent whole plant (WPS) 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 WPS 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 the lowest. Control tissues were negative for the presence of the 14 kDa or the 44 kDa proteins. Coefficients of variation (CV) were lacking for data presented and need to be included in order to adequately assess the results of the ELISA assays. 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 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	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 : negative below 0.5% were evaluated in this study. **Classification: Acceptable**.	453834-01
Characterization of Cry34Ab1 and Cry35Ab1 from recombinant *Pseudomonas fluorescens* and transgenic maize	Cry34Ab1 and Cry35Ab1 proteins were separated from maize leaf extracts and from microbial production sources on SDS-PAGE gels, 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 kD - Cry34Ab1; 44 kD Cry35Ab1). A second protein band reacted with the Cry35Ab1 specific antibody in the microbially produced protein preparation at approximately 40 kD, which is presumably a degradation product from protease activity. This band or signal was noted in the Western blot of the 3 of 6 maize leaf extracts representing the six events, but at	457904-01

	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.**	
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	Southern blots were used to discern the number of insertions from each of the two 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 results 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 represent 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 kb) suggests that the T-DNA insertion is the result of a single insertional event. A 9.4 kb signal in E4497.1.10 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 E4497.1.10 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 is a consequence of the position of the cleavage site for the *StuI* enzyme being within the *pat* ORF. Genomic DNA from control plants (non-transgenics) were 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**.	457902-02
Product characterization data for *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins expressed in transgenic maize plants (PHP17662)	Genes encoding the PAT, Cry34Ab1 and Cry35Ab1 proteins were transferred into the T-DNA region of the disarmed *Agrobacterium 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 kD) and Cry35Ab1 (44 kD) 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 48h of feeding on the transgenic maize plants, WCRW larvae had evidence of cell lysis, blebbing and vacuolization, as would be expected from intoxication with □-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.	457906-01

	Amino acid sequences of the three proteins and micrographs of histopathological midgut responses were presented. **Classification: Acceptable**.	
Quantitative ELISA analysis of Cry34Ab1 and Cry35Ab1 proteins expressed in maize plants transformed with the vector PHP17662	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, 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 Cry35Ab1were 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 through the use of 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 ng Cry34Ab1 protein / mg total extractable protein (TEP). Since data for water content exists for the tissue types sampled, the conversion to ng Cry34Ab1 protein / mg 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
Probe MOA Studies to assess potential for protein synthesis inhibition by *B.t.* PS149B1 Cry34Ab1/Cry35Ab1 proteins in a rabbit reticulocyte assay: re-examination of lab notebook data	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 inhibitory proteins (RIP). This was largely based upon comparison to a BSA control and PBS buffer control. A second experiment, with a different treatment regime and altered protocol concluded that there was some RIP activity associated with the PS149B1 proteins. Further, digesting the 14 kDa protein with trypsin may also reduce translation a little. The two experiments used different amounts of BSA (*i.e.*, 1x and 2x) and this added variable further confounds the interpretation of results. The lack of proper controls and different treatment regimes in the experiments makes 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	459428-01

	or similarity between the d-endotoxins of *Bacillus thuringiensis* or *B. 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.**	
Thermolability of PS149B1 binary delta-endotoxin	Results indicate that the PS149B1 proteins, or at least one of them, is 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 registrant 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
Heat lability of individual proteins of the PS149B1 binary ICP	The percent growth inhibition of larvae as observed in a Southern Corn Rootworm feeding assay was used as a measure or indicator that the Cry 34Ab1 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 suggests 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
Summary of heat lability studies with Cry34Ab1/Cry35Ab1 Slide presentation summarizing Cry34Ab1/Cry35Ab1 heat inactivation studies	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 utilizes the decrease in growth of larval SCRW, measured as mass, as a means of assessing activity remaining in the Cry protein(s), but does not give a direct correlation with protein concentration. Nonetheless, the assay provides for a determination as to the conformational changes (denaturation) that occur following heat treatment. The two studies previously submitted (MRID #455845-01, 453584-01) regarding heat stability of the Cry34Ab1 and Cry35Ab1 proteins demonstrate that these proteins are inactivated at $\leq$90 °C and $\leq$ 60 °C, respectively. The two current submissions serve to clarify the lability of the proteins to heat. Differences in the execution of the two studies complicate the interpretation and comparison of the results, however, the feeding bioassays using the southern corn rootworm indicate that the proteins are no longer active following their respective heat treatments. This is taken as evidence of protein denaturation. The SCRW assay is 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 #455845-01). Additionally, the presence of salts and	458086-01 458602-01

	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**.	
Independent Laboratory Validation Pioneer Hi-Bred International, Inc. 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 ng/mg. The reported data satisfied the requirements of U.S. EPA Guideline OPPTS 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 limit of quantitation (LOQ), and interferences were minor. **Classification: Acceptable.**	461239-01
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 independent laboratory validation (ILV) data for the determination of Cry35Ab1 insecticidal crystal protein in corn matrices and to support the stated limit of quantitation (LOQ) of 0.06 ng/mg. The reported data satisfy the requirements of U.S. EPA Guideline OPPTS 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 relative standard deviation (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 PCR analysis of total genomic DNA from collections of strains of *B. thuringiensis* 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 *B. thuringiensis* 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 No. 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 *cry34Ab1*, *cry35Ab1*, and *pat* Genes: Chile Locations	The data from this study indicate 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.	461239-04

	Classification: Acceptable.	
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 (bICP), 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 also with the efficacy profiles obtained with purified bICP 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 *B.t.* 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 *B.t*. transgenes. The results were also compared with results from a previously submitted study on Cry34Ab1 and Cry35Ab1 isolated from *B.t.* 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 is 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
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 *E. 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	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	461239-08

and DAS-59122-7	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.**	
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
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 (IC_{50}) with a concentration of 0.182 nM. In contrast, Cry34Ab1 and Cry35Ab1, as well as bovine serum albumin (BSA) used as a negative control, showed no inhibition of protein synthesis at concentrations up to 2880 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

II. B. HUMAN HEALTH ASSESSMENT

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

1. Mammalian Toxicity and Allergenicity Assessment

Based upon the human health data provided, there is a 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 this protein.

a) 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 158.740(b)(2)(i)). 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 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 of Cry34Ab1 alone [(MRID 452422-07, reviewed in Edelstein, 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 three 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 LD_{50} for the test substance in male CD-1 mice is greater than 5000 mg/kg. Since the test substance contained Cry34Ab1 at 54% purity, the acute oral LD_{50} for the pure Cry34Ab1 protein is greater than 2700 mg/kg.

In an oral toxicity of Cry35Ab1 alone [(MRID 452422-08, reviewed in Edelstein, 2005a)] , Cry35Ab1 produced from microbial culture was administered to five male mice (5000 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 two 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 is greater than 5000 mg/kg. Since the test substance contained Cry35Ab1 at 37% purity, the acute oral LD_{50} for the pure Cry35Ab1 protein is greater than 1850 mg/kg.

Finally, in an oral toxicity of Cry34Ab1 and Cry35Ab1 combined [(MRID 452422-09, reviewed inWozniak, 2001], a mixture of the microbially produced Cry34Ab1 and Cry35Ab1 proteins (5000 mg test material, containing 482 mg pure Cry34Ab1 and 1520 mg pure Cry35Ab1 (corresponding to an equimolar ratio), per kg body weight) was administered by oral gavage to five female and five 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 two 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 is greater than 5000 mg/kg body weight, corresponding to 2000 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 465847-01, reviewed in Edelstein, 2005c). 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, Roy D., et al. ``Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (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 to known toxic proteins available in public protein data bases.

b) 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 glycosylatedand can be present at high concentrations in the food. In the past, EPA has also considered heat stability in assessing allergenicity potential; however, the FIFRA Scientific Advisory Panel at a March 1-2, 2005 meeting 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 weigh-of-evidence approach.

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

Amino acid sequence. A comparison of amino acid sequences of Cry34Ab1 and Cry35Ab1 with known allergens (MRID 452422-05, reviewed in Wozniak, 2001) 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, Cry 34Ab1 is present at a concentration of approximately 50 ng/mg in grain from Event 59122-7, and Cry 35Ab1 is present at a concentration of approximately 1 ng/mg in grain from Event 59122-7 (MRID 461239-04, reviewed in Edelstein, 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 452422-12, reviewed in Wozniak, 2001) 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 mg/mL; pH 1.2; 37 C) with a pepsin to protein substrate ratio of approximately 20:1, mol/mol (equivalent to 60:1, 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 Cry 35Ab1 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 described above that used the time-to-disappearance endpoint, Dow submitted a second study on the in vitro digestibility of Cry34Ab1 in SGF using a kinetic approach (MRID 455845-02, reviewed in Wozniak, 2002b) . 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 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 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 (MRIDs 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 463886-01, reviewed in Edelstein, 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 Scientific Advisory Panel (SAP) meeting, http://www.epa.gov/oscpmont/sap/#march, to address the scientific issues that arose during the human health safety assessment of Cry34Ab1 and Cry35Ab1. The SAP report (SAP, 2005) is summarized below and in Edelstein (2005b). 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 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 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 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 465568-01, reviewed in Edelstein, 2005c).

Glycosylation. Cry34Ab1 and Cry35Ab1 expressed in corn were shown not to be glycosylated (MRID 461239-06, reviewed in Edelstein, 2004b) .

Conclusion. Considering all of the available information 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, and Cry34Ab1 is digested at a moderate rate in SGF; and 5) Cry34Ab1 and Cry35Ab1 are not glycoslyated 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 since using several time points is "inherently more accurate than the single point assay." However, the Panel pointed out that many of the statements and assumptions Dow made about the kinetic model are incorrect. In particular, Dow 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 states that "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 does not specify what a "significant fraction" is but points 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 states 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 states 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 discusses 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 HPLC). Typically, registrants 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 points 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 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 notes that "there are no data indicating that crystal proteins are allergens."

2. Aggregate Exposures

In examining aggregate exposure, section 408 of the 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. However, oral toxicity testing showed no 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.

3. Cumulative Effects

Pursuant to FFDCA section 408(b)(2)(D)(v), EPA has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity, resulting from the plant-incorporated protectants, we conclude that there are no cumulative effects for the Cry34Ab1 and Cry35Ab1 proteins.

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

a) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry34Ab1 and Cry35Ab1 proteins include the characterization of the expressed the 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 proteins 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 acute oral toxicity data submitted supports the prediction that the Cry34Ab1 and Cry35Ab1 proteins would be non-toxic to humans. As mentioned above, when proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, Roy D., et al. ``Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (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 Bacillus thuringiensis products from which these plant-incorporated protectants were derived. (See 40 CFR 158.740(b)(2)(i)). 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 the observed effects and clarify the source of these effects (Tiers II and III).

Cry34Ab1 and Cry35Ab1 proteins 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. However, data submitted 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 all of the available information 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 glycoslyated when expressed in maize; 4) Cry34Ab1 and Cry35Ab1 will only be present at low levels in food; and 5) Cry35Ab1 is rapidly digested in SGF, and 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 Scientific Advisory Panel (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 (DNA, RNA) which comprise genetic material encoding these proteins and their regulatory regions. The genetic material (DNA, RNA), necessary for the production of the Cry34Ab1 and Cry35Ab1 proteins have been exempted under the blanket exemption for all nucleic acids (40 CFR 174.475).

b) Infants and Children Risk Conclusions

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

In addition, FFDCA section 408(b)(2)(C) also provides that EPA shall apply an additional tenfold margin of safety for infants and children in the case of threshold effects to account for prenatal and

postnatal toxicity and the completeness of the 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, the provision requiring an additional margin of safety does not apply. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply.

c) Overall Safety Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the 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 above, no toxicity to mammals has been observed, nor any indication of allergenicity potential for the plant-incorporated protectants.

5. Other Considerations

a) Endocrine Disruptors

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

b) Analytical Method(s)

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 (MRIDs 461239-01 and 461239-02, reviewed in Edelstein, 2004b).

c) Codex Maximum Residue Level

No Codex maximum residue level exists for the plant-incorporated protectants *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for its production in corn.

6. Tolerance Exemptions

The data submitted and reviewed for Cry34Ab1 and Cry35Ab1 support the petition for a tolerance exemption for *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production in corn,

7. Supporting Data

Table 3. Submitted Data Supporting Human Health Assessment

Study Title	Summary	MRID #
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.	Based upon the search requirements that at least 8 contiguous amino acids are identical to a known allergenic sequence, PS149B1 13.6 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	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 two 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 5000 mg/kg and greater than 2000 mg/kg for an equimolar mixture (1:3) of the pure proteins. **Classification: Acceptable.**	452422-09
In vitro digestibility of PS149B1 proteins	*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 BSA positive control was rapidly digested (within 1 min) 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 to be 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 ICP proteins 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	BSA, 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-lac, was detectable and appeared to change little during the assay time frame. When averaged across the three duplicate gels containing Cry34Ab1, the protein	455845-02

	appears 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 is rapidly degraded in the SGF using this assay and detection methodology. The conditions of the assay are biologically appropriate in temperature, pH, and chemical makeup of the digestive solution. The first order decay rate kinetics accurately portray the digestion of Cry34Ab1. **Classification: Acceptable.**	
SDS-PAGE sensitivity analysis for Cry35Ab1 in support of the simulated gastric fluid digestion study MRID# 452422-12	GelCode Blue staining of the SDS-PAGE gel used to electrophorese Cry35Ab1 samples indicated that the amount of protein remaining which was visualized by dye binding, 15.6 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	The rate of digestion of allergenic and non-allergenic proteins in simulated gastric fluid (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 min.). **Classification: Supplemental.**	461239-20
Digestion Efficiency of Allergens and Non-Allergens in Simulated Gastric Fluid	The digestion of seven allergens and eight non-allergens was assessed in simulated gastric fluid (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
PS149B1 14 kDa Protein: Acute Oral Toxicity Study in CD-1 Mice	PS149B1 14 kDa protein (Cry34Ab1; 54% pure) was evaluated for acute oral toxicity. Five male CD-1 mice received 5000 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 three 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 is greater than 5000 mg/kg; since the test substance contained PS149B1 14 kDa protein (Cry34Ab1) at 54% purity, the acute LD_{50} for the pure protein is greater than 2700 mg/kg. **Classification: Acceptable.**	452422-07

PS149B1 44 kDa Protein: Acute Oral Toxicity Study in CD-1 M	PS149B1 44 kDa protein (Cry35Ab1; 37% pure) was evaluated for acute oral toxicity. Five male CD-1 mice received 5000 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 two 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 is greater than 5000 mg/kg; since the test substance contained PS149B1 44 kDa protein (Cry35Ab1) at 37% purity, the acute LD_{50} for the pure protein is greater than 1850 mg/kg. **Classification: Acceptable.**	452422-08
Lack of Cry34Ab1/Cry35Ab1 Co-Association in Solution	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
Evaluation of the Sequence Similarities of the Cry34Ab1, Cry35Ab1, and PAT Proteins with the Public Protein Sequence Datasets	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, five of which are Cry proteins from *Bacillus thuringiensis* 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, seven of which are similar or identical Cry proteins from *B. thuringiensis*. 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

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C. Environmental Assessment

Background

Dow AgroSciences LLC and Pioneer Hi-Bred International Inc. have requested a registration for *Bacillus thuringiensis* Cry34/35Ab1 binary protein and the genetic material necessary for its production in all corn lines and varieties. This protein is intended to control 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.

The Agency has 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 proteins 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 Scientific Advisory Panel (SAP) recommendations, consultations with scientific experts, and public comments on Plant-Incorporated Protectant (PIP) regulation.

I. Non-Target Wildlife Hazard Assessment

A. The Hazard Assessment Process

The Agency assesses the toxicity of a Cry protein (Bt endotoxin) to representatives of potentially exposed non-target organisms by a tiered testing system starting with Tier I single species maximum hazard dose laboratory data using mortality as the end point. This approach was developed for the Agency by the American Institute of Biological Sciences and approved in 1996 as an acceptable ecological hazard assessment method by a FIFRA Scientific Advisory Panel for microbial pesticides and microbial toxins, and by the December 9, 1999 SAP for protein Plant-Incorporated Protectants (PIPs). The methods were last published as the Harmonized OPPTS Testing Guidelines (EPA 712-C-96-280, February 1996). The guidelines include (but are not limited to) bacteria and their toxins as defined in 40 CFR 152.20. The guidelines apply to microbes and microbial toxins when used as pesticides, including both those that are naturally occurring, and those that are strain-improved, either by natural selection or by deliberate genetic manipulation (Cry34/35Ab1 binary proteins in corn, being bacterial toxins, also fall under these testing guidelines).

The guidelines in Tier I reflect a maximum hazard approach to testing. The OPPTS Harmonized Testing Guidelines (Series' 850 and 885) utilize the tier testing scheme to ensure, to the greatest extent possible, that only the minimum data sufficient to make scientifically sound regulatory decisions will be required. The Agency believes that the Tier I maximum hazard dose testing requirement represents a reasonable approach to evaluating hazards related to the use of biological pesticides, and is one in which negative results allow a high degree of confidence in the safety of the test agents. The Agency expects that most of the plant-incorporated Bt Cry proteins require testing only in the first tier for short-term hazard assessment. Long range adverse effects have to be ascertained by higher-tier long-term field testing. The October 2000 SAP and the National Academy of Sciences (NAS 2000) recommended testing non-target organisms directly in the field. This approach, together with an emphasis on testing of invertebrates found in the corn fields, was also recommended by the August 2002 SAP and was supported by several public comments.

The maximum hazard dose approach for Tier I testing is based on a safety factor times the maximum amount of active ingredient expected to be available to terrestrial and aquatic plants and animals in the environment (i.e., the expected environmental concentration, or EEC). Therefore, data that establishes an LC_{50}, ED_{50}, or LD_{50} that is greater than the maximum hazard dosage level (*e.g.* LD_{50} >10 x EEC) is sufficient to evaluate adverse effects.

The Guidelines call for testing of a single group or groups of test animals at the maximum hazard dose. When the active ingredient is a toxin, the appropriate endpoint would be death of the test organism. Each treatment and control group shall contain at least 10 test animals. When there is only one treatment group, at least 30 animals must be tested at that treatment level. The guidelines provide that the duration of all Tier I tests be about 30 days long. Some test species, notably non-

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

If there are no effects at the maximum hazard dose, lower dose testing is not necessary. If, however, significant toxic effects are noted at the maximum hazard dose level, the Harmonized Guidelines call for testing of multiple groups at sequentially lower doses in order to establish a definitive LD_{50} with confidence limits and quantify the hazard. Sufficient doses and test organisms are required to determine an LD_{50} value and, if necessary, the No Observeable Effect Level (NOEL), or reproductive and behavioral effects such as feeding inhibition, weight loss, etc. are evaluated. Appropriate statistical methods are to be used to express trends and to evaluate the significance of differences in data obtained from different test groups. The statistical methods used must reflect the current state-of-the-art with appropriate statistical power.

On December 9, 1999, the Agency presented the maximum hazard dosing approach to testing of protein PIPs and for possible new data requirements to a FIFRA Scientific Advisory Panel for their recommendations. The December 1999 SAP was generally supportive of the Agency's testing and hazard evaluation. The Panel also recommended more testing of non-target invertebrates closely related to the target species and of species likely to be present in the field of the GM crops. In addition, the October 2000 SAP recommended appropriate field testing should be conducted for non-target organisms. The August 2002 SAP and certain public comments also agreed with this approach with some additions. It was recommended that the choice of appropriate indicator organisms for testing be based on the potential field exposure as deduced from data on Cry protein activity and expression in the plant. The SAP thought that appropriately chosen single species Tier I laboratory tests showing no detrimental effects are sufficient to make a short-term hazard assessment and that field studies be conducted when these tests show toxicity (as higher Tier testing described in the OPPTS Microbial Testing Guidelines) but that proper multi-year commercial field studies with appropriate statistical power are needed to determine long-term ecological effects. The December 9, 1999, SAP, the August 2002 SAP, and several public comments noted that the maximum hazard approach to non-target species testing was not statistically appropriate for determination of No Observeable Effect Levels (NOELs). This comment is in agreement with the Agency's OPPTS Testing Guideline discussed above.

Bt Cry endotoxins are proteins and, unlike inorganic chemicals, do not have the potential to bioaccumulate and thereby result in delayed effects. An accumulation through the food chain is therefore not expected to take place, and there are no data to support this possibility for protein substances. The basic biological properties of proteins also make Bt Cry proteins readily susceptible to metabolic, microbial, and abiotic degradation once they are ingested or excreted into the environment. Although there are reports of soil binding under certain circumstances, the bound Cry proteins are also reported to be rapidly degraded by microbes upon elution from soil. The same sources also report that Bt proteins in the soil of Bt corn fields have no detectable effect on soil

invertebrates or culturable microbial flora. In addition, Bt Cry proteins do not have any characteristics in common with persistent, bioaccumulative chemicals that are transferred through the food chain. Therefore, chronic effects testing of protein substances is not routinely performed.

B. Ecological Effects 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 Coleoptera ssp. 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, honeybee and lady beetle larva maturation studies.

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, honeybees 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 § 158.740), 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 applicant to ascertain effects of Cry34/35Ab1 on beneficial decomposer species because prolonged exposure to Cry34/35Ab1 proteins in soil was a possibility. Honeybee 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. As per the OPPTS 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 of these studies are presented here in both tabular (Table 4) and in a more descriptive format. The complete review record of the submitted data can be found in the individual Data Evaluation Reports (DERs).

Table 4. Tabular results of non-target wildlife and soil fate studies.

Guideline No.	Study	Results	MRID
885.4150	Wild Mammal Testing, Tier I	Mammalian wildlife exposure to Cry34/35Ab1 protein is considered likely; however, the Cry34/35Ab1 toxicity data for Human Health Assessment 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 is not required.	NA
885.4050	Poultry Feeding Study	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
850.1075	Freshwater Fish Testing	There were no treatment-related deaths or clinical signs observed in Rainbow trout fed 100 mg PS149B1 IC binary protein kg^{-1} diet for 8 days. Based on these observations, the 8-day LD_{50} for Rainbow trout is $\geq$ 100 mg PS149B1 IC protein. No fresh water fish hazard is expected from commercial cultivation of Cry34/35Ab1 corn. Classification: Supplemental.	457904-03
850.1010	Aquatic Invertebrate Acute Toxicity Test (*Daphnia magna*)	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 L^{-1} 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.	457904-04
885.4280	Estuarine and Marine Animal Testing, Tier I	The Estuarine and Marine animal studies are not required for this product because the Cry34/35Ab1 is not intended for direct application into the estuarine or marine environment and the very low to no potential for exposure to Cry34/35Ab1 protein from field corn.	NA
885.4300	Nontarget Plant Studies, Tier I	The active ingredient is an insect toxin (Bt endotoxin) that is non-toxic to aquatic and terrestrial plants. Consequently, nontarget plant studies have been waived for this product.	NA

Guideline No.	Study	Results	MRID
885.4380	Honey Bee Larva Testing	The LC_{50} for honeybee larvae and adult bee emergence was found to be >5.6 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 honeybee larvae and adult bee emergence is anticipated. Classification: Acceptable.	453407-01
885.4340	Parasitic Hymenoptera Larva Testing	The LC_{50} for parasitic Hymenoptera was determined to be >280 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 Cry34/35Ab 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.	457904-07
885.4340	Lady Beetle Larval Feeding Study (*C. maculata*)	Significant growth inhibition (80% growth reduction in 7 day study) was observed in *C. 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 (MRIDs 452422-10 and 452422-11); Supplemental (MRID 461239-12), because the study was terminated prior to pupation.	452422-10 452422-11 461239-12
885.4340	Collembola Chronic Dietary Toxicity Study	The LC_{50} for collembola was found to be >12.7 mg PS149B1 a.i./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	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

Guideline No.	Study	Results	MRID
N/A	Insecticidal Activity Spectrum Study	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 WCR were not sensitive to the binary toxins, however. Rootworms (NCR, WCR and SCR) were the most susceptible to the PS149B1 proteins. ECB and Corn ear worm (CEW) demonstrated some inhibition of growth at higher concentrations of the test substance than the rootworm larvae. Black cut worm (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 GI50, were as follows: (most susceptible) WCR, NCR, SCR > ECB, CEW >> WCR adult, CLA, BCW (least susceptible). Classification: Acceptable.	452422-04
154-35	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.	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.	452422-14
N/A	Endangered Species Impact Assessment	Pioneer Hi-Bred 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	Nontarget Invertebrate Ecological Risk Assessment	Submitted data indicates 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 US Fish and Wildlife Service is expected from the proposed Cry34/35Ab1 CRW resistant corn registration. Classification: Acceptable.	461239-13

1. Non-target Wildlife Testing and Hazard Assessment

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 for Human Health Assessment 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 hazard assessment

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 Cry34/35 product registration.

i. Broiler study (MRID 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 libidum* during days 1-20 (Phase I) and a grower/finisher ration containing approximately 63% wet weight corn was provided *ad libidum* 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 further, that long-term exposure to Cry34/35 corn is not expected to pose a hazard to avian wildlife.

c. Aquatic species testing

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 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 OPPTS microbial testing guidelines.

This limit test was conducted at a target concentration of 100 mg PS149B1 IC binary protein kg^{-1} 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 thirty 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 sub lethal 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 is $\geq$ 100 mg PS149B1 IC protein kg^{-1} diet fed at 10 % approximate fish wet body weight day^{-1}. No statistical test was performed because it was a single dose (limit) test. Biological loading exceeded guideline parameters of 0.5 g fish L^{-1}.

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

ii. Aquatic invertebrates (MRID 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 PS149B1 added to water, at a target concentration of 100 mg PS149B1 ICP L^{-1}. The study is procedurally sound and no treatment mortality or behavior changes were reported between the dosed and control replicates during the 48-hr 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.

iii. Estuarine and Marine Animals

The estuarine 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.

iv. Terrestrial and Aquatic Plants

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

d. Non-Target Insect Testing

i. Effects on Honey Bee Larvae (MRID 453407-01)

An acceptable study was conducted based on OPPTS Series 885-4380, Honey bee testing Tier I. This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 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 Cry34/35 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 457904-05)

The study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 885-4340 Non-target Insect Testing, Tier 1.

A dietary toxicity study was conducted to evaluate the effect that *Bacillus thuringiensis* strain PS14B1 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) PS14B1 protein, which is approximately equivalent to 10X the maximum protein concentration present in PS14B1 corn pollen.

Based on this test, the LC_{50} for adult parasitic Hymenoptera exposed to dietary Cry PS14B1 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 *Nasonia vitripennis* is expected from exposure to Cry34/35 corn.

The *Nasonia vitgripennis* study was found acceptable by the Agency. However, the August 27, 2002 SAP concluded that the parasitic Hymenoptera (*Nasonia 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* or *Macrocentrus grandii*), should be considered for future studies.

iii. Green Lacewing Larva Testing (MRID 457904-07)

An acceptable study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 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 LD_{50} for green lacewing larvae exposed to PS149B1 in diet is >280 ppm. Based on these results it is not expected that the green lacewing will be adversely affected when exposed to Cry34/35 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 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 applicant 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 below in Table 5, the Agency has requested that the applicant submit this study as a condition of registration.

iv. Lady Beetle Testing

The Cry34/35 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 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 a.i./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 a.i./mL treatment group then the control group. Mortality and abnormal behavior was not treatment related. The study shows that "the dietary LC50 value for ladybird beetles exposed to PS149B1 ICP was determined to be greater than 280 µg a.i./mL, which was the highest concentration tested.

MRID 452422-11
A tri-trophic interaction study between field corn expressing PS149B1, corn leaf aphids (*Rhopalosiphum maidis*), and lady beetle (*Colemegilla 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 461239-12
Two diet assays were conducted (MRID 461239-12): a Tier I assay in which lady beetle larvae (*Coleomegilla maculata* DeGeer) were fed purified Cry34/35Ab1 protein mixed with artificial diet, and 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 1 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 lady beetle larvae'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 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 the product characterization section of this BRAD).

Conclusion:
Submitted data show that Cry34/35Ab1 protein is toxic to *C. maculata* at dose levels which 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 1 testing because beetles found within this family play important ecological and economic roles within agroecosystems, including corn fields. As noted in Table 5 below, the Agency is requesting that the applicant submit this study as a condition of registration.

v. Collembola Feeding Study (MRID 457904-06)

This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 885-4240 Freshwater Aquatic Invertebrate Testing, Tier I.

Juvenile collembola (*Folsomia candida*) were fed diets consisting of purified PS149B1 protein (purity = 54% 14kDa protein and 37% 44kDa protein) mixed with dry granulated Brewer's yeast at single treatment level of 12.7 mg a.i./kg PS149B1. 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 Φg/g, which is significantly lower than the treatment level used in this test.

This study adequately addresses potential concerns for Cry34/35Ab1 protein to collembola (*Folsomia candida*), a representative of beneficial soil insect species. The results of this study demonstrate that Cry34/35 proteins found in transgenic corn pose 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 which 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 453602-01
This study complied with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Parts 160 and 792; Organization for Economic Development (OECD) Principles of Good Laboratory Practice; and Japan Ministries of Agricultural Forestry and Fisheries (MAFF), with certain exceptions that did not affect the integrity of the test. The testing was conducted based on OPPTS Series 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 *Bacillus thuringiensis*.

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 Cry34/35 Ab1 corn plants.

vii. Insecticidal Activity Spectrum Study (MRID 452422-04)

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 WCR were not sensitive to the d-endotoxins, however. Rootworms (NCR, WCR and SCR) were the most susceptible to the PS149B1 proteins. ECB and Corn ear worm (CEW) demonstrated some inhibition of growth at higher concentrations of the test substance than the rootworm larvae. Black cut worm (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 GI50, were as follows: (most susceptible) WCR, NCR, SCR > ECB, CEW >> WCR adult, CLA, BCW (least susceptible).

viii. Field Evaluation of Cry34/35Ab1 Corn Exposure on Non-target Invertebrates (MRID 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, NE and Johnston, IA). The experimental design was 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 1st and 2nd generation CRW adults; and 5) non-transgenic maize hybrid with no corn rootworm control measures taken. Treatment plots covered approximately 2800 sq. ft. (70’ x 40’) and were separated by 18 ft. 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 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.

Conclusion:
According to submitted data, Cry34/35Ab1 corn does not adversely impact the abundance of non-target invertebrates found in corn fields. However, plot size (70' × 40') was 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 nontarget arthropods. However, supplemental studies, which employ larger plot sizes, more replicates, and more samples per plot, are recommended for further verification of long

range no adverse effect finding. In response to these concerns, as requested by the Agency in Table 5 below, the applicant is preparing additional field census data that will be submitted to the Agency as a condition of the 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 which 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.

2. Soil Fate (MRID 452422-14)

Soil organisms may be exposed to Cry34/35 protein through contact with corn plant roots (by direct feeding), corn plant root exudates, incorporation of above-ground plant tissues into soil following harvest, or by soil-deposited pollen. Some evidence suggests that 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). And 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 as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 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 one 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 µL) of these dilutions were applied to the surface of small wells (1.5 cm^2 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_{50s} 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.

Conclusion:
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 should 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 Cry34/35 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 5 below, the Agency has requested that the applicant submit these studies as a condition of registration.

3. Effects on Soil Microorganisms

Published studies on the impact of transgenic Cry producing plants indicate that adverse effects on soil microorganisms are unlikely. No effects have been seen due to the protein itself, and only a minimal, transient increase and shift in microbial populations was attributed to the transgenic cotton

plant tissue rather than the Cry protein expressed in that tissue. No adverse effects have been observed in a similar season-long field study with Cry3A potato. There are several ongoing USDA and EPA/ORD funded research projects addressing the effects of Cry protein crops on soil microbial flora. In the event that any adverse information becomes available from this research, the Agency will take appropriate action.

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

The Agency has evaluated the potential for horizontal gene transfer (hgt) from Bt crops to soil organisms and has considered possible risk implications if it occurred. Several experiments published in the scientific literature have been conducted to assess the likelihood of hgt, and have been unable to detect gene transfer under typical gene exchange conditions. Horizontal gene transfer to soil organisms has only been detected under conditions designed to favor transfer. In addition, the genes that have been engineered into the 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. No transfer of these genes or toxin to other microorganisms or plants has been detected. Therefore, the Agency concluded that hgt is at most an artificial event, and the traits engineered into the Bt crops are already present in soil bacteria or are unlikely to have selective value for soil microorganisms. In considering these data the Agency further concludes that there is no significant risk from hgt from the transgenes found in the Cry34/35Ab1 producing corn.

5. Gene Flow and Weediness Potential

The movement of transgenes from the host plant into weeds has been a significant concern for the Agency due to the possibility of novel exposures to the pesticidal substance. The Agency has determined that there is no significant risk of gene capture and expression of Cry34/35Ab1 protein by wild or weedy relatives of corn in the U.S., its possessions or territories. In addition, the USDA/APHIS 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 the Bt endotoxins by wild or weedy relatives of corn, cotton, and potatoes in the U.S., its possessions or territories. *B.t.* 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 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 *Zea mays*. Some *Zea* spp., such as the teosintes, are known to be interfertile with maize and are discussed as potential recipients of pollen directed gene flow from maize. This issue is of particular concern based upon the increased planting of genetically modified maize. Therefore, the Agency conducted a reevaluation in early 2000, the results of which are reported here.

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

Zea mays is a wind-pollinated, monoecious, annual species with imperfect flowers. This means that spatially separate tassels (male flowers) and silks (female flowers) are found on the same plant, a feature that limits inbreeding. A large variety of types are known to exist (*e.g.*, dent, flint, flour, pop, sweet) and have been selected for specific seed characteristics through standard breeding techniques. Maize cultivars and landraces are known to be diploid (2n = 20) and interfertile to a large degree. However, some evidence for genetic incompatibility exists within the species (*e.g.*, popcorn x dent crosses; Mexican maize landraces x Chalco teosinte). *Zea mays* has been domesticated for its current use by selection of key agronomic characters, such as a non-shattering rachis, grain yield and resistance to pests. The origin of corn is thought to be in Mexico or Central America, based largely on archaeological evidence of early cob-like maize in indigenous cultures approximately 7200 years ago.

A recent study has indicated that cross-pollination of commercial maize cultivars at 100 ft downwind from the source of genetically modified maize was 1 % and this proportion declined exponentially to 0.1 % at 130 ft and further declined to 0.03 % at 160 ft. At 1000 ft, the farthest distance measured, no cross-pollination was detected (Jemison and Vayda, 2000). For production of Foundation Seed, a distance of 660 ft 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 and South America, but three occur within the U.S. In the Manual of Grasses of the United States, A. S. Hitchcock (revisions by Agnes Chase; 1971) reports the presence of three species of *Tripsacum* in the continental United States: *T. dactyloides*, *T. floridanum* and *T. 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 IL and KS; 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; *Zea 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 *Zea 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; Chet 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 *Zea 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). *Zea 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, USDA-ARS, Woodward, OK, a geneticist working on improvement of grasses, he stated that relatively few accessions of *T. dactyloides* will cross with maize and the majority of progeny are not fertile or viable even in those that do. In controlled crosses, if the female parent is maize, there is a greater likelihood of obtaining viable seed. When these hybrids have been backcrossed to maize in attempts to introgress *Tripsacum* genes for quality enhancement or disease resistance, the *Tripsacum* chromosomes are typically lost in successive generations. In many instances where hybridization has been directed between these two species, the resultant genome is lacking in most or all of the chromosomal complements of one of the parent species in subsequent generations.

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

Dr. DeWald concluded that the possibility of maize contributing genetic material to Eastern Gama Grass through random pollen flow in agricultural or natural situations is extremely remote based upon his experience trying to create hybrids under the best of conditions. He also felt that no other known grass species present in the continental U.S. would interbreed with commercial maize populations (*i.e.*, be recipients of pollen-directed gene flow). This is in agreement with Holm *et al*. (1979) who determined that none of the sexually compatible relatives of corn in the U.S. are considered to be serious, principal, or common weeds in the U.S.

c. *Zea* species - Teosintes - General Biology

Teosintes, specifically *Z. mays* ssp. *mexicana* (Schrader) Iltis, *Z. mays* ssp. *parviglumis* Iltis and Doebley*, Z. mays* ssp. *huehuetenangensis* (Iltis and Doebley) Doebley*, Z. luxurians* (Durieu and Ascherson) Bird*, Z. perennis* (Hitchc.) Reeves and Mangelsdorf and *Z. 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, *Zea mays* ssp. *mexicana* (annual teosinte) and *Zea 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. *Zea 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 (Keith Bradley, personal communication, 2000; David Hall, personal communication, 2000; Richard 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 (John 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, 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 *Zea 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 *Zea mays* is not considered to be a significant agricultural or environmental risk. The growth habits of teosintes are such that the potential for serious weedy propagation and development is not biologically plausible in the United States.

d. Conclusions

The potential for pollen-directed gene flow from maize to Eastern Gama Grass is extremely remote. This is evidenced by the difficulty with which *Tripsacum dactyloides* x *Zea 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 *Zea mays* ssp. *mays*. None of these plants are known to harbor weedy characteristics and none of the native teosinte species, subspecies or races are considered to be aggressive weeds in their native or introduced habitats. In fact, many are on the brink of extinction where they are indigenous and will be lost without human intervention (*i.e*., conservation measures). Further, none of the landraces or cultivated lines of *Zea mays* are considered to have weedy potential and are generally considered to be incapable of survival in the wild as a result of breeding practices (*i.e*., selection) during domestication of the crop.

C. Impacts on Endangered Species (MRID 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 Cry34/35 corn pollen has shown no toxicity at the expected environmental concentration rates (EEC) to mammals, birds,

plants, aquatic species, insect 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, it appears that 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, the EPA determined that exposure of American burying beetle to harmful levels of Cry34/35 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 Cry34/35 protein would take place. The main reasons for the lack of exposure are geographical and habitat limitations. These species are located in non-corn production areas and/or their habitat does not encompass agricultural areas.

Likewise, other insect species in the orders Diptera, Hemiptera, Lepidoptera, Odonata and Orthoptera that are listed as endangered/threatened species are found in dune, meadow/prairie or open forest habitats and are not closely associated with row crop production often times due to the specificity of the habitat of their host plants. The reviewed toxicological data shows the relative insensitivity of a range of insects from non-Coleopteran orders to the Cry34/35Ab1 proteins indicating that the Cry34/35Ab1 maize 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 maize. Many of the endangered and threatened beetles occur in cave or aquatic habitats. Since movement of Cry34/35Ab1 in soil 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 the field were deposited in a 1 ha pond with 2 m depth and located $\exists$1 m 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.

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

D. Environmental Assessment Summary (MRID 461239-13)

The Agency is using a Maximum Hazard Dose Tiered system for biopesticide non-target wildlife hazard assessment. When no adverse effects at the maximum hazard dose are observed, 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 listed by the US 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 proteins 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 the 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 nonspecific 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 (IPM) for secondary pests such as aphids and leafhoppers. The overall result of cultivation of corn expressing Cry34/35Ab1 proteins 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, 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 crop. 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.

Conclusions:

This risk assessment finds no hazard to the environment at the present time from cultivation of Cry34/35Ab1 protein expressing corn for a time-limited registration.

E. 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. However, 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 which may result from long-term use of this product. The Agency does not believe that this data requirement was reasonably foreseeable by the applicant at the time of application.

Table 5. 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 also be conducted based on recommendations of the August, 2002 SAP presented here in summary form in the conclusion section of the review of MRID No. 461239-14 above.
Soil fate studies	Additional long range soil degradation field studies should also be conducted including the parameters outlined by the August 2002 SAP presented here in summary form in the conclusion section of the review of MRID No. 452422-14 above.

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II. D. INSECT RESISTANCE MANAGEMENT

A. SUPPORTING INFORMATION FOR DEVELOPMENT OF THE CRY34/35 EVENT DAS-59122-7 CORN DURABILITY PLAN

1) PEST BIOLOGY

A clear understanding of pest biology is essential to the development of a sound IRM plan. Cry 34/35 Bt corn was developed to control three target pest species: western corn rootworm (*Diabrotica virgifera virgifera* LeConte, WCR), northern corn rootworm (*D. Barberi* Smith & Lawrence, NCR) and Mexican corn rootworm (*D. V. zeae* Krysan and Smith, MCR). Key factors believed to influence

CRW adaptation to Cry 34/35 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. SCRW, also a minor pest of corn in the US, is not identified as a target pest of Cry34/35-protected corn.

Life Cycle/Univoltism
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 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.

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

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

In their discussion of adult dispersal factors (reference to model discussion), the registrant 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 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 registrant also references 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).

BPPD 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, MCRW biology and ecology could improve understanding of resistance risks and lead to improvements in the IRM strategies. Specific areas to be examined include: 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 Agency 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).

2) MODE OF ACTION AND CROSS-RESISTANCE

Cry34Ab1 (14-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 by 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 pesticides, 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 Vegetative Insecticidal Proteins (VIPs), Storer and Lefko (2003, MRID 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). 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). 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 WCR (Storer, 2003b).

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 *B. 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 Cry45Ab1 proteins and other registered Cry and VIP proteins. 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) 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 WCR (Storer, 2003b).

BPPD 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.

3) 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, 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 the 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 (SAP, 1998; 2001). 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 (see Storer, 2003b, MRID# 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# 461239-19).

Given the limited availability of information on the genetics of resistance to Cry 34/35, the IRM plan utilizes "worst case" assumptions of resistance potential in the modeling simulations of target pest adaption to Cry 34Ab1/Cry35Ab1 corn (Storer 2003b, MRID# 461239-19). These assumptions include the following: 1) all resistance to Cry 34Ab1/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 Cry 34Ab1/Cry35Ab1. BPPD agrees with this approach.

BPPD 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 mid-gut 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 registrants 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 registrant 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 registrants further study the "repellent" or behavioral mode of action and its implications in the selection of resistance.

4) 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/Pioneer conducted adult feeding studies with WCRW and Cry34/35 corn tissue. These studies indicated that adult WCRW feeding (solely) on Cry34/35Ab1 above-ground 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 "25 X" high dose definition. These data for dose were directly applied to CRW models to evaluate the durability of the IRM plan.

BPPD 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. However, 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.

5) 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; and Siegfried and Mullin, 1990) has been augmented by the work of Oyediran et al. (2004) and Clark and Hebert (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 Cry 34/35 corn fields, it is assumed that some percentage of target pests develop on alternate pant 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 (2003a, b) makes the assumption that 0.5% of larvae attacking Cry 34/35 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. 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 Cry 34/35 production regions, etc.);
- CRW movement between maize and non-maize plants at different points in target pest life cycle;
- impact that movement between Cry 34/35 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-Cry 34/35 corn will function to delay monogenic[3] resistance evolution by: a) reducing population-wide selection pressure for resistance; and b) providing a

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

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 Cry 34/35 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 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 cm **(**Hibbard et al., 2003; Hibbard et al., 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 Cry 34/35 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 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

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 place adjacent to or with 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 a integrated pest management program. For a detailed discussion of structured refuge specific for Cry34/25 corn, see the "Analysis Of Dow/Pioneer's Proposed Cry34/35 Event 59122-7 Corn Durability Plan" section later in this document.

6 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, MRID 461239-19). The product durability plan based on the simulated modeling is discussed in Storer and Lefko (2003, MRID 461239-18). 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 461239-19) is described in detail in Storer (2003a). A brief overview is presented in this review. 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 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 discussion above, "4. Dose"). 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 discussion above, "4. Dose"). 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# 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 distance 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 lowers, 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 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: $RRA = (1/Y \ln q_\gamma/q_0)/ 0.327$; where, the q_γ 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 20000 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 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 2000 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# 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

<u>Western corn rootworm</u>

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, RRA), 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 much 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 MON863, EPA Reg. No. 524-528) and YieldGard® Plus Corn (expresses both the Cry3Bb1 and Cry1Ab proteins, EPA Reg. No. 524-545). The FIFRA Scientific Advisory Panel (SAP) concluded that MON863 expressed a moderate dose to control rootworm (SAP, 2002). For the simulations, MON863 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 (EPA, 2003). 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

9. *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 the 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 increase 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.

10. *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 the 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 rotation 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 (2003a and b) 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. 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 >99 model years of durability if the refuge is fixed (blocks).

BPPD Analysis of Proposed Computer Models

BPPD agrees with Dow and Pioneer's assessment of CRW adaptation using the Storer simulation models (Storer 2003a, b) and Onstad et al's simulation model (Onstad et al., 2003). 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) 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 (2003a and b) 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) 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.

7) TECHNOLOGY ADOPTION

Product durability is affected by market penetration. Modeling efforts (Storer 2003b, MRID# 461239-19) make the conservative assumption of complete adoption (i.e. all corn acres are planted in Cry34Ab1/35Ab1 corn) because the registrants do not wish the durability of the product to depend upon the presence of competing products (Storer and Lefko (2003, MRID# 461239-18). The registrants state, 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# 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 in which to base a conservative IRM plan to manage the Cry34Ab1/Cry35Ab1 corn durability.

B. ANALYSIS OF DOW/PIONEER'S PROPOSED CRY34/35 EVENT 59122-7 CORN DURABILITY PLAN

Dow and Pioneer proposed a Cry34/35 Event 59122-7 corn durability plan 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 above). 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.

1) STRUCTURED REFUGE

The guidelines for structured refuge address the following: refuge size, refuge deployment (proximity of the refuge to the Cry 34/35 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 they wanted to ensure that the structured refuge would remain productive for the grower, while serving as an effective reservoir of susceptible target pests. The registrants' 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 the Mexican corn rootworm (MCRW) (Clark et al., 2001), the plan should also be applicable to, 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) indicate that the plan would be effective at managing region-wide resistance evolution if only 75% of farms correctly implement the refuge.

<u>Dow and Pioneer's Proposed Structured Refuge Guidelines</u>

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 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 below. At least four crop rows wide should be required.) Modeling (Storer, 2003b) 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 in (Storer, 2003b, MRID 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-resistance WCRW.

- Banded application of soil insecticide is permitted in the refuge. As discussed above, 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, b) 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 above, insecticide seed treatments have been shown to have little impact on the number or timing of adult emergence and are therefore acceptable for used 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 IPM 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, b) 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, b), this plan would be more conservative for WCRW. Since WCRW is related to the Mexican corn rootworm (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 one 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 sub-lethal 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. 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 (2003b) 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) 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 both lepidopteran-protected *Bt* corn hybrids and 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.

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, other than adult corn rootworm. Dow and Pioneer propose 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 (2^{nd} 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 (e.g., Sutter et al. 1991, Cormier and Martel, 1997). There is ongoing research that suggests that there 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, 2003; 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.

2) 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 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 registrant is 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 registrants 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 registrant will confirm that plants under question were expressing Cry 34/35Ab1 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/Pioneer is also working to develop a "high-throughput diagnostic screen" which uses Cry34/35 seedlings to determine discriminating dose. The registrant states 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 registrant argues 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 Cry 34/35 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 Cry 34/35 adoption rates.

BPPD 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/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 registrants 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. Registrants 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 registrant 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 registrant states that it 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 registrants 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 registrants 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 registrants should submit to EPA an appropriate sampling protocol as part of its monitoring plan.
2. The registrants should provide EPA a description of its resistance monitoring plan by January 31, 2006. The description would include: sampling (number of locations and samples per locations), sampling methodology, bioassay methodology, standardization procedures (including QA/AC 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 registrants should develop an appropriate discriminating or diagnostic dose assay by January 31, 2008
4. The registrants should follow-up on grower, extension specialist or consultant reports of unexpected damage or control failures for corn rootworm.
5. The registrants should provide EPA with an annual resistance monitoring report.

3) REMEDIAL ACTION

The remedial action plan is designed as a tiered approach for mitigating WCRW, NCRW, and MCRW resistance development to the Cry34/35Ab1 protein. 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 registrants will instruct affected growers to use alternate pest control measures such as adulticide treatment, crop rotation the following year, or use of 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 progreny 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 LC50 of the test population exceeds the upper limit of the 95% confidence interval for the LC50 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 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 levels until baseline sensitivity is determined (see discussion above "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.

4) 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: 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 registrants 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 Analysis of Grower Education and Compliance Assurance Program

Grower education and compliance are critical to the success of the trait durability plan. The registrants' grower education plans are acceptable. However, the registrants 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 plant to be fully effective depends on the level of technology adoption (see discussion above under "Analysis of 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.

ANNUAL REPORTS

It is required that the registrants 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).

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II. E. EPA PUBLIC INTEREST FINDING AND BENEFITS ASSESSMENT

EPA has reviewed the public interest document for Event DAS-59122-7 and concludes that conditional registration under 3(c)(7)(C) of the Cry34Ab1 and Cry35Ab1 proteins and the genetic materials necessary for their production in corn is in the public interest. Cry34/35Ab1 CRW-protected corn hybrids (Event DAS-59122-7) 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 such as energy savings resulting from reduced chemical insecticide use.

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 volume 51, No. 43 (OPP-32500; FRL-2977-2) title *Conditional Registration of New Pesticides*. There is a presumption that registration of a pesticide chemical is in the public interest if one of the following criteria is met: i) the use is for a minor crop; (ii) the use is a replacement for another pesticide that is of continuing concern to the Agency; (iii) the use is one for which an emergency exemption under FIFRA Section 18 has been granted for lack of an alternative pest control method, or (iv) 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: i) there is a need for the new chemical that is not being met by currently registered pesticides; ii) the new pesticide is comparatively less risky to health or the

environment than currently registered pesticides; or iii) the benefits (including economic benefits) from the use of the new active ingredient exceed those of alternative registered pesticides and other available non-chemical techniques.

Dow AgroSciences and Pioneer 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 Bt corn product on the market (the first is 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.

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 Sadeghi, 1991), behavior changes in NCRW (extended diapause) and WCRW (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 MON863 (expresses the Cry3Bb1 protein). This review concerns the second CRW-protected Bt corn product submitted for registration, Dow AgroSciences' (DAS) and Pioneer's DAS-59122-7, which produces the Cry34Ab1 and Cry35Ab1 insecticidal proteins (originally isolated from *Bacillus thuringiensis* strain PS149B1). Similar to event MON863 corn hybrids, event DAS-59122-7 Bt corn is targeted against the western corn rootworm (WCRW, *D. virgifera virgifera*, LeConte*),* northern corn rootworm (NCRW, *Diabrotica barberi*, Smith and Lawrence), and Mexican corn rootworm (MCRW, *D. virgifera zeae*, Krysan and Smith).

Efficacy

In 2003, DAS 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 registrant 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 (1000 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 6, 7, and 8). 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 6. 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, 3=three or more nodes eaten
[2]Within each column, means that are followed by the same letter are not significantly different.

Table 7. 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, 3=three or more nodes eaten
[2]Within each column, means that are followed by the same letter are not significantly different.

Table 8. 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, 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 registrant completed a NCRW trial that compared the efficacy of Cry34/35 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 (1000 WCRW/plants). Root ratings (Tables 6 and 7) 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 9).

Table 9. 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, 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 10).

Table 10. 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

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 (1000 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, Sugarcek, 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.

Yield and Agronomic Performance
Several yield and agronomic performance studies were completed by the registrants. Each evaluation is described below.

In 2003, Pioneer conducted a yield trial (in conjunction with 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 oz/1000 row ft). 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, 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, 12 treatments per replicate, and four rows per treatment. At plant growth stage V2, plants were artificially infested with WCRW eggs at a rate of 1000 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 (1000 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 seven locations, and the Dow evaluations at 12 and 13 locations (Dow completed two 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.

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.

Economic Benefits
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 DAS 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 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. 2000-2003) 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 form 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 such as 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 11-13 were revised by the registrant, 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 11). 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 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/a), liquid ($16-$23/a) and seed treatment options ($37-$40/a) (Table 12).

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 13). 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 11. 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/a[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/bu	$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 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, & interest (Duffy & Smith 2003) for 150 bu./a production.
[5] Fuel adjustment: 50% of normal combine cost @ $7.87/acre (Duffy & 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, & handling) reduced $0.16/bu for yields below 150 bu/a.
[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 12. 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/a[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/bu	$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 root rating (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, & interest (Duffy & Smith 2003) for 150 bu./a production.
[5] Fuel adjustment: 50% of normal combine cost @ $7.87/acre (Duffy & 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, & handling) reduced $0.16/bu for yields below 150 bu/a.
[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 13. 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/a[3]	$37.50	$37.50	$37.50	$37.50	$37.50
CRW premium	0	$13.13 - 16.88	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/bu	$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 root 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, & interest (Duffy & Smith 2003) for 150 bu./a production.
[5] Fuel adjustment: 50% of normal combine cost @ $7.87/acre (Duffy & 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, & handling) reduced $0.16/bu for yields below 150 bu/a.
[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 .

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; MRID 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 11-13).

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 11). In contrast, slightly higher returns are predicted in corn-soybean rotations with high rootworm pressure (Table 12). 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.

Practical Benefits

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.

BPPD Review

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

Market Adoption and Pesticide Use Reduction

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 because: 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.

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). And CRW

infested acreage is projected to increase from approximately 31.8 million acres in 2005 to 39 million acres by 2013 (Table 14).

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 USDA/NASS and DOANE 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: organophosphates (chlorpyrifos, tebupirimphos, tebufos, methyl parathion, and chlorethoxyfos), pyrethroids (tefluthrin, cyfluthrin, bifenthrin, lambda cyhalothrin), carbamates (carbofuron), and pyrazoles (fipronil) (Table 15). 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 14. Projected acreage with corn rootworm infestation and breakdown of associated CRW control practices for the years 2000 to 2013. 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 15. 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	. ---------- 1000 lb a.i. ----------											
Colorado	7		- 2	12			1		-	218		125
Illinois	810	2105	747	120	–		68	57	205	466	1117	–
Indiana	299	267	621	62			36	31	93	558	–	473
Iowa	1044	1248	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	4750	5153	2638	1037	–	–	558	432	454	4681	2787	1072
Total - as reported in USDA		5341	3024					522	523		3200	1660

[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

Direct and Indirect Economic Benefits

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 Cry34/35 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 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 Cry34/35 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.'s (2002) 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.

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 registrants' 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 registrants' conclusions on assumptions 1, 2, and 4. However, assumption 3, 'energy for application', may be over estimated[b]. A value within the range of 15,730 to 25,220 BTU/acre may be more accurate than the registrants' estimate of 94,000 BTU/acre.

[b] 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 33.5×10^6 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

Human Health Benefits

Event DAS 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 (See Agency's Incident Data Base, http://www.opp.gov/pesticides).

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.

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 Cry34/35 Human Health and Product Characterization chapters for more information on potential human health effects.)

Environmental Benefits

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," six as "highly toxic," one 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 16), 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

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done using a 50-ft boom sprayer pulled by a 60-hp tractor that covers 25.6 acres/hr. 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/hp-hr). 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 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/hr]. Based on 25.6 acres/hour, this is equivalent to 15,730 Btu/acre.

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.

BPPD Review
See BPPD's Cry34/35Ab1 CRW-protected corn hybrid Ecological Risk Chapter for more information on the potential for ecological effects.

Table 16. 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

Insect Resistance Management Benefits
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 chemical insecticides – pyrethroids, organophosphates, carbamates, 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.* MON863). To ensure the long-term efficacy of Cry34/35Ab1 CRW-protected corn hybrids, an insect resistance management plan should be implemented.

BPPD Review
See BPPD's Cry34/35Ab1 IRM chapter 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.

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III. Terms and Conditions of the Registration

1) The subject registrationswill automatically expire on midnight September 30, 2010 . Based on the Agency's review of the data submitted and cited in support of this application, the Agency anticipates at this time that an expiration date five years from the initial date of registration for this product would be appropriate.

2) The subject registrations will be limited to Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (plasmid insert PHP17662) in Event DAS-59122-7 corn use in field corn.

3) Submit/cite all data required for registration of your product under FIFRA § 3(c)(5) when the Agency requires registrants of similar products to submit such data.

4) 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.

5) 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 protocol is due within 90 days of the date of registration. 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.

6) Submit laboratory toxicity tests with *Orius insidiosus* (minute pirate bug), carabid (ground beetle), within 24 months of the date of registration. Protocols are due within 120 days of the date of registration.

7) 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 protocol is due within 90 days of the date of registration. A final report is due September 30, 2009.

8) The following insect resistance management data are recommended.

Additional research on corn rootworm pest biology and ecology, genetics and mechanisms of resistance, functional dominance, fitness costs, cross-resistance potential, dose (including the role of density-dependence), and mode of action are recommended to evaluate the sustainability of the insect resistance management durability plan and confirm the assumptions made in the simulation models. Research reports should be provided to the Agency once the research is completed and at least nine months prior to the expiration of this registration.

Should you wish to amend the refuge treatment option to allow independent treatment of the refuge for pests other than corn rootworms, data would be required regarding the impact of independent treatment of the refuge for other pests (not corn rootworm, e.g., corn borers, spider mites) on corn rootworm resistance management.

9) You must commit to do the following Insect Resistance Management Program which has the following elements:

a) Requirements relating to creation of a non-(corn rootworm-protected PIP) corn refuge in conjunction with the planting of any acreage of commercial Cry34/35Ab1 *Bt* corn;

b) Requirements for the registrants to prepare and require Cry34/35Ab1 *Bt* corn users to sign "grower agreements" which impose binding contractual obligations on the grower to comply with the refuge requirements;

c) Requirements for the registrants to develop, implement, and report to EPA on programs to educate growers about IRM requirements;

d) Requirements for the registrants to develop, implement, and report to EPA on programs to evaluate and promote growers' compliance with IRM requirements (the Cry34/35Ab1 Compliance

Assurance Program (CAP) must integrate with the Cry1 CAPs);

e) Requirements for the registrants to develop, implement, and report to EPA on monitoring programs to evaluate whether there are statistically significant and biologically relevant changes in target insect susceptibility to Cry34/35Ab1 proteins in the target insects;

f) Requirements for the registrants to develop, and if triggered, to implement a "remedial action plan" which would contain measures the registrants would take in the event that any insect resistance was detected as well as to report on activity under the plan to EPA;

g) Submit annual reports on units sold by state (units sold by county level will be made available to the Agency upon request), IRM grower agreements results, compliance assurance program including the education program, and resistance monitoring on or before January 31st each year beginning in 2007.

9a. Refuge Requirements

Grower agreements (also known as stewardship agreements) will specify that growers must adhere to the refuge requirements as described in the grower guide/product use guide and/or in supplements to the grower guide/product use guide.

1. *Refuge size*. The use of Cry34/35 corn from event DAS 59122-7 requires an accompanying 20% refuge

2. *Refuge location.* The rootworm refuge is 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 may be managed in such a way that there is little or no yield loss to rootworms, but must be managed in a way that it is sufficiently productive of susceptible rootworm adults. The in-field refuge options may be planted as a single block or as a series of strips measuring at least four (4) crop rows wide.

- Seed mixtures of Cry34/35 and refuge corn are not permitted.

- If the refuge is planted on rotated ground, then Cry34/35 corn must also be planted on rotated ground.

- 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).

- 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.

- 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.

- 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.

- Growers are encouraged 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 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.

9b. Grower Agreements

1] Persons purchasing the *Bt* corn product must sign a grower agreement. The term "grower agreement" refers to any grower purchase contract, license agreement, or similar legal document.

2] The grower agreement and/or specific stewardship documents referenced in the grower agreement must clearly set forth the terms of the current IRM program. By signing the grower agreement, a grower must be contractually bound to comply with the requirements of the IRM program.

3] The registrant must develop a system (equivalent to what is already approved for Cry1F Bt corn) which is reasonably likely to assure that persons purchasing the *Bt* corn product will affirm annually that they are contractually bound to comply with the requirements of the IRM program. The proposed system will be submitted to EPA by January 31, 2006.

4] The registrant must use grower agreements and submit to EPA by January 31, 2006 a copy of that

agreement and any specific stewardship documents referenced in the grower agreement. If either registrant wishes to change any part of the grower agreement or any specific stewardship documents referenced in the grower agreement that would affect either the content of the IRM program or the legal enforceability of the provisions of the agreement relating to the IRM program, thirty days prior to implementing a proposed change, the registrant must submit to EPA the text of such changes to ensure that it is consistent with the terms and conditions of the amendment.

5] The registrant must establish a system (equivalent to what is already approved for Cry1F Bt Corn) which is reasonably likely to assure that persons purchasing the *Bt* corn sign grower agreement(s), and must provide by January 31, 2006 a written description of that system.

6] The registrant shall maintain records of all *Bt* corn grower agreements for a period of three years from December 31st of the year in which the agreement was signed.

7] Beginning on January 31, 2007 and annually thereafter, the registrant shall provide EPA with a report showing the number of units of its Cry34/35Ab1 corn seeds sold or shipped and not returned, and the number of such units that were sold to persons who have signed grower agreements. The report shall cover the time frame of the twelve-month period covering the prior August through July.

8] The registrant must allow a review of the grower agreements and grower agreement records by EPA or by a State pesticide regulatory agency if the State agency can demonstrate that confidential business information, including names, personal information, and grower license number, will be protected.

9c. IRM Education and IRM Compliance Monitoring Programs

1] The registrants must implement a comprehensive, ongoing IRM education program designed to convey to *Bt* Cry34/35Ab1 corn users the importance of complying with the IRM program. The program shall include information encouraging *Bt* Cry34/35Ab1 corn users to pursue optional elements of the IRM program relating to refuge configuration and proximity to *Bt* Cry34/35Ab1 corn fields. The education program shall involve the use of multiple media, e.g. face-to-face meetings, mailing written materials, EPA reviewed language on IRM requirements on the bag or bag tag, and electronic communications such as by Internet, radio, or television commercials. Copies of the materials will be provided to EPA for its records for the first year of commercialization (2006 growing season) by January 31, 2007. The program shall involve at least one written communication annually to each *Bt* Cry34/35Ab1 corn user separate from the grower technical guide. The communication shall inform the user of the current IRM requirements. The registrants shall coordinate their education programs with educational efforts of other registrants and other organizations, such as the National Corn Grower Association and state extension programs.

2] Annually, the registrant shall revise, and expand as necessary, its education program to take into account the information collected through the compliance survey required under paragraph 6] and

from other sources. The changes shall address aspects of grower compliance that are not sufficiently high.

3] Beginning January 31, 2008 and annually thereafter, the registrant must provide EPA any substantive changes to its grower education activities as part of the overall IRM compliance assurance program report. The required features of the compliance assurance program are described in paragraphs 4]-15] below.

4] The registrant must design and implement an ongoing IRM compliance assurance program designed to evaluate the extent to which growers purchasing its Cry34/35Ab1 *Bt* corn product are complying with the IRM program and that takes such actions as are reasonably needed to assure that growers who have not complied with the program either do so in the future or lose their access to the Cry34/35Ab1 *Bt* corn product. The registrant shall coordinate with other *Bt* corn registrants in designing and implementing its compliance assurance program and integrate the Cry34/35Ab1 CAP with the Cry1 CAPs. The registrant must prepare and submit by January 31, 2006 a written description of their compliance assurance program including a summary of the program implemented in the 2006 growing season. Other required features of the program are described in paragraphs 5] - 15] below.

5] The registrant must establish and publicize a "phased compliance approach," i.e., a guidance document that indicates how the registrant will address instances of non-compliance with the terms of the IRM program and general criteria for choosing among options for responding to any non-compliant growers. The options shall include withdrawal of the right to purchase Cry34/35Ab1 *Bt* corn for an individual grower or for all growers in a specific region. An individual grower found to be significantly out of compliance two years in a row would be denied sales of the product the next year. Similarly, seed dealers who are not fulfilling their obligations to inform/educate growers of their IRM obligations will lose their opportunity to sell Cry34/35Ab1 *Bt* corn.

6] The IRM compliance assurance program shall include an annual survey conducted by an independent third party of a statistically representative sample of Cry34/35Ab1 *Bt* corn growers who plant the vast majority of all corn in the U.S. and in areas in which the selection intensity is greatest The survey shall consider only those growers who plant 200 or more acres of corn in the Corn-Belt and who plant 100 or more acres of corn in corn-cotton areas.. The survey shall measure the degree of compliance with the IRM program by growers in different regions of the country and consider the potential impact of non-response. The sample size and geographical resolution may be adjusted annually, based upon input from the independent marketing research firm and academic scientists, to allow analysis of compliance behavior within regions or between regions. The sample size must provide a reasonable sensitivity for comparing results across the U.S.

7] The survey shall be designed to provide an understanding of any difficulties growers encounter in implementing IRM requirements. An analysis of the survey results must include the reasons, extent, and potential biological significance of any implementation deviations.

8] The survey shall be designed to obtain grower feedback on the usefulness of specific educational tools and initiatives.

9] The registrant shall provide a written summary of the results of the prior year's survey (together with a description of the regions, the methodology used, and the supporting data) to EPA by January 31 of each year, beginning with 2007. The registrant shall confer with EPA on the design and content of the survey prior to its implementation.

10] Annually, the registrant shall revise, and expand as necessary, its compliance assurance program to take into account the information collected through the compliance survey required under paragraphs 6] through 8] and from other sources. The changes shall address aspects of grower compliance that are not sufficiently high. The registrant must confer with the Agency prior to adopting any significant changes.

11] The registrant shall conduct an annual on-farm assessment program. The registrant shall train its representatives who make on-farm visits with Cry34/35Ab1 *Bt* corn growers to perform assessments of compliance with IRM requirements. There is no minimum corn acreage size for this program. Therefore, growers will be selected for this program from across all farm sizes. In the event that any of these visits result in the identification of a grower who is not in compliance with the IRM program, the registrant shall take appropriate action, consistent with its "phased compliance approach," to promote compliance.

12] The registrant shall carry out a program for investigating legitimate "tips and complaints" that its growers are not in compliance with the IRM program. Whenever an investigation results in the identification of a grower who is not in compliance with the IRM program, the registrant shall take appropriate action, consistent with its "phased compliance approach."

13] If a grower, who purchases Cry34/35Ab1 *Bt* corn for planting, was specifically identified as not being in compliance during the previous year, the registrant shall visit with the grower and evaluate whether that the grower is in compliance with the IRM program for the current year.

14] Beginning January 31, 2007 and annually thereafter, The registrants shall provide a report to EPA summarizing the activities carried out under their compliance assurance program for the prior year including changes to the grower education program, and the plans for the compliance assurance program during the current year. The report will include information regarding grower interactions (including, but not limited to, third-party grower survey, on-farm visitation program, verified tips and complaints, education programs (e.g., grower meetings and letters), the extent of non-compliance, corrective measures to address the non-compliance (phased-compliance program), and any follow-up actions taken.

15] The registrant and the seed corn dealers for the registrant must allow a review of the compliance

records by EPA or by a State pesticide regulatory agency if the State agency can demonstrate that confidential business information, including the names, personal information, and grower license number of the growers will be protected.

9d. Insect Resistance Monitoring

The Agency is imposing the following conditions for this product:

The registrants must monitor for Cry34Ab1/35Ab1 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.

1. The registrants must provide EPA its resistance monitoring plan for approval. A preliminary plan must be submitted to the Agency by January 31, 2006 consisting of a description of the steps to be taken to establish corn rootworm baseline sensitivity and damage guidelines. A detailed resistance monitoring plan must be submitted to the Agency for review by January 31, 2008. This plan must include: baseline sensitivity data, sampling (number of locations, samples per locations), sampling methodology and life-stage sampled, bioassay methodology, standardization procedures (including QA/QC provisions), detection technique and sensitivity, the statistical analysis of the probability of detecting resistance, and an interim description of rootworm damage guidelines.

2. The registrants must develop and validate an appropriate discriminating or diagnostic dose assay by January 31, 2010. Further you must provide BPPD with a detailed explanation and validation (steps for) of the "high-throughput diagnostic screen" if it is to be considered an acceptable diagnostic dose assay.

3. You must finalize rootworm damage guidelines and submit these to BPPD by January 31, 2010.

4. The registrants must follow-up on grower, extension specialist or consultant reports of unexpected damage or control failures for corn rootworm.

5. The registrants must provide EPA with an annual resistance monitoring report by January 31st of each year beginning with 2008, reporting on populations collected the previous year.

9e. Remedial Action Plans

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

resistance to target pests is confirmed. However, the levels of "expected" damage cannot be identified until baseline sensitivity is determined. EPA requires that the registrants establish the baseline sensitivity by January 31, 2008, so that expected levels of crop damage and target pest resistance can be established, and a remedial action plan initiated when needed.

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

d. implicated corn plant roots were expressing Cry34/35Ab1 proteins at the expected levels;
e. the seed used was not mixed with non-Cry34/35Ab1 seed
f. 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;
g. the level of damage exceeds guidelines for expected damage.

If resistance is "suspected", the registrants will instruct affected growers to use alternate pest control measures such as adulticide treatment, crop rotation the following year, or use of 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":

f. the proportion of larvae that can feed and survive on Cry34/35Ab1 roots from neonate to adult is significantly higher than the baseline proportion (currently being established);
g. the LC50 of the test population exceeds the upper limit of the 95% confidence interval for the LC50 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;
h. the ability to survive is heritable;
i. Cry34/35Ab1 plant assays determine that damage caused by surviving insects would exceed economic thresholds;
j. if subsequent collections in the affected field area demonstrate similar bioassay results.

3. **Response to Confirmed Resistance:** When resistance is "confirmed", the following steps will be taken:EPA will receive notification within 30 days of confirming resistance;

f. affected customers and extension agents will be notified about confirmed resistance;
g. affected customers and extension agents will be encouraged to employ alternative CRW control measures;
h. sale and distribution of Cry34/35Ab1 corn in the affected area will cease immediately;
i. a long-term resistance management action plan will be devised according to the characteristics of the resistance event and local agronomic needs.

10) Annual Reports:

The registrant must provide annual reports to EPA on its Cry34/35Ab1 PIP expressed in corn based on the following table.

Report	Description	Due Date
Annual Sales	Units sold by state (county information is available upon request by the Agency)	January 31st each year beginning in 2007
Grower Agreement	Number of units of *Bt* corn seeds shipped or sold and not returned, and the number of such units that were sold to persons who have signed grower agreements	January 31st each year beginning in 2007
Grower Education (part of the Compliance Assurance Program Report, except for the 2006 growing season)	Education program for the 2006 growing season. Subsequent changes to the grower education program must be included in the annual compliance assurance program report.	January 31, 2007. Annual changes January 31st,each year beginning in 2008 as part of the Compliance Assurance Program Report
Proposed Compliance Plan	Written description of Compliance Assurance Program	January 31, 2006
Compliance Assurance Program	Compliance Assurance Program Activities and Results: third-party grower survey, on-farm visitation program, phased-compliance report, tips and complaints, and grower education programs	January 31st each year starting in 2007
Insect Resistance Monitoring Plan	Description of the steps to be taken to establish corn rootworm baseline sensitivity and damage guidelines	January 31, 2006

Report	Description	Due Date
Insect Resistance Monitoring Plan	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	January 31, 2008
Insect Resistance Monitoring	Submission of rootworm damage guidelines	January 31, 2010
Insect Resistance Monitoring	Development of diagnostic dose assay/high through-put screen	January 31, 2008
Insect Resistance Monitoring	Annual report of the insect resistance monitoring program. Results of monitoring and investigations of damage reports	August 31st each year beginning in 2008

Additional reports are due as described in the following table:

IRM Grower Agreements	Proposed system to assure growers sign grower agreements	January 31, 2006
IRM Affirmation Plan	System to assure annual affirmation by growers of their IRM obligations	January 31, 2006
Changes to Grower Agreement and/or IRM documents	Current grower agreement(s) and any specific stewardship documents	At least 30 days before any changes related to IRM are expected to be imposed.
Grower Agreement	Submission of grower agreement and any specific stewardship documents referenced in the grower agreement	January 31, 2006

IV. Regulatory Position for Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 Proteins and the Genetic Material Necessary for Their Production (Plasmid Insert PHP 17662) in Event DAS-59122-7 Corn

BACKGROUND

Active Ingredient

Cry34Ab1 and Cry35Ab1 proteins are from *Bacillus thuringiensis* 149B1 and have activity against certain beetles.

Registration Application and Public Comments

On September 1, 2004, EPA announced receipt of Dow and Pioneer's Cry34/35Ab1 products pursuant to the provisions of section 3(c)(4) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (69 FR 53436). Four comments were received. Three of the comments were from grower associations in favor of registration. They cited corn farmers' need for new products and technology, IRM benefits, reduction in chemical inputs, environmental benefits, and improved farmer profitability. They also cited the need for market competition for Bt corn rootworm products to provide more choice and lower costs. The fourth comment was against registration, but provided no substantive rationale, stating the "bt is harmful" and "neighbors should always be asked for permission for any test use."

REGULATORY RATIONALE

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

BPPD determined that it is appropriate to conditionally register the Cry34/35Ab1 products under Section 3(c)(7)(C) because insufficient time has elapsed since the imposition of the data requirements for:

1) Independent laboratory analytical method validation.
2) Field degradation studies evaluating accumulation and persistence of Cry34/35Ab1 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 or cited data sufficient for BPPD to determine that conditional registration of Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (plasmid insert PHP 17662) in Event DAS-59122-7 corn under FIFRA 3(c)(7)(C) will not result in unreasonable adverse effects to the environment, as discussed above. The applicants submitted and/or cited satisfactory data pertaining to the proposed use. The human health effects data and 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 ecological effects are likely to arise from the use of the product and that the risk of resistance developing to Cry34/35Ab1 proteins during the conditional registrations are not expected to be significant.

Registration of Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (plasmid insert PHP 17662) in Event DAS-59122-7 corn is in the public interest because:

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 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 corn trait on the market (the first is 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 Bt CRW-protected corn hybrids.

In view of these minimal risks and the clear benefits related to Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (plasmid insert PHP 17662) in Event DAS-59122-7 corn, BPPD believes that the use of the product during the limited period of the conditional registration will not cause any unreasonable adverse effects.

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

BPPD also believes, as explained in section II.E., that the third criterion for a FIFRA 3(c)(7)(C) conditional registration has been fulfilled because the use of Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (plasmid insert PHP 17662) in Event DAS-59122-7 corn under this registration is in the public interest.

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

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