BIOPESTICIDES REGISTRATION ACTION DOCUMENT

Bacillus thuringiensis modified Cry1Ab (SYN-IR67B-1) and Vip3Aa19 (SYN-IR102-7) insecticidal proteins and the genetic material necessary for their production in COT102 X COT67B cotton

U.S. Environmental Protection Agency
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I. Overview

A. Executive Summary

EPA has conditionally registered a new pesticide product containing Syngenta Seeds Inc.'s new active ingredients, *Bacillus thuringiensis* Vip3Aa19 (OECD Unique Identifier SYN-IR102-7) and modified Cry1Ab (OECD Unique Identifier SYN-IR67B-1) insecticidal proteins and the genetic material necessary for their production in COT102 X COT67B cotton. Syngenta has trademarked this product as VipCot -- the trademark name of VipCot will be used in this document to describe COT102 X COT67B cotton. 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 cotton plant-incorporated protectant, VipCot, produces its own insecticidal proteins within the cotton plant. These proteins were derived from *Bacillus thuringiensis* (Bt), a naturally occurring soil bacterium. The modified Cry1Ab and Vip3Aa19 proteins used in this product control lepidopteran pests of cotton.

On June 26, 2008, tolerance exemptions under 40 CFR Part 174 were approved for *Bacillus thuringiensis* modified Cry1Ab protein as identified under OECD Unique Identifier SYN-IR67B-1 in cotton (40 CFR 174.529) and Vip3Aa proteins in corn and cotton (40 CFR 174.501). The exemption for Vip3Aa is inclusive of the Vip3Aa19 protein and its use in cotton.

Benefits

Results of efficacy trials conducted in 2005 and 2006 show that VipCot cotton and its single event cotton isolines provide good protection against three major cotton pests: tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*), and pink bollworm (*Pectinophora gossypiella*). The Vip3Aa19 protein expressed in VipCot cotton has not been previously registered and provides a unique mode of action. When coupled with modified Cry1Ab in VipCot, the proteins have the potential to provide benefits for insect resistance management including: high-dose (for both proteins expressed together) against the major target pests, lack of cross-resistance (Vip3Aa19), and the potential to delay development of resistance in other cotton varieties expressing Cry toxins. As an additional registered Bt cotton product, VipCot will likely result in direct and indirect human and environmental health benefits by providing growers with an additional choice of Bt cotton option and the potential to increase grower choice and price competition, resulting in lower seed prices for consumers and higher adoption rates. Registration of VipCot may also result in further reduction of chemical insecticide use by growers.

Public Interest Finding

To grant a conditional registration under Section 3(c)(7)(C) of FIFRA, EPA must determine that such conditional registration will, *inter alia*, be in the public interest. EPA determines whether conditional registration of a pesticide is in the public interest in accordance with the criteria set forth at 51 Fed. Reg. 7628 (*Conditional Registration of New Pesticides*, March 5 1986). On the basis of analysis utilizing these criteria, EPA concludes that the use of VipCot protected cotton will be in the public interest, because it results in direct and indirect human and environmental health benefits by providing growers with an additional Bt cotton product which has the potential to extend the useful life of Bt cotton technology generally due to a novel mode of action (Vip3Aa19) and low likelihood of cross-resistance with other Bt Cry proteins.

Product Characterization

VipCot (COT102 x COT67B) was developed by conventional breeding of COT102 (Vip3Aa19) plants with COT67B (modified Cry1Ab) plants.

Event COT102 cotton, which was developed by *Agrobacterium*-mediated transformation of cotton using elements of a vector referred to as both pNOV3001 and pCOT1, expresses the insecticidal protein, Vip3Aa19 as well as a selectable marker, hygromycin B phosphotransferase (APH4). The Vip3Aa19 protein is intended to control several lepidopteran pests of cotton including *Heliothis virescens* (tobacco budworm, TBW), *Helicoverpa zea* (cotton bollworm, CBW), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), and *Trichoplusia ni* (cabbage looper). Vip3A is a vegetative (i.e., produced during the vegetative stage of bacterial growth) insecticidal protein from *Bacillus thuringiensis* (*Bt*), a gram positive bacterium commonly found in soil.

Event COT67B cotton, which was developed by *Agrobacterium*-mediated transformation of cotton using elements of vectors pNOV4641 and pNOV1914, expresses the insecticidal protein, modified Cry1Ab. This protein contains an additional 26 amino acid sequence at the C-terminus (termed the 'Geiser motif'). The modified Cry1Ab protein is intended to control several lepidopteran pests of cotton including *Heliothis virescens* (tobacco budworm), *Helicoverpa zea* (cotton bollworm), *Pectinophora gossypiella* (pink bollworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), and *Trichoplusia ni* (cabbage looper).

DNA characterization (i.e., Southern blot analysis) was used to confirm the integrity of the COT102 and COT67B inserts in the stacked product COT102 x COT67B. Samples from COT102 x COT67B cotton gave the same results as those observed for the individual events, indicating that the molecular characterization data provided for the individual events are also applicable to COT102 x COT67B.

Protein expression data, together with data indicating that there is no evidence of either a synergistic or antagonistic interaction between Vip3Aa19 and modified Cry1Ab in cotton bollworm or tobacco budworm, demonstrate that data on the individual events and individual proteins can be used to support the safety of the COT102 x COT67B (VipCot) combined product.

Human Health Assessment

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the modified Cry1Ab and Vip3Aa19 proteins. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because no toxicity to mammals has been observed, nor any indication of allergenicity potential for the plant-incorporated protectant.

Syngenta submitted four acute oral toxicity studies conducted on mice, which all indicated that Vip3Aa is non-toxic to humans. Three of the studies were conducted with microbially-produced Vip3Aa proteins with slight variations in amino acid sequence (1-2 amino acid differences), and one study was conduced with transgenic corn leaf tissue as the test material. No treatment-related adverse effects were observed in any of the studies. The oral LD_{50} for mice (males, females, and combined) was greater than 3675 mg Vip3Aa/kg body weight (the highest dose tested). For modified Cry1Ab, an acute oral toxicity study in mice indicated that the protein is non-toxic to humans. Groups of five male and five female mice were given 0 or 1830 mg/kg bodyweight microbially-produced modified Cry1Ab by oral gavage as a single dose. There were no effects on clinical condition, body weight, food consumption, clinical pathology, organ weight, or macroscopic or microscopic pathology that were attributed to the test substance.

Since Vip3Aa and modified Cry1Ab 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-evidence approach where the following factors are considered: source of the trait; amino acid sequence comparison with known allergens; and biochemical properties of the protein, including in vitro digestibility in simulated gastric fluid (SGF) and glycosylation. This approach is consistent with the approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." The allergenicity assessment for Vip3Aa and modified Cry1Ab is as follows:

1. Source of the trait. *Bacillus thuringiensis* is not considered to be a source of allergenic proteins.
2. Amino acid sequence. A comparison of the amino acid sequence of Vip3Aa19 and modified Cry1Ab with known allergens showed no significant sequence identity over 80 amino acids or identity at the level of eight contiguous amino acid residues.
3. Digestibility. The Vip3Aa and modified Cry1Ab proteins were digested rapidly in simulated

gastric fluid containing pepsin.

4. Glycosylation. Vip3Aa and modified Cry1Ab (expressed in cotton) were shown not to be glycosylated.
5. Conclusion. Considering all of the available information, EPA has concluded that the potential for Vip3Aa and modified Cry1Ab to be food allergens is minimal.

Environmental Assessment

The Agency concludes that for the VipCot cotton breeding stack (COT102 x COT67B, containing modified Cry1Ab and Vip3Aa19) no unreasonable adverse effects will result to the environment or any federally-listed threatened or endangered species from commercial cultivation of COT102 x COT67B cotton. This conclusion is based on prior assessments conducted on Vip3Aa and Cry1Ab proteins individually. Furthermore, the Agency has determined that Events COT102, COT67B, and VipCot cotton will have No Effect (NE) on endangered and/or threatened species listed by the US Fish and Wildlife Service (USFWS) and the National Marine Fisheries Services (NMFS), including mammals, birds, terrestrial and aquatic plants, and invertebrate species. Therefore, no consultation with the USFWS is required under the Endangered Species Act.

The Agency believes that cultivation of VipCot cotton may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, Bt cotton 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. Therefore, the overall result of cultivation of VipCot cotton, expressing Vip3Aa19 and modified Cry1Ab proteins, is that the number of chemical insecticide applications for non-target pest control will be reduced for management of multiple pest problems.

Insect Resistance Management

In order to reduce the possibility of the target pests developing resistance to Vip3Aa19 and modified Cry1Ab (as expressed in VipCot cotton), EPA is requiring Syngenta Seeds, Inc. to ensure that a portion of the planted acreage of this product be set aside where non-Bt cotton will be grown to serve as a "refuge." Under the established refuge strategy for Bt cotton, growers can choose from three structured refuge options:

Option 1: 95:5 external structured, unsprayed refuge; 150 ft wide, within ½ mile of edge of field.
Option 2: 80:20 external sprayed refuge; within 1 linear mile, preferably ½ mile, of edge of field.
Option 3: 95:5 embedded refuge; contiguous or within 1 mile^2 of field and 150 ft wide.

In addition to the refuge options above, growers of VipCot may participate in a community refuge plan in which multiple growers contribute to the overall required refuge acres by planting 20% external, sprayed or 5% external, unsprayed refuge.

BPPD has concluded that based on the modeling, dose, and efficacy studies, the requested refuge options 1-3 and community refuge plan are acceptable for VipCot cotton. Syngenta will also be required to develop and conduct a resistance monitoring program for Vip3Aa19 and modified Cry1Ab with the major target pests (cotton bollworm, tobacco budworm, and pink bollworm). Additional requirements for remedial action (in the event of resistance), grower education, compliance assurance, and annual reported will also be implemented for VipCot as terms of registration.

B. Use Profile

Pesticide Name: *Bacillus thuringiensis* Vip3Aa19 (OECD Unique Identifier SYN-IR102-7) and modified Cry1Ab (OECD Unique Identifier SYN-IR67B-1) insecticidal proteins and the genetic material necessary for their production in COT102 X COT67B cotton

Trade and Other Names: VipCot Cotton; COT102 X COT67B Cotton

OPP Chemical Code: 006499 (Vip3Aa19) and 006529 (modified Cry1Ab)

Basic Manufacturers: Syngenta Seeds, Inc.

Type of Pesticide: Plant-Incorporated Protectant

Uses: Cotton

Target Pest(s): tobacco budworm, cotton bollworm, pink bollworm

C. Regulatory History

Syngenta Seeds, Inc. was issued an Experimental Use Permit (EUP) for VipCot Bt cotton containing Vip3Aa19 (Event COT102) and modified Cry1Ab (Event COT67B) on April 26, 2007 (EPA Reg. No. 67979-EUP-7). These proteins were selected to provide protection of cotton from feeding damage caused by major lepidopteran pests including tobacco budworm, cotton bollworm, and pink bollworm. On April 26, 2007, EPA established a temporary exemption from the requirement of a tolerance for Vip3Aa19 (72 FR 26300, amended 72 CFR 40752; 40 CFR 174.501) in the food and

feed commodities of cotton. For the purpose of the EUP, modified Cry1Ab was determined to be covered under the permanent tolerance exemption for Cry1Ab in all crops (40 CFR 174.511). Both the EUP and temporary tolerance exemption were originally set to expire on May 1, 2008. However, Syngenta was granted an extension of both the EUP and temporary tolerance exemption (72 FR 68744) on November 27, 2007 which expire on May 1, 2009.

A separate EUP (EPA Reg. No. 67979-EUP-5) was previously issued to Syngenta for two Bt cotton events (Event COT202 and COT203) containing Vip3A. These two events were not part of the more recent VipCot EUP and have not been proposed for commercial registration. This EUP expired on March 31, 2006.

On December 14, 2006, Syngenta submitted an application (EPA Reg. No. 67979-O) to register VipCot (Event COT 102 x Event COT67B) under Section 3 of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). On April 5, 2007, Syngenta submitted a second application for a seed increase registration (EPA Reg. No. 67979-RR). This application was subsequently withdrawn by the registrant on January 7, 2008.

On September 6, 2007, Syngenta submitted a petition to EPA under the Federal Food, Drug, and Cosmetic Act (FFDCA), as amended by the Food Quality Protection Act of 1996 (FQPA), requesting a permanent tolerance exemption for Vip3Aa in all plants (PP 7F7254). A separate petition was submitted to request a permanent exemption for modified Cry1Ab in all plants on November 9, 2007 (PP 7F7290). After review of the supporting data, EPA determined that the permanent tolerance exemptions would be limited to corn and cotton (Vip3Aa) and cotton (modified Cry1Ab).

On June 26, 2008 (73 FR 45620 and 73 FR 40760), the Agency established permanent exemptions from the requirement of a tolerance for residues of the *Bacillus thuringiensis* Vip3Aa proteins in corn and cotton (40 CFR 174.501) and modified Cry1Ab protein as identified under OECD Unique Identifier SYN-IR67B-1 in cotton (40 CFR 174.529) when used as plant–incorporated protectants.

On June 26, 2008, a conditional registration was issued for VipCot Bt Cotton (EPA Reg. No. 67979-9).

II. Science Assessment

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

II.A. Product Characterization

II.A.1. Event COT102 Cotton (OECD Unique Identifier: SYN-IR102-7) Expressing Vip3Aa19

Event COT102 cotton, which was developed by *Agrobacterium*-mediated transformation of cotton using elements of a vector referred to as both pNOV3001 and pCOT1, expresses the insecticidal protein, Vip3Aa19 as well as a selectable marker, hygromycin B phosphotransferase (APH4). The Vip3Aa19 protein is intended to control several lepidopteran pests of cotton including, but not limited to, *Helicoverpa zea* (cotton bollworm/corn earworm), *Heliothis virescens* (tobacco budworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), and *Trichoplusia ni* (cabbage looper). Vip3A is a vegetative (i.e., produced during the vegetative stage of bacterial growth) insecticidal protein from *Bacillus thuringiensis* (*Bt*), a gram positive bacterium commonly found in soil.

Transformation System:
COT102 cotton was produced by *Agrobacterium tumefaciens*-mediated transformation of hypocotyls of *Gossypium hirsutum* L. cultivar Coker 312 with plasmid pNOV3001 (also referred to as pCOT1). Plasmid pNOV3001 (pCOT1) contains T-DNA with the *vip3Aa19* and *aph4* expression cassettes. The *vip3Aa19* expression cassette contains the *vip3Aa19* coding sequence under the regulation of the Act2 promoter and intron (derived from *Arabidopsis thaliana*), and NOS terminator (derived from *Agrobacterium tumefaciens*). The *aph4* expression cassette contains the *aph4* coding sequence under the regulation of the Ubq3 promoter and intron (derived from *Arabidopsis thaliana*) and the NOS terminator (derived from *Agrobacterium tumefaciens*). The *vip3Aa19* gene encodes a protein that differs from the Vip3Aa1 protein from *Bacillus thuringiensis* strain AB88 by one amino acid at position 284 (The *vip3Aa1* gene encodes lysine at position 284, and the *vip3Aa19* gene encodes glutamine). Vip3Aa19 confers resistance to several lepidopteran pests. The *aph4* gene encodes

hygromycin B phosphotransferase (APH4), an enzyme that catalyzes the phosphorylation of hygromycin and some related aminoglycosides. Expression of APH4 allows growth in the presence of hygromycin and was used as a selectable marker, enabling selection of transformed cells.

Characterization of the DNA Inserted in the Plant and Inheritance and Stability:
Characterization of the DNA isolated from event COT102 cotton using restriction enzyme digests and Southern blot analysis as well as DNA sequencing indicates that the DNA was inserted in the cotton genome at a single locus, and the insert contains one copy each of the *vip3Aa19* and *aph4* expression cassettes. There were no other detectable elements other than those associated with the respective cassettes. No backbone sequences from plasmid pNOV3001 (pCOT1) were detected in the cotton genome. Southern blot analysis and protein expression data also demonstrated the stability of the insert over multiple generations.

Protein Characterization:

The insecticidal protein produced in event COT102 cotton, designated as Vip3Aa19[a], is a variant of the naturally occurring Vip3Aa1 protein isolated from *Bacillus thuringiensis* strain AB88, differing from the Vip3Aa1 protein by one amino acid (Vip3Aa19 contains a glutamine at position 284, while Vip3Aa1 contains a lysine). Both proteins are 789 amino acids in length and have a molecular weight of approximately 89 kDa. Syngenta has also developed a transgenic corn variety, MIR162, that produces another variant, designated as Vip3Aa20, differing from the naturally occurring Vip3Aa1 protein by two amino acids; at position 284, Vip3Aa20 has the same amino acid substitution as Vip3Aa19 (i.e., K284Q), and in addition, at position 129, Vip3Aa20 contains an isoleucine, while Vip3Aa1 contains a methionine (M129I).

The following techniques were used to characterize and compare the plant-produced and the *E. coli*-produced Vip3Aa proteins: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, densitometry, mass spectrometry, glycosylation analysis, N-terminal amino acid sequencing, and insecticidal activity assays. Glycoslyation analysis indicated that the proteins are not glycoslyated. These analyses demonstrated the structural and functional similarity between the plant-produced Vip3Aa19 and the *E. coli*-produced Vip3Aa19, Vip3Aa20, and Vip3Aa1 proteins and justified the use of *E. coli*-produced proteins in toxicity studies.

Analytical Detection Methods:

Syngenta has provided a validation study for SeedChek Vip3A/FLCry1Ab, a lateral flow test kit that detects both Vip3A and Cry1Ab. The SeedChek Vip3A/FLCry1Ab lateral flow test kit was tested for the qualitative detection of modified Cry1Ab and Vip3A proteins in cotton seed and cotton leaf. The study showed that the SeedChek kit is able to detect Vip3A and Cry1Ab in both cotton seed and

[a] Prior to receiving the Crickmore designation of Vip3Aa19, the protein produced in COT102 was referred to as Vip3A or Vip3Aa.

cotton leaf. No unexpected cross reactivity with other transgenic varieties or nontransgenic controls was observed. An independent lab validation of this method is still needed.

Protein Expresson:
Expression level data were provided for Vip3Aa19 and APH4 in different plant tissues and at different growth stages in COT102.

Table 1. Mean Expression Levels of Vip3Aa19 and APH4 from COT102 Plant Tissues

Tissue Type	Vip3Aa19 (µg/g dry weight ± standard deviation)	APH4 (µg/g dry weight ± standard deviation)
Leaves*	44 ± 10 - 277 ± 41	< 0.42 – 8.2 ± 1.4
Squares	116 ± 22	2.2 ± 0.4
Flowers	162	1.68
Pollen	3.47	64.3
Bolls	19 ± 4	< 0.39
Whole Plants	25 ± 4	< 0.37
Seed	7 + 2	1.4 ± 0.3
Roots	16 ± 2	0.53 ± 0.11

*Ranges reflect means at different growth stages for leaves

The data submitted for product characterization for event COT102 cotton are summarized in Table 2 below.

Table 2. Product Characterization Data Submitted for Event COT102 Cotton (reviewed in Edelstein 2008 unless otherwise noted)

Study Type/Title	Summary	MRID #
Expression Levels/ Quantitation of VIP3A and APH4 Protein in Cotton Tissues and Whole Plants Derived from Transformation Event COT102[b]	Transgenic cotton plants (COT102) and a non-transgenic isoline (Coker 312) were grown concurrently in 2001 in Camilla, GA; Maricopa, AZ; and Idalou, TX. Ten whole transgenic plants (including roots) and two control plants were harvested approximately 2, 4, 9, 13, 15, and 22 week post-emergence (stages: four-leaf, squaring, first white bloom, peak bloom, first open boll, pre-harvest, respectively). Tissue extracts were analyzed for VIP3A and APH4 by ELISA. VIP3A protein was detected in COT102 whole plants, leaves, roots, squares, and bolls at all six developmental stages examined. VIP3A levels varied in all plant tissues, generally declined with time, but stayed constant in the roots. The highest levels were found in	45835801

[b] Study submitted with EUP request and reviewed in memorandum from C. Wozniak to L. Cole dated March 24, 2004.

Study Type/Title	Summary	MRID #
	leaves at the squaring stage (mean of 8.56 to 10.78 µg VIP3A/g fresh tissue). Low VIP3A levels were found in seed (mean of 2.51 to 3.23 µg VIP3A/g) and in pollen (1.09 µg VIP3A/g). VIP3A was not detected in cotton fiber or nectar. The protein marker, APH4, was detected in COT102 plants at low, non-quantifiable levels at some developmental stages in leaves, roots, bolls, squares, and whole plants and at quantifiable levels in pollen (2.25 µg APH4/g air-dried pollen). APH4 was not detected in cotton fiber or nectar. Geographic location appeared not to have a significant effect on VIP3A levels, but no statistical analysis was done. APH4 levels appears to be similar across locations, but the lack of data points in many instances and the detectable levels falling below the level of quantitation (LOQ) do not allow for any definitive conclusions to be made. The estimated amount of VIP3A/acre cotton varied considerably among the developmental stages with the greatest mean level found at the peak bloom stage (105.80 g VIP3A/acre based on whole plant VIP3A levels). **Classification: ACCEPTABLE**	
Characterization of Inserted DNA/Molecular Characterization and Genetic Stability of Event COT102[b]	Southern blot analysis and DNA sequencing suggest that event COT102 has one transgene insertion site with a single copy of intact *vip3A(a)* and *aph4* expression cassettes (containing one copy of the *vip3A(a)* gene, *aph4* gene, actin-2 promoter, and ubq3 promoter). DNA sequence alignment revealed an exact sequence match between the pCOT-1 vector and event COT102, and showed the lack of *Agrobacterium* sequence beyond the T-DNA borders. VIP3 protein expression measurement (by ELISA) of five generations of COT102 seedlings (F1, BC1F2, BC2F1, BC2F2, and BC3F1) showed that the *vip3A(a)* gene was stable across generations and segregated in a Mendelian fashion, consistent with a single transgene insertion site. MRID 458358-02 provided very scant experimental details. Insufficient experimental methods details were provided for the Southern blots, DNA cloning and sequencing, PCR analysis, and protein detection and segregation analysis by ELISA, precluding confirmation of their appropriateness by an independent reviewer. Sample Southern blots demonstrating the integration copy number and lack of rearrangements through appropriate restriction analyses must be provided in order to assess the results of this study. Further information is required regarding the number of plants utilized in the segregation and heritability analysis. **Classification: SUPPLEMENTAL,** upgradeable to acceptable pending submission of additional methods details and correction/clarification of typographic errors in Figure 1, Figure 2, and/or the text of MRID 458358-02. **Superseded by MRID 47017603**	45835802
Characteristics of *Bacillus thuringiensis* VIP3A Protein and VIP3A Cotton Plants Derived from Event COT102[b]	The *Bacillus thuringiensis* (*Bt*) VIP3A insect control protein as expressed in transgenic cotton seed confers protection against the bollworm complex and other lepidopteran cotton pests. The seeds are derived from transgenic cotton event COT102, which contains the insecticidal gene via plasmid vector pCOT1. The product active ingredient is ≤0.0015 % dry weight *Bacillus thuringiensis* VIP3A Protein and the genetic material necessary for its production (pCOT1 in cotton). The product also contains ≤0.0001% dry	45766501

Study Type/Title	Summary	MRID #
	weight marker protein and the genetic material necessary for its production (pCOT1 in cotton). VIP3A protein in transgenic cotton plants derived from Event COT102, is produced by a synthetic *vip3A(a)* gene, which encodes a polypeptide of 789 amino acids. The VIP3A toxin is proteolytically activated to a toxin core in the lepidopteran larval midgut and forms pores in the gut membranes of sensitive species. Several formulated microbial *Bt* products containing VIP3A-like proteins and the genetic components in plasmid pCOT1, as well as its expression analysis, are described in MRID 457665-01. **Classification: ACCEPTABLE.** The wide certified limits of the active ingredient need to be explained, although they are within the bounds covered by the acute oral toxicity studies submitted for review.	
Characterization of the active ingredient/Characterization of VIP3A Protein Produced in COT102-Derived Cotton and Comparison with VIP3A Protein Expressed in Both Maize (Corn) Derived from Event PACHA and Recombinant *Escherichia coli*[b]	VIP3A protein produced in cotton plants derived from transgenic cotton event "COT102" was characterized for its biochemical and functional similarity with VIP3A expressed in recombinant *Escherichia coli* and "Pacha" derived transgenic maize plants. Samples of purified VIP3A protein from *E. coli* and maize were dissolved in buffer for analysis by SDS-PAGE and Western blotting. VIP3A from cotton leaves was extracted following published procedures and prepared for SDS-PAGE and Western blotting. VIP3A proteins from all three sources were determined to have the predicted molecular weight of ca. 89,000 and cross-reacted immunologically with the same anti-VIP3A antibody. No evidence of any post-translational modification of VIP3A was observed in any of the three Vip3A protein sources. Peptides representing *ca*. 85% (673/789) of the complete VIP3A amino acid sequence were identified by mass spectral analysis of cotton produced VIP3A protein. Amino acid sequences corresponded identically to the predicted amino acid sequence of the VIP3A protein. Comparisons of bioactivity of *E. coli*-expressed and cotton-expressed VIP3A protein in larvae of four lepidopteran species demonstrated comparable activities, with the exception of the tobacco budworm bioassays (TBW). A 35% difference in mortality was noted in TBW assays comparing these two sources of test substance. In the absence of an in-depth statistical analysis, it is not possible to assign a particular factor as the causal agent in delimiting this result. Given that both test substances contain other constituents, it is difficult to assess the reason for this observation. TBW is considered as one of the least sensitive species of lepidopteran insects evaluated. A similar rank order of species sensitivity was found for both test solutions; FAW was the most sensitive to VIP3A, while CBW and TBW were the least sensitive. These data indicate that VIP3A proteins from recombinant *E. coli*, Pacha-derived maize and event COT102-derived cotton are substantially equivalent. **Classification: ACCEPTABLE**	45835812
Expression Level/ Analysis of Processed COT102 Cottonseed Products for Yield and Presence of Gossypol	Processing transgenic COT102 and control Coker 312 cotton seeds resulted in similar yields for the hulls, lint, kernels, refined oil, and de-fatted meal. Analysis of the refined oil and de-fatted meal (non-toasted and toasted) by ELISA detected VIP3A protein in COT102 meal but not in oil, and not in meal or oil from control seeds. Analysis of both COT102 and Coker 312	45835803

Study Type/Title	Summary	MRID #
and Vip3A Protein[b]	de-fatted meal for the plant toxin gossypol detected free gossypol (HPLC method) and total gossypol (free + protein-bound; spectrophotometric method). Refined oil had >100-fold lower levels of total gossypol than meal. MRID 45835803 provided inadequate and/or conflicting details for some experimental methods and results. **Classification: ACCEPTABLE. Submission of additional methods details and correction and/or clarification of the MRID 458358-03 text as listed under "Deficiencies" is, however, recommended to ensure adequate recording in the official record.** **The additional information was subsequently determined to be unnecessary because no adverse effects were observed in the nontarget studies.**	
The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab delta-endotoxin[c]	This publication (Lee *et al.*, 2003), which examined the differences in the mechanism of insecticidal activity of Cry1Ab and Vip3A, was submitted by the registrant to provide additional product characterization data, specifically Vip3A's mode of action. The submitted publication examined differences in the mechanism of insecticidal activity of Cry1Ab and Vip3A proteins. Ligand blotting showed that activated Cry1Ab and Vip3A-G (Vip3A proteolytically cleaved with lepidopteran gut juice) bound different receptor molecules in midgut of Tobacco hornworm (*Manducta sexta*, Linnaeus) and that Vip3A-G did not bind Cry1A receptors. Voltage clamping assays showed that Vip3A-G formed distinct pores in dissected midgut from *M. sexta* but not in the monarch butterfly (*Danaus plexippus*, Linnaeus). Cry1Ab and Vip3A both formed voltage-independent and cation-selective stable ion channels in planar lipid bilayers, but their primary conductance state and cation specificity differed. **Classification: ACCEPTABLE**	46880801
Characterization of Test Substance/Re-Characterization of Vip3A Protein Test Substance (Vip3A-0204)	The purpose of this study was to re-characterize the microbially produced test substance, VIP3A-0204. The purity, integrity, and bioactivity of the test substance were determined and compared with previous analyses after being stored *ca.* 15 months under desiccation at -20 °C. Total protein in VIP3A-0204 was quantified spectrophotometrically, and the purity was determined using SDS-PAGE followed by densitometric analysis. The integrity of the Vip3Aa19 protein in test substance VIP3A-0204 was determined using Western blot analysis, and bioactivity was assessed in insect feeding assays using freshly hatched first-instar *S. frugiperda* (fall army worm) larvae. This re-characterization study demonstrated that VIP3A-0204 largely retained its insecticidal activity (LC_{50} of 34 ng Vip3A/cm^2 diet surface vs. 45.1 initially) after storage for 15 months. The purity of test substance VIP3A-0204 was determined to be *ca.* 92% Vip3Aa19 by weight. Western blot analysis revealed a dominant immunoreactive band corresponding to the predicted molecular weight of Vip3Aa19 of *ca.* 89 kDa. These results are similar to those obtained in previous analyses, demonstrating that the	47017602

[c] Study submitted with EUP request and reviewed in memorandum from A. Waggoner to M. Mendelsohn dated February 8, 2007.

Study Type/Title	Summary	MRID #
	test substance is stable when stored desiccated at -20 °C for approximately 15 months. **Classification: ACCEPTABLE**	
Characterization of the inserted DNA/ Additional Molecular Characterization of Event COT102 Cotton by Southern Analysis	Molecular analysis of event COT 102 was performed using restriction enzyme digestion and Southern blot analysis to determine the number of inserts, copy number of functional elements, and the presence or absence of plasmid backbone sequences. This study also assessed the inheritance and stability of the insert. Data from the Southern analyses demonstrated that the BC4F1 generation of COT102 cotton: (1) contains a single intact insert; (2) contains a single copy of the *vip3Aa19* gene and the *aph4* gene; (3) contains a single copy of the Act2 promoter; (4) contains a single copy of the Ubq3 promoter; (5) does not contain any detectable backbone sequences from the transformation plasmid pCOT1; and (6) the insert is stably integrated into the cotton genome. These results are consistent with results from previous molecular analysis studies on event COT 102. **Classification: ACCEPTABLE**	47017603
Inheritance and Stability/ Stability of Vip3Aa19 and APH4 Protein Expression Across Multiple Generations of Event COT102 Cotton	The purpose of this study was to use ELISA to analyze the levels of expression of the Vip3Aa19 and hygromycin B phosphotransferase (APH4) proteins in leaves (collected at the 1st white bloom stage) of three generations (F1, BC1F1, and BC4F1) of Event COT102 cotton. The levels of Vip3Aa19 protein measured were comparable (ca. 60 µg/g dry weight) in all three generations analyzed. APH4 protein was detectable in all three generations analyzed, but the concentrations were below the limit of quantification (LOQ). The consistency of Vip3Aa19 and APH4 protein concentrations demonstrate the stability of transgenic protein expression across multiple generations of COT102 cotton at the 1st white bloom stage. **Classification: ACCEPTABLE**	47017609

II.A.2. Event COT67B Cotton (OECD Unique Identifier: SYN-IR67B-1) Expressing Modified Cry1Ab

Event COT67B cotton, which was developed by *Agrobacterium*-mediated transformation of cotton using elements of vectors pNOV4641 and pNOV1914, expresses the insecticidal protein, modified Cry1Ab. This protein contains an additional 26 amino acid sequence at the C-terminus (termed the 'Geiser motif'). The modified Cry1Ab protein is intended to control several lepidopteran pests of cotton including, but not limited to, *Helicoverpa zea* (cotton bollworm/corn earworm), *Heliothis virescens* (tobacco budworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), and *Trichoplusia ni* (cabbage looper).

Transformation System:

COT67B cotton was produced by *Agrobacterium tumefaciens*-mediated cotransformation of *Gossypium hirsutum* L. cultivar Coker 312 using transformation vectors pNOV4641 and pNOV1914, each carrying one T-DNA. Plasmid pNOV4641 contains a full-length *cry1Ab* gene that encodes a full-length Cry1Ab protein that is identical to the Cry1Ab protein produced by *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1, except that it contains an additional 26 amino acids, which Syngenta describes as the 'Geiser motif,' in the C-terminal portion of the protein. The *cry1Ab* gene is under the regulation of the Act2 promoter and intron (derived from *Arabidopsis thaliana*) and NOS terminator (derived from *Agrobacterium tumefaciens*). Plasmid pNOV1914 contains a *hygromycin B phosphotransferase* gene (aph4) derived from *Escherichia coli* that confers resistance to the antibiotic hygromycin B and was used as a selectable marker. The two-T-DNA system enabled Syngenta to separate the two inserts by traditional breeding. COT67B cotton contains only the T-DNA from plasmid pNOV4641 encoding the modified Cry1Ab protein; the T-DNA from pNOV1914 containing the *aph4* gene is absent.

Characterization of the DNA Inserted in the Plant and Inheritance and Stability:
Characterization of the DNA isolated from event COT67B cotton using restriction enzyme digests and Southern blot analysis as well as DNA sequencing indicates that the DNA was inserted in the cotton genome at a single locus, and the insert contains one copy of the *cry1Ab* gene. No backbone sequences from the transformation plasmid pNOV4641 were found in COT67B. The left border and the adjacent 13 bp of the insert along with 24 bp of the right border (RB) were deleted during the insertion of the T-DNA. However, such deletions are common during transformation and do not affect the functioning of the T-DNA itself. Additionally, the analysis showed that COT67B cotton does not contain the selectable marker gene, *hygromycin B phosphotransferase* (*aph4*), the Ubq3 promoter from the transformation plasmid pNOV1914, or any backbone sequences from pNOV1914. Inheritance and stability studies of the *cry1Ab* gene in COT67B verified that it is stably integrated into the cotton genome, segregating in an expected Mendelian fashion of 1:1.

Protein Characterization:
Event COT67B expresses a full-length Cry1Ab protein that is identical to the Cry1Ab protein produced by *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1, except that it contains an additional 26 amino acids (described by Syngenta as the 'Geiser motif') in the C-terminal portion of the protein. Syngenta states that the additional amino acids have been included because the insertion made fermentation in *Bacillus thuringiensis* more efficient, but they have no impact on insecticidal activity.

The following techniques were used to characterize and compare the plant-produced and the *E. coli*-produced modified Cry1Ab proteins: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, densitometry, mass spectrometry, glycosylation analysis, N-terminal amino acid sequencing, and insecticidal activity assays. Glycoslyation analysis indicated that the proteins are not glycoslyated. These analyses demonstrated the structural and functional similarity between the plant-produced and the *E. coli*-produced modified Cry1Ab proteins and justified the use of *E. coli*-produced protein in toxicity studies.

Analytical Detection Methods:
Syngenta has provided a validation study for SeedChek Vip3A/FLCry1Ab, a lateral flow test kit that detects both Vip3A and Cry1Ab. The SeedChek Vip3A/FLCry1Ab lateral flow test kit was tested for the qualitative detection of modified Cry1Ab and Vip3A proteins in cotton seed and cotton leaf. The study showed that the SeedChek kit is able to detect Vip3A and Cry1Ab in both cotton seed and cotton leaf, and no unexpected cross reactivity with other transgenic varieties or nontransgenic controls was observed. An independent lab validation of this method is still needed.

Protein Expresson:
Expression level data were provided for modified Cry1Ab in different plant tissues and at different growth stages in event COT67B cotton and summary results are provided in Table 3 below. The data were produced using an ELISA method.

Table 3. Mean Cry1Ab Expression levels in Event COT67B Cotton.

Tissue Type	Cry1Ab (µg/g dry weight ± standard deviation)*
Leaves	65 ± 9 – 158 ± 40
Squares	93 ± 13
Flowers	101
Pollen	12.1
Bolls	47 ± 7
Whole Plants	26 ± 2
Seed	29 ± 5
Roots	17 ± 1

*Range reflects means at different growth stages for leaves

Table 4 provides summaries of the product characterization studies and data provided.

Table 4. Product Characterization Data Submitted for Event COT67B (reviewed in Edelstein 2008 unless otherwise noted)

Study Type/Title	Summary	MRID #
Characterization of Inserted DNA/ Harper, B. (2006). Molecular characterization of	The purpose of this study was to determine the DNA sequence and contiguousness of the full length *cry1Ab* (*flcry1Ab*) gene present in Syngenta's COT67B cotton and its inheritance ratio across generations. COT67B cotton plants express a modified full length Cry1Ab *Bacillus*	46885901

[d] Reviewed in a memorandum from S. Matten to A. Reynolds dated April 4, 2007.

Study Type/Title	Summary	MRID #
Event COT67B cotton. Report No. SSB-125-06.[d]	*thuringiensis* protein (FLCry1Ab) that contain an additional 26 amino acids in the C-terminal portion of the protein described as the "Geiser motif." FLCry1Ab confers resistance to certain lepidopteran insects in cotton. The T-DNA insert (via the pNOV4641 plasmid) in COT67B cotton was analyzed by Southern blots and DNA sequencing. These analyses confirmed that there was a single, contiguous copy of the *flcry1Ab* gene present in COT67B. No backbone sequences from the transformation plasmid pNOV4641 were found in COT67B. The left border (LB) and the adjacent 13 bp of the insert along with 24 bp of the right border (RB) were deleted during the insertion of the T-DNA. However, such deletions are common during transformation and do not affect the functioning of the T-DNA itself. Additionally, COT67B cotton did not contain the selectable marker gene, hygromycin B phosphotransferase (*aph4*), or the Ubq3 promoter from the transformation plasmid pNOV1914 and was also free of any backbone sequences from pNOV1914. Inheritance studies of the *flcry1Ab* gene in COT67B verified that it is segregating in an expected Mendelian fashion of 1:1. **Classification: ACCEPTABLE**	
Expression Levels/ Hill, K. (2006). Quantification of Cry1Ab protein in Event COT67B cotton tissues and whole plants. Report No.SSB-022-06[d]	The purpose of this study was to quantify expression of Cry1Ab protein in Event COT67B-derived cotton plants. Quantifiable levels of Cry1Ab protein in Event COT67B-derived cotton plants were determined by enzyme-linked immunosorbent assay (ELISA) for various plant tissues and whole plants at five developmental stages in four locations. Corresponding, near-isogenic, non-transgenic control cotton plants were analyzed in parallel. As expected, Cry1Ab protein was detected in all COT67B plant tissues (i.e., young leaves, old leaves, roots, flowers, pollen, bolls) except fiber and nectar. The concentrations of Cry1Ab in COT67B were similar between the four locations for each tissue type at each time point, although no specific conclusions about differences between locations can be made from the data. Where the concentrations of Cry1Ab appeared variable, there were no consistent trends to indicate that the plants grown in a given location had higher or lower Cry1Ab concentrations. No statistical analysis was performed. Cry1Ab concentrations in most of the near-isogenic, nontransgenic control samples were either below the limit of detection (LOD) or below the limit of quantification (LOQ). The negative control seed from Quitman, GA was determined to have a low level of Cry1Ab (0.24 µg/g dw) that was likely due to contamination during processing or extraction. The average relative extraction efficiency for the various plant tissues analyzed varied between 70.7% for whole plants to 78.5% for pollen. The absolute amount of Cry1Ab in the cotton tissue samples is unknown and some Cry1Ab may be unextractable with the methods used. Extraction efficiency for the purposes of satisfying the analytical method would need to use a spike-recovery method. Several deviations from the protocol were noted by the study authors, but none of these affected the overall conclusions of the study. Across all growth stages, mean Cry1Ab concentrations (averaged across locations) measured in young leaves, old leaves and roots of COT67B	46885902

Study Type/Title	Summary	MRID #
	cotton ranged from 87.70 - 323.84, 194.02 - 255.74, and 12.61 - 56.56 µg/g dry weight (dw), respectively. Mean Cry1Ab concentrations measured in bolls (collected at 1st open boll), whole plants (collected at pre-harvest), and seed (collected at pre-harvest) averaged 45.24, 42.87, and 25.17 µg/g dw across locations, respectively. Cry1Ab concentrations in flowers and pollen collected at the Winnsboro, LA site at peak-bloom averaged 161.74 and 5.45 µg/g dw, respectively. Cry1Ab concentrations in nectar taken from the same cotton plants was not detectable (limit of detection = 0.0002 µg/mL). Cry1Ab concentrations in fiber samples collected at this site at pre-harvest was <0.02 µg/g dw The average Cry1Ab protein per acre and per hectare in pre-harvest COT67B plants collected from 4 sites was determined assuming a planting density of 50,000 plants/acre (123,500 plants/hectare). The average Cry1Ab protein concentration ranged from 46 to 183 g/acre (115 to 451 g/hectare). **Classification: ACCEPTABLE for the purposes of supporting the Experimental Use Permit**. Statistically-valid trends in the data (e.g., expression level differences between tissue types, across developmental stages, between locations) cannot be made. For a quantitative analysis, it is recommended that the expression data submitted to support the Section 3 registration include an appropriate statistical analysis. **Superseded by MRID 47017607.**	
Characterization of test substance/Characterization of Cry1Ab Test Substance FLCRY1AB-0103 and Certificate of Analysis	The purity, integrity, and bioactivity of *E. coli*-produced test substance FLCRY1AB-0103, containing modified full-length Cry1Ab, were determined initially and after *ca.* 5 months of being stored under desiccation at -20 °C. The purity of test substance FLCRY1AB-0103 was determined to be *ca.* 86%, both before and after storage, and Western blot analysis of the test substance showed a dominant immunoreactive band corresponding to the predicted molecular weight of *ca.* 133.5 kDa before and after storage. N-terminal sequencing confirmed that the first 12 amino acids of the test protein corresponded to the predicted N-terminal sequence of Cry1Ab. The test substance was insecticidally active and had a 72-hour LC_{50} of 3.7 ng Cry1Ab/cm^2 diet surface against first instar larvae of the European corn borer. Re-analysis of FLCRY1AB-0103 *ca.* 5 months after the initial analysis demonstrated that the test substance retained insecticidal activity when stored desiccated at -20 °C. **Classification: ACCEPTABLE**	47017604
Characterization of test substance/Re-Characterization of Cry1Ab Test Substance FLCRY1AB-0103	The purpose of this study was to re-characterize the purity, integrity, and bioactivity of microbially produced test substance FLCRY1AB-0103 (containing modified full-length Cry1Ab) after storage at -20 °C for *ca.* 14 months. Total protein in test substance FLCRY1AB-0103 was quantified spectrophotometrically by measuring its absorption at 280 nm (A_{280} method). The purity of test substance FLCRY1AB-0103 was calculated from the total sample weight and the total protein as determined by the A_{280}	47017605

Study Type/Title	Summary	MRID #
	method in conjunction with densitometry data after electrophoretic separation. The integrity of the Cry1Ab protein in test substance FLCRY1AB-0103 was determined using Western blot analysis. Bioactivity of the Cry1Ab protein in FLCRY1AB-0103 was assessed in insect feeding assays using freshly hatched first-instar *O. nubilalis* (European corn borer) larvae. The results demonstrated that the test substance remained intact and retained insecticidal activity during this storage period. **Classification: ACCEPTABLE**	
Characterization of Expressed Substance/ Characterization of the Cry1Ab Protein Produced in Event COT67B-Derived Cotton Plants and Comparison with Cry1Ab Protein Produced in Recombinant *Escherichia coli*	The purpose of this study was to use various biochemical and functional parameters to demonstrate the biochemical equivalence between the Cry1Ab protein expressed in transgenic Event COT67B cotton and the Cry1Ab protein contained in test substance FLCRY1AB-0103 prepared from an *E. coli* over-expression system. Cry1Ab protein was extracted from COT67B cotton plant tissue and its apparent molecular weight, immunoreactivity, glycosylation status, and bioactivity were compared to the Cry1Ab protein from test substance FLCRY1AB-0103. In addition, the microbial- and plant-derived Cry1Ab proteins were analyzed by peptide mass mapping and the N-terminal amino acid sequence of Cry1Ab from test substance FLCRY1AB-0103 was determined. The Cry1Ab proteins from COT67B and from microbially-derived test substance FLCRY1AB-0103 both had an apparent molecular weight of *ca.* 133.5 kDa, and both reacted with anti-Cry1Ab antibodies, as shown by Western blot analysis. Also, both the protein extract from COT67B and FLCRY1AB-0103 showed strong insecticidal activity against *O. nubilalis* (European corn borer). There was no evidence of post-translational glycosylation of Cry1Ab protein from COT67B or from microbially-derived test substance FLCRY1AB-0103. Peptide mass mapping analysis provided additional evidence of the identity of the insecticidal protein expressed in COT67B cotton and in test substance FLCRY1AB-0103. Based on the results of this study it can be concluded that Cry1Ab protein produced in recombinant *E. coli* (test substance FLCRY1AB-0103) is a suitable surrogate for Cry1Ab expressed in COT67B cotton. **Classification: ACCEPTABLE**	47017608
Expression levels/Stability of Cry1Ab Protein Expression Across Multiple Generations of Event COT67B Cotton	The purpose of this study was to use ELISA to analyze the levels of expression of the modified Cry1Ab protein in leaves (collected at open boll stage) of the F1, BC1F1, and BC4F1 generations of Event COT67B cotton. Identical plant tissues from two near-isogenic, nontransgenic cotton plants (cotton line 2429) from the BC1F1 and BC4F1 generations were concurrently sampled and analyzed to identify any potential background effects of the plant matrix on the ELISA. The levels of Cry1Ab protein measured in the three generations of COT67B cotton were comparable (~60 µg/g dry weight). The consistency of the Cry1Ab protein concentrations demonstrates the stability of transgenic protein expression across multiple generations of COT67B cotton at the open boll stage. **Classification: ACCEPTABLE**	47017610

Study Type/Title	Summary	MRID #
Expression Levels/ Analysis for the Presence of Cry1Ab Protein in Linters, Toasted Cottonseed Oil from Processed Seed of Event COT67B Cotton Expressing Full-Length Cry1Ab Protein	The purpose of this study was to quantify Cry1Ab protein in linters, defatted toasted cottonseed meal, and once-refined cottonseed oil derived from COT67B, and to determine Cry1Ab protein concentrations in the fuzzy seed used to generate these processed fractions. Quantification was carried out using an enzyme-linked immunosorbent assay (ELISA). The Cry1Ab extraction efficiencies were >69% for fuzzy seed, linters, and defatted toasted cottonseed meal from COT67B. The mean concentrations of Cry1Ab protein (corrected for extraction efficiency) in fuzzy seed, linters, and defatted toasted cottonseed meal from COT67B were 25.1, 9.6, and 47.5 µg Cry1Ab/g, respectively. Cry1Ab was not detectable in the once-refined oil from COT67B (limit of detection = 0.003 µg Cry1Ab/ml). Cry1Ab concentrations in all cottonseed samples from Coker 312 (negative control) were below the limit of detection. **Classification: ACCEPTABLE**	47017611

II.A.3. COT102 x COT67B Cotton (OECD ID No SYN-IR102-7 x OECD ID No. SYN-IR67B-1) Expressing Vip3Aa19, APH4, and Modified Cry1Ab

COT102 x COT67B was developed by conventional breeding of COT102 plants with COT67B plants.

DNA characterization (*i.e.*, Southern blot analysis) was used to confirm the integrity of the COT102 and COT67B inserts in the stacked product COT102 x COT67B. Samples from COT102 x COT67B cotton gave the same results as those observed for the individual events, indicating that the molecular characterization data provided for the individual events are also applicable to COT102 x COT67B.

Analytical Detection Methods:
Syngenta has provided a validation study for SeedChek Vip3A/FLCry1Ab, a lateral flow test kit that detects both Vip3A and Cry1Ab. The SeedChek Vip3A/FLCry1Ab lateral flow test kit was tested for the qualitative detection of modified Cry1Ab and Vip3A proteins in cotton seed and cotton leaf. The study showed that the SeedChek kit is able to detect Vip3A and Cry1Ab in both cotton seed and cotton leaf, and no unexpected cross reactivity with other transgenic varieties or nontransgenic controls was observed. An independent lab validation of this method is still needed.

Protein Expresson:
Protein expression levels were provided for Vip3Aa19, APH4, and modified Cry1Ab in different plant tissues from COT102 x COT67B cotton, and means are shown below in Table 5. The protein levels are similar to those observed in plant tissue from cotton from the individual events.

Table 5. Mean Expression Levels of Vip3Aa19, APH4, and Modified Cry1Ab from COT102 x COT67B Plant Tissues

Tissue Type	Vip3Aa19 (µg/g dry weight ± standard deviation)*	APH4 (µg/g dry weight ± standard deviation)*	Cry1Ab (µg/g dry weight ± standard deviation)*
Leaves	55 ± 7 – 239 ± 46	<0.41 – 6.3 ± 1.3	70 ± 14 – 185 ± 63
Squares	132 ± 18	2.1 ± 0.5	94 ± 10
Flowers	148	1.80	121
Pollen	3.06	74.7	10.7
Bolls	21 ± 4	< 0.43	42 ± 7
Whole Plants	25 ± 7	< 0.40	29 ± 7
Seed	7 ± 1	1.6 ± 0.4	27 ± 4
Roots	11 ± 3	0.46 ± 0.05	20 ± 4

*Ranges reflect means at different growth stages for leaves

These data, together with data indicating that there is no evidence of either a synergistic or antagonistic interaction between Vip3Aa19 and modified Cry1Ab in cotton bollworm or tobacco budworm (reviewed in the ecological risk assessment memo for this product), demonstrate that data on the individual events and individual proteins can be used to support the safety of the COT102 x COT67B combined product.

Table 6. Product Characterization Data Submitted for COT102 x COT67B (reviewed in Edelstein 2008 unless otherwise noted)

Study Type/Title	Summary	MRID #
Characterization of Inserted DNA/ Comparative Southern Analysis of Stacked COT102 x COT67B	Molecular analyses (restriction enzyme digests and Southern blots) were performed to compare the integrity of the transgenic inserts in the cotton lines Event COT102 cotton and Event COT67B cotton with the transgenic inserts in stacked COT102 x COT67B cotton, which was produced by conventional plant breeding of COT102 and COT67B. The Southern blot data demonstrated the predicted molecular organization of the *vip3Aa19 and aph4* genes from COT102 cotton and the *cry1Ab* gene from COT67B cotton. The DNA hybridization patterns from each single event cotton line were identical to those in stacked COT102 x COT67B cotton, demonstrating that the integrity of the transgenic inserts was retained when the component lines were combined into the COT102 x COT67B cotton. **Classification: ACCEPTABLE**	47017606
Expression Levels/ Comparison of Transgenic Protein Expression in Event	The purpose of this study was to use an enzyme-linked immunosorbent assay (ELISA) to analyze tissues from cotton plants derived from transformation Event COT102, Event COT67B and from COT102 x COT67B in order to compare the concentrations of Vip3Aa19, hygromycin	47017607

Study Type/Title	Summary	MRID #
COT102, Event COT67B, and Stacked COT102 x COT67B Cotton Lines	B phosphotransferase (APH4), and Cry1Ab proteins produced in the transgenic plants. For the Vip3Aa19 and APH4 proteins, the concentrations and patterns of expression were generally similar between the COT102 line and the COT102 x COT67B line. Likewise, for the modified Cry1Ab protein, the concentrations and patterns of expression were generally similar between the COT67B line and the COT102 x COT67B line. Some statistically significant differences were seen in some tissues at certain sampling stages, but these differences were not consistent by genotype and/or were not consistent across the growing season. **Classification: ACCEPTABLE**	
Analytical Detection Method/Analytical Detection Method for the Detection of Vip3A and FLCry1Ab Protein in Cotton Tissues Derived from COT102 x COT67B Cotton (VipCot Cotton)	The SeedChek Vip3A/FLCry1Ab lateral flow test kit was tested for the qualitative detection of modified Cry1Ab and Vip3A proteins in cotton seed and cotton leaf. The study showed that the SeedChek kit is able to detect Vip3A and Cry1Ab in both cotton seed and cotton leaf, and no unexpected cross reactivity with other transgenic varieties was observed. **Classification: ACCEPTABLE**	47074101

II.B. Human Health Assessment

II.B.1. Human Health Assessment of Vip3Aa

Note: EPA's human health assessment was conducted for Vip3Aa proteins, which include the Vip3Aa19 protein as expressed in cotton.

A. Mammalian Toxicity and Allergenicity Assessment

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

Syngenta has submitted acute oral toxicity data demonstrating the lack of mammalian toxicity at high levels of exposure to Vip3Aa proteins. These data demonstrate the safety of Vip3Aa at a level 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 testing and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR Sec. 158.2140) For microbial products, further toxicity testing (Tiers II & III) and residue data are triggered by significant adverse acute effects in studies such as the mouse oral toxicity study, to verify the observed adverse effects and clarify the source of these effects.

Syngenta submitted four acute oral toxicity studies conducted on mice. Three of the studies were conducted with microbially-produced Vip3Aa proteins (Vip3Aa1, Vip3Aa19, and Vip3Aa20) with slight variations in amino acid sequence (1-2 amino acid differences), and one study was conducted with transgenic corn leaf tissue expressing Vip3Aa19 as the test material. No treatment-related adverse effects were observed in any of the studies. The results of these studies showed that the oral LD_{50} for mice (males, females, and combined) was greater than 3675 mg/kg body weight (the highest dose tested) for the tested Vip3Aa proteins.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad et al., 1992). Therefore, since no acute effects were shown to be caused by the Vip3Aa19 and Vip3Aa20 proteins, even at relatively high dose levels, they are not considered toxic. (This is also true of the Vip3Aa1 protein that was tested.) Further, amino acid sequence comparisons showed no similarities between Vip3Aa19 and Vip3Aa20, on the one hand, and known toxic proteins in protein databases, on the other hand, that would raise a safety concern.

Since Vip3Aa is a protein, allergenic potential was also considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a weight-of-evidence approach where the following factors are considered: source of the trait; amino acid sequence comparison with known allergens; and biochemical properties of the protein, including in vitro digestibility in simulated gastric fluid (SGF) and glycosylation. This approach is consistent with the approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." The allergenicity assessment for Vip3Aa follows:

1. Source of the trait. *Bacillus thuringiensis,* the microorganism from which Vip3Aa proteins are derived, is not considered to be a source of allergenic proteins.
2. Amino acid sequence. A comparison of the amino acid sequence of Vip3Aa19 and Vip3Aa20 with known allergens showed no significant sequence identity over 80 amino acids or identity at the level of eight contiguous amino acid residues.
3. Digestibility. Both Vip3Aa19 and Vip3Aa20 proteins are digested rapidly in simulated gastric fluid containing pepsin.
4. Glycosylation. Both Vip3Aa19 and Vip3Aa20 were shown not to be glycosylated.

Considering all of the available information on Vip3Aa19 and Vip3Aa20, EPA concludes that the potential for these specific proteins to be food allergens is minimal. Moreover, as further explained below, EPA believes these data and the other submitted data demonstrating a lack of mammalian toxicity at high levels of exposure to Vip3Aa19 and Vip3Aa20 can be extrapolated to cover Vip3Aa more generally.

Vip3Aa is the designation assigned to a closely-related group of similar insecticidal proteins isolated from Bacillus thuringiensis. The specific variants referred to throughout this document (i.e., Vip3Aa19 and Vip3Aa20) are isolates of Vip3Aa protein. All Vip3Aa proteins (there are 25 known Vip3Aa proteins and there are sequences available for 19 of these) are highly related. Indeed, the amino acid sequence of all the Vip3Aa proteins can only vary up to 5% to be considered a part of the Vip3Aa group. With respect to the 19 Vip3Aa proteins for which sequences are available, they vary by less than 28 amino acids out of the 789 amino acids that make up the protein. This level of sequence similarity makes that group of 19 Vip3Aa protein variants 96% identical overall. The sequence identity between any two individual sequences is even higher. For example, the sequences of the protein variants tested by Syngenta (i.e., Vip3Aa19 and Vip3Aa20) are over 99.7% identical. Finally, as to the few amino acid differences that do exist between the Vip3Aa variants, these differences do not alter the surrounding sequence, rarely occur as contiguous amino acids, and are often substitutions with similar chemical side groups indicating similar chemical functionality. Therefore, EPA finds that none of the Vip3Aa variants would be expected to have significant amino acid sequence identity -- which is defined as either 35% identity over an 80 amino acid stretch and, for allergens, at the level of eight contiguous amino acids -- with a toxin, an anti-nutrient or an allergen.

This conclusion is further supported by EPA's overall safety assessment that includes other considerations such as the source of the trait, digestibility and glycosylation. As noted above, *Bacillus thuringiensis* (from which the Vip3Aa proteins are derived) is not considered to be a source of allergenic proteins. Furthermore, since all the Vip3Aa proteins have extremely homogenous structural similarities (as explained above), they are highly likely to show similar biochemical characteristics in terms of digestibility and glycosylation. So, as is the case for both Vip3Aa19 and Vip3Aa20, EPA expects that all Vip3Aa proteins will be rapidly digested under simulated gastric conditions and will not be glycosylated. The Vip3Aa proteins were only shown not to be glycosylated in cotton and corn, similarly it is unlikely to be glycosylated in any other crops because in order for a protein to be glycosylated, it needs to contain specific recognition sites for the enzymes involved in glycosylation, and the mechanisms of protein glycosylation are similar in different plants (Lerouge et al., 1998). Thus, EPA reasonably expects that because the data on Vip3Aa in cotton and corn demonstrate a lack of protein glycosylation, it will not be glycosylated in any other plants.

Finally, it is also highly relevant here that microbial pesticide products, which are distinct from plant-incorporated protectant pesticide products, containing *Bacillus thuringiensis* and its components (which could include microbially-expressed Vip3Aa proteins) are already exempt from the requirement for a tolerance under 40 CFR part 180.1011.

Accordingly, EPA believes that the foregoing supports EPA's reasonable certainty of no harm finding not only for the Vip3Aa19 and Vip3Aa20 protein variants, but also for all other closely-related members of the Vip3Aa designation as described using the Crickmore classification system (Crickmore et al., 2007).

B. Aggregate Exposures

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

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue (i.e., the Vip3Aa proteins) and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectant's chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectant is contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. In addition, even if exposure can occur through inhalation, the potential for Vip3Aa to be an allergen is low, as discussed above. Although the allergenicity assessment focuses on potential to be a food allergen, the data also indicate a low potential for Vip3Aa to be an inhalation allergen. Exposure via residential or lawn use to infants and children is also not expected because the use sites for Vip3Aa proteins are agricultural. Oral exposure, at very low levels, may occur from ingestion of processed products and, theoretically, drinking water. However oral toxicity testing showed no adverse effects.

C. Cumulative Effects

Pursuant to FFDCA section 408(b)(2)(D)(v), EPA has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity from exposure to Vip3Aa proteins, we conclude that there are no cumulative effects for the Vip3Aa proteins.

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

1) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for Vip3Aa proteins includes the characterization of representative Vip3Aa proteins, as well as the acute oral toxicity studies, amino acid sequence comparisons to known allergens and toxins, and in vitro digestibility of the representative Vip3Aa proteins. The results of these studies were used to evaluate human risk, and the validity, completeness, and reliability of the available data from the studies were also considered.

Adequate information was submitted to show that the Vip3Aa test materials derived from microbial cultures were biochemically and functionally equivalent to the proteins produced by the plant-incorporated protectant ingredient in the plants. Microbially produced proteins were used in the studies so that sufficient material for testing was available.

The acute oral toxicity data submitted for the representative Vip3Aa proteins support the prediction that Vip3Aa proteins will 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 et al., 1992). Since no treatment-related adverse effects were shown to be caused by the representative Vip3Aa proteins, even at relatively high dose levels, Vip3Aa proteins are not considered toxic. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing or residue data is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.2140). For microbial products, further toxicity testing (Tiers II and III) and residue data are triggered when significant adverse effects are seen in studies such as the acute oral toxicity study. Further studies verify the observed adverse effects and clarify the source of these effects.

Residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity. However, data submitted demonstrated low levels of the representative Vip3Aa proteins in corn and cotton tissues.

Since Vip3Aa are proteins, potential allergenicity is also considered as part of the toxicity assessment. Considering all of the available information, including that (1) Vip3Aa originates from a non-allergenic source; (2) Vip3Aa19 and Vip3Aa20 have no sequence similarities with known allergens; (3) Vip3Aa19 and Vip3Aa20 are not glycosylated; (4) Vip3Aa19 and Vip3Aa20 are rapidly digested in simulated gastric fluid; and (5) the data developed for Vip3Aa19 and Vip3Aa20 can be extrapolated to all Vip3Aa proteins due to the extremely high level of structural similarity that exists between and among Vip3Aa proteins, EPA has concluded that the potential for Vip3Aa to be an allergen is minimal.

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 representative Vip3Aa proteins, as well as the minimal potential to be a food allergen, demonstrates the safety of Vip3Aa at levels well above possible maximum exposure levels anticipated.

The genetic material necessary for the production of the plant-incorporated protectant active ingredient include the nucleic acids (DNA, RNA) that encode these proteins and regulatory regions. The genetic material (DNA, RNA), necessary for the production of Vip3Aa proteins has been exempted from the requirement of a tolerance under 40 CFR 174.507 (“Nucleic acids that are part of a plant-incorporated protectant”).

2) Infants and Children Risk Conclusions

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

In this instance, based on all the available information, the Agency concludes that there is a finding of no toxicity for Vip3Aa proteins. Thus, there are no threshold effects of concern and, as a result, the provision requiring an additional margin of safety does not apply. Further, the considerations of consumption patterns, special susceptibility, and cumulative effects do not apply.

3) 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 Vip3Aa proteins. 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 Vip3Aa proteins.

E. Other Considerations

1) Endocrine Disruptors

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

2) Analytical Method(s)

A validated lateral flow enzyme-linked immunosorbent assay (ELISA) protocol has been provided to the Agency for detecting Vip3Aa in cotton as well as a qualitative ELISA method for detecting Vip3Aa in corn.

3) Codex Maximum Residue Level

No Codex maximum residue level exists for the plant-incorporated protectant *Bacillus thuringiensis* Vip3Aa proteins and the genetic material necessary for their production in corn and cotton.

F. Tolerance Exemptions

The data submitted and reviewed for Vip3Aa support the petition for an exemption from the requirement of tolerance for *Bacillus thuringiensis* Vip3Aa proteins when used as plant–incorporated protectants in or on the food and feed commodities of corn and cotton.

G. Supporting Data

The human health studies submitted to support the safety of Vip3Aa are summarized in Table 7 below.

Table 7. Summary of Vip3Aa Human Health Data (reviewed in Edelstein 2008 unless otherwise noted)

Study Type/Title	Summary	MRID #
Summary of Mammalian Toxicology Data for the VIP3A and APH4 Proteins Produced by Transgenic VIP3A Cotton Event COT102[2]	No significant adverse effects were observed in male and female mice dose by gavage at approximately 3675 mg VIP3A/kg body weight (the highest dose tested) and the LD_{50} for pure VIP3A protein was >3675 mg/kg body weight. The LD_{50} for pure APH4 protein in male and female mice was >774 mg/kg body weight. The allergen database compiled by Syngenta needs to be better defined or described in order to ascertain the number and types of allergens searched for homology. **Classification: SUPPLEMENTAL.** **Note: this is a summary of multiple studies and is therefore superseded by the individual studies summarized below, which provide additional information, including the requested information on the SBI allergen database.**	45766502
Acute Oral Toxicity/ Acute Oral Toxicity of Vip3A Protein in Mice[2]	Eleven male and 11 female HSD:ICR albino mice were dosed with VIP3A protein (Lot no. VIP3A-0196 containing ~ 32% by weight VIP3A protein). The mice were quarantined for 5 days and fasted approximately 16 hours prior to dosing. The test material (5050 mg/kg body weight) was dosed as a 12.5 % w/v suspension in 2 % w/v carboxymethyl cellulose (CMC) in distilled water by gavage (Table 1). The dose volume was 40.4 mL/kg and was divided into 2 parts administered approximately one hour apart. The control group was treated with 2 % w/v CMC in the same manner as the test animals. Body weights were recorded prior to dosing, on days 7 and 14 or at death. The test animals were observed for clinical signs of toxicity at least three times post-dosing and at least daily thereafter for 14 days. All decedent or euthanized animals were necropsied. One control male (No. 17-M) was found dead on day 2. All other mice survived the study. With the exception of one female (No. 10-F) that failed to gain weight during the first week, all surviving animals gained weight during the study. In the	45766503

Study Type/Title	Summary	MRID #
	vehicle control group (*i.e.*, CMC treated), there was no affect on weight gain. The oral LD_{50} for males, females, and combined was greater than 5050 mg/kg (or > 1616 mg VIP3A protein/kg body weight). **Classification: SUPPLEMENTAL.** The VIP3A protein used in this study differs from the VIP3A protein present in COT102 cotton by a two amino acids, one at position 2 (aspartate replaces asparagine), another at position 284 (lysine replaces glutamine). **Note: this study provides additional support for the conclusion that Vip3Aa proteins are non-toxic to mammals.**	
Acute Oral Toxicity/ Single Dose Oral Toxicity Study with VIP3A-0199 in Mice[2]	Twenty-seven male and 27 female CD-1® (ICR)BR mice were dosed with VIP3A protein (Batch VIP3A-0199 containing ~ 54% by weight VIP3A protein), produced in an *E. coli* over-expression system. The VIP3A protein used as the test material differs from that present in cotton Event COT102 by a single amino acid; glutamine substituted for lysine (Q284K). The mice were quarantined for 16 days and fasted approximately 4 hours prior to dosing. The test material (5000 mg/kg body weight) was dosed as a suspension of 200 mg/mL in 0.5% w/v carboxymethyl cellulose (CMC) in deionized water by gavage (Table 1). The dose volume was 25 mL/kg. The control group was treated with 0.5 % w/v CMC in the same manner and volume as the test animals. Body weights were recorded prior to dosing, and on day 8 for animals designated to be sacrificed on day 15, and on each animals's respective day of necropsy (days 1, 2, or 15). The animals were observed for clinical signs of toxicity approximately 1, 2.5, 4, and 6 hours post dosing and at least daily until sacrifice. Animals were observed for any abnormal behavior, changes in posture or clonic / tonic movements. Mortality was observed twice daily. All animals were necropsied after sacrifice. The organ weight of the brain, kidneys, liver with drained gallbladder, and stomach were recorded and organ to body weight and organ to brain weight were calculated. Histopathology was performed on brain, gallbladder, heart, intestines (cecum, colon, duodenum, ileum, jejunum, and rectum), kidneys, lesions, liver, lung, and stomach. All animals survived prior to the scheduled sacrifice. All animals sacrificed on day 15 had normal body weight gains. All control and a few test animals sacrificed on day 1 and one male test and some control animals sacrificed on day 2 lost weight prior to sacrifice. No significant differences considered to be test material related in organ/body weight or organ/brain weight between control and test animals were found. The oral LD_{50} for males, females, and combined was greater than 5000 mg/kg (or > 2700 mg VIP3A protein/kg body weight). **Classification: SUPPLEMENTAL -** The test material for this study, VIP3A-0199, differs in sequence by one amino acid (Q284K) from that form of the protein which is present in COT102. **Note: this study provides additional support for the conclusion that Vip3Aa proteins are non-toxic to mammals.**	45766504
Acute Oral Toxicity/ Acute Oral Toxicity	The test animals (Sixteen male and 16 female Crl-1® (ICR)BR mice) were quarantined for 9 days and fasted approximately 4 hours prior to dosing.	45766505

Study Type/Title	Summary	MRID #
Study with Test Substance VIP3A-0100 Protein in Mice[2]	The test material (5000 mg/kg body weight) was dosed as a suspension of 196 mg/mL in 0.5% w/v carboxymethyl cellulose (CMC) in deionized water by gavage. The dose volume was 25.5 mL/kg. The control group was treated with 0.5% w/v CMC in the same manner as the test animals. Body weights were recorded prior to dosing and on days 8 and 15 for animals designated to be sacrificed on day 15. The animals were observed for clinical signs of toxicity approximately 1, 2.5, 4, and 6 hours post dosing and at least daily until sacrifice. Mortality was observed twice daily. All animals were necropsied after sacrifice. The organ weight of the brain, kidneys, liver with drained gallbladder, and stomach were recorded and organ to body weight and organ to brain weight were calculated. Histopathology was performed on brain, gallbladder, heart, intestines (cecum, colon, duodenum, ileum, jejunum, and rectum), kidneys, lesions, liver, lung, and stomach. All animals sacrificed on day 15 had normal body weight gains. No test material related macroscopic alterations were noted. In addition, no significant differences related to the test material in organ/body weight or organ/brain weight between control and test animals were found. The oral LD_{50} for males, females, and combined was greater than 5000 mg/kg (or > 3675 mg VIP3A protein/kg body weight). **Classification: Acceptable**	
Acute Oral Toxicity/ Single Dose Oral Toxicity Study with VIP3A-Enriched Maize (Corn) Leaf Protein (LPPACHA-0199) in Mice[2]	VIP3A-Enriched Maize (Corn) Leaf Protein (Sample Lot. No. LPPACHA-0199 containing ~ 0.36% by weight VIP3A protein) was prepared from transgenic VIP3A maize (corn) leaves. The mice were quarantined for at least 7 days and fasted approximately 4 hours prior to dosing. The test material (5000 mg/kg body weight) was dosed as a suspension of 250 mg/mL in 0.5% w/v carboxymethyl cellulose (CMC) in deionized water by gavage (Table 1). The dose volume was 20 mL/kg. The control group was treated with Control Maize (Corn) Leaf Protein, Batch LPPACHA-0199C in 0.5% w/v CMC in deionized water at a concentration of 250 mg/mL in the same manner as the test animals. Body weights were recorded prior to dosing, and on days 7, 14, or at death. The test animals were observed for clinical signs of toxicity at least three times post-dosing and at least daily thereafter for 14 days. All decedent or euthanized animals were necropsied. All mice survived the study, gained weight and appeared normal during the study. The oral LD_{50} for males, females, and combined was greater than 18 mg/kg VIP3A protein/kg body weight. The net concentration of VIP3A (18 mg / kg body weight) is significantly lower than the prescribed 2000 to 5000 mg / kg body weight suggested in the guideline requirements. At this concentration and with the mix of other proteins present in the leaf preparation, no toxicity was evident in the test animals. **Classification: SUPPLEMENTAL.** Information is supportive, but not part of guideline requirements; no further information required. **Note: this study provides additional support for the conclusion that Vip3Aa proteins are non-toxic to mammals.**	45766506
In Vitro Digestibility of VIP3A Protein Under	VIP3A from recombinant maize (field corn) plants was prepared as sample LPPACHA-0199 by extracting the leaves of recombinant corn plants and	45835805

Study Type/Title	Summary	MRID #
Simulated Mammalian Gastric Conditions [2]	concentrating the VIP3A by ammonium sulfate precipitation, dialysis of the resulting salt, and lyophilization of the collected protein. ELISA showed VIP3A constituted ~0.36 % by weight of the sample and retained insecticidal activity against sensitive lepidopteran species. VIP3A from *E. coli* was prepared as sample VIP3A-0100 in an *E. coli* strain BL21DE3pLysS over-expression system. The synthetic *vip3A(a)* gene was cloned into the inducible over-expression pET-3a® vector. Following collection, purification, dialysis, and lyophilization, the sample was estimated by ELISA to contain ~73.5% VIP3A by weight and it retained its insecticidal activity against sensitive lepidopteran species. The reactions were initiated by the addition of 80 µL of LPPACHA-0199 or VIP3A-0100 to 320 µL of simulated gastric fluid containing pepsin incubated at 37°C. Immediately after sample addition, an aliquot was removed and quenched with an equal volume of Laemmli buffer (pH not reported) and inactivated at >75°C for 10 minutes. Additional aliquots were removed and treated as above following 2, 5, 10, 20, 30, and 60 minutes of incubation. Digestion of the protein samples was evaluated using SDS-PAGE and Western blotting. The digestion of VIP3A protein in a simulated gastric environment proceeds at a rapid rate and demonstrates the lability of this protein to conditions typical of a monogastric mammalian stomach. The presence of a small amount of immunoreactive protein (approximately 6 to 9 kD) indicates that a portion or domain of the protein is less readily digested in this environment, although these bands do degrade beyond the point of immunorecognition with time. Results of this study indicate VIP3A protein, whether isolated from recombinant corn plants or from genetically modified *E. coli*, will be rapidly digested in a simulated gastric environment. **Classification: ACCEPTABLE**	
Amino acid sequence comparison/ Vip3Aa19: Assessment of amino acid sequence homology with known toxins. Report No. SSB-122-06[4]	The purpose of this study was to determine if Vip3Aa19 had any significant amino acid sequence homology to known protein toxins. No relevant similarities between the Vip3Aa19 query sequence and known protein toxins were found other than with other insect-specific vegetative insecticidal proteins of *B. thuringiensis*. **Classification: Acceptable; Supersedes MRID 457665-02**	46885903
Amino acid sequence comparison/ Vip3Aa19: Assessment of amino acid sequence homology with known allergens. Report No. SSB-130-06[4]	The purpose of this study was to determine if Vip3Aa19 had any significant amino acid sequence homology to known protein allergens. Vip3Aa19 had no significant amino acid sequence homology to known or putative allergenic proteins. **Classification: Acceptable; Supersedes MRID 457665-02**	46885906
Amino acid sequence comparison/ Vip3A as	The purpose of the study was to determine if Event MIR162 Vip3A protein had any significant amino acid sequence homology to known or putative	46864808

Study Type/Title	Summary	MRID #
expressed in Event MIR162 maize: Assessment of amino acid sequence homology with known toxins[3]	protein toxins. The database identified 32 entries with E values below 6 x 10^{-6}, of which 30 were vegetative insecticidal proteins of *B. thuringiensis* and had E values of 0.0 to 1 x 10^{-10}. Two proteins were identified as rhoptry proteins from *Plasmodium yoelii*, a pathogen that causes malaria in rodents *via* erythrocyte binding and invasion (Ogun and Holder, 1996). Despite the pathogenic nature of *P. yoelii*, the low overall sequence similarity between MIR162 Vip3A and the rhoptry proteins (3.9 or 11.4% overall amino acid sequence identity) suggests that the E values are of no biological significance (Doolittle, 1990). Furthermore, a global protein alignment (Myers and Miller, 1988) demonstrates that there are no more than three contiguous identical amino acids between Vip3A and the rhoptry proteins. Therefore, no relevant similarities between the Event MIR162 Vip3A query sequence and known protein toxins were found. **Classification: ACCEPTABLE**	
Amino acid sequence comparison/ Vip3A as expressed in Event MIR162 maize: Assessment of amino acid sequence homology with known allergens[3]	The purpose of this study was to determine if Event MIR162 Vip3Aa20 had any significant amino acid sequence homology to known or putative protein allergens. No significant sequence homology was found between any sequential MIR162 Vip3A 80-amino acid peptides and any entry in the SBI Allergen Database. No alignments of eight or more contiguous identical amino acids were identified between MIR162 Vip3A and proteins in the SBI Allergen Database. Therefore, no significant amino acid sequence homology was found between the MIR162 Vip3A and any known or putative protein allergens. **Classification: ACCEPTABLE**	46864809
Analysis of Vip3A or Vip3A-Like Proteins in Six Different Commercial Microbial *Bacillus thuringiensis* Products	The purpose of this study was to determine whether Vip3A or Vip3A-like proteins are detectable and quantifiable in commercial formulations of *Bacillus thuringiensis* (Bt)-based microbial insecticide products. ELISA (enzyme-linked immunosorbent assay) and Western blot analyses were used to detect and analyze Vip3A or Vip3A-like proteins in the formulations. Vip3A or Vip3A-like proteins were detected in all six commercial products, with concentrations ranging from a low of *ca*. 2.0 µg/g product to a high of *ca*. 209 µg/g. Those products showing the highest protein concentrations were all derived from the *kurstaki* subspecies of *B. thuringiensis*. **Classification: ACCEPTABLE**	47017613
Amino acid sequence comparison/ Vip3Aa19: Assessment of amino acid sequence homology with known allergens	Two amino acid sequences comparisons of Vip3Aa19 with known allergens were conducted using the Syngenta Biotechnology, Inc (SBI) Allergen Database. The results indicate that Vip3Aa19 has no significant amino acid sequence homology to known or putative allergenic proteins based on a search for greater than 35% sequence identity over successive 80-amino acid peptides and a search for eight or more contiguous identical amino acids. **Classification: ACCEPTABLE**	47017617

II.B.2. Human Health Assessment of Modified Cry1Ab Containing 26 Additional Amino Acids

A. Mammalian Toxicity and Allergenicity Assessment

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

Syngenta has submitted acute oral toxicity data demonstrating the lack of mammalian toxicity at high levels of exposure to the pure modified Cry1Ab protein containing the additional 26 amino acid 'Geiser motif'. The 26 amino acid sequence is found at the C-terminus of the pro-toxin portion of the modified Cry1Ab protein. The pro-toxin is enzymatically cleaved in the insect gut to produce active Cry1Ab. These toxicity data demonstrate the safety of the product at a level well above maximum possible exposure levels that are reasonably anticipated in the crop. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing and residue data is similar to the Agency position regarding toxicity testing and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.2140). For microbial products, further toxicity testing (Tiers II & III) and residue data are triggered by significant adverse acute effects in studies such as the acute oral toxicity study, to verify the observed adverse effects and clarify the source of these effects.

An acute oral toxicity study in mice indicated that modified Cry1Ab is non-toxic to humans. Groups of five male and five female mice were given 0 or 1830 mg/kg bodyweight microbially-produced modified Cry1Ab by oral gavage as a single dose. There were no effects on clinical condition, body weight, food consumption, clinical pathology, organ weight, or macroscopic or microscopic pathology that were attributed to the test substance.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad et al., 1992). Therefore, since no acute effects were shown to be caused by modified Cry1Ab, even at relatively high dose levels, the modified Cry1Ab protein is not considered toxic.

Since modified Cry1Ab is a protein, allergenic potential was also considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a weight-of-evidence approach where the following factors are considered: source of the trait; amino acid sequence comparison with known allergens; and biochemical properties of the protein, including in vitro digestibility in simulated gastric fluid (SGF) and glycosylation. This approach is consistent with the approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." The allergenicity assessment for modified Cry1Ab follows:

1. Source of the trait. *Bacillus thuringiensis* is not considered to be a source of allergenic proteins.
2. Amino acid sequence. A comparison of the amino acid sequence of modified Cry1Ab with known allergens showed no significant sequence identity over 80 amino acids or identity at the level of eight contiguous amino acid residues.
3. Digestibility. Modified Cry1Ab was rapidly digested in simulated gastric fluid containing pepsin.
4. Glycosylation. Modified Cry1Ab expressed in cotton was shown not to be glycosylated.
5. Conclusion. Considering all of the available information, EPA has concluded that the potential for modified Cry1Ab to be a food allergen is minimal.

Although modified Cry1Ab was only shown not to be glycosylated in cotton, it is unlikely to be glycosylated in any other crops because in order for a protein to be glycoslyated, it needs to contain specific recognition sites for the enzymes involved in glycosylation, and the mechanisms of protein glycosylation are similar in different plants (Lerouge et al., 1998).

B. Aggregate Exposures

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

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for 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 protectant is contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. In addition, even if exposure can occur through inhalation, the potential for modified Cry1Ab to be an allergen is low, as discussed above. Although the allergenicity assessment focuses on potential to be a food allergen, the data also indicate a low potential for modified Cry1Ab to be an inhalation allergen. Exposure via residential or lawn use to infants and children is also not expected because the use sites for the modified Cry1Ab protein is agricultural. Dietary exposure may occur from ingestion of processed cotton products but is expected to be very low because the already low expression levels in the seed and would be reduced further by the heat and pressure used for processing. Also, dietary exposure may theoretically occur through exposure in drinking water because plant stubble may release modified Cry1Ab protein into ground water upon decay. This protein would not be expected to survive in the soil due to microbial degradation, adherence to soil components and removal upon drinking water treatment procedures. In addition, oral toxicity testing showed no adverse effects.

C. Cumulative Effects

Pursuant to FFDCA section 408(b)(2)(D)(v), EPA has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity from the plant-incorporated protectant, EPA concludes that there are no cumulative effects for the modified Cry1Ab protein.

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

1) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the modified Cry1Ab protein includes the characterization of the expressed modified Cry1Ab protein in cotton, as well as the acute oral toxicity study, amino acid sequence comparisons to known allergens, and in vitro digestibility of the protein. The results of these studies were used to evaluate human risk, and the validity, completeness, and reliability of the available data from the studies were also considered.

Adequate information was submitted to show that the modified Cry1Ab test material derived from microbial culture was biochemically and functionally equivalent to the protein in the plant. Microbially produced protein was used in the safety studies so that sufficient material for testing was available.

The acute oral toxicity data submitted support the prediction that the modified Cry1Ab protein would be non-toxic to humans. As mentioned above, when proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad et al., 1992). Since no treatment-related adverse effects were shown to be caused by the Cry1Ab protein, even at relatively high dose levels, the modified Cry1Ab protein is not considered toxic. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing and residue data is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.2140). For microbial products, further toxicity testing and residue data are triggered when significant adverse effects are seen in studies such as the acute oral toxicity study. Further studies verify the observed adverse effects and clarify the source of these effects.

Residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity. However, data submitted demonstrated low levels of the modified Cry1Ab protein in cotton tissues.

Since Cry1Ab is a protein, potential allergenicity is also considered as part of the toxicity assessment. Considering all of the available information (1) modified Cry1Ab originates from a non-allergenic source; (2) modified Cry1Ab has no sequence similarities with known allergens; (3) modified Cry1Ab is not glycosylated; and (4) modified Cry1Ab is rapidly digested in simulated gastric fluid; EPA has concluded that the potential for modified Cry1Ab to be an allergen is minimal.

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 modified Cry1Ab protein, as well as the minimal potential to be an allergen, demonstrate the safety of the product at levels well above possible maximum exposure levels anticipated.

The genetic material necessary for the production of the plant-incorporated protectant active ingredient include the nucleic acids (DNA, RNA) that encode these proteins and regulatory regions. The genetic material (DNA, RNA) necessary for the production of the modified Cry1Ab protein has been exempted from the requirement of a tolerance under 40 CFR 174.507 ("Nucleic acids that are part of a plant-incorporated protectant").

2) Infants and Children Risk Conclusions

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

In this instance, based on all the available information, the Agency concludes that there is a finding of no toxicity for the modified Cry1Ab protein. Thus, there are no threshold effects of concern and, as a result, the provision requiring an additional margin of safety does not apply. Further, the considerations of consumption patterns, special susceptibility, and cumulative effects do not apply.

3) 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 modified Cry1Ab protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because, as

discussed above, no toxicity to mammals has been observed, nor any indication of allergenicity potential for the plant-incorporated protectant.

E. Other Considerations

1) Endocrine Disruptors

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

2) Analytical Method(s)

A lateral flow enzyme-linked immunosorbent assay (ELISA) protocol has been provided to the Agency for detecting modified Cry1Ab in cotton. This analytical method will be independently validated as a condition of registration for cotton product(s) containing modified Cry1Ab.

3) Codex Maximum Residue Level

No Codex maximum residue level exists for the plant-incorporated protectant *Bacillus thuringiensis* modified Cry1Ab protein.

F. Tolerance Exemptions

The data submitted and reviewed for modified Cry1Ab support the petition for an exemption from the requirement of tolerance for *Bacillus thuringiensis* modified Cry1Ab protein containing the additional 26 amino acid sequence when used as a plant-incorporated protectant in or on the food and feed commodities of cotton.

G. Supporting Data

The human health studies submitted to support the safety of modified Cry1Ab are summarized in Table 8 below.

Table 8. Summary of Modified Cry1Ab Human Health Data (reviewed in Edelstein 2008 unless otherwise noted)

Study Type/Title	Summary	MRID #
Acute oral toxicity (OPPTS 870.1100)/ FLCRY1AB-0103: Single Dose Oral	Groups of five male and five female mice were given 0 or 1830 mg/kg bodyweight microbially-produced modified Cry1Ab (FLCRY1AB-0103) by oral gavage as a single dose. There were no effects on clinical condition, body weight, food consumption, clinical pathology, organ weight, or	47017614

Toxicity Study in the Mouse (AM7516/Regulatory/Report)	macroscopic or microscopic pathology that were attributed to the test substance. **Classification: ACCEPTABLE**	
In vitro digestibility/ *In vitro* digestibility of full-length Cry1Ab protein (test substances FLCRY1AB-0103 and IAPCOT67B-0106) under simulated mammalian gastric conditions	The *in vitro* digestibility in simulated gastric fluid of the modified Cry1Ab protein as expressed in COT67B and from a bacterial source was investigated. No intact full-length modified Cry1Ab protein from bacterial- or plant-derived sources was found one minute after incubation in simulated gastric fluid. An immunoreactive polypeptide fragment (~ 60,000 Da) in the digestion mixture was visible in the 5 minute sample in the plant-derived source and in the 10 minute sample in the bacterial-derived source. The study results indicate that the full-length Cry1Ab protein is rapidly digested in simulated gastric fluid; a 60 kDa fragment is formed, which also appears to be digestible, but at a slower rate. **Classification: ACCEPTABLE**	47017615
Heat stability/ Effect of temperature on the stability of full-length Cry1Ab protein	The effect of temperature on the bioactivity of modified Cry1Ab was investigated. Heating of *E. coli*-derived modified Cry1Ab (FLCRY1AB-0103) at 65˚C or 95˚C for 30 minutes substantially decreased or eliminated the insecticidal activity of the protein. No significant effect on the protein's insecticidal properties was found following incubation for 30 minutes at temperatures ≤37˚C. **Classification: ACCEPTABLE**	47017616
Amino acid sequence comparison/ Full-length Cry1Ab as expressed in Event COT67B: Assessment of amino acid sequence homology with known allergens	Two amino acid sequences comparisons of modified Cry1Ab with known allergens were conducted using the Syngenta Biotechnology, Inc (SBI) Allergen Database. The results indicate that modified Cry1Ab has no significant amino acid sequence homology to known or putative allergenic proteins based on a search for greater than 35% sequence identity over successive 80-amino acid peptides and a search for eight or more identical contiguous amino acids. **Classification: ACCEPTABLE**	47017619

II.B.3. Human Health Assessment of Hygromycin B Phosphtransferase (APH4)

The hygromycin B phosphotransferase (APH4) protein expressed in COT102 x COT67B is covered by the exemption from the requirement of a tolerance at 40 CFR 174.526 Hygromycin B phosphtransferase (APH4) marker protein in all plants; exemption from the requirement of a tolerance.

Summary of new data submitted for APH4

MRID# 47017618—APH4 (Entrez Database accession No. CAA85741): Assessment of Amino Acid Sequence Homology with Known Allergens:
Two amino acid sequences comparisons of APH4 with known allergens were conducted using the Syngenta Biotechnology, Inc (SBI) Allergen Database. The results indicate that APH4 has no

significant amino acid sequence homology to known or putative allergenic proteins based on a search for greater than 35% sequence identity over successive 80-amino acid peptides and a search for eight or more contiguous amino acids.
Classification: ACCEPTABLE

II.B.4. References

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Waggoner, A., 2007. Memorandum from A. Waggoner to M. Mendelsohn dated February 8, 2007.

Wozniak, C. 2004. EPA Review of the product characterization, environmental fate and human health toxicity studies for the insecticidal protein VIP3A, as expressed in upland cotton, for control of lepidopteran insect pests, and the selective marker protein APH4. Memorandum from C.

Wozniak to L. Cole dated March 24, 2004.

II. C. Environmental Hazard Assessment

Note: EPA's environmental assessment was conducted for Vip3Aa proteins, which include the Vip3Aa19 protein as expressed in cotton. "Full-length Cry1Ab" (FLCry1Ab) refers to the modified Cry1Ab (OECD Unique Identifier SYN-IR67B-1) protein in VipCot.

Background

Vip3A is a novel class of recently discovered insecticidal proteins that occur naturally in *Bacillus thuringiensis* (*Bt*), a gram-positive soil bacterium (Estruch, *et al.* 1996). The vegetative insecticidal proteins are produced during vegetative bacterial growth and are secreted as soluble proteins into the extracellular environment. Syngenta Seeds, Inc. has developed Event COT102, a cotton line that expresses an insect control protein, known as Vip3Aa. In addition, Syngenta Seeds, Inc. has also developed Event COT67B, a cotton line that expresses a *Bt* insect control Cry protein, known as full-length Cry1Ab. These proteins are intended to control several lepidopteran pests of cotton including: *Helicoverpa zea* (cotton bollworm), *Heliothis virescens* (tobacco budworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), and *Trichoplusia ni* (cabbage looper).

Syngenta Seeds, Inc. is requesting a Sec. 3 registration for *Bt* insect control protein Vip3Aa as expressed in Event COT102 cotton, full-length Cry1Ab (hereafter, referred to as FLCry1Ab) as expressed in Event COT67B cotton, and its associated breeding stack, COT102 x COT67B [also known as VipCot, EPA Reg. No. 67979-O] cotton (which combines Vip3Aa and FLCry1Ab proteins), crossed via traditional breeding. An experimental use permit (EUP) was granted by the Agency to conduct field tests on Event COT102, Event COT67B, and its associated breeding stack COT102 x COT67B (Matten, 2007).

Event COT102 cotton specifically expresses Vip3Aa19[e], a variant of the naturally occurring Vip3Aa1 protein isolated from *Bacillus thuringiensis* strain AB88, differing from the Vip3Aa1 protein by one amino acid. The same protein variant present in Event COT102 cotton is also expressed as Vip3Aa19 in Syngenta's experimental Event Pacha corn. The Agency previously determined that "all proteins designated as Vip3Aa are more than 95% identical," and "there is sufficient information to support the safety of all Vip3Aa proteins, provided that they do not have any significant sequence similarity with known allergens" (Edelstein, 2008). Therefore, in addition to the data reviewed in this report, all the previously submitted data developed for Vip3Aa protein can be cited in support of the registration of Event COT102.

Although Vip3Aa protein shares no homology with FLCry1Ab or other known Cry proteins, extensive testing by Syngenta has established that Vip3Aa has demonstrated a similar toxicity

[e] Prior to receiving the Crickmore designation of Vip3Aa19, the protein produced in Events COT102 and Pacha were referred to as VIP3A, Vip3A or Vip3Aa.

against larvae of certain lepidopteran species, including key pests of cotton. While the modes of action differ between the two proteins, the general symptoms displayed by sensitive lepidopteran larvae following ingestion of Vip proteins resembles that caused by Cry proteins (i.e., cessation of feeding, loss of gut peristalsis, overall paralysis of the insect, and death) (Yu, *et al*, 1997). Since the effects of Vip and Cry proteins are considered similar, the studies submitted on non-target organisms for Event COT102 were conducted and evaluated according to the same environmental risk assessment criteria of previously reviewed PIP products containing Cry protein.

FLCry1Ab protein expressed in COT67B cotton and native Cry1Ab protein are both derived from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 (*B.t.k.*). FLCry1Ab differs from the naturally occurring Cry1Ab protein in that FLCry1Ab contains 26 additional consecutive amino acids (described as the 'Geiser motif") in the C-terminal portion (Geiser *et al*., 1986). The 'Geiser motif' is also expressed in another registered PIP cotton product containing Cry1Ac. FLCry1Ab protein in Event COT67B is also similar to the truncated protein variants of Cry1Ab as expressed in transgenic maize. The Agency previously determined that Syngenta's Event Bt11 corn produces a truncated Cry1Ab protein that has the same insecticidal active region of amino acids as FLCry1Ab produced in COT67B cotton (Matten, 2007). In addition, there are numerous laboratory studies, field studies, and scientific literature on the mode of action of Cry1Ab protein, Cry1Ab-expressing maize and Cry1Ac-expressing cotton (US EPA, 2001b; Naranjo *et al*., 2005; Romeis *et al*., 2006; Cattaneo *et al*., 2006; and Torres and Ruberson, 2007). These data provide a large weight-of-evidence that these proteins demonstrate very similar insecticidal activity against several lepidopteran cotton pests at concentrations found in transgenic plants. Furthermore, the Agency also determined that field efficacy data submitted with the registration application (MRID No. 470176-33) and reports provided with the Public Interest Document (MRID No. 470176-35) demonstrate a similar insecticidal spectrum of the truncated and full-length Cry1Ab proteins (Martinez, 2008). Therefore, the effects of truncated Cry1Ab proteins are considered predictive of the effects of FLCry1Ab protein as expressed in COT67B cotton to non-target organisms for the purposes of the environmental risk assessment.

The Agency has conducted an environmental risk assessment of COT102 and COT67B cotton lines expressing Vip3Aa and FLCry1Ab proteins. The general topics covered include gene flow to related wild plants, potential of weediness, effects on wildlife, and fate of Vip3Aa and Cry1Ab proteins in the environment. The assessment is based on data submitted to the Agency during the development of the cotton lines, additional data submitted for registration, Federal Insecticide Fungicide and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) recommendations, consultations with scientific experts, and public comments on Plant-Incorporated Protectant (PIP) regulation.

II. C. 1. Environmental Risk Assessment for COT102 and COT67B (lepidopteran active)

A. Tiered Testing and Risk Assessment Process

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

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

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

Testing methods which utilize the tiered approach were last published by the EPA as Harmonized OPPTS Testing Guidelines, Series 850 and 885 (EPA 712-C-96-280, February 1996)[g]. These

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

[g] OPPTS Testing Guidelines, Series 850 and 885 website:
http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/885Microbial_Pesticide_Test_Guidelines/Series

guidelines, as defined in 40 CFR 152.20, apply to microbes and microbial toxins when used as pesticides, including those that are naturally occurring, and those that are strain-improved, either by natural selection or by deliberate genetic manipulation. Therefore, PIPs containing microbial toxins are also covered by these testing guidelines.

The Tier I screening maximum hazard dose (MHD) approach to environmental hazard assessment is based on some factor (whenever possible >10) times the maximum amount of active ingredient expected to be available to terrestrial and aquatic non-target organisms in the environment (EEC)[h]. Tier I tests serve to identify potential hazards and are conducted in the laboratory at high dose levels which increase the statistical power to test the hypotheses. Elevated test doses, therefore, add certainty to the assessment, and such tests can be well standardized. The Guidelines call for initial screening testing of a single group or several groups of test animals at the maximum hazard dose level. The Guidelines call for testing of one treatment group of at least 30 animals or three groups of 10 test animals at the screening test concentration. The Guidelines further state that the duration of all Tier I tests should be approximately 30 days. Some test species, notably non-target insects, may be difficult to culture and the suggested test duration has been adjusted accordingly. Control and treated insects should be observed for at least 30 days, or in cases where an insect species cannot be cultured for 30 days, until negative control mortality rises above 20 percent.

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

When screening tests indicate a need for additional data, the OPPTS Harmonized Guidelines call for testing at incrementally lower doses in order to establish a definitive LD_{50} and to quantify the hazard. In the definitive testing, the number of doses and test organisms evaluated must be sufficient to

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

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

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

determine an LD_{50} value and, when necessary, the Lowest Observed Effect Concentration (LOEC), No Observed Adverse Effect Level (NOAEL) , or reproductive and behavioral effects such as feeding inhibition, weight loss, etc. In the final analysis, a risk assessment is made by comparing the LOAEC to the EEC; when the EEC is lower than the LOAEC, a no risk conclusion is made. These tests offer greater environmental realism, but they may have lower statistical power. Appropriate statistical methods, and appropriate statistical power, must be employed to evaluate the data from the definitive tests. Higher levels of replication, the number of test species, and/or repetition are needed to enhance statistical power in these circumstances.

Data that shows less than 50 % mortality at the maximum hazard dosage level – (i.e. LC_{50}, ED_{50}, or LD_{50} >10 X EEC) is sufficient to evaluate adverse effects, making lower field exposure dose definitive testing unnecessary. It is also notable that the recommended >10X EEC maximum hazard dose level is a highly conservative factor. The published EPA Level of Concern [LOC] is 50% mortality at 5X EEC [k] (US EPA, 1998).
Validation: The tiered hazard assessment approach was developed for the EPA by the American Institute of Biological Sciences (AIBS) and confirmed in 1996 as an acceptable method of environmental hazard assessment by a FIFRA Scientific Advisory Panel (SAP) on microbial pesticides and microbial toxins. The December 9, 1999 SAP agreed that the Tiered approach was suitable for use with Plant-Incorporated Protectants (PIPs); however, this panel recommended that, for PIPs with insecticidal properties, additional testing of beneficial invertebrates closely related to target species and/or likely to be present in GM crop fields should be conducted. Testing of *Bt* Cry proteins on species not closely related to the target insect pest was not recommended, although it is still performed to fulfill the published EPA non-target species data requirements. In October 2000, another SAP also recommended that field testing should be used to evaluate population-level effects on non-target organisms. The August 2002 SAP, and some public comments, generally agreed with this approach, with the additional recommendation that indicator organisms should be selected on the basis of potential for field exposure to the subject protein (US EPA, 2000, 2001a, 2002, and 2004).

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

[k] The established peer and EPA Science Board reviewed guidance on screening test levels of concern is 50% mortality at 5X environmental concentration. The appropriate endpoints in high dose limit/screening testing are based on mortality of the treated, as compared to the untreated (control) non-target organisms. A single group of 30 test animals may be tested at the maximum hazard dose.

Conclusion: The tiered approach to test guidelines ensures, to the greatest extent possible, that the Agency requires the minimum amount of data needed to make scientifically sound regulatory decisions. The EPA believes that maximum hazard dose Tier I screening testing presents a reasonable approach for evaluating hazards related to the use of biological pesticides and for identifying negative results with a high degree of confidence. The Agency expects that Tier 1 testing for short-term hazard assessment will be sufficient for most studies submitted in support of PIP registrations. However, if long range adverse effects must be ascertained, then higher-tier longer-term field testing will be required As noted above, the October 2000 SAP and the National Academy of Sciences (NAS, 2000) recommended testing non-target organisms directly in the field. This approach, with an emphasis on testing invertebrates found in corn fields, was also recommended by the August 2002 SAP and was supported by several public comments. Based on these recommendations, the Agency has required field studies on long term invertebrate population/community and Cry protein accumulation in soils as a condition of registration due to the lack of baseline data on the potential for long-term environmental effects from the cultivation of PIP-producing plants.

Since the commercialization of *Bt* crops, the number of field studies published in scientific literature in combination with the post-registration field studies submitted to the Agency has accumulated to a level where empirical conclusions can be made. As a result, the issue of long range effects of cultivation of these Cry proteins on the invertebrate community structure in *Bt* crop fields has since been adequately addressed. Specifically, a meta-analysis[1] of the data collected from 42 field studies indicated that non-target invertebrates are generally more abundant in *Bt* cotton and *Bt* maize fields than in non-transgenic fields managed with insecticides (Marvier, *et al*., 2007). In addition, a comprehensive review of short and long term field studies on the effects of invertebrate populations in *Bt* corn and cotton fields indicated that no unreasonable adverse effects are taking place as a result of wide scale *Bt* crop cultivation (Sanvido, *et al.* 2007). Another review of field tests published to date concluded that the large-scale studies in commercial *Bt* cotton have not revealed any unexpected non-target effects other than subtle shifts in the arthropod community caused by the effective control of the target pests (Romeis *et al*., 2006). Slight reductions in some invertebrate predator populations are an inevitable result of all pest management practices, which result in reductions in the abundance of the pests as prey.

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

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

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

B. Environmental Exposure Assessment

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

For the purposes of the non-target organism (NTO) studies submitted in support of Event COT102 and Event COT67B, the test material dose levels were based on the estimated concentration of Vip3Aa and full-length Cry1Ab protein expressed in the tissue(s) that NTO would most likely be exposed to in the environment (see Matten, 2007; Edelstein, 2008 for protein expression levels). The Agency has determined that the NTOs most likely to be exposed to the Vip3Aa and FLCry1Ab protein in transgenic cotton fields were beneficial insects feeding on cotton pollen. Consequently, test material dose levels were based on the maximum level of measured protein expression in pollen (3.47 ug/g dwt for Vip3Aa and 12.1 ug/g dwt for Cry1Ab). The principal route of Vip3Aa and full-length Cry1Ab protein exposure for soil-dwelling organisms (such as collembola, earthworms, and/or rove beetles) is assumed to be from decomposing plant tissue and plant exudates in soil. Consequently, the dose levels of the test material were based on the maximum level of estimated protein expression in the soil environment.

C. Non-Target Wildlife Hazard Assessments for Event COT102 and Event COT67B

Two separate SAP reports (October 2000 and August 2002) recommended that non-target testing of *Bt* Cry proteins should focus on invertebrate species exposed to the crop being registered. Following SAP recommendations, the EPA determined that non-target organisms with the greatest exposure potential to Cry protein in transgenic corn fields are beneficial insects, which feed on corn pollen and nectar, and soil invertebrates, particularly Lepidoptera species. The Agency recommended using this same approach for testing the effects of Vip protein in Event COT102 and Cry protein in Event COT67B on beneficial insects in transgenic cotton fields. Therefore, toxicity testing using the maximum hazard dose on representative beneficial organisms from several taxa was performed in support of both Section 3 FIFRA cotton registrations. The toxicity of the Vip3Aa and Cry1Ab have been evaluated on several species of invertebrates including the lady beetle, minute pirate bug,

collembola, daphnia, honey bee, rove beetle, and/or earthworm. Reproductive and developmental observations were also examined in the lady beetle, rove beetle, minute pirate bug, and honeybee studies.

As previously noted, Vip3Aa protein in Event COT102 and Cry1Ab protein in Event COT67B are very host specific, conferring toxic effects on cotton bollworm, tobacco budworm, fall armyworm, beet armyworm, and cabbage looper. Despite the October 2000 and August 2002 SAP's recommendations against testing of non-target species not related to susceptible target pests, EPA has completed a risk assessment on a range of non-target wildlife to comply with the Agency's published non-target data requirements. In the absence of PIP-specific risk assessment guidance, EPA requires applicants for PIP registrations to meet the 40 CFR Part 158 data requirements for microbial toxins. These requirements include birds, mammals, plants, and aquatic species. In addition, earthworm, springtail, and/or rove beetle studies were voluntarily submitted to the Agency to ascertain the potential effects of Vip3Aa and FLCry1Ab proteins on beneficial decomposer species.

The October 2000 SAP recommended that while actual plant material is the preferred test material, bacterial-derived protein is also a valid test substance, particularly in scenarios where test animals do not normally consume cotton plant tissue and where large amounts of Cry protein (Cry protein concentrations that exceed levels present in plant tissue) are needed for maximum hazard dose testing. For Event COT102, an insect feeding study, which compared the relative potency of plant-derived Vip3Aa protein in both Event COT102 cotton and Event Pacha corn to the microbial-derived proteins, indicated that plant-derived protein was similar in toxicity to the microbial-derived protein (MRID No. 458358-12 and Edelstein, 2008). Similarly, for Event COT67B, an insect feeding study, which compared the relative potency of plant-derived FLCry1Ab protein in COT67B cotton to the microbial-derived protein, indicated that plant-derived protein was similar in toxicity to the microbial-derived protein (MRID No. 470176-08 and Edelstein, 2008). Therefore, these data indicate that the microbial-derived proteins for each event are substantially equivalent to the plant-derived proteins expressed in cotton plants based on the similar insecticidal activity for studying any potential toxicity on NTOs for the purposes of the environmental risk assessment.

The Agency has also determined that toxicity studies using corn-derived plant material rather than cotton-derived plant material is acceptable because cotton contains gossypol and other possible plant toxicants that may adversely affect non-target organisms. Furthermore, the non-target species in the cotton agroecosystem are comparable to those in corn; Specifically for Vip3Aa protein toxicity tests, Event COT102 cotton expresses the same *vip3A(a)* gene as is expressed in Event Pacha corn, and the expression level of pollen of Event Pacha corn is much higher than that of Event COT102 cotton.

In support of the COT102 registration, test substances used in the submitted studies included bacterial-produced purified Vip3Aa19 and Vip3Aa1 protein, in addition to Vip3Aa19 as expressed in COT102 cotton pollen and Event Pacha maize grain, pollen, and leaves. Likewise, in support of

the COT67B registration, test substances used in the submitted studies included bacterial-produced purified full-length Cry1Ab and truncated Cry1Ab protein, in addition to Cry1Ab protein as expressed in Event Bt11 maize grain, pollen, and leaves. The individual results for each study on ecological effects for Vip3Aa and Cry1Ab are summarized in Tables 9 and 10, respectively. The results are also presented in a more descriptive format in subsequent sections of the risk assessment document. Full reviews of each study for each event can be found in the individual Data Evaluation Reports (DERs/MRID#s).

Table 9. Summary of environmental effects studies and waiver justifications for COT102 submitted to comply with data requirements published in 40 CFR § 158.2150(d).

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
Avian dietary testing, broiler chicken, *Gallus domesticus*	885.4050	Vip3Aa19 maize grain (Event Pacha)	A 49-day dietary study showed no adverse affects to broiler chickens when fed a 50% diet composed of Event Pacha maize grain (containing VIP3A). Therefore, the NOEC was 0.588 µg VIP3A/g corn feed and the LC_{50} was > 0.588 µg VIP3A/g feed corn grain. **Classification: Acceptable**	470176-23
Avian injection testing	885.4100	N/A	**Acceptable waiver rationale**	N/A
Avian oral testing, bobwhite quail, *Colinus virginianus*	850.2100	Microbial Vip3Aa1 (VIP3A-0198)	A 14-day study showed no adverse effects to bobwhite quail from VIP3A-0198, after a single oral dose via gavage. The NOEL was 400 mg VIP3A/kg and the LD_{50} was > 400 mg VIP3A/kg bird body weight. **Classification: Acceptable**	457665-08
Wild mammal testing	885.4150	N/A	**Acceptable bridging rationale to acute oral toxicity test on mice (MRID No. 457665-05).**	N/A
Freshwater fish testing, channel catfish, *Ictalurus punctatus*	885.4200	Vip3Aa19 maize grain (FFPACHA-0100)	A 30-day study showed no adverse effects on juvenile catfish after exposure to Vip3Aa protein from Event Pacha corn grain. Therefore, the NOEC was 7.10 µg Vip3Aa19/g fish feed and the LC_{50} was > 7.10 µg Vip3Aa19/g **Classification: Acceptable**	470176-24
Freshwater aquatic invertebrate testing, water flea, *Daphnia magna*	885.4240	Vip3Aa19 maize pollen (PHOPACHA-0199)	In a 48-hour static renewal limit bioassay, VIP3A maize pollen (containing 83.8 µg VIP3A protein/g) had no adverse effects on the survival of *Daphnia magna,* when suspended in 120 mg pollen/L. The LC_{50} was > 83.8 µg VIP3A protein/g. **Classification: Unacceptable.** The 885 Series Guidelines call for a 21 day study. The submitted 48 hour acute study is inadequate.	457921-01
Estuarine and marine animal	885.4280	N/A	**Acceptable waiver rationale**	N/A

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
testing				
Non-target plant testing	885.4300	N/A	**Acceptable waiver rationale**	N/A
Non-target insect testing, minute pirate/insidious flower bug , *Orius insidiosus*	885.4340	Microbial Vip3Aa19 (VIP3A-0104)	*Orius insidiosus* nymphs fed a meat-based diet containing microbial-derived 7.25 mg Vip3Aa19 protein/ g diet showed no adverse effects after 21 days. The NOEC was 7.25 mg Vip3Aa19 protein/ g and the LC_{50} was > 7.25 mg Vip3Aa19 protein/ g. **Classification: Acceptable**	468648-14
Non-target insect testing, pink-spotted lady beetle, *Coleomegilla maculata*	885.4340	Vip3Aa19 maize pollen (PHOPACHA-0100)	*Coleomegilla maculata* adults were fed a diet containing 5.0% VIP3A maize pollen (containing 144.8 µg VIP3A protein/g pollen) for 21 days with no adverse effects observed. The NOEC was 7.24 µg VIP3A protein/g pollen and the LC_{50} was > 7.24 µg/g VIP3A/g pollen. **Classification: Acceptable**	457665-09
Non-target insect testing, seven-spotted ladybird beetle, *Coccinella septempunctata*	885.4340	Microbial Vip3Aa19 (VIP3A-0204)	*C. septempunctata* adults fed a 50% sucrose diet containing 7250 µg Vip3Aa19 protein/g diet for showed no adverse effects after 15 days. The NOEC was 7250 µg Vip3Aa19 protein/g diet and the LC_{50} was > 7250 µg/g Vip3Aa19 protein/g diet. **Classification: Acceptable**	468848-02
Non-target insect testing, green lacewing, *Chyrsoperla carnea*	885.4340	Microbial Vip3Aa19 (VIP3A-0104)	*Chyrsoperla carnea* larvae fed a meat-based diet containing 7250 µg Vip3Aa19 protein/g diet showed no adverse effects. The NOEC of 7250 µg Vip3Aa19 protein/g diet and the LC_{50} was > 7250 µg Vip3Aa19 protein/g diet at day 14, when the control mortality reached 20%. There were no statistically significant differences between the VIP3A-0104 group and the negative control group. **Classification: Acceptable**	468848-15
Non-target insect testing, collembolan, *Folsomia candida*	885.4340	Vip3Aa19 maize leaves (LLPACHA-0100)	Collembola were fed a diet containing 50% yeast and 50% leaf tissue for 28 days. No statistically significant effects on survival or reproduction were found among the test and negative control groups. The NOEC was 43.2 µg Vip3Aa19 protein/g diet and the LC_{50} was > 43.2 µg Vip3Aa19 protein/g diet. **Classification: Acceptable**	458358-10
Honeybee testing, Honeybee larvae, *Apis mellifera*	885.4380	Vip3Aa19 maize pollen (PHOPACHA-0199)	Three-to-five day old honeybee larvae were administered a single dose of *ca.*2 mg of pollen moistened with a drop of 30% sucrose solution (containing the equivalent of 168 µg of Vip3Aa) in their individual brood cells. After 19 days, there were no significant differences between the treatment and control groups in survival to capping, survival to emergence of adults, and the behavior and morphology of the emerged adults. The NOEL was	458358-09

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
			83.8 µg Vip3Aa19 protein/g diet and the LD_{50} was > 83.8 µg Vip3Aa19 protein/g diet. **Classification: Acceptable**	
Earthworm toxicity, *Eisenia foetida*	OECD Guideline 207, 850.6200	Vip3Aa19 maize leaves (LPPACHA-0199)	Adult earthworms were exposed to artificial soil containing 3.60 µg VIP3A protein/g soil for 14 days. No mortality or differences in body weights were observed in the test group. The NOEC was 3.60 µg VIP3A protein/g soil and the LC_{50} > 3.60 µg VIP3A protein/g soil. **Classification: Acceptable**	457921-02
Soil fate and degradation	885.5200	Vip3Aa19 maize leaves (LPPACHA-0199)	Results of this degradation study indicate that the DT_{50} of 16 mg/g concentration of the Vip3Aa19 test material protein do not persist in various types of soil from 6 days to 12.6 days via measuring the loss of bioactivity in black cutworm. **Classification: Acceptable**	470176-30

Table 10. Summary of environmental effects studies and waiver justifications for COT67B submitted to comply with data requirements published in 40 CFR § 158.2150 (d).

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
Avian dietary testing, broiler chicken, *Gallus domesticus*	885.4050	Bt11 maize grain	A 42-day dietary study showed no deleterious effects on broiler chicken survival or carcass yield when fed a 50% diet composed of Bt11 maize grain (containing Cry1Ab). **Classification: Acceptable**	4565251-01
Avian injection testing	885.4100	N/A	**Acceptable waiver rationale**	N/A
Avian oral testing, bobwhite quail, *Colinus virginianus*	850.2100	Bt176 Maize leaf protein (LP176-0194)	A 14-day study on bobwhite quail showed no adverse effects after a single oral dose of Bt176 grain, containing Cry1Ab. The NOEL was 140 mg Cry1Ab/kg bodyweight and the LD_{50} was > 140 mg Cry1Ab/kg bodyweight. **Classification: Acceptable**	433236-09
Wild mammal testing	885.4150	N/A	**Acceptable bridging rationale to acute oral toxicity test on mice (MRID No. 47017614)**	N/A
Freshwater fish testing, channel catfish, *Ictalurus punctatus*	885.4200	Microbial FLCry1Ab (FLCRY1AB-0103)	A 30-day study showed no adverse effects to juvenile channel catfish. The NOAEC was 7.10 µg FLCry1Ab/g fish feed and the LC_{50} was > 7.10 µg FLCry1Ab/g fish feed. **Classification: Acceptable**	470176-25
Freshwater aquatic invertebrate,	885.4240	Bt176 maize pollen (PHO176-0194)	In a 48-hour static renewal limit bioassay, Event 176 maize pollen containing 12.36 µg Cry1Ab protein/g had no adverse effects on the survival of *Daphnia magna*,	433236-10