UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM FEB 07 2008

SUBJECT: Review of Human Health and Product Characterization Data for Registration of *B. thuringiensis* Modified Cry1Ab and Vip3Aa19 Proteins and the Genetic Material Necessary for their Production in COT67B x COT102 Cotton

TO: Alan Reynolds
Regulatory Action Leader
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511P)

FROM: Rebecca L. Edelstein, Ph.D., Chemist /s/
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511P)

THROUGH: John L. Kough, Ph.D., Biologist /s/
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511P)

ACTION REQUESTED: To review the human health and product characterization data for registration of COT67B x COT102 cotton and conduct a risk assessment

RECOMMENDATION:
Syngenta has submitted a registration application request for event COT67B x COT102 cotton, as well as petitions for permanent tolerance exemptions for modified Cry1Ab protein containing an additional 26 amino acids and Vip3Aa in or on all crops. The product characterization and human health data submitted on event COT67B x COT102 cotton are sufficient to support the requests. An independent lab validation of the analytical detection method for Vip3Aa and modified Cry1Ab is still needed.

DATA REVIEW RECORD

Active Ingredient:	*Bacillus thuringiensis* modified Cry1Ab containing an additional 26 amino acids and Vip3Aa19 insecticidal proteins and the genetic material necessary for their production in cotton
Product Name:	COT67B X COT102 Cotton
Company Name:	Syngenta Seeds, Inc.—Field Crops—NAFTA
ID No:	67969-O
Chemical Number:	006499 and 006529
Decision Number:	373333
DP Barcode:	337037
MRID/Study Titles:	47017602—Re-Characterization of Vip3A Protein Test Substance (Vip3A-0204) 47017603—Additional Molecular Characterization of Event COT102 Cotton by Southern Analyses 47017604—Characterization of Cry1Ab Test Substance FLCRY1AB-0103 and Certificate of Analysis 47017605—Re-Characterization of Cry1Ab Test Substance FLCRY1AB-0103 47017606—Comparative Southern Analyses of Stacked COT102 x COT67B Cotton 47017607—Comparison of Transgenic Protein Expression in Event COT102 and COT67B Cotton and Stacked COT102 x COT67B Cotton Lines 47017608—Characterization of the Cry1Ab Protein Produced in Event COT67B-Derived Cotton Plants and Comparison with Cry1Ab Protein Produced in Recombinant *Escherichia coli* 47017609—Stability of Vip3Aa19 and APH4 Protein Expression Across Multiple Generations of Event COT102 Cotton 47017610—Stability of Cry1Ab Protein Expression Across Multiple Generations of Event COT67B Cotton 47017611—Analysis for the Presence of Cry1Ab Protein in Linters, Toasted Cottonseed Oil from Processed Seed of Event COT67B Cotton Expressing Full-Length Cry1Ab Protein 47017613—Analysis of Vip3A or Vip3A-Like Proteins in Six Different Commercial Microbial *Bacillus thuringiensis* Products 47017614—FLCRY1AB-0103: Single Dose Oral Toxicity Study in the Mouse 47017615—*In vitro* Digestibility of Full-Length Cry1Ab Protein (Test Substances FLCRY1AB-0103 and IAPCOT67B-0106) Under Simulated Mammalian Gastric Conditions 47017616—Effect of Temperature on the Stability of Full-Length Cry1Ab

Protein
47017617—Vip3Aa19: Assessment of Amino Acid Sequence Homology with Known Allergens
47017618—APH4 (Entrez Database accession No. CAA85741): Assessment of Amino Acid Sequence Homology with Known Allergens
47017619—Full-Length Cry1Ab as Expressed in Event COT67B Cotton: Assessment of Amino Acid Sequence Homology with Known Allergens
47074101—Analytical Method for the Detection of Vip3A and FL Cry1Ab Protein in Cotton Tissues Derived from COT102 x COT67B Cotton (VipCot Cotton)

BACKGROUND:

Syngenta Seeds, Inc. previously submitted EUP requests to conduct field tests on Event COT102, Event COT67B, and Event COT102 x Event COT67B. 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, for the control of several lepidopteran pests of cotton. COT102 cotton also expresses a selectable marker, hygromycin B phosphotransferase (APH4). Event COT67B cotton, which was developed by *Agrobacterium*-mediated transformation of cotton using two binary vectors, pNOV4641 and pNOV1914, expresses a full-length Cry1Ab protein containing an additional 26 amino acids (referred to by Syngenta as the 'Geiser motif'). This protein is intended to control a number of lepidopteran pests. *Bacillus thuringiensis* VIP3Aa19 protein in cotton has a temporary exemption from the requirement of a tolerance, which will expire on May 1, 2008 (40 CFR 174.501). Hygromycin B phosphotransferase (APH4) marker protein in all plants is exempt from the requirement of a tolerance when used as a plant-incorporated protectant inert ingredient (40 CFR 174.526). *Bacillus thuringiensis* Cry1Ab protein in all plants is exempt from the requirement of a tolerance when used as a plant-incorporated protectant in all food commodities (40 CFR 174.511). It was previously determined that this tolerance exemption was sufficient to cover the small amounts of modified Cry1Ab protein present in food as a result of the EUPs for COT67B and COT102 x COT67B. However, because the Cry1Ab protein produced from these events has an additional 26 amino acids that are not present in any naturally occurring Cry1Ab protein, EPA has determined that a new tolerance exemption, specific for the modified protein, is required for registration.

For registration of Event COT102 x Event COT67B cotton, Syngenta has submitted petitions for permanent tolerance exemptions for Vip3Aa in or on all crops and modified Cry1Ab containing an additional 26 amino acids in or on all crops. Syngenta is relying on previously submitted data (reviewed in memoranda dated March 24, 2004 from C. Wozniak to L. Cole, February 8, 2007 from A. Waggoner to M. Mendelsohn, and April 4, 2007 from S. Matten to A. Reynolds) to support the tolerance exemption for Vip3Aa but has submitted new data, reviewed in this memorandum, to support the tolerance exemption for modified Cry1Ab. The tolerance exemption for APH4 discussed above covers the APH4 protein produced in Event COT102 x Event COT67B.

Product characterization and human health data submitted for registration of COT102 x Event

COT67B and for establishing permanent tolerance exemptions for Vip3Aa and modified Cry1Ab containing an additional 26 amino acids are reviewed in this memorandum. Studies relevant to the risk assessment that were reviewed previously are also discussed and included in the data tables, and footnotes are provided with citations to the previous reviews.

RISK ASSESSMENT

PRODUCT CHARACTERIZATION

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[1], 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 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)

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

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

Study Type/Title	Summary	MRID #
Expression Levels/ Quantitation of VIP3A and APH4 Protein in Cotton Tissues and Whole Plants Derived from Transformation Event COT102[2]	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 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**	45835801
Characterization of Inserted DNA/Molecular	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,	45835802

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

Characterization and Genetic Stability of Event COT102[2]	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**	
Characteristics of *Bacillus thuringiensis* VIP3A Protein and VIP3A Cotton Plants Derived from Event COT102[2]	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 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.	45766501
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	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	45835812

Recombinant *Escherichia coli*[2]	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**	
Expression Level/ Analysis of Processed COT102 Cottonseed Products for Yield and Presence of Gossypol and Vip3A Protein[2]	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 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.**	45835803
The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab delta-endotoxin[3]	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	46880801

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

	state and cation specificity differed. **Classification: ACCEPTABLE**	
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 test substance is stable when stored desiccated at -20 °C for approximately 15 months. **Classification: ACCEPTABLE**	47017602
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

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 containing an additional 26 amino acids. 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 1. Mean Expression Levels of Cry1A.105 and Cry2Ab2 from MON 89034 Plant Tissues

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

Table 4. Product Characterization Data Submitted for Event COT67B

Study Type/Title	Summary	MRID #
Characterization of Inserted DNA/ Harper, B. (2006). Molecular characterization of Event COT67B cotton. Report No. SSB-125-06.[4]	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 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**	46885901
Expression Levels/ Hill, K. (2006). Quantification of Cry1Ab protein in Event COT67B cotton tissues and whole plants. Report No.SSB-022-06[4]	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	46885902

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

	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 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	47017605

	the total sample weight and the total protein as determined by the A_{280} 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
Expression Levels/ Analysis for the Presence of Cry1Ab	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	47017611

Protein in Linters, Toasted Cottonseed Oil from Processed Seed of Event COT67B Cotton Expressing Full-Length Cry1Ab Protein	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**	

Event 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 ± 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 Event COT102 x COT67B

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 COT102, Event COT67B, and Stacked COT102 x COT67B Cotton Lines	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 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**	47017607
Analytical Detection Method/Analytical Detection Method for the Detection of Vip3A and FLCry1Ab Protein in	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	47074101

Cotton Tissues Derived from COT102 x COT67B Cotton (VipCot Cotton)	cross reactivity with other transgenic varieties was observed. **Classification: ACCEPTABLE**	

Human Health Assessment of Vip3Aa

Background

Vip3Aa is a vegetative (i.e., produced during the vegetative stage of bacterial growth) insecticidal protein from *Bacillus thuringiensis* (*Bt*) with insecticidal activity to several lepidopteran pests. The insecticidal protein produced in event COT102 cotton, designated as Vip3Aa19, 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; K284Q). 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 *Bt* delta endotoxin nomenclature committee assigns a quaternary numerical rank (e.g., 1, 19, or 20) to each independently sequenced gene; therefore, some toxins with different quaternary ranks may be identical. All proteins designated as Vip3Aa are more than 95% identical. EPA has determined that 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.

Section 408(c)(2)(A)(i) of the FFDCA allows EPA to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if EPA determines that the exemption is "safe." Section 408(c)(2)(A)(ii) of the FFDCA defines "safe" to mean that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." This includes exposure through drinking water and in residential settings, but does not include occupational exposure. Pursuant to section 408(c)(2)(B), in establishing or maintaining in effect an exemption from the requirement of a tolerance, EPA must take into account the factors set forth in section 408(b)(2)(C), which require EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance and to "ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue...." Additionally, section 408(b)(2)(D) of the FFDCA requires that the Agency consider "available information concerning the cumulative effects of a particular pesticide's residues" and "other substances that have a common mechanism of toxicity." EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, EPA determines the toxicity of pesticides. Second, EPA examines exposure to the pesticide through food, drinking

water, and through other exposures that occur as a result of pesticide use in residential settings.

1. Toxicological Profile

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

Mammalian Toxicity and Allergenicity Assessment

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, 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).

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, Roy D., et al., "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)). Therefore, since no acute effects were shown to be caused by Vip3Aa proteins, even at relatively high dose levels, the Vip3Aa is not considered toxic. Further, amino acid sequence comparisons showed no similarities between Vip3Aa and known toxic proteins in protein databases 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* 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. The Vip3Aa protein was digested rapidly in simulated gastric fluid containing pepsin.
4. Glycosylation. Vip3Aa was shown not to be glycosylated.
5. Conclusion. Considering all of the available information, EPA has concluded that the potential for Vip3Aa to be a food allergen is minimal.

The amino acid sequence analysis was only performed on two Vip3Aa proteins, Vip3Aa19 and Vip3Aa20. The amino acid sequence of Vip3Aa proteins can vary up to 5%, and although very unlikely, it is possible that another Vip3Aa protein could have sequence identity with an allergen at the level of eight contiguous amino acids. Therefore, EPA is including the restriction on this tolerance exemption that it only applies to Vip3Aa proteins with no significant amino acid similarity with known allergens.

Although Vip3Aa was only shown not to be glycosylated in cotton and corn, it is unlikely to be glycosylated in any other crops because in order for a protein to be glycoslyated, it needs to contain specific recognition sites for the enzymes involved in glycosylation, and the mechanisms of protein glycosylation are similar in different plants (Lerouge, P. Cabanes-Macheteau, M., Rayon, C., Fichette-Lainé, A-C., Gomord, V., and Faye, L., "N-Glycoprotein biosynthesis in plants: recent developments and future trends," *Plant Molecular Biology* **38**: 31-48, 1998).

2. Aggregate Exposures

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

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for 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 the Vip3Aa protein is 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.

3. Cumulative Effects

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

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

a) Toxicity and Allergenicity Conclusions

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

Adequate information was submitted to show that the 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 support the prediction that the Vip3Aa 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, Roy D., et al., "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)). Since no treatment-related adverse effects were shown to be caused by the Vip3Aa protein, even at relatively high dose levels, the Vip3Aa protein is not considered toxic. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing or residue data is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.2140). 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 Vip3Aa in corn and cotton tissues.

Since Vip3Aa is a protein, potential allergenicity is also considered as part of the toxicity assessment. Considering all of the available information (1) Vip3Aa originates from a non-allergenic source; (2) Vip3Aa19 and Vip3Aa20 have no sequence similarities with known allergens; (3) Vip3Aa is not glycosylated; and (4) Vip3Aa is rapidly digested in simulated gastric fluid; 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 the Vip3Aa protein, as well as the minimal potential to be a food allergen, demonstrate the safety of the product at levels well above possible maximum exposure levels anticipated.

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 Vip3Aa protein has been exempted from the requirement of a tolerance under 40 CFR 174.507 Nucleic acids that are part of a plant-incorporated protectant.

b) Infants and Children Risk Conclusions

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

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

c) Overall Safety Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Vip3Aa protein, provided it has no significant amino acid similarity with known allergens. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed, nor any indication of

allergenicity potential for the plant-incorporated protectant.

5. Other Considerations

a) Endocrine Disruptors

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

b) Analytical Method(s)

A validated lateral flow enzyme-linked immubsorbent 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.

c) Codex Maximum Residue Level

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

Proposed Language for Tolerance Exemption:
Bacillus thuringiensis Vip3Aa proteins in all plants; exemption from the requirement of a tolerance.
Residues of *Bacillus thuringiensis* Vip3Aa proteins in all plants are exempt from the requirement of a tolerance when used as plant-incorporated protectants in all food commodities provided that the Vip3Aa protein does not have any significant amino acid similarity with any known allergens.

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

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**, upgradable to Acceptable with the submission of further information on the SBI allergen database. **Note: this is a summary of multiple studies and is therefore superseded by the**	45766502

	individual studies summarized below, which provide additional information, including the requested information on the SBI allergen database.	
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 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 single amino acid at position 2 (aspartate replaces asparagine). **Note: this study provides additional support for the conclusion that Vip3Aa proteins are non-toxic to mammals.**	45766503
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	45766504

	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.**	
Acute Oral Toxicity/ Acute Oral Toxicity Study with Test Substance VIP3A-0100 Protein in Mice[2]	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. 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**	45766505
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	45766506

	of guideline requirements; no further information required. **Note: this study provides additional support for the conclusion that Vip3Aa proteins are non-toxic to mammals.**	
In Vitro Digestibility of VIP3A Protein Under Simulated Mammalian Gastric Conditions [2]	VIP3A from recombinant maize (field corn) plants was prepared as sample LPPACHA-0199 by extracting the leaves of recombinant corn plants and 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**	45835805
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

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

Human Health Assessment of Modified Cry1Ab Containing 26 Additional Amino Acids

Background

EPA has previously established an exemption from the requirement of a tolerance for *Bacillus thuringiensis* Cry1Ab protein in all plants when used as a plant-incorporated protectant in all food

commodities (40 CFR 174.511). Event COT67B cotton produces 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. The additional amino acids have no impact on insecticidal activity, as they are in the protoxin portion of the protein, which is cleaved off during toxin activation. However, because Cry1Ab proteins in nature do not contain these additional amino acids, EPA has determined that the tolerance exemption at 40 CFR 174.511 does not cover this modified protein and is therefore establishing a new tolerance exemption for the modified Cry1Ab protein.

Section 408(c)(2)(A)(i) of the FFDCA allows EPA to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if EPA determines that the exemption is "safe." Section 408(c)(2)(A)(ii) of the FFDCA defines "safe" to mean that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." This includes exposure through drinking water and in residential settings, but does not include occupational exposure. Pursuant to section 408(c)(2)(B), in establishing or maintaining in effect an exemption from the requirement of a tolerance, EPA must take into account the factors set forth in section 408(b)(2)(C), which require EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance and to "ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue...." Additionally, section 408(b)(2)(D) of the FFDCA requires that the Agency consider "available information concerning the cumulative effects of a particular pesticide's residues" and "other substances that have a common mechanism of toxicity." EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, EPA determines the toxicity of pesticides. Second, EPA examines exposure to the pesticide through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings.

Toxicological Profile

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

Mammalian Toxicity and Allergenicity Assessment

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'. These data demonstrate the safety of the product at a level well above maximum possible exposure levels that are reasonably anticipated in the crop. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing and residue data is

similar to the Agency position regarding toxicity testing and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 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, Roy D., et al., "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)). Therefore, since no acute effects were shown to be caused by 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, P. Cabanes-Macheteau, M., Rayon, C., Fichette-Lainé, A-C., Gomord, V., and Faye, L., "N-Glycoprotein biosynthesis in plants: recent

developments and future trends," *Plant Molecular Biology* **38**: 31-48, 1998).

2. Aggregate Exposures

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

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for 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. Oral exposure, at very low levels, may occur from ingestion of processed corn products and, theoretically, drinking water. However, oral toxicity testing showed no adverse effects.

3. Cumulative Effects

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

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

Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the 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, Roy D., et al., "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (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.

b) Infants and Children Risk Conclusions

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

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

In this instance, based on all the available information, the Agency 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.

c) Overall Safety Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the 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.

5. Other Considerations

a) Endocrine Disruptors

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

b) Analytical Method(s)

A validated lateral flow enzyme-linked immubsorbent assay (ELISA) protocol has been provided to the Agency for detecting modified Cry1Ab in cotton.

c) Codex Maximum Residue Level

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

References

Sjoblad, R. D., McClintock, J. T., and Engler, R., "Toxicological Considerations for Protein Components of Biological Pesticide Products," *Reg. Toxicol. Pharmacol*. 15(1), **1992**, 3-9.

Table 8. Summary of Modified Cry1Ab Human Health Data

Study Type/Title	Summary	MRID #
Acute oral toxicity	Groups of five male and five female mice were given 0 or 1830 mg/kg	47017614

(OPPTS 870.1100)/ FLCRY1AB-0103: Single Dose Oral Toxicity Study in the Mouse (AM7516/Regulatory/Report)	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 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

HUMAN HEALTH ASSESSMENT 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

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