BIOPESTICIDES REGISTRATION ACTION DOCUMENT

Bacillus thuringiensis Cry3Bb1 Corn

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

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[1]Currently with USDA's CSREES.

[2]Currently with USDA's APHIS/BRS.

I. Overview

A. Use Profiles

- **Pesticide Names:**

1) *Bacillus thuringiensis* Cry3Bb1 Protein and the Genetic Material Necessary for its Production (Vector ZMIR13L) in Event MON863 Corn
Trade and Other Names: Corn Event MON863, YieldGard Rootworm™
OPP Chemical Code: 006484

2) *Bacillus thuringiensis* Cry3Bb1 Protein and the Genetic Material Necessary for its Production (Vector ZMIR13L) in Event MON 863 Corn and *Bacillus thuringiensis* Cry1Ab Delta-Endotoxin and the Genetic Material Necessary for its Production in Corn
Trade and Other Names: YieldGard® Plus Corn
OPP Chemical Codes: 006484 and 006430

3) *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material necessary for its production (Vector ZMIR39) in Event MON 88017 corn (OECD Unique Identifier: MON-88Ø17-3)
Trade and Other Names: MON 88017 Corn
OPP Chemical Codes: 006502

4) (*Bacillus thuringiensis* Cry3Bb1 protein and the genetic material necessary for its production (Vector ZMIR39) in Event MON 88017 corn (OECD Unique Identifier: MON-88Ø17-3) and *Bacillus thuringiensis* Cry1Ab Delta-Endotoxin and the Genetic Material Necessary for its Production in Corn
Trade and Other Names: MON 88017 x MON 810Corn
OPP Chemical Codes: 006502 and 006430

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

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

- **Uses: Field Corn**

- **Target Pest(s):**

Cry3Bb1 Only Products - Western Corn Rootworm, Northern Corn Rootworm, and Mexican Corn Rootworm

Cry3Bb1 and Cry1Ab Combined Products - European Corn Borer, Southwestern Corn Borer, Southwestern Cornstalk Borer, Southern Cornstalk Borer, Sugarcane Cornstalk Borer, Corn Earworm, Fall Armyworm, Stalk Borer, Western Corn Rootworm, Northern Corn Rootworm, and Mexican Corn Rootworm

B. Public Comments

Over 900 comments were received by EPA in response to announcements regarding MON 863 (YieldGard Rootworm) and MON 863 x MON 810 (YieldGard Plus). Comments were received from private citizens, independent farmers, farming cooperatives, farm industry representatives, trade organizations, advocacy groups, academic researchers, and government officials. Comments included handwritten letters, typed letters, e-mails, faxes, and slide presentations from the SAP meeting. Two response to comment documents were prepared and are available on the U.S. Federal Government's Regulations.gov website.

One comment was received by EPA in response to the FR announcement regarding MON 88017 and MON 88017 x MON 810 from a private citizen, who asked that Monsanto executives be fed MON 88017 and MON 88017 x MON 810 corn for five years before EPA makes a safety determination, The Agency recognizes that some individuals believe that genetically modified crops and food should be banned completely but has nonetheless concluded that the registration of these products will pose no unreasonable adverse effects on the environment.

To access materials in the electronic docket for YieldGard Rootworm and YieldGard Plus referenced above: click on http://www.regulations.gov select the "All Documents" radio button, and type the docket number EPA-HQ-OPP-2004-0182 into the "Keyword or ID" field. To access materials in the electronic docket for MON 88017 and MON 88017 x MON 810, follow the instructions above, but use docket number EPA-HQ-OPP-2004-0228.

II. Science Assessment

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

A. Product Characterization

Product characterization is critical to understanding the way in which the product was made and the unique characteristics that need to be assessed. The product characterization data provide information on the specific transformation systems used for each product, on the actual DNA inserted into the plant, on the inheritance and stability of these traits in the plant, on biochemical characteristics of the *Bt* Cry3Bb1 protein and on *Bt* Cry3Bb1 protein expression levels for various plant tissues.

Transformation system: Cry3Bb1 plant-incorporated protectants, except for MON 88017, were transformed into corn tissue via a method employing bombardment of particles coated with DNA encoding the intended insert. MON 88017 was produced by the Agrobacterium-mediated transformation of corn cells with the plasmid vector PV-ZMIR39. Each plasmid description includes a reference to the strains of *Bacillus thuringiensis* used as the source of the DNA sequence for the toxin protein. In addition, the sources for marker proteins, promoters, terminators and enhancers, as well as the fragment size, orientation and any modifications to the original DNA sequence to enhance expression in the plant are given. All the other DNA sequences introduced to improve or restrict expression of the introduced traits are also described. Finally, the plasmid discussion includes a description of any modifications made to the DNA (e.g., codon modifications to improve eukaryotic expression).

Characterization of the DNA Inserted in the Plant: Inserted DNA is characterized with Southern blot data of the DNA in the plant genome. The analysis usually consists of DNA isolation from the transformed plant, digestion of this DNA with several different endonucleases and hybridization of these restriction endonuclease fragments with labeled-DNA which is complementary to the introduced traits. This analysis includes not only probes specific for the entire insert, but also probes

recognizing just the coding regions of the traits or DNA elements outside the coding region. Polymerase chain reaction (PCR) assays utilizing various specific and non-specific primers, genome walking, cosmid libraries and DNA sequencing have also been employed with sensitive Southern blotting techniques to more completely describe the inserted DNA and surrounding regions. The information available from these blots can indicate the presence of all the elements of the expected insert as well as information about the possibility of deletions and other errors associated with DNA introduction by transformation. Comparison of Southern blots of genomic DNA, digested using a range of restriction endonucleases, can also reveal the copy number of the genes introduced and suspected linkage of the traits. Alternatively, the intensity of the radioactive label from binding the probe DNA can also estimate the number of insert copies incorporated in the plant genome.

Inheritance and Stability after Transformation: The data generated for this endpoint examine progeny from crosses between selected elite lines with the transformed *Bt* expressing line, looking for the independent segregation of the introduced traits in the progeny. Traditional breeding work done during the development of the plant line by backcrossing can reveal the linkage of the introduced traits as well as changes in trait expression. The inheritance data is the ratio of progeny expressing the hemizygous trait based on expected Mendelian inheritance. Stability data implies an examination of either the expression of the trait or tracking of the DNA itself over several plant generations. One of the main concerns with stability is spontaneous loss of the inserted DNA or loss of efficacy due to gene silencing. Neither MON 863 nor MON 88017 showed independent assortment of the introduced traits with their marker protein genes (*nptII* and *cp4 epsps*, respectively). This indicates that in both MON 863 and MON 88017 the Cry3Bb1 and marker protein traits were on the same chromosome and closely linked (crossover events were not detected). The submissions that covered characterization of the actual DNA insert and stability/inheritance data are listed in the MRIDs for each product. These submissions are acceptable and fulfill this data requirement.

Protein Characterization and Expression: Data has been presented to demonstrate that the protein expressed from the inserted DNA is similar to what was produced in the source bacterium and is active as expected against the intended target insect. Some protein characterization data demonstrate that microbially produced *Bt* protein is the equivalent to that expressed in the plant. This apparent scientific tautology (where plant produced protein is the same as microbial protein is the same as the plant produced protein) has been used to justify the use of the microbially-produced protein as a test substance in toxicity tests. Because the expression level of these proteins is so low in plants, and the maximum hazard dose acute oral toxicity test is required as part of the human health risk assessment for these proteins, the ability to produce the protein in an industrial microbe is essential. The acute oral test requires between 2000 and 5000 mg of protein per kg bodyweight of test animal. Isolating the amount of purified protein required to dose several animals from *Bt*-expressing plants would be a tremendous burden involving harvesting and processing large volumes of plant material (ecological effects testing differs and is addressed in the ecological effects section of this document). Proper characterization of the equivalency between these microbial proteins and plant expressed proteins

provides an alternative to purifying the test material as the plant-produced protein from large volumes of tissue.

Much of the characterization data describes the procedures used to isolate the protein or a highly *Bt* protein enriched fraction of plant extract. The tests done to support the equivalence of microbial and plant-produced *Bt* protein include: molecular sizing by SDS-PAGE and western blot analysis; immunorecognition using ELISA and western blot analysis; N-terminal amino acid sequencing; MALDI-TOF analysis of protein digests; confirmation of the lack of glycosylation in the plant-produced protein; and bioactivity against a range of insects (often pest species including the target pest). Since the issues surrounding non-target effects are considered essential for the ecological effects assessment, these non-target pest tests are also covered in the ecological effects assessment.

The *Bt* protein expression level in various tissues throughout the growing season have been determined for both MON 863, MON810xMON863, MON 88017, MON88017x MON810. However, the data for MON 863 was presented on a fresh weight basis and data in terms of dry weight leaf, root, pollen, seed, and whole plant are required. These data were submitted and are currently under review (MRID No. 464799-02). Also, to support MON 88017 x MON 810, expression level data regarding Cry1Ab protein levels in MON 810 and MON 88017 x MON 810 young root and forage root have been required. These data were submitted and are currently under review (MRID No. 470045-01).

Residue Analytical Methods

Independent laboratory method validation (under OPPTS Guidelines OPPTS 860.1340) and EPA laboratory method validation were required to complete the database for Cry3Bb1 corn. The extraction and detection method as described for Cry3Bb1 protein appears to be adequate for analysis of Cry3Bb1 protein in corn grain. The independent laboratory validation study (MRID No. 463942-01) was received and found acceptable. Validation by EPA's Biological and Economic Analysis Division laboratory is pending and is necessary to fully validate the method.

Cry3Bb1 BACKGROUND

<u>MON 863</u>

Cry3Bb1 protein is a delta-endotoxin from *Bacillus thuringiensis* spp. *kumamotoensis* and has activity against certain beetles. The wild-type *cry3Bb1* gene was modified to enhance the protein's activity against the corn root worm complex. Two Cry3Bb1 variants were engineered for expression in the bacterium *Bacillus thuringiensis* strains EG11098 and EG11231. Cry3Bb1 protein resulting from these strains differed from wild-type Cry3Bb1 protein by 5 and 4 amino acid substitutions (Table1). Corn was genetically modified to express the Cry3Bb1.11231 protein (resulting in corn line MON 853) or the Cry3Bb1.11098 protein (resulting in corn line MON863). At the 5' end of the *cry3Bb1* gene's reading frame, the vectors used for making MON 853 and MON 863 corn coded for

an additional amino acid residue due to creation of a restriction enzyme site necessary to construct the vectors.

Data based on Cry3Bb1.11098 and Cry3Bb1.11231 proteins supported an exemption from the requirement of a food tolerance and a tolerance exemption (40CFR§180.1214) was published May 11, 2001.

Monsanto subsequently submitted additional data regarding the MON 863 corn line. The vector used to transform MON 863 corn coded for an arginine residue at position 349 instead of glutamine (as previously thought) within the *cry3Bb1* gene's reading frame. Since the bacterially produced protein used in human health safety studies had the glutamine at position 349 and not arginine (as produced in MON 863), Monsanto generated another package of characterization and toxicology data for this variant, Cry3Bb1.11098(Q349R), since this protein is produced by the MON 863 corn line rather than the Cry3Bb1.11098 protein.

The Agency has reviewed the additional data submitted by Monsanto in connection with MON863 and concluded that the data provided supports the contention that the Cry3Bb1.11098, Cry3Bb1.11098 (Q349R) and Cry3Bb1.11231 proteins are variants of the Cry3Bb1 protein. Since these variants do not differ significantly from the Cry3Bb1 protein in terms of biochemical or toxicological characteristics, the Cry3Bb1.11098, Cry3Bb1.11098(Q349R) and Cry3Bb1.11231 protein variants are all covered by the exemption from the requirement of a food and/or feed tolerance (40CFR§180.1214).

MON 88017

The Cry3Bb1 protein produced in MON 88017 is a variant of the wild-type Cry3Bb1 protein from *B.t.* subsp. *kumamotoensis* that protects the roots of corn plants from feeding damage caused by the coleopteran pest, corn rootworm (*Diabrotica* sp.). The Cry3Bb1.pvzmir39 variant produced in MON 88017 differs in its amino acid sequence by seven amino acids from the wild type Cry3Bb1 protein and by a single amino acid from the Cry3Bb1.11098(Q349R) protein in MON 863. The Cry3Bb1 protein variants in MON 88017 and MON 863 share an amino acid sequence identity of >99.8%, differing from one another by only one of 653 amino acids at position 166 in MON 863, where glycine is present instead of aspartic acid. (See Table 1.)

MON 88017 also expresses the 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium sp.* strain CP4 (CP4 EPSPS) which confers tolerance to glyphosate, the active ingredient in Roundup® herbicides.

All physicochemical characteristics (which includes immunoreactivity, amino acid sequence, molecular weight, and glycosylation status) in the Cry3Bb1 protein in MON 88017 were found to be similar with the Cry3Bb1 protein in MON 863. Moreover, the protein expression, functional activity, and field efficacy data of Cry3Bb1 protein in MON 88017 were compared to MON 863 and

found to be functionally equivalent. Based on substantial similarity of Cry3Bb1 protein produced in MON 88017 and the Cry3Bb1 protein produced in the registered MON 863 corn product (YieldGard Corn Rootworm), human health data previously submitted for MON 863 were bridged to MON 88017.

Table 1. Cry3Bb1 Protein Variants: Amino Acid Sequence Percent Identities and Position Differences

Cry3Bb1 Variant [c]	% Identity Wild-type	Amino Acid Positions [ab]						
		2	165/ 166	231/232	311/ 312	313/ 314	317/318	348/ 349
Bacterial-produced protein								
Wild-type	*na*	*na*	D	H	S	N	E	Q
Cry3Bb1.11231	99.4	*na*	D	R	L	T	K	Q
Cry3Bb1.11098	99.2	*na*	G	R	L	T	K	Q
Cry3Bb1.11098 (Q349R)	98.9	A	G	R	L	T	K	R
Cry3Bb1.pvzmir39	99.1	A	D	R	L	T	K	R
Plant-produced protein / product								
Cry3Bb1.11231 / MON 853	99.2	A	D	R	L	T	K	Q
Cry3Bb1.11098 (Q349R) /MON 863	98.9	A	G	R	L	T	K	R
Cry3Bb1.pvzmir39 /MON 88017	99.1	A	D	R	L	T	K	R

[a] A - alanine; G = glycine, D= aspartic acid; R = arginine; H = histidine; L = leucine; S = serine; T = threonine; N = asparagine; K = lysine; E = glutamic acid; and Q = glutamine; *na* = not applicable

[b] The *B.t.*- produced Cry3Bb1 protein variants contain 652 amino acids. The *E.coli*- and plant-produced Cry3Bb1 protein variants contain 653 amino acids due to the insertion of an alanine residue at position 2, resulting from the assembly of the *cry3Bb1* gene into the *E.coli*- or plant transformation vector.

[c] All Cry3Bb1 protein variants, except for Cry3bb1.pvzmir39, are discussed in Astwood, et al., MRID 454240-09 and the Wild-type Cry3Bb1 is discussed in Donovan et al., 1992.

MON 88017 x MON 810

The product characterization and protein expression analyses for Cry3Bb1 and Cry1Ab insect control proteins and the genetic material necessary for their production in maize (corn) plants derived from MON 88017 x MON 810 (which were combined through traditional plant breeding) were found to be similar and functionally equivalent to Cry3Bb1 protein expressed in MON 88017 and to Cry1Ab

protein expressed in MON 810. Toxicological and allergenicity data can be bridged to support the finding that there is a reasonable certainty of no harm to exposure of Cry3Bb1 and Cry1Ab proteins expressed in MON 88017 x MON 810 to humans.

GUIDELINE NO	STUDY	RESULTS	MRID NO.
885.11	Product Characterization (Transformation System and Inheritence and Stability After Transformation)	MON853, MON860, MON862, and MON863 were produced by the incorporation of one of three constructs [PV-ZMIR12L (MON862), PV-ZMIR13L (MON863) or PV-ZMIR14L (MON853 & MON860)] via a particle bombardment mechanism.. The *cry3Bb1* and *nptII* genes were stably introduced into the corn genomes, as determined by at least three generations of greenhouse and field studies. Acceptable.	448779-01
885.11	Product Characterization (DNA Characterization)	The data presented in this submission describe the DNA insert for event MON 863. The data provided support the finding that event MON 863 contains 1 intact copy of the insert which encodes for both Cry3Bb1 and NPTII proteins. ACCEPTABLE	454240-02 451568-02
885.11	Product Characterization (DNA Characterization) **YieldGard Plus** Confirmation of the molecular identity of YieldGard® and corn rootworm protected combines trait corn hybrid MON 810 x MON 863 by Southern blot analysis.	The corn hybrid MON 810 x MON 863 containing the two transformation events, MON 810 (*cry1Ab*) and MON 863 (*cry3Bb1*) was examined for the presence of these two genes encoding -endotoxins in the resulting hybrid. Probes for the *cry1Ab* and *cry3Bb1* genes were obtained from previous studies and corresponded to the first 900 bp (*cry1Ab*) or the entire length of the gene (*cry3Bb1*). The radiolabelled (^{32}P) probe for *cry1Ab* hybridized to restricted DNA samples on nylon membranes and resulted in a lack of any signal detection for the samples from MON 846 (non-transgenic) and MON 863 samples, but did detect the presence of *cry1Ab* in the MON 810 and MON 810 x MON 863 hybrid plant samples. When DNA samples were probed with the *cry3Bb1* sequence, hybridization confirmed the presence of this gene in MON 863 plants and the MON 810 x MON 863 hybrid, but again failed to detect the presence of this gene in the negative control MON 846. Plasmid DNA from plasmids containing either gene separately did react positively with the appropriate probes when the restricted plasmid DNA was co-electrophoresed with MON 846 DNA and hybridized with the respective probe. From these results it is evident that the hybrid MON 810 x MON 863 contains the *cry1Ab* and *cry3Bb1* genes. Additionally, the restriction patterns noted on the Southern blot provided suggest that there have been no major alterations or rearrangements in the conventional cross of these two events (hybrids) for these two gene inserts. CLASSIFICATION: ACCEPTABLE	457917-01

GUIDELINE NO	STUDY	RESULTS	MRID NO.
885.11	Product Characterization Transformation System,. Inheritence and Stability After Transformation, DNA Characterization **Event MON 88017**	MON 88017 was produced by the Agrobacterium- mediated transformation of corn cells with the plasmid vector PV-ZMIR39. In this procedure, only the DNA present between the left and right borders was transferred into the host corn cells. No detectable backbone sequence were detected. This plasmid vector contains two gene cassettes within the borders: 1) the cry3Bb1 coding region regulated by the enhanced 35S plant promoter, the wtCAB leader, the rice actin intron and the tahsp17 3' polyadenylation sequence; and 2) the cp4 epsps coding region (containing a chloroplast transit peptide sequence) regulated by the rice actin promoter and intron, and the NOS 3' polyadenylation sequence. Molecular characterization experiments (including: genomic DNA isolation, restriction enzyme digestion, Southern blot analyses, and polymerase chain reaction and sequence analysis) were conducted on MON 88017. The results demonstrate that MON 88017 contains: a single DNA integration locus, one copy of the DNA insert at the site of integration, an intact cry3Bb1 cassette and an intact cp4 epsps cassette. No detectable plasmid backbone sequences and no additional elements, linked or unlinked to intact cassettes, from the transformation vector PV-ZMIR39 were found. Stability of the insert was also confirmed by the maintenance of the expected Southern fingerprint across seven breeding generations and that no backbone sequence from the transformation vector PV-ZMIR39 was detected. Finally, PCR and DNA sequence analyses confirmed the 5' and 3' insert-to-plant junctions, the organization of the elements within the insert, and the complete DNA sequence of the insert in corn event MON 88017; thereby, confirming that only Cry3Bb1 and CP4 EPSPS proteins are encoded by the insert in MON 88017. These analyses also confirmed that the encoded amino acid sequence was identical to that of the Cry3Bb1 protein produced in MON 863, except for the expected difference of one amino acid. CLASSIFICATION: ACCEPTABLE	461817-02
885.11	Product Characterization- Confirmation of the Identity of **MON 88107 x MON 810** Corn by Southern blot Analysis	Southern blot analysis was utilized to confirm the presence of the event-specific fingerprints for MON 88017 and MON 810 in MON 88017 x MON 810 corn. Specific Southern blot fingerprints of MON 88017 and MON 810 have been previously described and were used as comparison. In the verification of the presence of the MON 88017 event-specific fingerprint in MON 88017 x MON 810, test and control DNA samples were digested with the restriction enzyme Xba I, while PV-ZMIR39 served as a positive hybridization control. The blot was probed with four 32P-labeled probes that spanned the entire T-DNA sequence. As expected, no hybridization was observed in the non-transgenic control corn line, while plasmid PV-ZMIR39 produced the expected bands resulting from hybridization of the T-DNA probes to the plasmid. MON 810 produced a faint band of approximately ~3.3 kb with probes for PV-ZMIR39. This band is the result of hybridization of the P-e35S element on the probes for the T-DNA portion of the PV-ZMIR39 with the same 323 bp segment of the P-e35S promoter of the MON 810. Most importantly, MON 88017 and MON 88017 x MON 810 produced the expected ~5.5 and 7.4 kb bands that are	461850-02

GUIDELINE NO	STUDY	RESULTS	MRID NO.
		unique to MON 88017. These results verify that the MON 88017 event-specific fingerprint is present in MON 88017 x MON 810. In the verification of the presence of the MON 810 event-specific fingerprint in MON 88017 x MON 810, Test and control DNA samples were digested with the restriction enzyme EcoR I and Nco I, while PV-ZMBK07 served as a positive hybridization control. The blot was probed with a ~900 bp portion of the cry1Ab coding region. As expected, the DNA from the non-transgenic corn control line and MON 88017 showed no detectable hybridization bands, while plasmid PV-ZMBK07 positive control produced the expected bands resulting from hybridization of the T-DNA probes to the plasmid. MON 810 and hybrid MON 88017 x MON 810 each produced a band of ~2.8 kb. The size of the MON 810 event specific band had previously been reported as ~3.1 kb. Upon review of the reported genomic flanking sequences and the insert sequence of MON 810, it was determined that the expected size of the border fragment produced when digesting MON 810 genomic DNA with EcoR I and Nco I is ~2.8 kb and not 3.1 kb as previously described. This because the molecular weight markers used in this study provided a more accurate estimate of the actual size of this border fragment which is consistent with the described DNA sequence information. Nevertheless, these results verify that the MON 810 event-specific fingerprint is also present and unchanged in MON 88017 x MON 810. CLASSIFICATION: ACCEPTABLE	
885.11	Product Characterization (Sequencing & Immunoreactivity)	The N-terminal sequence analysis and the immunoreactivity to Cry3Bb1 polyclonal antisera confirm the relationship of Cry3Bb1.11098 and Cry3Bb1.11231 to wild-type Cry3Bb1. Further confirmatory data include protein molecular weight analysis and bioactivity. There are some amino acid changes (four or five) in the two test proteins compared to wild-type. However, these changes do not appear to significantly affect the bioactivity nor the immunoreactivity of the variant proteins. Based upon the data submitted, the two proteins produced by fermentation - Cry3Bb1.11098 and Cry3Bb1.11231 - have been confirmed as Cry3Bb1 protein variants. CLASSIFICATION: Acceptable.	454240-03
885.11	Product Characterization (Protein Equivalence)	Based upon the data provided, it appears that both the Cry3Bb1.11098 and NPTII proteins produced in event MON 863 have equivalent molecular weights and antigenic properties with these same proteins produced in *B.t.* and *E. coli* respectively. ACCEPTABLE	451568-03 454240-05
85.11	Product Characterization (Protein Equivalence)	This report compares the physical (MW, N-terminal sequencing) and functional (bioassay) characteristics of Cry3Bb1.11098 and Cry3Bb1.11231 proteins produced in *E. coli* and CRW protected corn. The data provided show that the proteins have equivalent molecular weight, immunological reactivities, N-terminal sequences and comparable LC_{50} values. This data supports the determination of the equivalence of the bacteria- and plant-produced proteins, and the use of the bacterially-produced proteins to support registration of the CRW corn product. CLASSIFICATION: Acceptable.	454240-04

GUIDELINE NO	STUDY	RESULTS	MRID NO.
885.11	Product Characterization (Protein Equivalence)	Two genetic variants designated as *cry3Bb1.11098* and *cry3Bb1.11231* produce the -endotoxin proteins Cry3Bb1.11098 and Cry3Bb1.11231, respectively. Cry3Bb1.11098 differs from the wild type *B.t.* protein by 5 amino acids, while the Cry3Bb1.11231 protein differs by 4 amino acids. The *cry3Bb1.11098* gene was used to develop maize line MON 863 and variant *cry3Bb1.11231* was used in the development of MON 853 for control of the corn rootworm complex. Further manipulations during cloning and insertion into the maize genome brings the total amino acid differences for these two transformants to seven and five for the 11098 (MON 863) and 11231 (MON 853) Cry3Bb1 proteins, respectively. Cry3Bb1 protein was purified from event MON 863 grain by immunoaffinity chromatography and then analyzed by N-terminal sequencing and MALDI-TOF. Trypsin fragments subjected to MALDI-TOF / MS provided for identification or verification of 38 % of the total protein by mass matching when coupled with sequencing of 29 N-terminal amino acids. Data from MALDI-TOF / MS and N–terminal sequencing indicate that the deduced amino acid sequences of Cry3Bb1.11098, as present in MON 863 and in *B.t.* strain EG11098, are accurate. A comparison of functionality and physicochemical characteristics strongly suggests that the two protein variants are nearly equivalent. Proteins from the fermentation of *B.t.* strains EG11098 and EG11231 were used for mammalian and ecotoxicology studies as well as in assays relying on immunorecognition of proteins. These proteins are considered as biologically suitable for these studies based upon structural data indicating only minor changes in the shape of the -endotoxin proteins. Classification: Acceptable.	454240-10
885.11	Product Characterization (Amino Acid Sequencing)	Transformation event MON 863 (maize) produces the 74 kDa Cry3Bb1.11098 protein for control of the corn rootworm complex. Modifications to this protein for expression *in planta* bring the differences between the wild type and MON 863 expressed variant to seven amino acids. Grain from event MON 863 was used as a source of Cry3Bb1.11098 protein for MALDI-TOF / MS and N-terminal sequence analyses. Of the 653 amino acids present in the 74 kDa form of the Cry3bb1 protein, 225 were identifiable as to position based upon mass matching. Three fragments from the N–terminal region of the protein were also among those matched, representing 43 amino acids. One fragment included the N–terminus indicating the loss of the terminal methionine and the acetylation of the alanine added at position two. This potentially explains the difficulty in sequencing the N-terminus of the 66 kDa form of the protein eluted from PVDF blots. Protein samples obtained from elution off of PVDF membranes of both the 74 kDa and 66 kDa proteins were subjected to Edman degradation chemistry, but the larger peptide revealed no sequence data, presumably due to blockage of the terminal amino acid residue. When the bacterially produced version of this protein was subjected to N–terminal sequencing procedures, N–terminal sequence data was obtained successfully. The presumed reason for this rests with the post-translational modifications that are typical of eukaryotes (*e.g.*, plants) which are lacking in prokaryotes (*e.g.*, bacteria). Such modification could explain the blockage noted during the attempt to sequence the N-terminus of the corn-derived Cry3Bb1.11098 protein. Classification: Acceptable.	454240-11

GUIDELINE NO	STUDY	RESULTS	MRID NO.
885.11	Product Characterization (Protein Equivalence)	MALDI-TOF analysis of the microbial and corn Cry3B1.11098(Q349R) proteins yielded an agreement of from 42 to 50 amino acid fragments predicted from the theoretical sequence. The N-terminus of the microbial form lacked the terminal methionine which is commonly cleaved in expressed proteins. The corn form was apparently not only lacking the terminal methionine but the N-terminal alanine residue was acetylated as indicated by a 42 Dalton greater weight. The N-terminal amino acid sequence analyses were flawed in that unequivocal determinations were not possible due to the presence of multiple residues in most cycles. However, by comparison to the expected sequences, several different start sites for N-terminal sequencing could be detected. In the *E. coli* Cry3Bb1, the sequence started at both position 2 and 32. In the corn Cry3Bb1, three different starts were detected at position 19, 25 and 36. The immunoblot analysis gave similar positive band patterns that indicated the Cry3Bb1 protein produced in both corn and *E. coli* had essentially the same electrophoretic mobility and immunoreactivity. The positive bands themselves were sometimes rather broad (74-66kDa) but no series of distinct bands could be discerned from the photographs provided. The molecular weight and purity analyses for the corn and microbial extracts indicate that the microbially produced samples were nearly twofold higher purity in Cry3Bb1 proteins compared to the corn extracts. The purity for Cry3Bb1 was 92.6% and 53.9% for microbial and corn extracts, respectively. Total protein concentrations for the two extracts were determined as 0.58 mg/ml and 0.46 mg/ml for microbial and corn extracts, respectively, by colormetric assays. The glycosylation analysis for the Cry3Bb1 extracts gave no positive carbohydrate staining regions for either the microbial or corn samples in the expected regions for Cry3Bb1 protein. The results of the bioassays for the two Cry3Bb1extracts against Colorado potato beetle larvae (table 5 attached) indicate that there was a dose/response in all tests and the LC_{50} values were similar and had overlapping 95% confidence intervals. Classification: Acceptable.	455382-01

GUIDELINE NO	STUDY	RESULTS	MRID NO.
885.11	Product Characterization (Protein Levels)	The protein titer data provided for MON860 and MON853 shows the ranges of Cry3Bb1 protein in various parts of the plant, as well as geographical variation. Overall, based upon the ranges provided, there appears to be significant variation between the samples analyzed on different days post-planting and at different sites. The registrant mentions a potential difference between decreasing titer in MON 853 and mid-season increasing titer in MON 860. However, such a determination cannot be made based upon the data provided in this submission. Even if such a trend was supported by additional data for MON 860, the difference in the protein titers is much smaller than the variation seen for MON 853 on days 44, 55 and 100 post-planting. Ranges of Cry3Bb1 protein levels in MON853 in microgram Cry3bb1 protein per gram of fresh weight tissue were 7.01 - 68.98 (leaf), 1.66 -17.64 (root), and 1.23 - 29.06 (above ground whole plant). Ranges of Cry3Bb1 protein levels in MON860 in microgram Cry3bb1 protein per gram of fresh weight tissue were 32.61-91.11 (leaf), 2.24 -10.33 (root), and 0.63-13.95 (above ground whole plant). ACCEPTABLE	449043-02
885.11	Product Characterization (Protein Levels)	The protein titer data provided shows the ranges of Cry3Bb1 protein in various parts of the plant, as well as geographical variation. Overall, based upon the ranges provided, there appears to be significant variation between the samples analyzed on different days post-planting and at different sites. Ranges of Cry3Bb1 protein levels in MON863 in microgram Cry3bb1 protein per gram of fresh weight tissue were 30-93 (leaf), 49-86 (grain), 30-93 (pollen), 3.2-66 (root), and 13-54 (above ground whole plant). CLASSIFICATION: Acceptable.	454240-01 451568-02
885.11	Product Characterization (Protein Levels)	The levels of proteins Cry3Bb1 and NPTII were determined in tissues of transgenic corn MON 863 generated in five 2003 U.S. field trials. The two proteins were quantitated by validated enzyme-linked immunosorbent assays (ELISA) and presented in terms of dry tissue weight (dwt, µg/g). Although there were some data shortcomings (several samples were analyzed outside the demonstrated storage stability time frame), the results are unlikely to be compromised because the samples were stored at -80° C. Furthermore, the results were found consistent with previously submitted tissue expression data (from MON 863 corn grown in 1999 field trials). The highest Cry3Bb1 levels were in leaf, whole plant, and root samples, which had, respectively, mean levels of 180-240, 130-340, and 140-290 µg/g dwt. Levels of Cry3Bb1 tended to be lower at the V10-V12 growth stage in whole plants and roots than for the earlier stages (V2-V3, V4-V5, V6-V7). Forage and forage root had similar Cry3Bb1 levels (mean of 55 and 80 µg/g dwt, respectively), and the lowest Cry3Bb1 levels were in pollen, stover, senescent root, and grain (mean of 20-35 µg/g dwt). NPTII protein was only measured in grain, where it was not detected. Protein expression levels of Cry3Bb1 and NPTII have been established at various stages of development. CLASSIFICATION: ACCEPTABLE.	464799-02
885.11	Product Characterization (Protein Levels) **YieldGard Plus**	The hybrid maize resulting from the MON 863 x MON 810 cross represents the test substance for this study. This dual trait or stacked trait hybrid (also known as MT-02) results from the	457917-02

GUIDELINE NO	STUDY	RESULTS	MRID NO.
	Cry3Bb1, Cry1Ab and NPTII protein levels in the dual trait maize hybrid MON 863 x MON 810 produced in Argentina field trials conducted during the 1999-2000 growing season.	contribution of the *cry3Bb1* and *nptII* genes from MON 863 and the *cry1Ab* gene from MON 810. The Cry3Bb1 protein from MON 863 is also known as Cry3Bb1.11098, but will be referred to in this review as simply Cry3Bb1. Reference protein Cry3Bb1standard for the ELISA was produced by fermentation of *Bacillus thuringiensis* encoding Cry3Bb1.11231 and stored as a lyophilized powder. Cry1Ab protein, produced in *E. coli*, was trypsinized and established as the reference standard. The coding sequence for *nptII* was cloned and transferred to *E. coli* for fermentation. Four field sites within the province of Buenos Aires (Argentina) were used to culture plants for sample generation: Fontezuela 1, Fontezuela 2, Salto and Rojas. Young leaves, pollen, mature root, over-season root, grain and forage samples were taken for ELISA analysis. For each protein, a validated double antibody sandwich ELISA has been developed. Antibodies to these proteins were raised in rabbits and Protein A selected. The trypsinized core of Cry1Ab was used rather than the full length protein for antibody generation. For Cry3Bb1 and NPTII protein levels, a tissue specific correction factor was used to optimize the accuracy and reduce bias in the estimation of protein content of samples based upon extraction efficiency and recovery. Samples for forage, grain and root analysis of Cry3Bb1 protein content were stored longer than their intended period of known stability in some instances. An increase in apparent Cry3Bb1 protein levels resulted from the estimated 10 to 20 % instability evidenced in these samples stored beyond their intended dates of analysis. The net result is that the Cry3Bb1 levels reported may be overestimated by as much as 20 %. Data reported from forage samples were not adjusted as presented for this instability factor. Root tissues were estimated to exhibit a 20 to 30 % instability which resulted in a decrease in apparent Cry3Bb1 levels in roots. Hence, the Cry3Bb1 levels reported may be underestimated by up to 30 %. Data from grain samples were estimated to be overestimated by up to 20 % due to instability in stored samples. The reported root and grain data were not adjusted, as reported, for this instability factor. Data regarding the stability or instability of extracts stored beyond their intended expiration dates were not presented. The ranges of protein levels observed for Cry3Bb1, Cry1Ab and NPTII were similar across all four sites for the MON 863 x MON 810 hybrid and the MON 863 or MON 810 hybrids as appropriate for the transgene in question. Averages for the Cry3Bb1 and Cry1Ab proteins were, however, somewhat higher in the MON 863 x MON 810 hybrid versus the single trait hybrids. NPTII protein levels and ranges in the MON 863 x MON 810 hybrid and the MON 863 hybrid were similar. The highest levels of expression of Cry3Bb1 or Cry1Ab were found to occur in pollen for Cry3Bb1 (79.6 g/g FWT) and in leaf (17.9 g/g FWT) for Cry1Ab. CLASSIFICATION: ACCEPTABLE	
885.11	Product Characterization (Protein Levels) Cry3Bb1 and CP4 EPSPS Protein Levels in Corn Tissues Collected from **MON 88017** Corn Produced in U.S. Field Trials conducted in 2002	Tissues samples were collected at various times throughout the growing season from MON 88017 corn grown in U.S. field trials at three field sites in 2002 and were analyzed for Cry3Bb1 and CP4 EPSPS protein levels using validated ELISA methods. The mean Cry3Bb1 levels across three field sites for leaf, whole plant and root tissue harvested throughout the growing season ranged from 260-570, 220-500 and 100-370 µg/g dwt, respectively. The mean protein levels for pollen, forage, silk, grain, and stover tissue were 25, 95, 380, 15, and 88 µg/g dwt, respectively. The level of	461817-03

GUIDELINE NO	STUDY	RESULTS	MRID NO.
		Cry3Bb1 protein in tissue samples from the control hybrid H1200902 were below the LOQ or LOD for each tissue assay. The mean CP4 EPSPS protein levels for leaf and root tissues throughout the growing season ranged from 150-220 and 70-150 µg/g dwt, respectively. The mean CP4 EPSPS protein levels for pollen, forage and grain tissue were 390, 57, and 5.8 µg/g dwt, respectively. CP4 EPSPS protein levels were not assessed in whole plant or silk tissue. The level of CP4 EPSPS protein in tissue samples from the control hybrid H1200902 were below the LOQ or LOD for each tissue assay, with the exception of one OSL-2 (overseason leaf) tissue sample, which gave an unexpected positive result of 150 µg/g dwt. Therefore, these data establish the protein levels of Cry3Bb1 and CP4 EPSPS proteins on a fresh weight and dry weight basis in the various tissues throughout the growing season. Summaries of the ELISA method validation results were presented for accuracy, matrix interferences and parallelism, precision, and sensitivity. The extraction efficiency, spike and recovery values are within their respective acceptance criteria. No matrix interferences were detected when the sample extracts were analyzed. The plant-produced Cry3Bb1 protein and the E. coli-produced Cry3Bb1 met the parallelism acceptance criteria, indicating immunological equivalence between the two proteins. The inter- and intra-assay precision were accessed and the coefficients of variation were no more than 21%. The limit of detection and limit of quantification for the Cry3Bb1 assay ranged from 0.0073 to 0.032 µg/g fwt and from 0.039 to 0.051 µg/g fwt, respectively. The limit of detection and limit of quantification for the CP4 EPSPS assay ranged from 0.050 to 0.18 and from 0.10 µg/g fwt to 0.28 µg/g fwt, respectively. Although the protein levels were reported less than or equal to the LOD and LOQ on a fresh weight basis, the LOD and LOQ values were not reported on a dry weight basis. However, the low value of the LODs and LOQs for both assays indicated optimal sensitivity. CLASSIFICATION: ACCEPTABLE	

GUIDELINE NO	STUDY	RESULTS	MRID NO.
	Product Characterization (Protein Levels) Cry3Bb1 and CP4 EPSPS Protein Levels in Corn Tissues Collected from MON 88017 x MON 810 Corn Produced in U.S. Field Trials conducted in 2002	The levels of Cry3Bb1, Cry1Ab and CP4 EPSPS proteins in corn tissues of MON 88017 x MON 810 were assessed by enzyme-linked immunosorbent assay (ELISA) in samples collected from field trails conducted at three sites (IA, IL, and NE) in the U.S. during 2002. Overseason leaf, overseason whole plant, overseason root, pollen, silk, forage, forage root, grain, stover and senescent root tissues were collected from each replicated plot at all field sites. The levels of Cry3Bb1 and Cry1Ab protein in tissue samples from the control hybrid H1200902 were below the LOQ or LOD for each tissue assay. The mean Cry3Bb1 protein levels in MON 88017 x MON 810 corn for root tissue harvested at the V2-V3 stage ranged from 140 - 350 µg/g dwt. The mean Cry3Bb1 protein levels for leaf, pollen, grain and forage tissues were 670, 27, 9.3, and 100 µg/g dwt, respectively. The mean CP4 EPSPS protein levels in MON 88017 x MON corn for grain and forage tissues were 4.3 and 51 µg/g dwt, respectively. Finally, the mean Cry1Ab protein levels in MON 88017 x MON 810 corn for leaf, grain, and forage tissues were 110, 0.39, and 14 µg/g dwt, respectively. The mean Cry1Ab protein level for pollen was below the limit of detection (LOD, 0.090 µg/g fwt). Therefore, the results demonstrate the range and mean Cry3Bb1 and Cry1Ab expression levels in each tissue of MON 88107 x MON 810 were similar to the corresponding levels in the single-trait events MON 88017 and MON 810, respectively. Finally, the levels of the inert ingredient CP4 EPSPS protein produced in MON 88017 x MON 810 were similar to the levels observed for the same CP4 EPSPS protein produced in MON 88017. Summaries of the ELISA method validation results were also presented for accuracy, matrix interferences and parallelism, precision, and sensitivity. The extraction efficiency, spike and recovery values are within their respective acceptance criteria. No matrix interferences were detected when the sample extracts were analyzed. The plant- and E. coli-produced Cry3Bb1 proteins met the parallelism acceptance criteria, indicating immunologically equivalence between the two proteins. Matrix interferences and parallelism data were not presented for Cry1Ab. The inter- and intra-assay precision were accessed and the coefficient of variations were no more than 21%. The limit of detection (LOD) and limit of quantification (LOQ) for the Cry3Bb1 assay ranged from 0.0073 to 0.032 µg/g fwt and from 0.039 to 0.051 µg/g fwt, respectively. The LOD and LOQ for the CP4 EPSPS assay ranged from 0.050 to 0.18 µg/g fwt and from 0.10 to 0.28 µg/g fwt, respectively. Finally, the LOD and LOQ for the Cry1Ab assay ranged from 0.090 to 1.1 µg/g fwt and from 0.13 to 2.0 µg/g fwt, respectively. CLASSIFICATION: ACCEPTABLE	461850-03
885.11	Protein Characterization and Human Health Assessment **MON 88017**	This report is a summary of product characterization studies and the human health assessment for the plant-incorporated protectant *Bacillus thuringiensis* (*B.t.*) Cry3Bb1 protein produced in MON 88017 and also includes the detailed protein characterization data for Cry3Bb1 produced in MON 88017. The human health	461817-01

GUIDELINE NO	STUDY	RESULTS	MRID NO.
		assessment is based on the substantial similarity of the Cry3Bb1 protein produced in MON 88017 to the currently registered Cry3Bb1 protein produced in MON 863 (EPA Reg. No. 524-528). The Cry3Bb1 protein produced in MON 88017 was isolated and purified from corn grain and characterized to determine its immunoreactivity, amino acid sequence, molecular weight, glycosylation status, and functional activity based on insect bioassay. Field Efficacy: Field efficacy was estimated by artificially infesting plots of MON 88017 and MON 863 corn with WCRW during 2002 at seven locations (in IA, IL, NE, and SD) and 2003 at five locations (in IA and IL). Root damage ratings (RDR) were statistically evaluated and MON 88017 and MON 863 were statistically similar across all sites (RDR of 0.12) compared to the non-transgenic hybrid control RDR of 1.47, thus, indicating equivalence for field efficacy between the two Cry3Bb1 protein variants against corn rootworm larvae. Immunoreactivity/ Western Blot: Previous characterization studies conducted with other Cry3Bb1 protein variants have shown that the full-length Cry3Bb1 protein with a predicted molecular weight of 74.4 kDa, when processed in the corn plant, produces variants with molecular weights of ~66 and ~55 kDa. For MON 88017, four immunoreactive bands migrating at approximately 77, 65, 55, and 46 kDa were observed for the plant- produced Cry3Bb1 protein sample. The ~55 kDa peptide was attributed to the *B.t.* tryptic core protein (Schnepf et al., 1998) and the 46 kDa band was considered a degradation product and not further analyzed due to its smaller size. The immunoreactivity between the *E. coli*- and plant-produced Cry3Bb1 proteins were also evaluated by densitometric analysis of the bands for different exposures (15 and 30 secs) of the Western blot and showed both proteins were >90% identical. Amino Acid Sequence/ MALDI-TOF: The three bands were also analyzed separately by MALDI-TOF mass spectrometry and produced 20, 20, and 15 peptide mass fragments, respectively, which were matched to the theoretical tryptic peptide mass map for the Cry3Bb1 protein. These masses were used to assemble a combined coverage map and 44% confirmation of the anticipated amino acid sequence was observed. The presence of the expected amino acid differences from the sequence of the wild-type Cry3Bb1 at positions 232, 312, 314, 318, and 349 were confirmed as well as the single amino acid difference at position 166 between the Cry3Bb1 proteins produced in MON 88017 and MON 863. The Cry3Bb1 variants in MON 88017 and MON 863 each contain 653 amino acids and share an amino acid sequence identity of 99.8%. The only difference occurs at position 166 in MON 863, where glycine is present instead of aspartic acid. (See Table 1.) Molecular Weight and Purity: The molecular weight and purity of the Cry3Bb1 protein produced in MON 88017 was determined using densitometric analysis of a Colloidal Brilliant Blue G stained SDS-PAGE gel. The full-length Cry3Bb1 protein in the plant-produced sample had an estimated average molecular weight of 77.2 kDa; whereas the molecular weight of the full-length *E. coli* produced Cry3Bb1 protein was estimated to be 77.7 kDa. The	

GUIDELINE NO	STUDY	RESULTS	MRID NO.
		difference in molecular weight between the plant- and *E. coli*-produced Cry3Bb1 proteins was 1%. The average molecular weights of the additional Cry3Bb1 protein fragments were estimated to be 66.2 and 55.4 KDa. The purity value for the plant-produced Cry3Bb1 protein was determined to be 66.1%. The Cry3Bb1 protein produced in MON 88017 was also analyzed for covalently bound carbohydrate moieties. Glycosylation: No carbohydrate positive band was observed in the plant-produced Cry3Bb1 sample and therefore, no post-translational glycosylation of the Cry3Bb1 protein had occurred, when expressed in corn. Structural Similarity: The structure of the wild-type Cry3Bb1 protein has been determined at a resolution of 2.4 Å by X-ray crystallographic analysis (Galitsky et al., 2001). Results showed that the Cry3Bb1 protein has three distinct regions: Domain I (residues 64-294) consisting of a seven-helix bundle which forms a left-handed helix surrounding a central helix; Domain II (residues 295-503) consists of three antiparallel β-sheets; and Domain III (residues 504-652) has a sandwich structure with two antiparallel β-sheets. X-ray crystallographic analyses of Cry3Bb1 protein variants showed only minor changes to the basic tertiary structure as a result of the amino acid substitutions (Described in Table 4, Astwood et al., 2001, MRID No. 454240-09). For the Cry3Bb1 protein produced in MON 88017, the tertiary structure of this protein is expected to be same as that of the wild-type and other Cry3Bb1 protein variants due to the minor number and types of amino acid substitutions. CLASSIFICATION: ACCEPTABLE	
885.11	Product Characterization **MON 88017** Functional Equivalence Evaluation of the Product Performance, Efficacy and Biological Equivalency of Two Cry3Bb1 Protein Variants against Susceptible Coleopteran Species	The functional equivalence of the two Cry3Bb1 variants (Cry3Bb1.11098(Q349R) produced in MON 863 and (Cry3Bb1.pvzmir39) produced in MON 88017 was evaluated by comparing their biological activity against two susceptible species of insects, larvae of the Colorado potato beetle (CPB), Leptinotarsa decemlineata and larvae of the Western corn rootworm (WCRW), Diabrotica virgifera virgifera. The biological activity of the two Cry3Bb1 protein variants against CPB or WCRM larvae was determined by a diet-incorporation insect bioassay, which measured the LC50 values. The mean LC50's for CPB larvae were 0.95 µg/mL (ranging from 0.79-1.11 µg/mL) of diet for the Cry3Bb1.11098(Q349R) protein and 0.84 µg/mL of diet (ranging from 0.64- 1.06 µg/mL) for the Cry3Bb1.pvzmir39 protein. For WCRW larvae, the mean LC50's were 100 µg/mL of diet (ranging from 73.2-137 µg/mL) for the Cry3Bb1.11098(Q349R) protein and 139 µg/mL of diet (ranging from 74.6 - 231 µg/mL) for the Cry3Bb1.pvzmir39 protein. Therefore, both CPB and WCRW bioassays indicate that there are no significant differences in the biological activity between the two protein variants Cry3Bb1.11098(Q349R) and Cry3Bb1.pvzmir39 against the susceptible coleopteran insect species tested. Furthermore, the two protein variants are functionally equivalent in biological activity against the CPB and WCRW. CLASSIFICATION: ACCEPTABLE	461817-04

GUIDELINE NO	STUDY	RESULTS	MRID NO.
885.11	Product Characterization and Human Health Assessment of the Plant-Incorporated Protectant B.t. Cry3Bb1 and Cry1Ab proteins produced in **MON 88017 x MON 810** corn	Summaries of the confirmation of the identity of MON 88017 x MON 810 by Southern blot analysis and the levels of Cry3Bb1, Cry1Ab and CP4 EPSPS proteins in corn tissues of MON 88017 x MON 810 were assessed. More detailed descriptions of these results can be found in MRID Nos. 461850-02 and 461850-03.	461850-01
885.11	Product Characterization (Agronomic Performance)	The data included in this submission appear to support the agronomic equivalency of corn event MON 863 hybrids. Results of the study show that there are some differences in the properties of the transgenic plants versus the control lines used in the tests. Some of the variation identified included a variety of differences in corn ear height, plant height, weight, grain moisture and yield, but in each case, the difference was small. However, based upon the data provided, it appears that none of these differences would have a significant agronomic impact on the crops and are likely similar to typical differences seen in different plant lines and/or those differences caused by differing ecological effects.	453484-03
860.134	Validated Method for Extraction and Direct ELISA Analysis of Cry3Bb1in Corn Grain	The extraction and detection method as described for Cry3Bb1 protein appears to be adequate for analysis of Cry3Bb1 protein in corn grain. However, this method must be validated by both an independent laboratory and the EPA Biological and Economic Analysis Division laboratory before it can be considered a valid method. Acceptable, pending validation as described.	453731-01
860.134	Validated Method for Extraction and Direct ELISA Analysis of Cry3Bb1in Corn Grain	The *TraitChek*™ Cry3Bb lateral flow strip test and the *SeedChek*™ Cry3Bb enzyme-linked immunosorbent assay (ELISA) were evaluated to verify performance as qualitative detection kits for Cry3Bb protein in corn seed and leaves by an independent, third party laboratory (Strategic Diagnostics, Inc.). The *TraitChek*™ strip uses an immunochromatographic format with colloidal gold conjugated antibody, while the *SeedChek*™ test is a simultaneous dual monoclonal antibody ELISA. CLASSIFICATION: ACCEPTABLE- Satisfies the EPA Residue Chemistry Guidelines OPPTS 860.1340(c)(6) Residue Analytical methods and PR Notice 96-1. However, the EPA's Analytical Method Laboratory located in Fort Meade (Maryland) will have to independently validate Monsanto's lateral flow strip test and ELISA protocol for accuracy, precision, and sensitivity.	463942-01

Comparison of Cry1Ab and Cry3Bb1 protein levels in single trait hybrids and YieldGard® Plus Corn

	Average Cry1Ab Protein Levels (µg/g fwt) (Range)		Average Cry3Bb1 Protein Levels (µg/g fwt) (Range)	
Tissue Type and Collection Time (Days Post-Planting)	**MON 863 x MON 810**	**MON 810**	**MON 863 x MON 810**	**MON 863**
Young Leaf (≈18)	**17.9 (14.1 - 27.5)**	**13.0 (9.8 - 15.4)**	**46.7 (35.5 - 53.2)**	**30.0 (21.3 - 47.2)**
Forage (≈90)	**7.9 (3.9 - 11.9)**	**5.6 (3.0 - 8.2)**	**23.6 (6.7 - 39.7)**	**12.8 (<0.22 - 28.8)**
Grain (≈117)	**0.84 (0.63 - 1.2)**	**0.46 (0.24 - 0.77)**	**61.1 (38.5 - 83.1)**	**43.7 (<0.096 - 84.1)**
Pollen (≈60)	**<0.08 (<0.08 - 0.18)**	**<0.08 (<0.08)**	**79.6 (65.1 - 96.5)**	**60.4 (29.7 - 90.7)**
Mature Root (≈90)	**N/A**	**N/A**	**19.7 (6.0 - 41.7)**	**16.2 (<0.76 - 49.8)**
Over-season Root (≈46)	**N/A**	**N/A**	**22.0 (N/A)**	**20.0 (N/A)**

Cry3Bb1 and Cry1Ab protein levels in MON 88017 & MON 88017 x MON 810 tissues.

Tissue type	**Growth stage**	**Cry3Bb1 Protein Levels (µg/g dwt)**	**Cry1Ab Protein Levels (µg/g dwt)**
		MON 88107 x MON 810 **Mean (SD)** **[Range], n=9**	**MON 88017** **Mean (SD)** **[Range], n=9** **MON 88017 x MON 810** **Mean (SD)** **[Range], n=9** **MON 810** **Mean (SD)** **[Range], n=9**
Young leaf	V2-V3	670 (130) [550-920]	570 (170) [230-820] 110 (17) [85-140] 100 (12) [89-130]

Young root	V2-V3	350 (150) [88-560]	370(80) [240-510] ND ND
Pollen	R1	27 (5.7) [NA-34]	25 (4.2) [17-32] NA NA
Forage	R4-R6 (early dent)	100 (23) [71-150]	95 (19) [75-130] 14 (2.1) [11-17] 14(3.4) [8.4-19]
Forage root	R4-R6 (early dent)	140 (29) [89-180]	130 (29) [98-170] ND ND
Grain	R6	9.3 (3.4) [3.9-13]	15 (3.6) [10-22] 0.39 (0.13) [0.16-0.63] 0.43 (0.091) [0.27-0.54]

ND=not determined; NA=not applicable, as levels were below the level of detection.
Data from pg. 14, MRID #46185001

B. Human Health Assessment

1. Background

The basic premise relied on for the toxicology assessment is the fact that all the *Bt* plant-incorporated protectants are proteins. Proteins are commonly found in the diet and, except for a few well described phenomena, present little risk as a mammalian hazard.

Several types of data are required for the *Bt* plant-incorporated protectants to provide a reasonable certainty that no harm will result from the aggregate exposure to these proteins. The information is intended to show that the *Bt* protein behaves as would be expected of a dietary protein, is not structurally related to any known food allergen or protein toxin, and does not display any oral toxicity when administered at high doses. These data consist of an *in vitro*

digestion assay, amino acid sequence homology comparisons and an acute oral toxicity test. The acute oral toxicity test is done at a maximum hazard dose using purified protein of the plant-incorporated protectant as a test substance. Due to limitations of obtaining sufficient quantities of pure protein test substance from the plant itself, an alternative production source of the protein is often used such as the *Bacillus thuringiensis* source organism or an industrial fermentation microbe. The justification for employing this alternative source of pure protein is the equivalence data discussed above under product characterization.

EPA believes that protein instability in digestive fluids and the lack of adverse effects using the maximum hazard dose approach in general eliminate the need for longer-term testing of *Bt* protein plant-incorporated protectants. Dosing of these animals with the maximum hazard dose, along with the product characterization data should identify potential toxins and allergens, and provide an effective means to determine the safety of these protein. The adequacy of the current testing requirements was discussed at the June 7, 2000 Scientific Advisory Panel (SAP) meeting. In their final report, the SAP agreed in principle with the methods used by EPA to assess the toxicity of proteins expressed in plants especially the maximum hazard dose approach.

a. In vitro Digestibility Assay

The intent of this assay is to demonstrate that the *Bt* protein is degraded into small peptides or amino acids in solutions that mimic digestive fluids. Usually only gastric fluid is tested since Cry protein is known to be stable in intestinal fluid. In order to track the breakdown, the proteins were added to a solution of the digestive fluids and a sample was either removed or quenched at given time points (usually at time 0, one to several minutes later and one hour later). The time point samples were then electrophoresed on either an SDS-PAGE gel and further analyzed by western blot or tested in a bioassay against the target pest.

As has been stated in several public fora, the *in vitro* digestibility test is basically a test to confirm the biochemical characteristic of instability of the protein in the presence of digestive fluids. The digestibility test is not intended to provide information on the toxicity of the protein or imply that similar breakdown will happen in all human digestive systems. The *in vitro* digestibility assay may also provide information about the potential of a protein to be a food allergen. The *in vitro* digestion assays confirm that the protein is being broken down in the presence of typical digestive fluids and is not unusually persistent in the digestive system. One of the limitations of the test is that it usually only tracks protein breakdown to fragments still recognized by the immunological reagents employed.

b. Amino Acid Homology

An additional characteristic that is considered as an indication of possible relation to a food allergen are a protein's amino acid sequence when compared to known food allergens.

c. Acute Oral Toxicity

One of the bases for addressing the toxicity of proteins primarily through the use of acute oral toxicity is that, when demonstrated to be toxic, proteins are toxic at low doses (Sjoblad, *et al*., 1992). Therefore, when no effects are shown to be caused by the protein plant-incorporated protectants, even at relatively high dose levels in the acute oral exposure, the proteins are not considered toxic.

2. Cry3Bb1 Revised Assessment

Pursuant to section 408(b)(2)(D) of 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.

Data have been submitted demonstrating the lack of mammalian toxicity at high levels of exposure to the pure Cry3Bb1 proteins. These data demonstrate the safety of the productsat levels well above maximum possible exposure levels that are reasonably anticipated in the crops. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.740(b)(2)(i)). For microbial products, further toxicity testing and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study, to verify the observed effects and clarify the source of these effects (Tiers II and III).

Three acute oral studies were submitted for Cry3Bb1 proteins. These studies were done with three variants of the Cry3Bb1 protein engineered with either four or five internal amino acid sequence changes to enhance activity against the corn rootworm. The acute oral toxicity data submitted support the prediction that the Cry3Bb1 protein would be non-toxic to humans. Male and female mice (10 of each) were dosed with 36, 396, or 3,780 milligrams/kilograms bodyweight (mg/kg bwt) of Cry3Bb1 protein for one variant. The mice were dosed with 38.7, 419, or 2,980 mg/kg bwt of Cry3Bb1 protein for the second variant. The mice were dosed with 300, 900, or 2,700 mg/kg bwt of Cry3Bb1 protein for the third variant. In one study, two animals in the high dose group died within a day of dosing. These animals both had signs of trauma probably due to dose administration (i.e., lung perforation or severe discoloration of lung, stomach, brain and small intestine). No clinical signs were observed in the surviving

animals and body weight gains were recorded throughout the 14-day study for the remaining animals. Gross necropsies performed at the end of the study indicated no findings of toxicity attributed to exposure to the test substance in any of the three studies. No other mortality or clinical signs attributed to the test substance were noted during either study.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, Roy D., et al. ``Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)). Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry3Bb1 proteins are not considered toxic. Further, amino acid sequence comparisons showed no similarity between Cry3Bb1 proteins to known toxic proteins available in public protein data bases.

Since Cry3Bb1 are proteins, allergenic sensitivities were considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by acid and proteases, may be glycosylated and present at high concentrations in the food.

Data have been submitted that demonstrate that the Cry3Bb1 protein is rapidly degraded by gastric fluid in vitro. In a solution of simulated gastric fluid (pH 1.2 - U.S. Pharmacopeia), complete degradation of detectable Cry3Bb1 protein occurred within 30 seconds. Insect bioassay data indicated that the protein loss insecticidal activity within 2 minutes of incubation in SGF. Incubation in simulated intestinal fluid resulted in a~59 kDa protein digestion product. A comparison of amino acid sequences of known allergens uncovered no evidence of any homology with Cry3Bb1, even at the level of 8 contiguous amino acids residues.

The potential for the Cry3Bb1 proteins to be food allergens is minimal. Regarding toxicity to the immune system, the acute oral toxicity data submitted support the prediction that the Cry3Bb1 proteins would be non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, Roy D., et al. ``Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)). Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry3Bb1 proteins are not considered toxic.

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 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. Oral exposure, at very low levels, may occur from ingestion of processed corn products and, potentially, drinking water. However a lack of mammalian toxicity and the digestibility of the plant-incorporated protectants have been demonstrated. The use sites for the Cry3Bb1 proteins are all agricultural for control of insects. Therefore, exposure via residential or lawn use to infants and children is not expected. Even if negligible exposure should occur, the Agency concludes that such exposure would present no risk due to the lack of toxicity demonstrated for the Cry3Bb1 proteins.

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 to these plant-incorporated protectants, we conclude that there are no cumulative effects for the Cry3Bb1 proteins.

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 Cry3Bb1 proteins include the characterization of the expressed Cry3Bb1 protein in corn, as well as the acute oral toxicity, and in vitro digestibility of the proteins. The results of these studies were determined applicable to evaluate human risk and the validity, completeness, and reliability of the available data from the studies were considered.

Adequate information was submitted to show that the Cry3Bb1 test material derived from microbial cultures was biochemically and functionally similar to the protein produced by the plant-incorporated protectant ingredients in corn. Production of microbially produced protein was chosen in order to obtain sufficient material for testing.

The acute oral toxicity data submitted supports the prediction that the Cry3Bb1 proteins would be non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, Roy D., et al. ``Toxicological Considerations for Protein

Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)). Since no effects were shown to be caused by Cry3Bb1, even at relatively high dose levels (3,780 mg Cry3Bb1/kg bwt), the Cry3Bb1 proteins are not considered toxic. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial Bacillus thuringiensis products from which this plant-incorporated protectant was derived. See 40 CFR 158.740(b)(2)(i). For microbial products, further toxicity testing and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study to verify the observed effects and clarify the source of these effects (Tiers II and III).

Cry3Bb1 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.

Both available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children); and safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food additives, are generally recognized as appropriate for the use of animal experimentation data were not evaluated. The lack of mammalian toxicity at high levels of exposure to the Cry3Bb1 proteins demonstrate the safety of the product at levels well above possible maximum exposure levels anticipated in the crop.

The genetic material necessary for the production of the plant-incorporated protectants active ingredients are the nucleic acids (DNA, RNA) which comprise genetic material encoding these proteins and their regulatory regions. ``Regulatory regions'' are the genetic material, such as promoters, terminators, and enhancers, that control the expression of the genetic material encoding the proteins. DNA and RNA are common to all forms of plant and animal life and the Agency knows of no instance where these nucleic acids have been associated with toxic effects related to their consumption as a component of food. These ubiquitous nucleic acids, as they appear in the subject active ingredient, have been adequately characterized by the applicant. Therefore, no mammalian toxicity is anticipated from dietary exposure to the genetic material necessary for the production of the subject active plant pesticidal ingredients.

B. Infants and Children Risk Conclusions

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

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

In this instance, based on all the available information, the Agency concludes that there is a finding of no toxicity for the Cry3Bb1 proteins and the genetic material necessary for their production. Thus, there are no threshold effects of concern and, as a result, the Agency concludes there is no need to apply a tenfold margin of safety for infants and children and that not doing so will be safe for infants and children. Further, because of the Agency's no toxicity findings, the provisions requiring the Agency to assess the risk of the pesticide chemical residue to infants and children based on consumption patterns, special susceptibility, and cumulative effects are rendered moot.

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 Cry3Bb1 proteins and the genetic material necessary for their production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information.

The Agency has arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed for the plant-incorporated protectants.

Other Considerations

A. Endocrine Disruptors

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

B. Analytical Method(s)

Methods for extraction and direct ELISA analysis of Cry3Bb1 in corn grain have been submitted and found acceptable by the Agency.

C. Codex Maximum Residue Level

No Codex maximum residue levels exists for the plant-incorporated protectantsBacillus thuringiensis Cry3Bb1 protein and the genetic material necessary for its production in corn.

D. Occupational Exposure and Risk Characterization

Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Worker exposure to the Cry protein via seed dust is also expected

to be negligible because of the low amount of protein expressed in transformed plants. If such exposure should occur, the Agency concludes that such exposure would not be expected to present any risk due to the lack of toxicity. However, if any unreasonable adverse effects caused by exposure to Cry3Bb1 are identified, these effects must be reported to the Agency as described in Sec. 6(a)(2) of FIFRA.

E. Current Tolerance Exemption

Sec. 180.1214 Bacillus thuringiensis Cry3Bb1 protein and the genetic material necessary for its production in corn; exemption from the requirement of a tolerance.

Bacillus thuringiensis Cry3Bb1 protein and the genetic material necessary for its production in corn are exempt from the requirement of a tolerance when used as plant-incorporated protectants in the food and feed commodities of field corn, sweet corn and popcorn. Genetic material necessary for its production means the genetic material which comprise genetic material encoding the Cry3Bb1 protein and its regulatory regions. Regulatory regions are the genetic material, such as promoters, terminators, and enhancers, that control the expression of the genetic material encoding the Cry3Bb1 protein.

F. MON 88017 Considerations

The evaluation of mammalian toxicity of the Cry3Bb1 protein produced in MON 88017 is based on studies conducted with Cry3Bb1 protein variants (Cry3Bb1.11098 and Cry3Bb1.11231). The physicochemical characteristics of the Cry3Bb1 protein in MON 88017 were found to be similar with the Cry3Bb1 protein in MON 863. Moreover, the protein expression levels, insect bioactivity, and field efficacy data of Cry3Bb1 protein in MON 88017 were compared to MON 863 and found to be similar and functionally equivalent. Therefore, because Cry3Bb1 proteins produced in MON 88017 and MON 863 share an amino acid sequence identity of >99.8% and the aforementioned similarities, the data (including the acute mouse gavage studies and *in vitro* digestibility studies, previously submitted for MON 863) were bridged to support the finding that there is a reasonable certainty that no harm will result to people as the result of potential exposure to the Cry3Bb1 protein.

G. Stacked Product Considerations

MON 863 x MON810 and MON 88017 x MON810

The product characterization and protein expression analyses of Cry3Bb1 and Cry1Ab insect control proteins and the genetic material necessary for their production in maize (corn) plants derived from MON 88017 x MON 810 (which were combined through traditional plant breeding) were found to be similar and functionally equivalent to Cry3Bb1 protein expressed in MON 88017 and to Cry1Ab protein expressed in MON 810.

The product characterization and protein expression analyses of Cry3Bb1 and Cry1Ab insect control proteins and the genetic material necessary for their production in maize (corn) plants derived from MON 863 x MON 810 (which were combined through traditional plant breeding) were found to be similar and functionally equivalent to Cry3Bb1 protein expressed in MON 863 and to Cry1Ab protein expressed in MON 810.

Thus, toxicological and allergenicity data can be bridged to support the finding that there is a reasonable certainty of no harm to exposure of Cry3Bb1 and Cry1Ab proteins expressed in MON 863 x MON 810 and MON 88017 x MON 810 to humans. Further, the presence of two or more newly expressed proteins in a genetically engineered plant is not expected to routinely require additional toxicity testing if there are existing data to demonstrate the safety of the individual proteins and a food tolerance determination has been made. This rationale is similar to that employed for the current products which often consist of both an expressed pesticidal protein and a marker gene protein expressed in a single plant. There may be instances where information available on the mode of action of the proteins would indicate the likelihood that the combination of proteins should be evaluated and the Agency reserves the right to test combinations in those instances.

The data generated for the Cry1Ab and Cry3Bb1 proteins individually support a food tolerance determination for the combination since the mode of action for these proteins does not suggest an enhanced activity in combination for mammalian species. This lack of synergism is also suggested by the absence of enhanced responses in sensitive target species tested with the combination of Cry1Ab and Cry3Bb1proteins.

H. Consideration of MON863 90-Day Rat Feeding Study

The EPA and FDA did not request or receive the 90-day dietary study nor was there any formal review of the full study by either Agency. Both Agencies completed the safety evaluations for MON 863 corn and did not need the 90-day oral toxicity study to reach a finding on human dietary safety. However, other governments often request additional information or studies and summary information on these studies are frequently provided to the US governmental agencies on request. A summary of the study report was provided to the EPA.

The 90-day oral toxicity study (CV-2000-260) was performed by Convance Labs (Madison, WI) with high percentages of MON 863 corn (11and 33%) used to make the test rodents' diet. The study was done according to OECD guideline protocol 408 for a 90-day oral toxicity study and was performed under the good laboratory practices guidelines. These testing requirements, routinely used for regulatory purposes, are actually more stringent in quality control procedures and record keeping than studies routinely reported in the scientific literature. An appropriate isoline corn control (a corn hybrid similar to MON 863 but not expressing the beetle control protein), and reference corn varieties (six different non-transgenic, commercial corn varieties) were employed to address the possible effects of different corn compositions on rat nutrition.

The prepared diets were also analyzed to confirm that the proper test corn lines were used to make the animal feed. All test animals received diets specially prepared by Purina Mills formulated to contain 33% corn (conventional corn, MON 863 corn or a combination) and provide a balanced diet.

It is important that a balanced diet be provided rather than feeding the test animals 100% corn so that any abnormalities that occur can be assigned to the effect of the corn component itself rather than any dietary insufficiency. It is also important that more than one level of MON 863 corn in the diet be included to establish if a dose-response is present. The dose-response shows that, if an effect is seen at a low dose, a more dramatic and similar effect will also occur at a higher dose. While dose-response is a key component to a toxicology assessment, it is difficult to establish in a whole food study. This is one of the reasons that USEPA requests high dose purified protein toxicity studies for PIPs and compositional analysis is used for examining whole food.

The 90-day rat oral study with MON 863 was reviewed by both the Robert Koch Institute (RKI) as the German competent authority and subsequently the European Food Safety Authority (EFSA). As could be anticipated by having such a large study with numerous treatments and comparisons, some differences were found between treatments. In understanding the significance of these findings, it is important to be aware of the natural variation of biological systems in a population of test organisms and the range of responses to a treatment. As discussed in information provided by both the RKI and EFSA, the decreases in male kidney weight and some changes in hematological parameters (lymphocyte and reticulocyte counts) for the highest MON 863 treatment groups were significantly different compared to the control isoline corn group. However, when compared to the included reference corn variety treatments, the values found to be within the realm of normal biological variation. Since there were questions about possible kidney pathology, independent veterinary pathologists were asked to examine tissue samples and render an opinion. No significant findings relevant to an adverse toxicity determination were noted. Therefore, both RKI and EFSA found that there were no resultant concerns over the safety of the MON 863 due to the results of the 90-day toxicity test.

Since the earlier examination of the study summaries of the 90-day feeding study for MON 863, a more recent publication by G.E. Seralini et.al (2007) has reanalyzed the data from the study. The paper states that there are significant effects due to the MON 863 corn being present in the diet of the treated rodents. The authors postulate that new analyses of growth curves and blood chemistry parameters suggest significant hepatorenal effects. The Agency considers these new findings open to a different interpretation. The original studies did not demonstrate any changes in the blood chemistry parameters or body weight gains that showed dose dependent responses. In addition, the organs most likely to be impacted by these chemistry changes (liver and kidney) were examined histologically in the original reports by independent competent veterinary pathologists. The reports on these tissues indicated no signs of cellular toxicity were found in

the hepatic or renal tissues. Without signs of cellular tissue damage in the relevant organs the variations in blood chemistry should not be considered signs of toxicity.

REFERENCE

Seralini, G-E, Cellier, D., and de Vendomois, J.P. (2007), New Analysis of a Rat Feeding Study with a Genetically Modified Maize Reveals Signs of Hepatorenal Toxicity. Accepted for publication in *Arch. Environ. Contam. Toxicol.* (2007)

Guideline No	Study	Results	MRID
885.305	Acute Oral Toxicity	There did not appear to be significant adverse affects to animals dosed with Cry3Bb1 at corrected dose amounts of 38.7, 419, or 2980 mg/kg bodyweight. Two animals died during the study - animal #s 98035M3-007 and 98035F3-004 in the 2980 mg/kg treatment group. These deaths appeared to be the result of trauma from dosing rather than from the test substance. In addition, although, there were some minor weight loss and minor abnormal observations at gross necropsy, these occurred in both test and control groups and therefore do not appear to be Cry3Bb1.11098 protein exposure related. Based upon the data provided, the LD_{50} for Cry3Bb1.11098 is greater than 2980 mg/kg bodyweight in mice. ACCEPTABLE.	449043-06
885.305	Acute Oral Toxicity	There were no apparent adverse effects identified in mice dosed orally with 36, 396 and 3780 mg/kg bodyweight of Cry3Bb1.11231 protein. There was some minor weight loss in a few animals and some minor abnormal observations via gross necropsy, but these occurred in both the test and control groups and therefore do not appear to be Cry3Bb1.11231 protein exposure related. Based upon the data provided, the LD_{50} for Cry3Bb1.11231 is greater than 3780 mg/kg bodyweight in mice. ACCEPTABLE.	449043-05
885.305	Acute Oral Toxicity	There did not appear to be significant adverse affects to animals resulting from exposure to Cry3Bb1.11098(Q349R) at dose amounts of 300, 900 & 2700 mg/kg body weight. Observations included some minor clinical affects and a relatively insignificant lack of weight gain in two animals, however, these do not appear to be related to exposure to the test substance, because these occurred in the various test groups. Based upon the data contained in this submission, the LD_{50} for Cry3Bb1.11098(Q349R) is greater than 3200 mg/kg body weight in mice. CLASSIFICATION: Acceptable.	455382-02

Guideline No	Study	Results	MRID
	In vitro Digestibility	The tests performed in this study show that the Cry3Bb1 proteins are not stable to digestion in simulated gastric fluid. Incubation of Cry3Bb1.11098 and Cry3Bb1.11231 in SGF results in the loss of detectable protein by the 30 and 15 second observation points, respectively, as detected by SDS-PAGE. Insect bioassay data indicated that the protein loss insecticidal activity within 2 minutes of incubation in SGF. Incubation in the SIF resulted in a ~59 kDa digestion product that retained its insecticidal activity for at least 30 minutes. ACCEPTABLE. Neither Cry3Bb1.11098 nor Cry3Bb1.11231 appear to be stable to digestion in simulated gastric fluid for more than 30 seconds. However, there appears to be a discrepancy between the results discussion and figures 2 & 4. The Cry3Bb1.11098 protein appeared to be significantly digested within 15 seconds and completely digested in less than 30 seconds based upon the figures provided. This differs somewhat with the discussion provided by the registrant which indicates that the protein was digested to undetectable levels between 30 and 60 seconds. These do represent drastically different digestion times, but the discrepancy should be addressed. This study was conducted in accordance with Good Laboratory Practice guidelines with four exceptions as described in the submission. These exceptions should not have a significant impact on the outcome of the study. CLASSIFICATION: Acceptable.	449043-07
885.11	*In vitro* Digestibility	The tests performed in this study show that the Cry3Bb1 proteins are not stable to digestion in simulated gastric fluid. Incubation of Cry3Bb1.11098 and Cry3Bb1.11231 in SGF results in the loss of detectable protein by the 30 and 15 second observation points, respectively, as detected by SDS-PAGE. Insect bioassay data indicated that the protein loss insecticidal activity within 2 minutes of incubation in SGF. Incubation in the SIF resulted in a ~59 kDa digestion product that retained its insecticidal activity after at least 30 minutes incubation. CLASSIFICATION: Acceptable.	454240-06
885.11	*In vitro* Digestibility	The tests performed in this study show that the Cry3Bb1 proteins are degraded in simulated gastric fluid. Incubation of corn-produced and *E. coli*-produced Cry3Bb1 protein in SGF results in the loss of detectable protein by the 15 second observation point, as detected by SDS-PAGE. CLASSIFICATION: Acceptable.	455382-03

Guideline No	Study	Results	MRID
	In vitro digestibility	Simulated intestinal fluid activity was verified to be present and at a level deemed acceptable by SOP GEN-PRO-058-01. The gels provided indicate that the Cry3Bb1.11098 (Q349R) protein is present as a single band at 74 kDa which rapidly degraded to two bands of 68 and 57kDa at the first assay time point of 1 minute. The subsequent samples (from 5 minute to 24 hours) all gave a single 57 kDa band which did not appear to decrease in intensity. This lack of degration by intestinal fluids is similar to the majority of Cry proteins which are resistant to the action of trypsin. CLASSIFICATION: ACCEPTABLE.	455770-02
	Product Characterization (Heat Stability)	Heating the corn flour samples at 204°C for 30 minutes destroys both the immunoreactivity and insect bioactivity of the Cry3Bb1.11098 found in MON 863 corn. The Cry3Bb1 immunoreactivity was not detectable in both an immunoblot and ELISA format for MON 863. For MON 853, Cry3Bb1 was not recognizable in an immunoblot and reduced more than 1000-fold in an ELISA format. Since the rabbit anti-Cry3Bb1 antibody employed was polyclonal IgG, it is also suggestive that epitopes were destroyed and not just rendered unrecognized by alteration of the three dimensional configuration. CLASSIFICATION: ACCEPTABLE.	454240-07
885.11	Product Characterization (Heat Stability)	The immunoblot shows that extraction of the MON863 corn grain spiked with NPTII yielded an immunoreactive band that comigrated with the *E. coli* produced NPTII. The blot also showed that, regardless of the extraction buffer used, the heat treatment effectively removed any immunoreactive bands from the samples. The results suggest that, even if detectable levels of NPTII were present in MON863 corn grain, the heat treatment would remove them. Unfortunately, the use of a mouse monoclonal antibody limits the ability of this data to be extrapolated. A heat treatment significantly above the 95.8°C used for sample preparation for SDS-PAGE destroyed the epitope(s) recognized by the anti-NPTII antibody used. Classification: ACCEPTABLE	455382-09
885.11	Product Characterization (Toxin Database Comparison)	Several amino acid database comparison tools were employed to compare the amino acid sequence of Cry3Bb1.11098 and Cry3Bb1.11231 to known protein toxins. The TOXIN4 database was compiled to allow for comparison of Cry3Bb1.11098 and Cry3Bb1.11231 to these known toxin proteins. All of the protein similarities identified were to insecticidal protein, with no similarity to proteins known to be toxic to humans and/or animals. Based upon this data, it does not appear that Cry3Bb1.11098 nor Cry3Bb1.11231 share significant structural, biological or immunological similarity with known protein toxins other than those affecting insects. Classification: ACCEPTABLE	449043-08

Guideline No	Study	Results	MRID
885.11	Product Characterization (Allergen Database Comparison)	Several amino acid database comparison tools were employed to compare the amino acid sequence of Cry3Bb to known protein allergens and gliadins. The UPDATE2 database was compiled to allow for comparison of Cry3Bb1.11098 and Cry3Bb1.11231 to these proteins. The level of similarity identified does not indicate significant similarity to any of the proteins or gliadins contained in the database. In addition, no contiguous stretch of 8 identical amino acids was identified in either the FASTA or IDENTITYSEARCH algorithms suggesting a lack of immunological similarity. Based upon this data, it does not appear that Cry3Bb1 shares significant structural, biological or immunological similarity with known protein allergens or gliadins. Classification: ACCEPTABLE	449043-09 454240-08
	Safety Assessment of Cry3Bb1 Variants in Corn Rootworm Protected Corn	Plants transformed for corn rootworm control (Event MON 863) contained a total of seven amino acid changes within the Cry3Bb1.11098 -endotoxin when compared to the sequence as found in wild type *B. thuringiensis. B.t.* strains EG11231 and EG11098 contain variants of the Cry3Bb1 protein which differ from the wild type -endotoxin by 4 and 5 amino acids, respectively. Two further alterations in amino acid sequence were made for Cry3Bb1.11098 during cloning and insertion into the maize genome. Structural data indicate that these alleles of this protein maintained a very similar structure to the native form. The initial transformation event used to evaluate the rootworm protected maize was MON 853, which encodes Cry3Bb1 variant 11231. Protein produced by fermentation of *B. thuringiensis* cells expressing variant 11231 was used in toxicology studies for environmental and mammalian concerns. Functional and physicochemical equivalence between variant 11231 produced in *B.t.* and that produced in MON 853 were demonstrated. Maize was also transformed with variant 11098 resulting in transformation event MON 863. These two variants, 11098 and 11231, were shown to be physicochemically and functionally equivalent. The registrant stated that an examination of toxicity toward catfish, bobwhite quail, *Daphnia magna*, Collembola (*Folsomia candida*), adult and larval honeybees, a ladybird beetle, a green lacewing, a parasitic wasp, and earthworms resulted in a NOEC (No Observable Effect Concentration) being established which exceeded the concentration of Cry3Bb1 toxins expected in the maximum environmental exposure. NOECs surpassed maximum predicted environmental concentrations by 3 to 141 fold, hence, the risk to non-target organisms from the culture of MON 863 is indicated to be minimal. However, this aspect is the subject of another review and outside the purview of this report. Given the lack of known mechanisms of mammalian toxicity from *B.t.* -endotoxins, their widespread use in agriculture, the rapid digestibility of Cry3B proteins, their lack of homology to known toxins and allergens, and the safety of the microbial biopesticide Raven®, which expresses Cry3B proteins, the Cry3Bb1 protein is expected to have a reasonable certainty of causing no harm in its aggregate exposure.	454240-09

Guideline No	Study	Results	MRID
	Protein Characterization and Human Health Assessment **MON 88017**	This report is a summary of product characterization studies and the human health assessment for the plant-incorporated protectant *Bacillus thuringiensis* (*B.t.*) Cry3Bb1 protein produced in MON 88017 and also includes the detailed protein characterization data for Cry3Bb1 produced in MON 88017. The human health assessment is based on the substantial similarity of the Cry3Bb1 protein produced in MON 88017 to the currently registered Cry3Bb1 protein produced in MON 863 (EPA Reg. No. 524-528).	461817-01