10) Holleschak, G., R. E. Hileman and J. D. Astwood (2001). Amended report for MSL-16597 : Immuno-detectability of Cry3Bb1.11098 and Cry3Bb1.11231 proteins in the grain of insect protected corn events MON 863 and MON 853 after heat treatment. MSL-17223, an unpublished study conducted by Monsanto Company.

Study Title

Amended Report for MSL-16597: Immuno-detectability of Cry3Bb1.11098 and Cry3Bb1.11231 Proteins in the Grain of Insect Protected Corn Events MON 863 and MON 853 After Heat Treatment

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Study Completed On

Amendment 1
April 10, 2001

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Laboratory Project ID

Study Number: 99-01-39-32

Monsanto Report Number: MSL-17223

Statement of No Data Confidentiality Claim

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA 10(d)(l)(A), (B), or (C).

"We submit this material to the United States Environmental Protection Agency specifically under provisions contained in FIFRA as amended, and thereby consent to use and disclosure of this material by EPA according to FIFRA. In submitting this material to the EPA according to method and format requirements contained in PR Notice 86-5, we do not waive any protection of rights involving this material that would have been claimed by the company if this material had not been submitted to the EPA."

Company: Monsanto Company

Company Agent: Dennis P. Ward

Title: Regulatory Affairs Manager

Signature: _____ Date: 9 May 2001

Statement of Compliance

This study meets the GLP requirements for 40 CFR Part 160 (EPA) except for the following:

Submitter	Date 9 May 2001
Sponsor Representative	Date April 10, 2001
Study Director	Date 4/10/2001

Quality Assurance Statement

Reviews conducted by the QAU confirm that the final report reflects the raw data.

Following is a list of reviews conducted by the Monsanto Regulatory QAU on the study reported herein.

Dates Of Inspection / Audit	Phase	Date Reported To:	
		Study Director	Management
2/21/2000	Protocol Review	2/23/2000	2/23/2000
2/22/2000	Heating	2/23/2000	2/23/2000
3/14/2000	Data Audit	3/28/2000	3/28/2000
3/14/2000	Final Report Audit	3/28/2000	3/28/2000
04/04/2001	Amended Report Audit	04/10/2001	04/10/2001

Michelle Rathgeber

04-10-01
Date

Quality Assurance Specialist
Monsanto Regulatory, Monsanto Company

Signatures of Approval

Study Number: 99-01-39-32

Title: Amended Report for MSL-16597: Immunodetectability of Cry3Bb1.11098 and Cry3Bb1.11231 Proteins in the Grain of Insect Protected Corn Events MON 863 and MON 853 After Heat Treatment

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Study Start Date: February 22, 2000

Original Completion Date: April 3, 2000

Amended Report Completion Date: April 10, 2001

Records Retention: All study specific raw data, protocols, final reports and facility records will be retained at Monsanto-St. Louis.

Signatures of Approval (continued)

Amendments to Report:

This amendment modifies the final report, to reflect new Cry3Bb1 protein information. The following changes do not effect the quality or integrity of the data.

MSL-17223 Amended Report	MSL-16597 Original Report	Amendments
1. Title Page (Page 1)	1. Title Page (Page 1)	a. Added 'Amended Report for MSL-16597' in front of the study title. b. Changed Study completion date.
2. Quality Assurance Statement (Page 4)	2. Quality Assurance Statement (Page 4)	a. Added new line: 'Amended Report Audit' to the list.
4. Signatures of Approval (Page 5)	4. Signatures of Approval (Page 5)	a. Added 'Amended Report for MSL-16597' in front of the study title. b. Added the word 'Original' to the study completion date. c. Added 'Amended Report Completion Date'. d. Added a new page 'Signatures of Approval (continued)'. e. Added list of changes. f. Remove Signatures of Approval heading.
5. Pages 7 - 8	5. Pages 6 - 7	Table of Content - changed pagination.
7. Page 10	7. Page 9	Added minor changes to the summary.
6. Page 11	6. Page 10	Reworded second paragraph to reflect new protein information.
8. Page 17	8. Page 16	a. Added additional reference. b. Updated MSL number for Hileman 1999.

Study Director — Date: 4/10/2001

Sponsor Representative — Date: April 10, 2001

Table of Contents

Table

Figures

Appendices

Abbreviations and Definitions

B.t.	*Bacillus thuringiensis*
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
IPC	Insect protected corn
IgG	Immunoglobulin G
kDa	Kilodalton
MW	Molecular weight
NFDM	Non fat dried milk
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate buffered saline containing Tween 20
SDS	Sodium dodecylsulfate
SOP	Standard operating procedure

1.0 Summary

Genetically modified insect-protected corn (IPC) plants that produce the variants of *Bacillus thuringiensis* Cry3Bb1.11098 or Cry3Bb1.11231 protein have been designed by insertion of the *cry3Bb1.11098* or *cry3Bb1.11231* coding sequences into the corn genome that encode a 74 kDa protein. The Cry3Bb1 protein protects the plant from some root eating insects. The purpose of this study was to assess the immuno-detectability and bioactivity of the Cry3Bb1.11098 and Cry3Bb1.11231 proteins in grain of IPC events MON 863 and MON 853 after heating. This study will be used to support the registration of IPC corn events containing either the Cry3Bb1.11098 or the Cry3Bb1.11231 protein.

Grain from IPC events MON 853 and MON 863 and corresponding control lines MON 847 and MON 846 were ground to a fine powder and baked at 204 °C (399.2 °F) for 30 minutes to simulate a heat step used in food processing. Unbaked and baked grain of IPC events MON 853 and MON 863 and control lines were extracted with two different buffer solutions: phosphate buffered saline containing Tween [PBST; 1.54 mM KH_2PO_4, 154 mM NaCl, 5.11 mM Na_2HPO_4 and 0.05% (v/v) Tween-20] and 2× Laemmli buffer [500 mM Tris, 4% (w/v) SDS, 20% (v/v) 2-mercaptoethanol and 60 % (v/v) glycerol]. PBST represents a relatively mild aqueous extraction buffer with physiologic ionic strength and pH, whereas 2× Laemmli buffer represents a denaturing and reducing extraction buffer. Extracts were analyzed by immunoblotting and ELISA to evaluate the immuno-detectability of the Cry3Bb1 proteins.

After heating IPC flour, Cry3Bb1 protein was not detected using immunoblot analysis when extracted with PBST or 2× Laemmli buffer. Similarly, after baking, 0.4% and 0% of the Cry3Bb1 protein were detected by ELISA analysis in processed grain of IPC events MON 853 and MON 863, respectively. In contrast, the Cry3Bb1 protein was readily detected using immunoblot and ELISA analysis for unbaked IPC flour. A 74 kDa band was detected using immunoblot analysis for unbaked IPC flour, suggesting that the full-length protein was extracted from grain. These results were consistent with the observed loss of insecticidal activity against Colorado potato beetles (*Leptinotarsa decemlibeata*) in baked IPC flour.

2.0 Introduction

B. thuringiensis is a Gram-positive bacterium commonly present in soil. Many different strains of *B. thuringiensis* have been shown to produce crystal proteins or inclusion bodies that are effective in controlling certain orders and species of insect pests. *Bacillus thuringiensis* (*B.t.*) based products have been widely used as microbial pesticides since 1961 (McClintock, et al., 1995). Microbial pesticides have been commercially available and used as environmentally acceptable insecticides because they are specific for the targeted insect

pests and are typically harmless to other non-targeted organisms. *B.t.* proteins have been classified based on their insecticidal activity (*e.g.*, Cryl, Cry2, Cry3 and Cry4 proteins are toxic to lepidopteran, lepidopteran/dipteran, coleopteran and dipteran pests, respectively (Bravo, 1997). The Cry3B class protein has natural insecticidal activity against the coleopteran pest, corn rootworm, *Diabrotica* (Von Tersch et al., 1994).

The Cry3Bb1 protein nomenclature will be used in this report. Two variants of the wild type *cry3Bb1* coding sequence (GenBank Accession No. M89794) have been designed to encode for proteins with enhanced insecticidal activity against corn rootworm. These Cry3Bb1 protein variants were initially produced for investigation with a *B.t.* cloning vector and expression system. Protein variants, Cry3Bb1.11231 and Cry3Bb1.11098, were produced by the recombinant *B.t.* strains EG11231 and EG11098, respectively. Cry3Bb1.11231 and Cry3Bb1.11098 proteins differ in amino acid sequence from the wild type protein by a total of four and five amino acids, respectively. To facilitate optimum expression in plants, these coding sequences were further manipulated prior to their use in transforming corn (*Zea mays*). Corn events expressing variants of the *cry3Bb1.11231*and *cry3Bb1.11098* coding sequences produce proteins that differ in amino acid sequence from the wild type protein by five and seven amino acids, respectively (Hileman and Astwood, 2001). These sequences are predicted to encode a 74 kDa protein.

Product characterization and protein safety studies confirmed the food and feed safety of this insect protected corn product. Product characterization studies included: molecular characterization of the inserted DNA and estimation of Cry3Bb1 protein levels in key tissues. The safety of the introduced protein was confirmed by assessing its history of safe use, the extant database of toxicology studies with relevant Cry3 proteins, the absence of oral acute toxicity established by mouse gavage, protein digestibility *in vitro* and bioinformatics assessments demonstrating the absence of sequence similarities to known toxins and allergens. Nutrient composition of the food and feed components of IPC confirmed substantial equivalence when compared to traditional commercial corn varieties. The weight of evidence from these evaluations has led to the conclusion that foods and feeds derived from *cry3Bb1* expressing corn are as safe as traditional corn varieties. Environmental safety of the product was confirmed through specific studies with beneficial insects, fish, birds and other non-target organisms, as well as agronomic plant performance assessments conducted throughout field testing.

The level of accumulation of the Cry3Bb1.11098 and Cry3Bb1.11231 proteins in IPC tissues was determined to be low (≤ 0.1 % total protein). For comparison the zein seed storage protein represents 50% (Wilson, 1994) of total protein found in grain and rubisco represents 25% of the total protein in leaves. To obtain sufficient quantities of these Cry3Bb1 proteins for evaluation of food, feed and environmental safety, it was necessary to express the *cry3Bb1* genes in a separate fermentation of *Bacillus thuringiensis*. The

Cry3Bb1 proteins produced by fermentation exceeded 5 % of total protein to enabled facile purification and characterization. Subsequently, Cry3Bb1.11098 protein purified from fermentation was confirmed to be functionally and physicochemically equivalent to Cry3Bb1.11098 protein produced in IPC event MON 863; Cry3Bb1.11231 protein purified from fermentation was confirmed to be functionally and physicochemically equivalent to Cry3Bb1.11098 proteins produced in IPC MON 853. Likewise, the equivalence of the NPTII protein purified from *E. coli* fermentation has been previously confirmed to be functionally and physicochemically equivalent to NPTII protein produced in plants (Nida, 1994, Bartnicki et al., 1993b).

3.0 Purpose

Genetically modified insect protected corn (IPC) plants that produce the *Bacillus thuringiensis*-derived Cry3Bb1.11098 and Cry3Bb1.11231 proteins were designed by insertion of the *cry3Bb1.11098* or the *cry3Bb1.11231* genes into the corn genome. The purpose of this study was to assess the immuno-detectability and bioactivity of the Cry3Bb1.11098 or Cry3Bb1.11231 proteins in grain of IPC events MON 853 and MON 863 after heat treatment similar to corn flake manufacturing.

4.0 Test, Control and Reference Substances

4.1 Immuno-detectability of Cry3Bb1.11098 and Cry3Bb1.11231 Proteins Produced in IPC Grain of Events MON 863 and MON 853, respectively

4.1.1 *Test Substances.* The test substances were the grain of IPC events MON 863 and MON 853 that produce the Cry3Bb1.11098 and Cry3Bb1.11231 proteins, respectively. MON 863 grain (containing the Cry3Bb1.11098 protein) were obtained from plants grown in the field under Production Plan 99-01-39-08. The identity of MON 863 was confirmed by polymerase chain reaction (Study # 99-01-39-08) prior to initiation of this study. MON 853 grain (containing the Cry3Bb1.11231 protein) were obtained from plants grown in the field under Production Plan 99-01-39-13. The presence of cry3Bb1.11231 protein in MON 853 was confirmed by ELISA. Test substance corn grain was stored at room temperature.

4.1.2 *Control Substances.* The control substance for IPC event MON 863 is the non-transgenic grain from corn line MON 846. The control substance for IPC event MON 853 is the non-transgenic grain from corn line MON 847. These control substances do not contain the genetic material for Cry3Bb1 proteins. The absence of the *Cry3Bb1.11098* gene in the control line MON 846 corn grain was established by use of the polymerase chain reaction (PCR, Study 99-01-39-02 prior to initiation of this study. The absence of the Cry3Bb1.11231 protein in

MON 847 was confirmed by ELISA. Control corn grain were be stored at room temperature.

4.1.3 *Reference Substances.* The Cry3Bb1.11098 (lot 6312803) and Cry3Bb1.11231 (lot 6312812) protein reference substances were produced in and purified from *Bacillus thuringiensis* strains EG11098 and EG11231, respectively. These proteins were purified to greater than 90% purity using non-chromatographic methods and were characterized prior to initiation of this study (Hileman, et al., 1999).

5.0 Justification of the Analytical Methods Used

The selected heat treated method used in this study simulates the condition used for production of corn flakes for human consumption (Rooney and Serna-Saldivar, 1994). The method selected to assess the presence of Cry3Bb1.11098 and Cry3Bb1.11231 proteins in corn grain extracts are well accepted, commonly used and extensively referenced in scientific literature relevant to the purpose for which they are being employed (Deutcher, 1990). Specifically the immunoblot and ELISA analytical procedures were chosen to assess the effect of heat treatment on the immuno-reactivity/extractability of the Cry3Bb1 proteins because it is highly specific, highly sensitive and ideally suited for the detection of immuno-reactive polypeptides extracted from a complex plant matrix such as heat treated corn grain. The insect bioassay was design to test the bioactivity of the Cry3Bb1 proteins against Colorado potato beetle.

6.0 Methods

6.1 Immuno-detectability of Cry3Bb1.11098 and Cry3Bb1.11231 Proteins Produced in IPC Grain of Events MON 863 and MON 853, respectively

6.1.1 *Heating.* Grain from IPC event MON 853 and MON 863 and control line MON 846 and MON 847 were ground to a fine powder and subset fractions of these samples were heated at 204 °C in a programmable electric furnace to simulate the commercial processing of corn grain (Rooney and Serna-Saldivar, 1994).

6.1.2 *Extraction of Cry3Bb1 Proteins from Grain of IPC Events MON 853 and MON 863.* Heated and non-heated grain samples of IPC event MON 853, MON 863 and control lines MON 846 and MON 847 were homogenized separately in 15 mL plastic centrifuge tubes in PBS-T and Laemmli buffer. The tissue to buffer volume ratio was 1 g tissue to 10 mL of extraction buffer. Samples were homogenized with an Omni 2000 hand held homogenizer (Omni Intl., Waterbury,

CT) at high speed for ≈ 20 seconds. The samples were clarified by centrifugation for ≈ 10 min at 14,500 ×g and 500 µL supernatant was transferred to a fresh vial. Extracts prepared in PBST were diluted with an equal volume of 2× Laemmli buffer (Laemmli, 1970) and the extracts prepared in Laemmli buffer were diluted with an equal volume of milli Q water. All samples were stored in a –80 °C freezer until analyzed.

6.1.3 *SDS-PAGE and Immunoblotting*. Samples were heated for 3 min at 100 °C before electrophoresis. SDS-PAGE was performed on 4-20% polyacrylamide gradient gels according to SOP PB-EQP-005-01 using the mini gel system of NOVEX (San Diego, CA). Electrophoresis was conducted at constant voltage (150 V) for 1.5 h (until the dye front reached the bottom of the gel). Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membrane (0.45 µm, NOVEX) at 150 mA constant current for 1.5 hours. Non-specific sites on the membranes were blocked with 5% (w/v) NFDM in PBST for 45 min at room temperature. The immunoblot was subsequently incubated for 45 min at 25 °C with an anti-Cry3Bb1 wild type IgG polyclonal antibody (lot 783) at 1:5000 dilution in PBST containing 1% (w/v) NFDM. Rabbit IgG bound to blotted protein was probed using an approximately 1:7500 dilution of goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Sigma, Cat. No. A-9169, lot 39H4860) in PBST containing 1% (w/v) NFDM for 45 min. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system of Amersham Pharmacia Biotech (Cat. No. RPN 2106, Buckinghamshire, UK) and exposed to Hyperfilm™ ECL™ high performance chemiluminescence film (Amersham, Cat. No. RPN 3114H, Buckinghamshire, UK).

6.1.4 *ELISA*. Baked and unbaked grain from IPC event MON 853 and MON 863 and control lines MON 846 and MON 847 were submitted for an ELISA analysis in order to determine the extractable levels of the Cry3Bb1 protein. For each of the processed grain samples, ≈ 1g was weighed to a 15 mL conical tube and extracted with 10 mL of PBST with 0.1% BSA (w/v). The ELISA was performed according to SOP BR-ME-0059-04.

6.1.5 *Insect bioassay*. An insect bioassay was performed on baked and unbaked grain samples from IPC event MON 853 and MON 863 and control lines MON 846 and MON 847. For each of the IPC events and control lines, 1 and 2 g corn grain were weighed out to a 15 mL conical tube. The weighed samples were diluted with 2 mL of Milli Q water and further diluted with 8 mL of insect test diet. Cry 3Bb1 reference proteins were similarly prepared at final doses of 10 µg/mL, 16 test larvae were used for each assay point. The vehicle, Milli-Q water,

was used as the negative control. Colorado potato beetles (CPB, *Leptinotarsa decemlibeata*) larvae were grown on test diets and incubated at ≈ 28 °C for 1 week. The CPB were scored for larval mortality according to SOP BR-ME-0044-02.

7.0 Control of Bias and Quality Control Measures

All sample and assay tubes were clearly labeled to assure that the correct samples were analyzed. Appropriate controls and standards were included with each analysis.

8.0 Results and Discussion

The effect of heat treatment on the immunologically detectable levels assayed by immunoblot of Cry3Bb1.11098 and Cry3Bb1.11231 proteins were summarized in Figures 1 and 2. The heat treatment of 204 °C for 30 min resulted in loss in the immunologically detectable amounts of the Cry3Bb1 proteins. Extract were prepared in two separate buffers, either PBST or 2× Laemmli buffer. PBST represents a relatively mild extraction buffer having physiological ionic strength and pH, whereas 2× Laemmli buffer represents a stringent extraction buffer that contains detergent [SDS, 4% (w/v)] and reducing agent [2-mercaptoethanol, 10% (v/v)]. Immunoblotting results showed that baking caused the level of immuno-detectable Cry3Bb1.11098 and Cry3Bb1.11231 proteins to decline to non-detectable levels when extracts were prepared in both PBST and 2× Laemmli.

MON 853 and MON 863 were designed by insertion of a *cry3Bb1* gene that is predicted to encode a 74 kDa protein. The Cry3Bb1 proteins isolated from *Bacillus thuringiensis* (used as reference standards in Figures 1 and 2, lanes 3-5) represent proteolytically truncated products (Hileman et al., 1999). *Bacillus thuringiensis* expression of the *cry3Bb1* gene results in the accumulation of polypeptides that have observed molecular weights of ≈56 and ≈66 kDa (Holleschak et al., 1999). A 74 kDa band was detected using immunoblot analysis for unbaked IPC flour (Figures 1 and 2), suggesting that the full-length protein was extracted from grain.

Each blot had the same loading pattern. High range colored molecular weight markers and biotinylated molecular weight markers were loaded in lanes 1 and 2, respectively. Varying amounts of the *Bacillus thuringiensis*-produced protein standards were loaded in lanes 3, 4 and 5. Extracts from unbaked and baked samples of control grain were loaded in lanes 7, 9, 11 and 13. Extracts from unbaked and baked samples of grain from IPC events were loaded in lanes 6, 8, 10 and 12. The antibodies used to probe for the Cry3Bb1 proteins were very specific,

however there was one minor cross contaminating band observed for the blots for the Cry3Bb1.11098 protein in the control extracts which in no way interfered with data interpretation (Figures 1, lanes 6 to 10). The reduced immuno-reactivity of the Cry3Bb1.110908 and Cry3Bb1.11231 proteins due to baking is very dramatic and the visual loss in signal is clearly observed in the results obtained.
The levels of the Cry3Bb1 proteins in baked and unbaked grain of IPC events MON 853 and MON 863 assayed by ELISA were summarized in Table 1. After baking, 0.4% and 0% of the Cry3Bb1 protein were detected by ELISA analysis in processed grain of IPC events MON 853 and MON 863, respectively.

The mortality results from the Colorado potato beetle bioassay was analyzed to assess the effect of heating on grain from IPC events MON 853 and MON 863. Baked grain from both of the IPC events, MON 853 and MON 863 showed a loss in insecticidal activity (Figure 3). The insect bioassay data indicates that the functional activity of the Cry3Bb1 protein accumulated in corn grain is unstable when exposed to 204 °C for ≈30 minutes.

9.0 Conclusions

During the commercial processing of corn, the kernels are treated at high temperatures. The heat treatment used in this experiment was designed to provide a laboratory simulation of commercial corn grain processing. Heat treatment, similar to that used in grain processing, affects the ability to immunodetect the Cry3Bb1 proteins in PBST or 2× Laemmli buffer extracts of corn grain. A loss of immuno-reactivity was observed in both immunoblot and ELISA analyses. Finally, an insect bioassay against a susceptible pest, Colorado potato beetle was performed on both the baked and unbaked corn samples. These results clearly demonstrate the loss of activity of the Cry3Bb1 protein contained in baked grain of IPC events MON 853 and MON 863.

Procedures	Observations After Baking	
	Cry3Bb1.11098	**Cry3Bb1.11231**
Immunoblot	Not detected	Not detected
ELISA	Not detected	0.01 µg/g[1]
Insect Bioassay	15 out of 16 insects survived	All 16 insects survived

[1] The weight is based on baked corn flour

10.0 References

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Table 1. Direct ELISA Analysis of Cry3Bb1 Proteins in Baked and Unbaked Grain from IPC Events MON 853 and MON 863 and Control Lines MON 846 and MON 847.

Samples	ng/mL of Cry3Bb1 protein
Unbaked MON 846	< L.O.D.[2]
Baked MON 846	< L.O.D
Unbaked MON 847	< L.O.D.
Baked MON 847	< L.O.D.
Unbaked MON853	> 2560
Baked MON 853	> 10
Unbaked MON 863	> 2560
Baked MON 863	< L.O.D.

[2] Limit of detection is 1ng/mL

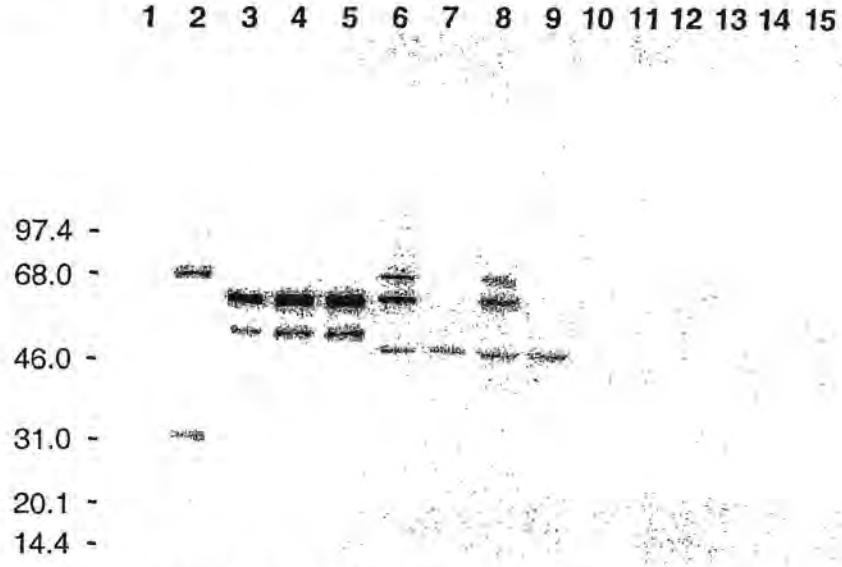

Figure 1. Immunoblot analysis of Cry3Bb1.11098 protein extract from grain of IPC event MON 863 and control line MON 846 and protein purified from *B.t.*. Proteins were separated by SDS-PAGE, electroblotted to nitrocellulose membranes, detected with polyclonal Cry3Bb1 wild type antisera followed by HRP conjugated to goat anti-rabbit IgG and developed using the ECL system. Lane 1 corresponds to Amersham rainbow high-range MW markers and lane 2 with Amersham biotinylated marker with MW (kDa) shown on the left.

Lane	Sample	Amount Loaded	Amount Observed
1	Protein MW Markers		1 µg/band
2	Biotinylated Protein MW Markers		–
3	Cry3Bb1.11098 from *B.t.*	1 µL	≈ 10 ng
4	Cry3Bb1.11098 from *B.t.*	2 µL	≈ 20 ng
5	Cry3Bb1.11098 from *B.t.*	3 µL	≈ 30 ng
6	PBS-T extract from **unbaked** MON 863	20 µL	–
7	PBS-T extract from **unbaked** MON 846	20 µL	–
8	Laemmli extract from **unbaked** MON 863	20 µL	–
9	Laemmli extract from **unbaked** MON 846	20 µL	–
10	PBS-T extract from **baked** MON 863	20 µL	–
11	PBS-T extract from **baked** MON 846	20 µL	–
12	Laemmli extract from **baked** MON 863	20 µL	–
13	Laemmli extract from **baked** MON 846	20 µL	–
14	Blank		
15	Blank		

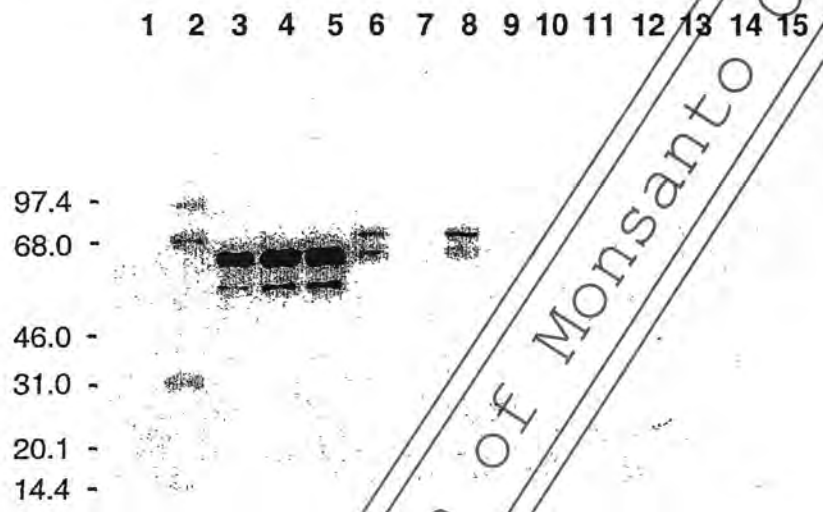

Figure 2. Immunoblot analysis of Cry3Bb1.11231 protein extract from grain of IPC event MON 853 and control line MON 847 and protein purified from *B.t.*. Proteins were separated by SDS-PAGE, electroblotted to nitrocellulose membranes, detected with polyclonal Cry3Bb1 wild type antisera followed by HRP conjugated to goat anti-rabbit IgG and developed using the ECL system. Lane 1 corresponds to Amersham rainbow high-range MW markers and lane 2 with Amersham biotinylated marker with MW (kDa) shown on the left.

Lane	Sample	Amount Loaded	Amount Observed
1	Protein MW Markers		1 µg/band
2	Biotinylated Protein MW Markers		–
3	Cry3Bb1.11231 from *B.t.*	1 µL	≈ 10 ng
4	Cry3Bb1.11231 from *B.t.*	2 µL	≈ 20 ng
5	Cry3Bb1.11231 from *B.t.*	3 µL	≈ 30 ng
6	PBS-T extract from **unbaked** MON 853	20 µL	–
7	PBS-T extract from **unbaked** MON 847	20 µL	–
8	Laemmli extract from **unbaked** MON 853	20 µL	–
9	Laemmli extract from **unbaked** MON 847	20 µL	–
10	PBS-T extract from **baked** MON 853	20 µL	–
11	PBS-T extract from **baked** MON 847	20 µL	–
12	Laemmli extract from **baked** MON 853	20 µL	–
13	Laemmli extract from **baked** MON 847	20 µL	–
14	Blank		
15	Blank		

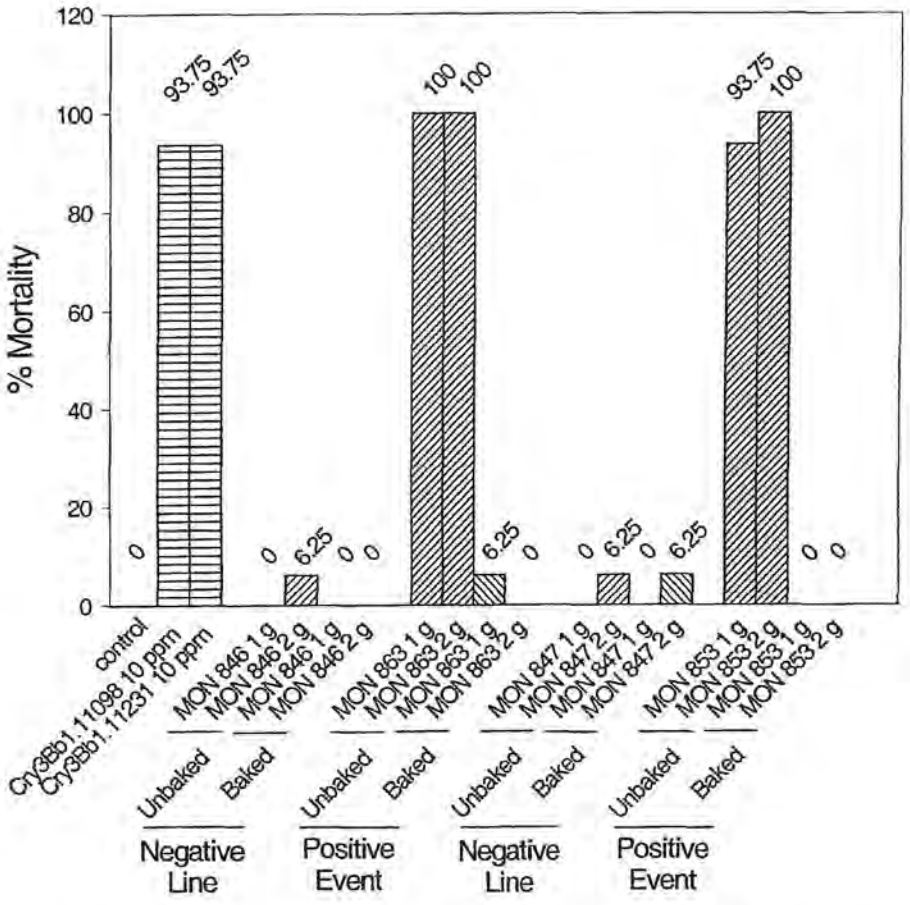

Figure 3. Insect bioassay demonstrating the loss of insecticidal activity on baked grain from IPC events MON 853 and MON 863. A functional assay was used to assess the activity against Colorado Potato beetle. *Bacillus thuringiensis*-produced Cry3Bb1.11098 and Cry3Bb1.11231 proteins were assayed at 10 µg/mL as a positive control. The percent mortality for each sample is labeled on the graph.

Appendix 1
List of Applicable Method SOPs

SOP Number	SOP Title
PB-EQP-005-01	SDS Polyacrylamide Gel Electrophoresis
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)
BR-ME-0044-02	Diet Incorporated Insect Bioassay for the Activity Measurement of *Bacillus thuringiensis* & Other Insecticidal Proteins
BR-ME-0059-04	Extraction and Direct ELISA Analysis of Cry3Bb1 in Corn Tissue

Appendix 2
Protocol and Amendments

Immuno-detectability of Cry3Bb1.11098 and Cry3Bb1.11231 Proteins in Insect Protected Corn Events MON 853 and MON 863 After Heat Treatment

Study #: 99-01-39-32

Study Title: Heat Stability of Cry3Bb1.11098 and Cry3Bb1.11231 Proteins in Insect Protected Corn Events MON 853 and MON 863

Sponsor:
Monsanto Company
Biotechnology Regulatory Sciences
700 Chesterfield Parkway North
St. Louis, MO 63198

Primary Testing Facility:
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Biotechnology Regulatory Sciences
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Study Director:
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[redacted]

Approved By:

Testing Facility Management Representative:

Date: Feb 21, 2000

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Study Director:

Date: 2.22.2000

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Date: Feb. 22, 2000

James D. Astwood
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[redacted]

[redacted]

1.0 Regulatory Compliance

1.1 GLP Compliance

This is a product characterization study as defined by section §160.135(b) of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards (40 CFR Part 160) intended to characterize the physical and/or chemical properties of a potential commercial product. This study will be conducted in compliance with all requirements of section §160.135(b).

2.0 Purpose

Genetically modified insect protected corn (IPC) plants have been produced that express the *Bacillus thuringiensis* Cry3Bb1.11098 and Cry3Bb1.11231 proteins by insertion of the *cry3Bb1.11098* or the *cry3Bb1.11231* genes into the corn genome. The purpose of this study is to assess the heat stability of the Cry3Bb1.11098 or Cry3Bb1.11231 proteins in grain of IPC events MON 853 and MON 863. This study will be part of the data used to support the commercialization of IPC corn events containing either the Cry3Bb1.11098 or the Cry3Bb1.11231 proteins.

3.0 Experimental Dates

Proposed experimental start date:	February 21, 2000
Proposed experimental termination date:	March 31, 2000

4.0 Experimental Design

Grain from IPC events MON 853 and MON 863 and the non-transgenic lines MON 846 and MON 847 will be ground to a fine powder and half of the samples will be baked at a target temperature of 204 °C for 30-35 minutes. This will simulate the initial processing procedure for food items containing corn prior to human consumption. Immunoblot analysis will be performed on both the baked and unbaked samples to assess the relative percent loss of Cry3Bb1 immunoreactivity for each IPC event resulting from this simulated processing.

[redacted]

4.1 *Test Substances.* The test substances are the grain of IPC events MON 863 and MON 853 that express the Cry3Bb1.11098 and Cry3Bb1.11231 proteins, repectively. MON 863 grain (containing the Cry3Bb1.11098 protein) will be obtained from plants grown in the field under Production Plan 99-01-39-08. The identity of MON 863 was confirmed by polymerase chain reaction (Study # 99-01-39-08) prior to initiation of this study. MON 853 grain (containing the Cry3Bb1.11231 protein) will be obtained from plants grown in the field under Production Plan 99-01-39-13. The identity of MON 853 will be confirmed by polymerase chain reaction. All grain will be stored at room temperature.

4.2 *Control Substances.* The control substance for IPC event MON 863 is the non-transgenic grain from corn line MON 846. The control substance for IPC event MON 853 is the non-transgenic grain from corn line MON 847. These control substances do not contain the genetic material for Cry3Bb1 proteins. The absence of the *Cry3Bb1.11098* gene in corn grain was established by use of the polymerase chain reaction (PCR, Study 99-01-39-02) for control line MON 846 prior to initiation of this study. The identity of MON 847 will be confirmed by polymerase chain reaction. Control corn grain will be stored at room temperature.

4.3 *Reference Substances.* The Cry3Bb1.11098 (lot 6312803) and Cry3Bb1.11231 (lot 6312812) protein reference substances were produced in and purified from *Bacillus thuringiensis* strains EG11098 and EG 11231, respectively. These proteins were purified to greater than 90% purity using non-chromatographic methods and were characterized prior to initiation of this study (Hileman, et al., 1999).

4.4 *Description of the Analytical Methods and Procedures.* Analytical tests will be used to characterize and compare the physical properties of the Cry3Bb1.11098 and Cry3Bb1.11231 proteins contained in baked and unbaked IPC grain to bacterial sources. Heat stability will be assessed using the immunoblot analytical procedure (described in Section 4.8.2). Data from this method will be used to compare the apparent molecular weights and assess the extent of the specific immunological recognition for the Cry3Bb1.11098 and Cry3Bb1.11231 proteins in baked and unbaked grain samples. Each gel/blot will be uniquely identified and the samples added to each lane documented.

4.5 *Justification of Analytical Methods.* The method selected to assess the physical equivalence of Cry3Bb1.11098 and Cry3Bb1.11231 proteins isolated from IPC and bacterial sources is an accepted method which is commonly used and extensively referenced in scientific literature relevant to the purpose for which they are being employed (Deutcher, 1990). The western blot analytical procedure was chosen to assess the effect of heat treatment on the immuno-

reactivity/extractability of the Cry3Bb1 proteins because it is highly specific, highly sensitive and ideally suited for the detection of immuno-reactive polypeptides extracted from a complex plant matrix such as heat treated corn grain.

4.6 *Procedure for Identification of the Test and Control Substances.* All samples will be labeled with the appropriate name (e.g. Cry3Bb1 or MON 863), storage condition, lot number, experimental date, expiration date (if any), study number and other information (e.g., aliquot number) necessary to uniquely identify each sample. Definitions of sample labels will be added to the data when relevant.

4.7 *Description of the Experimental Design.* Grain from IPC events MON 853, MON 863 and control lines MON 846 and MON 847 will be ground to a fine powder and brought up in 25 % (w/w) of water (Rooney and Serna-Saldivar, 1994). Samples of ground grain will be baked at a target temperature of 204°C (Rooney and Serna-Saldivar, 1994) for ≈ 30-35 min. Extracts will be prepared in appropriate buffers and subjected to SDS page analysis. Proteins separated by SDS-PAGE will be electrophoretically transferred to nitrocellulose membrane and non-specific sites on blots blocked using an appropriate protein solution. Blots will be subsequently treated with a series of appropriate antibodies, reagents and washes to specifically probe for the presence of Cry3Bb1.11098 or Cry3Bb1.11231 protein. Immunoreactive bands will be visualized using the Enhanced Chemiluminescence Kit (Amersham, RPN 2106) according to the directions provided by the manufacturer.

4.7.1 *Extraction of Cry3Bb1.11098 and Cry3Bb1.11231 proteins from grain.* Unbaked samples will be used to enable the comparison of heating on the extraction of the Cry3Bb1 proteins. Extraction will be performed using two separate buffers; the PBS extraction buffer represents a commonly used, neutral pH buffer and the Laemmli buffer represents a harsh extraction buffer that contains detergent and reducing agent. The baked and unbaked samples will be extracted in PBS and Laemmli buffer. Extracts prepared in PBS will be diluted with an equal volume of 2× Laemmli buffer and heated for 3-5 min at ≈100 °C. Extracts prepared in Laemmli buffer will also be heated for 3-5 min at ≈100 °C. Extracts prepared in both PBS and Laemmli buffer will be clarified by centrifugation and subjected to SDS-PAGE and immunoblot analysis.

4.7.2 *SDS-PAGE and Immunoblotting.* Laemmli extracts of the test, control and reference substances will be subjected to SDS-PAGE according to SOP PB-EQP-005-01 and subsequently blotted to nitrocellulose membrane according SOP GEN-PRO-002-03. Non-specific sites on the blots will be blocked using 5% non-

fat dry milk in Phosphate buffered saline containing Tween [(PBST) 10 mM Na_2HPO_4, 1 mM KH_2PO_4 pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.05% (v/v) Tween 20]. The blots will be probed with a 1:5000 dilution in 1% (w/v) nonfat dried milk (NFDM) in PBST of rabbit polyclonal antisera (Bleed 783) raised against purified Cry3Bb1 wild type protein . Unbound primary antibody will be removed using PBST washes. Bound primary antibody will be probed by incubation in a 1:7500 dilution of goat anti-rabbit IgG (Sigma, Cat. No. A-9169) in 1% (w/v) NFDM in PBST. Finally, immunoreactive bands will be visualized using an ECL kit (Amersham, Cat. No. RPN 2106) according to the manufacturer's instructions. Signals on the films corresponding to immunoreactive bands will be produced by exposing the ECL-treated membranes to X-ray film (Hyperfilm™ ECL, Amersham, Cat. No. RPN3114H). Films will be developed using a Konica SRX-101 film processor.

4.7.3 *Optional Analysis.* Densitometric analysis may be used to quantitate the Cry3Bb1 proteins observed on the immunoblot. A detailed description of the imaging and densitometry analysis will be included and archived with the raw data. Briefly, each visible band will be quantified as a percentage of the total protein in the test substance. The relative optical density will be calculated using a Bio-Rad model GS-700 imaging densitometer with the supplied Quantity One software.

5.0 Records to be Maintained

Records will be maintained of all sample transfers, analyses, the protocol and all deviations and amendments thereto and copies of all letters memoranda and other correspondence related to this study. These documents may include: photocopies, computer generated hard copies or hand-written notes that describe the procedures used to generate data for this study. Upon completion of the study, all study records and final report will be archived by Monsanto Company.

6.0 Changes to the Protocol

Planned changes to the protocol will be documented in the form of written protocol amendments and signed by the Study Director. Amendments become part of the protocol and will be archived with the protocol. All other changes will be in the form of written protocol deviations and will be filed with the raw data. All changes to the protocol will be addressed in the final report.

7.0 References

Deutcher, M. P. 1990. Guide to protein purification, Volume 182 Academic Press, Inc., New York.

Hileman, R. E., Pyla, P. D., Lee, T. C. and Astwood, J. D. 1999. Characterization of *B.t.* protein 11098 and *B.t.* protein 11231 produced by fermentation. Study number 98-01-39-05, MSL-15531, an unpublished study conducted by Monsanto Company.

Rooney, L. W. and Serna-Saldivar, S. O. 1994. Food uses of whole corn and dry-milled fractions. In Corn chemistry and technology, Watson, S. A. and Ramstad, P. E., eds. St. Paul, MN: American Association of Cereal Chemistry, Inc, pp. 399-429.

Appendix: SOP List

SOP Number	SOP Title
PB-EQP-005-01	SDS Polyacrylamide Gel Electrophoresis
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)

Study #/SOP#: 99-01-39-32 **Amendment # 1**

Date Change Implemented: 23 February 2000
Page No/s. &/or Section/s: Page 1
Amended as Follows:

Add:

Testing Facility for Insect Bioassay: Monsanto Company
800 North Lindbergh
St. Louis, MO 63141

Principal Investigator:

John W. Martin
Monsanto Company
800 North Lindbergh
St. Louis, MO 63141
Phone: (314) 694-7422
Fax: (314) 694-8774
email: john.w.martin@monsanto.com

Reason for Amendment and what impact will result from this change: John Martin will be assigned as the principal investigator of this study. John will be responsible for conducting the insect bioassays for this study. There is no impact on the quality of this study.

Date Change Implemented: 23 February 2000
Page No/s. &/or Section/s: n/a
Amended as Follows:

Add:

4.7.4 *Insect Bioassay:* An insect Bioassay will be performed on the baked and unbaked grain samples of insect protected corn event MON 853 and MON 863 and control lines MON 846 and MON 847. Colorado potato beetle (CPB, *Leptinotarsa decemlineata*) larvae will be grown on tests diets containing grain which express the Cry3Bb1 proteins and the Cry3Bb1 proteins purified from *Bacillus thuringiensis*.

Reason for Amendment and what impact will result from this change: The insect bioassay will be used to determine the effects of heating on IPC containing the Cry3Bb1.11098 or the Cry3Bb1.11231 proteins. The insect bioassay will be performed according to SOP BR-ME-0044-02. This revision to the protocol will positively affect the quality of the study by demonstrating the inactivity of the Cry3Bb1 proteins after heating.

Date Change Implemented: 28 February 2000
Page No/s. &/or Section/s: Page n/a
Amended as Follows:

Add:

4.7.5 *ELISA.* A Direct ELISA analysis will be performed on baked and unbaked corn grain extracts from IPC events MON 853, MON 863 and control lines MON 846 and MON 847. The ELISA analysis will be used to quantitate the levels of Cry3Bb1.11098 and Cry3Bb1.11231 protein in IPC corn grain.

Reason for Amendment and what impact will result from this change: The ELISA will be used to determined the extractable levels of the Cry3Bb1 proteins from corn grain. The ELISA will be performed according to SOP BR-ME-0059-04. The ELISA was not specified in the original version of the protocol and this analysis will enhance quality the study.

Date Change Implemented: 13 March 2000
Page No/s. &/or Section/s: Page 8
Protocol/SOP originally stated:
Appendix: SOP List

SOP Number	SOP Title
PB-EQP-005-01	SDS Polyacrylamide Gel Electrophoresis
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)

Amended as Follows:

Add to the SOP list BR-ME-0059-04 and BR-ME-0044-02.
Appendix: SOP List

SOP Number	SOP Title
PB-EQP-005-01	SDS Polyacrylamide Gel Electrophoresis
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)
BR-ME-0059-04	Extraction and Direct ELISA Analysis of Cry3Bb1 in Corn Tissue
BR-ME-0044-02	Diet Incorporation Insect Bioassay for the Activity Measurement of *Bacillus thuringiensis* & Other Insecticidal Proteins

Reason for Amendment and what impact will result from this change: The SOP appendix list was updated to include additional procedures for this study. This revision to the study specific protocol will positively affect the quality of the study.

Signatures of Approval

Study Director:

_____ **Date:** March 14, 2000

Sponsor Representative:

_____ **Date:** March 13, 2000

Testing Facilities Management Representative:

_____ **Date:** March 13, 2000

Reviewed by:

Principal Investigator:

_____ **Date:** MARCH 13, 2000

Signature of the Principal Investigator signifies review of the original protocol in addition to this amendment.

Monsanto Company Biotechnology Regulatory Sciences	SOP/Protocol Amendment Form SOP Ref.: GEN-POL-005

Study #/SOP#: 99-01-39-32 Amendment # 2

Date Change Implemented: 27 March 2000
Page No/s. &/or Section/s: Page 1

Protocol/SOP originally stated:

Study Title: Heat Stability of Cry3Bb1.11098 and Cry3Bb1.11231 Proteins in Insect Protected Corn Events MON 853 and MON 863

Amendment as Follows:

Study Title: Immuno-detectability of Cry3Bb1.11098 and Cry3Bb1.11231 Proteins in the Grain of Insect Protected Corn Events MON 863 and MON 853 After Heat Treatment

Reason for Amendment and what impact will result from this change:

This amendment will increase the clarity of the study. There was no impact on the quality of this study.

Signatures of Approval

Study Director:

Date: March 27, 2000

Sponsor Representative:

Date: March 27, 2000

Testing Facilities Management Representative:

Date: March 27, 2000