3.0 Sensitivity

Limits of Quantitation[5] and Detection[6]:

Tissue Type	Dilution	LOD (ng/ml)	LOD (µg/g fwt)	LOQ (ng/ml)	LOQ (µg/g fwt)
Forage	1:10	0.372	0.372	0.438	0.44
Leaf	1:30	0.379	0.568	0.438	0.66
Pollen	1:25	0.165	0.412	0.438	1.1
Root	1:15	0.338	0.254	0.438	0.33
Silk	1:20	0.275	0.275	0.438	0.44
Grain	1:50	0.105	0.262	0.438	1.1

5. The limit of detection (LOD) was calculated as the mean value plus three SD using the data generated with conventional sample extracts for each tissue type. The LOD value in "ng/ml" was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.
6. The limit of quantitation (LOQ) was calculated based on the lowest standard concentration. The "ng/ml" value was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.

4.0 Extraction Parameters[7]

Tissue Type	Tissue-to-Buffer Ratio	Extraction Buffer
Forage	1:100	1X TBA + 2 M Urea + 0.1 M DTT
Grain	1:50	1X TBA + 2 M Urea + 0.1 M DTT
Leaf	1:50	1X TBA + 2 M Urea + 0.1 M DTT
Pollen	1:100	1X SEBA + 10 mM NLS
Root	1:50	1X TBA + 2 M Urea + 0.1 M DTT
Silk	1:50	1X TBA + 2 M Urea + 0.1 M DTT

7. The Cry1A.105 protein was extracted from each tissue by adding the appropriate volume of Cry1A.105 Extraction Buffer, and shaking in a Harbil mixer. The extracted sample was clarified using a serum filter or by centrifugation (pollen tissue only).

Appendix 3. Summary of the Validation Results for the Cry2Ab2 Protein ELISA in Corn Matrices

1.0 Accuracy

1.1 Extraction Efficiency and Spike and Recovery

Extraction Efficiency acceptance criteria = 70 – 100%.
Spike and Recovery acceptance criteria = 70 – 130%.

Tissue Matrix	Tissue-to-Buffer Ratio	Extraction Efficiency[1]	Spike and Recovery[2]
Forage	1:100	92 %	90 – 92 %
Grain	1:50	73 %	84 – 88 %
Leaf	1:100	92 %	84 – 92 %
Pollen	1:25	N/A[3]	85 – 93 %
Root	1:50	94 %	82 – 87 %
Silk	1:50	93 %	83 – 90 %

1. Extraction efficiency for each tissue type was determined by comparing an aqueous extract to an extract in harsh buffer (e.g. 1X Laemmli buffer) on a western blot.
2. To evaluate the analytical accuracy of the ELISA, extracts prepared from each tissue type of conventional corn plants were spiked with known quantities of Cry2Ab2 protein at three concentrations spanning the range of the standard curve.
3. The extraction efficiency experiment was not completed for pollen tissue due to very low protein levels.

1.2 Matrix Effects

No matrix interferences (non-specific binding) were noted when sample extracts were analyzed at matrix dilutions stated below. Matrix Effects acceptance criteria = 70 – 130%.

Tissue	Minimal Dilution to Avoid Matrix Effects	Average Recovery Range
Forage	1:20	97 – 105 %
Grain	1:20	86 – 90 %
Leaf	1:20	84 – 93 %
Pollen	1:20	88 – 97 %
Root	1:20	94 – 104 %
Silk	1:20	85 – 95 %

1.3 Parallelism

Parallelism is defined to mean that the plant-produced Cry2Ab2 protein is immunologically equivalent to the *E. coli*-Cry2Ab2 protein standard. Parallelism acceptance criteria = 70 – 130%.

Tissue	Parallelism between 70-130%
Forage	110 – 122 %
Grain	111 – 122 %
Leaf	109 – 119 %
Pollen	N/A[4]
Root	93 – 100 %
Silk	101 – 117 %

[4] The parallelism experiment was not completed for pollen tissue due to very low protein levels.

2.0 Precision

Range of Quantitation: 0.219 – 7 ng/ml
Method for Curve Fit: 4-parameter

Intra-Assay Precision Acceptance Criteria: ≤ 15%
Inter-Assay Precision Acceptance Criteria: ≤ 25%
Precition Profile Acceptance Criteria: Standards 1-5 ≤ 15%
Standard 6 ≤ 25%

Intra-Assay Precision[5]: 6.0 %
Inter-Assay Precision[5]: 21.2 %

5. The inter- and intra-assay precision were assessed by determining the CV of the concentration of Cry2Ab2 protein measured for the positive control sample from 21 independent ELISAs using one-way analysis of variance (ANOVA).

Precision Profile:

Standard Number	Concentration (ng/ml)	%CV (over 21 runs)
1	7	5.1
2	3.5	5.2
3	1.75	4.7
4	0.875	4.0
5	0.438	6.5
6	0.219	5.8

The total intra-assay precision based on the standard curve was calculated to be 5.2%.

3.0 Sensitivity

Limits of Quantitation[6] and Detection[7]:

Tissue Type	Dilution	LOD (ng/ml)	LOD (µg/g fwt)	LOQ (ng/ml)	LOQ (µg/g fwt)
Forage	1:20	0.095	0.191	0.219	0.44
Leaf	1:20	0.041	0.081	0.219	0.44
Grain	1:20	0.123	0.123	0.219	0.22
Pollen	1:20	0.109	0.055	0.219	0.11
Root	1:20	0.056	0.056	0.219	0.22
Silk	1:20	0.040	0.040	0.219	0.22

6. The limit of detection (LOD) was calculated as the mean value plus three SD using the data generated with conventional sample extracts for each tissue type. The LOD value in "ng/ml" was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.
7. The limit of quantitation (LOQ) was calculated based on the lowest standard concentration. The "ng/ml" value was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.

4) Extraction Parameters[8]

Tissue Type	Tissue-to-Buffer Ratio	Extraction Buffer
Leaf	1:100	1X Tris-Borate buffer
Grain	1:50	1X Tris-Borate buffer
Pollen	1:25	1X Tris-Borate buffer, pH 7.4
Forage	1:100	1X Tris-Borate buffer
Root	1:50	1X Tris-Borate buffer
Silk	1:50	1X Tris-Borate buffer

8. The Cry2Ab2 protein was extracted from each tissue by adding the appropriate volume of Cry2Ab2 Extraction Buffer, and shaking in a Harbil mixer. The extracted sample was clarified using a serum filter or by centrifugation (pollen tissue only).

AN ACUTE ORAL TOXICITY STUDY IN MICE WITH Cry1A.105 PROTEIN

FINAL REPORT

Guidelines

EPA-OPPTS (870.1100), OECD (401), EEC (B.1)

Author

Kimberly L. Bonnette, M.S., LATG

Study Completed on

November 4, 2005

Performing Laboratory

Charles River Laboratories
Preclinical Services
640 North Elizabeth Street
Spencerville, OH 45887

Study No.

EUF00081

Monsanto Study No.

CRO-2005-050

Submitted to:

Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, MO 63167

Study No. EUF00081
Monsanto Study No. CRO-2005-050

The text below applies only to use of the data by the United States Environmental Protection Agency (US EPA) in connection with the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

1. STATEMENT OF *NO* DATA CONFIDENTIALITY CLAIMS

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)(1)(A), (B), or (C).

"We submit this material to the United States Environmental Protection Agency specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by EPA strictly in accordance with FIFRA. By submitting this material to EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by the company notwithstanding this submission to EPA"

Company: Monsanto Company

Company Agent: _____

Title: _____

Signature: _____ Date: _____

2. COMPLIANCE STATEMENT

This study was conducted in compliance with the Good Laboratory Practice Standards as described by the EPA (40 CFR Part 160).

Kimberly L. Bonnette, M.S., LATG
Study Director/Author
Charles River Laboratories

Date 11/4/05

Terry A. Kaempfe, B.S.
Sponsor
Monsanto Company

Date 11/8/05

Submitter
Monsanto Company

Date _____

3. QUALITY ASSURANCE STATEMENT

This study has been inspected by the Quality Assurance Unit to assure conformance with the Good Laboratory Practice (GLP) regulations promulgated by the EPA (40 CFR Part 160). Reports were submitted in accordance with Standard Operating Procedures as follows:

QA INSPECTION DATES

Dates of Inspection	Phase(s) Inspected	Dates Findings Submitted to: Study Director	Study Director's Management
06/09/05	Protocol Review	08/03/05	08/03/05
06/28/05	Protocol Amendment Review	08/03/05	08/03/05
06/30/05	Dosing	08/03/05	08/03/05
08/03/05	Data Audit	08/03/05	08/03/05
08/03/05	Draft Report Review	08/03/05	08/03/05
09/28/05	Revised Draft Report Review	09/28/05	09/28/05
10/26/05	Revised Draft Report Review	10/26/05	10/26/05
11/04/05	Final Report Review	11/04/05	11/04/05

The final report has been reviewed to assure that it accurately describes the materials and methods, and the reported results accurately reflect the raw data.

Matthew D Bruns Date 11/4/05

Matthew D. Bruns, B.S.
Associate Quality Assurance Auditor

4. INTELLECTUAL PROPERTY RIGHTS

5. TABLE OF CONTENTS

6. SUMMARY

The acute oral toxicity of Cry1A.105 protein was evaluated in CD-1 mice. Three groups of animals received the vehicle control, the protein control [bovine serum albumin (BSA)], or the test protein (Cry1A.105 protein) by oral gavage as indicated below:

Group No.	Treatment[a]	Analytically-Determined Dose Level (mg/kg)	No. of Animals	
			Male	Female
1	Vehicle Control	0	10	10
2	Protein Control (BSA)	1998	10	10
3	Cry1A.105 protein	2072	10	10

[a]A total dose volume of 66.6 mL/kg was administered on Day 0 as two separate 33.3 mL/kg doses.

Following dosing, the mice were observed daily, weighed weekly, and food consumption was measured weekly. A gross necropsy examination was performed on all animals at the time of death or scheduled euthanasia (day 14).

No test article-related mortality was observed. Clinical observations noted during the 14-day post-dosing period included transient incidences of few feces in all three dose groups and raised area of the urogenital area in single male animals from the protein control and test protein groups.

There were no statistically significant differences in body weight, body weight changes or food consumption between the three groups during the study.

No treatment-related gross pathological findings were observed at necropsy on day 14.

Under the conditions of this test, no test article-related mortality or other toxicity was observed in the Cry1A.105 protein group. Therefore, the acute oral LD50 of Cry1A.105 protein in mice is greater than 2072 mg/kg.

7. INTRODUCTION

This study was performed to assess the potential toxicity of Cry1A.105 protein in CD-1 mice when administered by gavage as a single oral dose. This study was performed at Charles River Laboratories, 553 North Broadway, Spencerville, Ohio. The protocol was signed by the Study Director on June 9, 2005 (GLP initiation date). The in-life phase of the study was initiated with test article administration on June 30, 2005 (day 0), and concluded with necropsy on July 14, 2005.

8. MATERIALS AND METHODS

8.1. Experimental Protocol

This study was performed in general conformance with US EPA, Health Effects Test Guidelines, OPPTS 870.1100, Acute Oral Toxicity, December 1998; the OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects, Subsection 401, February 24, 1987; and the EEC Part B: Methods for the Determination of Toxicity, B.1, No. L 383 A/110, December 29, 1992.

8.2. Test Article, Control Article and Vehicle Control

The test article, control article (protein control) and vehicle control were defined as follows:

Sponsor's Identification	Monsanto Lot Number
Vehicle Control - Carbonate-bicarbonate with reduced glutathione (pH 10-11)	7622132-A
Test Control Article - Bovine Serum Albumin, BSA (powder)	B59628
Test Article – *E. coli*-produced Cry1A.105 protein	20-100073

The dosing solutions were prepared by Monsanto Company from the above materials and identified as follows:

Sponsor's ID	Assigned CRL ID	Physical Description	Receipt Date	Expiration Date
Vehicle Control - Carbonate-bicarbonate buffer with reduced glutathione (pH 10-11) Monsanto Lot No.: 7622132-A Purity: None provided	V05.002.EUF	Clear colorless liquid (solution)	06/29/05	06/14/06
Test Control Dosing Solution (TCDS) – Solubilized BSA Monsanto Lot No.: B59628-C Purity: 98%	S05.023.EUF	Clear pale yellow liquid (solution)	06/29/05	06/28/06
Test Dose Solution (TDS) – Concentrated *E. Coli*-produced Cry1A.105 protein Monsanto Lot No.: 20-100073-C Purity: 92%	S05.024.EUF	Pale yellow liquid (suspension)	06/29/05	06/28/06

The vehicle control and test control dosing solutions were stored frozen at -70°C until use. The test dose solution (TDS) was stored refrigerated, as required by the Sponsor. The Sponsor was responsible for any necessary evaluations related to chemical composition, strength, purity, stability and any other data required by EPA 40 CFR 160.105. The Principal Investigator for the preparation and analysis of the dosing solutions was Thomas Lee. The preparation analyses of the dosing solutions were performed by Monsanto Company, 800 North Lindbergh Blvd., St. Louis, MO 63167. Certificates of Analysis for the BSA and Cry1A.105 protein were provided by the Sponsor and are presented in Appendix 1.

8.3. Retention Sample

The Sponsor was responsible for maintaining a retention sample of each test article.

8.4. Dosing Solutions Disposition

All unused dosing solutions were returned frozen (on dry ice) to the Sponsor following completion of the in-life phase.

8.5. Method of Dose Preparation and Analyses

Dosing solutions were prepared and analyzed by the Sponsor according to the study specific work procedure. The dosing solutions were administered as received within one day of receipt. The TCDS and vehicle control were removed from the freezer and allowed to thaw at room temperature for approximately 2.5 hours, and then subsequently gently stirred for approximately 30 minutes to 1.5 hours prior to dosing and continuously under room

temperature conditions until completion of dosing. The TDS was removed from the refrigerator for dosing and gently stirred at room temperature for approximately 30 minutes prior to the first dose. The dosing solutions were stored refrigerated between doses and gently stirred continuously at room temperature for approximately 20 minutes prior to the second dose. The TDS was determined by visual inspection to be a suspension while the TCDS was determined to be a solution. Therefore, homogeneity samples were collected from the TDS container and no homogeneity samples were collected from the TCDS. Samples collected for analysis are summarized in the following table:

Time	Dosing Solution	Samples for Analysis	
		Concentration	Homogeneity
Pre-dose	TDS	500 µL	Top – 250 µL Middle – 250 µL Bottom – 250 µL
	TCDS	500 µL	-------
	Vehicle Control	Not Sampled	-------
Post-dose[a]	TDS	500 µL	-------
	TCDS	500 µL	-------
	Vehicle Control	Not sampled	-------

[a]Approximately four hours after pre-dose sample.

8.6. Sample Shipment

All samples (dosing aliquots and remaining unused dosing solutions) were immediately frozen (maintained on dry ice in a -20°C freezer until transfer later in the day to a -70°C freezer) subsequent to sampling/dosing completion. All analytical samples were transported frozen (on dry ice) to Monsanto Company as soon as practical. In addition, the bulk dosing solution containers of each received material were returned to Monsanto Company on dry ice by overnight courier for analysis. Results of the analyses are included in the Analytical Report presented in Appendix 2.

8.7. Animals and Animal Husbandry

8.7.1. Description, Identification and Housing

Young adult, Crl: CD-1®(ICR)BR (VAF/Plus®) mice were received on June 21, 2005, at the Testing Facility from Charles River Laboratories, Inc., Stone Ridge, New York. Upon receipt, metal ear tags displaying unique identification numbers were used to individually identify the animals. Cage cards displaying the study number, animal number and sex were affixed to each cage. The animals were housed individually in suspended stainless steel cages. All housing and care were based on the standards recommended by the Guide for the Care and Use of Laboratory Animals [1].

8.7.2. Environment

The animal room temperature and relative humidity ranges were 73-76°F (23-24°C) and 38-58%, respectively. Environmental control equipment was monitored and adjusted as necessary to minimize fluctuations in the animal room environment. Light timers were set to maintain a 12-hour light/12-hour dark cycle and room ventilation was set to produce 10-15 air changes/hour. The animal room temperature and relative humidity were recorded a minimum of once daily.

8.7.3. Food

PMI Certified Rodent Meal #5002 (PMI Nutrition International) was provided *ad libitum* to the animals throughout the study (except during fasting). The lot number and expiration date of each batch of diet used during the study were recorded. The feed was analyzed and certified by the supplier for nutritional components and environmental contaminants. Dietary limitations for various environmental contaminants, including heavy metals, pesticides, polychlorinated biphenyls and total aflatoxin are set by the manufacturer. Within these limits, contaminants which may have been present were not expected to compromise the purpose of this study. Results of the dietary analyses (Certificates of Analysis) are provided by the manufacturer for each lot of diet. These are maintained by the Testing Facility.

8.7.4. Water

Municipal tap water treated by reverse osmosis was available *ad libitum* throughout the study. The purified water was supplied by an automatic watering system. Monitoring of the drinking water for contaminants is conducted by the testing laboratory and the records are available for inspection. Within generally accepted limits, contaminants that may have been present were not expected to compromise the purpose of this study. The water meets the standards specified under the EPA National Drinking Water Regulations (40 CFR Part 141).

8.7.5. Acclimation

Upon receipt, the animals were removed randomly from the shipping cartons, examined by qualified personnel, identified with metal ear tags and then acclimated to the laboratory conditions for a minimum of five days. The animals were observed daily for overt physical or behavioral abnormalities, general health/moribundity and mortality.

8.7.6. Animal Selection

Only healthy animals were chosen for study use. On the day of dosing, prior to randomization, at least 30 animals of each sex were weighed and examined in detail for adverse clinical signs. Animals determined to be suitable as test subjects were assigned randomly to groups based on body weights. The animal numbers and the respective body weight values were entered into the computer. The criteria for acceptance of the randomization was homogeneity of groups by body weight, which was met. Disposition of animals not selected for study was documented in the study records. Females were nulliparous and nonpregnant. The male animals were approximately 8 weeks of age and

weighed 26.7-30.5 g and the female animals were approximately 9 weeks of age and weighed 24.4-27.9 g prior to fasting.

9. EXPERIMENTAL PROCEDURES

9.1. Dosing

On day 0, the animals chosen for use on study were weighed and fasted approximately 2-3 hours prior to dose administration. The dosing solutions were administered orally as two doses separated by 4 hours (± 20 minutes) using a ball tipped stainless steel gavage needle attached to a syringe at the following levels:

Group No.	Treatment	Target Dose Level (mg/kg)	Dose Volume (mL/kg)	No. of Animals	
				Male	Female
1	Vehicle Control	0	66.6 (33.3/dose)	10	10
2	BSA (Protein Control)	1000 (500/dose)	66.6 (33.3/dose)	10	10
3	Cry1A.105 protein (Test Protein)	1000 (500/dose)	66.6 (33.3/dose)	10	10

Individual doses were calculated based on the animal's nonfasted (day 0) body weight. Animals were returned to *ad libitum* feeding after dosing.

9.1.1. Body Weights

Individual body weights were obtained for the animals prior to fasting (day 0), prior to dosing on day 0 and for all surviving animals on days 7 and 14. The animal that was euthanized moribund was weighed prior to necropsy on day 1.

9.1.2. Food Consumption

Individual food consumption was measured on days 0, 7 and 14.

9.1.3. Detailed Clinical Observations

Study animals were observed for clinical abnormalities two times on study day 0 (post-dose) and daily thereafter (days 1-14). The study day 0 observations occurred approximately 30 minutes following the first and second dosing. Clinical observations were performed according to Appendix 3 and included, but were not limited to, changes in the skin and fur, eyes and mucous membranes, respiratory system, circulatory system, autonomic and central nervous systems, including tremors and convulsions, changes in level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypies or bizarre behavior. A general health/mortality check was performed twice daily (in the morning and in the afternoon).

9.2. Unscheduled and Scheduled Euthanasia

On day 1, one male Cry1A.105 protein animal (A1885) was euthanized moribund after it was observed with clinical signs consistent with a mechanical dosing injury (wobbly gait, labored breathing, cool to touch, few feces, pale eyes). The dosing injury was confirmed at necropsy. On day 14, all surviving animals were euthanized by carbon dioxide inhalation. All animals that died or were euthanized were necropsied. Body cavities (cranial, thoracic, abdominal and pelvic) were opened and examined. No organ weights were collected. The whole animal was retained in 10% neutral buffered formalin for possible future examination. The lungs and GI tract were flushed with 10% neutral buffered formalin prior to immersion. No tissues were removed but left intact.

9.3. Protocol Deviations

No protocol deviations occurred during the study.

10. DATA ACQUISITION AND ELECTRONIC RECORDS

Electronic data were recorded on a Compaq Alpha Server DS10 utilizing the Toxicology Analysis System Customized, GenToxicology Module, Version 1.0.0 or higher. The study number assigned to this study was EUF00081. The computer study number used to collect data for the study phases was EUF81. The tables within the report display the applicable computer study number.

11. ANALYSIS OF DATA

Less than 50% mortality occurred during the study, therefore, the LD50 was estimated to be greater than the administered dose.

Inferential statistical analyses were performed by the Testing Facility's Alpha DS-10 computer system. Body weights, body weight changes, and food consumption were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for group-wise comparisons to the protein control group, when appropriate. Summary data tables display calculated group means, standard deviations (S.D.), and group sizes (N), as appropriate. All statistical comparisons were two-tailed with a minimum level of significance of 5% ($p < 0.05$).

Body weight means and standard deviations were calculated (as appropriate).

12. MAINTENANCE OF RAW DATA AND RECORDS

All original paper data, electronic records, tissues, tissue blocks, slides and reports will be archived on-site at the Testing Facility for a period of three years from issuance of the final report. The Sponsor will be contacted prior to final disposition of these items. Monsanto will archive the dosing solution characterization, dosing solution preparation and analysis data and corresponding sub report in the Monsanto Regulatory Archives.

13. RESULTS

13.1. Test and Control Article Analysis

Report: Appendix 2

Group No.	Identification	Target Dose Level (mg/kg)	Analytically-Determined Dose Level (mg/kg)
1	Vehicle Control	0	--
2	BSA (Protein Control)	1000	1998
3	Cry1A.105 protein (Test Protein)	1000	2072

13.2. Mortality

Summary Data: Table 1
Individual Data: Appendix 4

Mortality occurred during the study as follows:

Test Material	No. Dead/No. Dosed	
	Males	Females
Vehicle Control	0/10	0/10
BSA (Protein Control)	0/10	0/10
Cry1A.105 protein (Test Protein)	1/10[a]	0/10

[a]The moribund euthanasia required on day 1 was attributed to a mechanical dosing error (perforated esophagus confirmed during necropsy examination).

13.3. Clinical Observations

Summary Data: Table 1
Individual Data: Appendix 4

Vehicle control group: Clinical abnormalities observed included transient incidences of few feces and feces small in size in one female animal.

Protein control group: Clinical abnormalities observed included a raised area in the urogenital area in one male and transient incidences of few feces in three females.

Cry1A.105 protein group: The most notable clinical observations in the surviving animals included transient incidences of few feces in two males and one female and a raised area in the urogential area of one male. Several clinical observations were noted on day 1 in one male which was later sacrificed moribund and confirmed by necropsy to have a perforated esophagus. These included wobbly gait, labored breathing, cool to the touch, pale eyes, decreased reactivity to handling, increased lacrimation and urine stain (findings consistent with a mechanical dosing error).

13.4. Body Weight Data

Summary Data: Table 2 and Table 3
Individual Data: Appendix 5 and Appendix 6

Vehicle control group: Two female animals exhibited slight body weight loss during the study day 7 to 14 interval; however, the final weights exceeded the initial fasted body weights. All other animals gained weight throughout the test period.

Protein control group: One female animal exhibited body weight loss during the study day 0 to 7 interval. One male animal exhibited body weight loss during each interval (study day 0 to 7 and study day 7 to 14). An additional male animal and two female animals exhibited body weight loss during the study day 7 to 14 interval. All other animals from this group gained weight throughout the test period.

Cry1A.105 protein group: Body weight loss was noted for one male and one female during the study day 0 to 7 interval and one male and one female during the study day 7 to 14 interval. Two males and one female did not exceed the initial body weight by study termination. All other animals from this group gained/maintained weight throughout the test period.

There were no statistically significant differences in body weight or body weight change among the three groups during the study.

13.5. Food Consumption Data

Summary Data: Table 4
Individual Data: Appendix 7

No statistical differences were observed in the food consumption data during the study.

13.6. Gross Necropsy

Summary Data: Table 5
Individual Data: Appendix 8

Vehicle control group: Enlarged lymph nodes were noted in one female animal at study termination on day 14. Additional findings of periovarian cyst were noted in 3/10 females.

Protein control group: Gross necropsy observations included foci on the stomach of one female and enlarged lymph nodes in one female. An additional finding included skin scabbing overlying the left preputial gland in one male.

Cry1A.105 protein group: Findings among the animals sacrificed at study termination on day 14 included dark red areas on the lungs and liver of one female animal and periovarian cyst(s) in 2/10 females. Gross necropsy observations noted in the animal that was sacrificed moribund included a perforated esophagus supporting the conclusion that the animal was injured during dosing.

The periovarian cysts noted in the vehicle control and the Cry1A.105 groups are common findings in this strain of mouse and are not considered significant. Since there was no pattern of response, the single occurrences of enlarged lymph nodes in the vehicle control group and dark red areas on the lungs and liver in the Cry1A.105 group are not considered to be related to treatment.

14. CONCLUSION

Under the conditions of this test, no test article-related mortality or other toxicity was observed in the Cry1A.105 protein group. Therefore, the acute oral LD50 of Cry1A.105 protein in mice is greater than 2072 mg/kg.

Kimberly L. Bonnette, M.S., LATG
Study Director

Date 11/4/05

15. REPORT REVIEW

Rusty E. Rush, M.S., LAT, DABT
Director, Toxicology

Date 11-4-05

16. REFERENCE

1. Guide for the Care and Use of Laboratory Animals, DHHS Publication No. (NIH) 96-03, 1996.

17. TABLES

Table 1. Summary of Survival and Clinical Observations

TABLE 1

AN ACUTE ORAL TOXICITY STUDY IN MICE

MALES — SUMMARY OF SURVIVAL AND CLINICAL OBSERVATIONS (FREQUENCY/ANIMALS)

GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry1A.105)
LEVEL (MG/KG):	0	1000	1000
DAY 0 to 14			
NORMAL			
WITHIN NORMAL LIMITS	160/10	153/10	142/10
EUTHANIZED MORIBUND	0/ 0	0/ 0	1/ 1
SCHEDULED EUTHANASIA	10/10	10/10	9/ 9
ACTIVITY			
WOBBLY GAIT	0/ 0	0/ 0	2/ 1
REACTIVITY TO HANDLING - DECREASED	0/ 0	0/ 0	2/ 1
RESPIRATION			
LABORED BREATHING	0/ 0	0/ 0	2/ 1
EXCRETA/EMESIS			
FEW FECES	0/ 0	0/ 0	4/ 2

TABLE 1

AN ACUTE ORAL TOXICITY STUDY IN MICE

MALES

SUMMARY OF SURVIVAL AND CLINICAL OBSERVATIONS (FREQUENCY/ANIMALS)

GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry1A.105)
LEVEL (MG/KG):	0	1000	1000
BODY			
COOL TO TOUCH	0/ 0	0/ 0	2/ 1
RAISED AREA - UROGENITAL REGION	0/ 0	7/ 1	2/ 1
URINE STAIN - UROGENITAL REGION	0/ 0	0/ 0	1/ 1
EYE(S)			
EYE(S) APPEAR PALE	0/ 0	0/ 0	1/ 1
LACRIMATION - INCREASED	0/ 0	0/ 0	2/ 1
FIRST DOSING OBS			
ANIMAL STRUGGLED DURING DOSING	1/ 1	2/ 2	0/ 0
SECOND DOSING OBS			
ANIMAL STRUGGLED DURING DOSING	2/ 2	3/ 3	7/ 7

TABLE 1

AN ACUTE ORAL TOXICITY STUDY IN MICE

FEMALES SUMMARY OF SURVIVAL AND CLINICAL OBSERVATIONS (FREQUENCY/ANIMALS)

GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry1A.105)
LEVEL (MG/KG):	0	1000	1000
DAY 0 to 14			
NORMAL			
WITHIN NORMAL LIMITS	158/10	151/10	159/10
SCHEDULED EUTHANASIA	10/10	10/10	10/10
EXCRETA/EMESIS			
FEW FECES	2/ 1	9/ 3	1/ 1
FECES SMALL IN SIZE	1/ 1	0/ 0	0/ 0
FIRST DOSING OBS			
ANIMAL STRUGGLED DURING DOSING	1/ 1	0/ 0	0/ 0

Table 2. Summary of Body Weight Data

TABLE 2

AN ACUTE ORAL TOXICITY STUDY IN MICE

MALES

SUMMARY OF BODY WEIGHT DATA (GRAMS)

	GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry1A.105)
	LEVEL (MG/KG):	0	1000	1000
DAY 0	MEAN	27.0 t	27.4	27.3
	S.D.	1.03	0.99	0.89
	N	10	10	10
DAY 7	MEAN	29.3 t	29.4	28.4
	S.D.	0.95	1.43	1.67
	N	10	10	9
DAY 14	MEAN	30.7 t	30.1	29.7
	S.D.	1.17	2.54	2.11
	N	10	10	9

STATISTICAL KEY: t=ANOVA/TUKEY-KRAMER

TABLE 2

AN ACUTE ORAL TOXICITY STUDY IN MICE

FEMALES

SUMMARY OF BODY WEIGHT DATA (GRAMS)

GROUP:		VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry1A.105)
LEVEL (MG/KG):		0	1000	1000
DAY 0	MEAN	24.6 t	24.6	24.8
	S.D.	0.98	0.85	0.93
	N	10	10	10
DAY 7	MEAN	26.5 t	25.9	25.7
	S.D.	1.18	1.52	1.49
	N	10	10	10
DAY 14	MEAN	27.1 t	26.4	26.3
	S.D.	1.62	1.48	1.60
	N	10	10	10

STATISTICAL KEY: t=ANOVA/TUKEY-KRAMER

Table 3. Summary of Body Weight Changes

TABLE 3

AN ACUTE ORAL TOXICITY STUDY IN MICE

MALES

SUMMARY OF BODY WEIGHT CHANGES (GRAMS)

GROUP:		VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry1A.105)
LEVEL (MG/KG):		0	1000	1000
DAY 0 TO 7	MEAN	2.3 t	2.0	1.1
	S.D.	0.90	0.91	1.67
	N	10	10	9
DAY 7 TO 14	MEAN	1.4 t	0.7	1.3
	S.D.	0.92	1.92	0.89
	N	10	10	9

STATISTICAL KEY: t=ANOVA/TUKEY-KRAMER

TABLE 3

AN ACUTE ORAL TOXICITY STUDY IN MICE

FEMALES

SUMMARY OF BODY WEIGHT CHANGES (GRAMS)

GROUP:		VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry1A.105)
LEVEL (MG/KG):		0	1000	1000
DAY 0 TO 7	MEAN	1.9 t	1.2	0.9
	S.D.	0.48	1.28	0.90
	N	10	10	10
DAY 7 TO 14	MEAN	0.6 t	0.5	0.6
	S.D.	0.72	1.00	0.63
	N	10	10	10

STATISTICAL KEY: t=ANOVA/TUKEY-KRAMER

Table 4. Summary of Food Consumption Data (Grams/Animal/Day)

TABLE 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

MALES SUMMARY OF FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

	GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry1A.105)
	LEVEL (MG/KG):	0	1000	1000
DAY 0 to 7	MEAN	4.5 t	4.6	4.6
	S.D.	0.20	1.01	0.43
	N	10	10	9
DAY 7 to 14	MEAN	4.4 t	4.9	5.4
	S.D.	0.35	0.98	1.47
	N	10	10	9

Statistical key: t=ANOVA/TUKEY-KRAMER

TABLE 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

FEMALES

SUMMARY OF FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

	GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry1A.105)
	LEVEL (MG/KG):	0	1000	1000
DAY 0 to 7	MEAN	4.5 t	4.6	4.5
	S.D.	0.58	1.25	0.91
	N	10	10	10
DAY 7 to 14	MEAN	4.9 t	5.0	5.6
	S.D.	0.81	0.99	1.51
	N	10	10	10

STATISTICAL KEY: t=ANOVA/TUKEY-KRAMER

Table 5. Summary of Gross Necropsy Observations

TABLE 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS

GROUP:		VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry1A.105)
LEVEL (MG/KG):		0	1000	1000
MALES: TOTAL NUMBER EXAMINED		10	10	10
WITHIN NORMAL LIMITS		10	9	8
ESOPHAGUS	N	0	0	1
PERFORATION	N	0	0	1
GENERAL COMMENT	N	0	0	1
FINAL CLINICAL OBSERVATION NOT APPARENT POSTMORTEM	N	0	0	1
HAIRCOAT	N	0	0	1
WET MATTING	N	0	0	1
SKIN	N	0	1	0
SCABBING	N	0	1	0

TABLE 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS

GROUP: / LEVEL (MG/KG):		VEHICLE CONTROL 0	PROTEIN CONTROL (BSA) 1000	TEST PROTEIN (Cry1A.105) 1000
FEMALES: TOTAL NUMBER EXAMINED		10	10	10
WITHIN NORMAL LIMITS		6	8	8
LIVER	N	0	0	1
DARK RED LOBE(S)	N	0	0	1
LUNG	N	0	0	1
DARK RED AREA(S)	N	0	0	1
LYMPH NODE, MANDIBULAR	N	1	1	0
ENLARGED	N	1	1	0
OVARY	N	3	0	2
PERIOVARIAN CYST(S)	N	3	0	2

TABLE 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS

GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry1A.105)
LEVEL (MG/KG):	0	1000	1000
FEMALES: TOTAL NUMBER EXAMINED	10	10	10
STOMACH N	0	1	0
FOCI N	0	1	0

18. APPENDICES

Appendix 1. Certificates of Analysis

Analytical Protein Standard Certificate of Analysis

JUN 2 9 2005

MONSANTO ANALYTICAL PROTEIN STANDARDS

Sample Information:

Name of APS	**APS Lot Number**	**Recertification Date**
E. coli-produced Cry1A.105 protein	20-100073	August 31, 2005
Common or Alias Name(s)	**Historical APS Lot Number(s)**	**Storage Requirements (until use)** –80 °C
—	—	
Source: Fermentation of *Escherichia coli* containing the pMON96851 expression plasmid		**Comment(s)** None
Additional Background Information: —		

Characteristic	Method	Assay Date	Result
Concentration	Amino Acid analysis	10 February 2005	1.2 mg/mL (total protein)
Purity	SDS-PAGE/Densitometry	14 February 2005	92%
Molecular weight	SDS-PAGE/Densitometry	14 February 2005	131.5 – 58.1 kDa
Molecular weight	MALDI-TOF MS	18 February 2005	133604.32 Da
Identity	Immunoblot	18 February 2005	Confirmed
Identity	N-terminal sequence	18 February 2005	Confirmed 131.5 KDa – MDNNPNINE(C)IPY(N) 88.1 KDa – MDNNPNINEXIP(Y)(N)
Identity	MALDI-TOF MS	17 February 2005	Confirmed sequence 42.6% coverage of expected sequence
Activity	Insect Bioassay	10 March 2005	EC_{50} = 5.8 ng Cry1A.105/mL diet

Buffer composition: 25 mM CAPS, pH ~10.3, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT
Physical description: Clear solution
Short-term storage stability (28 days) was evaluated during the certification process. Based upon the criteria provided in Characterization Plan 20-100073, no significant degradation was observed for samples stored at 4°C, –20°C and –80°C.
Purity corrected concentration is 1.1 mg/mL (1.2 mg/mL × 0.92 ≈ 1.1 mg/mL)

Joan M Rejda-Heath
Quality Assurance Specialist — May 6, 2005 Date

Monte C Rinehart
Testing Facility Management — 5/6/05 Date

Analytical Protein Standards Officer — 5/6/2005 Date

Exact Copy of Original as of 6/21/05 Date
Certified By _____ Initials or Signature

Study No. EUF00081
Monsanto Study No. CRO-2005-050

CALBIOCHEM®

JUN 2 9 2005

Certificate of Analysis

Albumin, Bovine Serum, Fraction V, Fatty Acid-Free, Nuclease- and Protease-Free

Product Number: 126609 **Lot Number:** B59628

Molecular Weight: 66,000 **CAS Number:** 9048-46-8

TEST	RESULTS
Appearance	Off-white powder
Solubility	Clear, colorless solution (1% in H_2O)
Purity by SDS-PAGE	98%
Loss on drying	1.2%
Heavy Metals	< 0.1 ppm
pH	6.9
Sulfated ash	0.6%
Nuclease	None detected
Protease	None detected
Free Fatty Acid	0.001%

Storage and Handling	REFRIGERATOR (+4°C)

Ying Peng, Ph.D, Director, QA/QC

10-May-2004
Date

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE.

USA and Canada
Tel (800) 628-8470
technical@calbiochem.com

Germany
Tel 0800 100 3496
techservice@merckbiosciences.de

United Kingdom and Ireland
Freefone 0800 622935
Toll Free 1800 409445
customer.service
@merckbiosciences.co.uk

All Other Countries
Contact Your Local Distributor
www.calbiochem.com
technical@calbiochem.com

A Brand of EMD Biosciences, Inc., an Affiliate of Merck KGaA, Darmstadt, Germany
www.calbiochem.com

Printed off The internet by TCC on 6/14/05

Appendix 2. Analytical Chemistry Report

Analytical Sub-Report Title

Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with *E. coli*-Produced Cry1A.105 Protein

Authors

Thomas C. Lee, Ph.D., Steven L. Levine, Ph.D. and Elena A. Rice, Ph.D.

Analytical Sub-Report Completed On

November 02, 2005

Performing Laboratory

Monsanto Company
800 North Lindbergh Boulevard
Saint Louis, Missouri 63167

Laboratory Project ID

MSL-20000
Charles River Study #: EUF00081
Monsanto Study #: CRO-2005-050

The text below applies only to use of the data by the United States Environmental Protection Agency (US EPA) in connection with the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

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)(1)(A), (B), or (C).

"We submit this material to the United States Environmental Protection Agency specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by EPA strictly in accordance with FIFRA. By submitting this material to EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by the company notwithstanding this submission to EPA."

Company: Monsanto Company

Company Agent: _____

Title: _____

Signature: _____ Date: _____

Statement of Compliance

This study meets the US EPA Good Laboratory Practices specified in 40 CFR Part 160 (EPA).

Submitter: _____ Date: _____

Sponsor Representative: Terry A. Kaempfe Date: 11/2/05
Terry A. Kaempfe, B.S.

Principal Investigator: Thomas C. Lee Date: 11/2/05
Thomas C. Lee, Ph.D.

Quality Assurance Statement

Analytical Sub-Report Title: Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with *E. coli*-Produced Cry1A.105 Protein

Charles River Study No. EUF00081
Monsanto Study No. CRO-2005-050

Reviews conducted by the Quality Assurance Unit confirm that the final analytical sub-report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

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

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
06/28/2005	Dose Preparation	07/12/2005	07/12/2005
08/01/2005	Purity/Molecular Weight Determination	08/02/2005	08/02/2005
10/17/2005	Raw Data Audit	10/31/2005	10/31/2005
10/17/2005	Draft Report Audit	10/31/2005	10/31/2005

Joan M. Rejda-Heath — November 2, 2005
Date

Quality Assurance Unit
Monsanto Regulatory, Monsanto Company

Study Information

Charles River/Monsanto Study Number:	EUF00081/CRO-2005-050
Analytical Sub-Report Title:	Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with *E. coli*-Produced Cry1A.105 Protein
Facilities:	Monsanto Company 800 North Lindbergh Boulevard Saint Louis, Missouri 63167, USA
Study Director:	Kimberly L. Bonnette, M.S., LATG
Principal Investigator:	Thomas C. Lee, Ph.D.
Contributors:	Tallis Brown Changjian Jiang, Ph.D. Steve Levine, Ph.D. Richard Thoma Josh Uffman
Study Specific Work Procedure Initiation Date:	June 9, 2005
Analytical Sub-Report Completion Date:	November 02, 2005
Records Retention:	All Study-Specific Work Procedure raw data, Study-Specific Work Procedure plan and amendments, final sub-report and facility records were retained at Monsanto, St. Louis.
Disposition of Remaining Dosing Solutions:	Dosing samples were returned to Monsanto and disposed at the close of the study.

Signature of Analytical Sub-Report Approval

The results reported in this Analytical Sub-Report accurately reflect the data generated under Study-Specific Work Procedure number CRO-2005-050.

Principal Investigator: Thomas C. Lee Date: 11/2/05

Table of Contents

Tables

Figures

Appendices

Abbreviations[1]

AAA	Amino acid analysis
APS	Analytical Protein Standard
BSA	Bovine serum albumin
BW	Body weight
CEW	Corn earworm
CFR	Code of Federal Regulations
COA	Certificate of analysis
CV	Coefficient of variance
EC_{50}	The level of the test article that produces a 50% growth inhibition
E. coli	*Escherichia coli*
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
MW	Molecular weight
MWCO	Molecular weight cutoff
NIST	National Institute of Standards and Technology
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard operating procedure
TDS	Test dosing solution
TCDS	Test control dosing solution
US EPA	United States Environmental Protection Agency
VC	Vehicle control

[1] Standard abbreviations, e.g. units of measure, concentration, mass, time., are used without definition according to the format described in "Instructions to Authors" in The Journal of Biological Chemistry.

1.0 Summary

This analytical sub-report describes the formulation and subsequent analyses of the test dosing solution (TDS), containing the *E. coli*-produced Cry1A.105 protein, and the test control dosing solution (TCDS), containing bovine serum albumin (BSA). The Cry1A.105 and BSA proteins were characterized prior to the mouse gavage study. The TDS and the TCDS were used in a mouse acute oral toxicity study performed at Charles River Laboratories, Inc. (Spencerville, OH).

The Cry1A.105 protein (APS lot 20-100073) was dialyzed into 1 mM carbonate-bicarbonate, pH 10.15-10.32, and 0.125 mM reduced glutathione, and then lyophilized. The TDS was prepared by resuspending the lyophilized powder in 1 mM carbonate-bicarbonate buffer, pH 10.26. The TCDS was prepared by dissolving the BSA protein in 25.8 mM carbonate-bicarbonate, pH 10.28, and 3.1 mM reduced glutathione. A vehicle control (VC) dose containing 25.8 mM carbonate-bicarbonate, pH 10.28, and 3.1 mM reduced glutathione was also prepared. Dosing materials were transported on dry ice (TCDS and VC) or wet ice (TDS) to the testing facility. Ten female and ten male mice were each administered two doses on the same day. All doses were administered at a rate of 33.3 ml/kg body weight (BW). In order to assess the concentration and stability of the dosing solutions, samples containing the TDS and TCDS were collected prior to administration to mice (pre-dose) and after administration of the final dose (post-dose). Aliquots of the TDS were also taken pre-dose to assess the homogeneity of the dosing solution. After the gavage, dosing samples were returned to Monsanto on dry ice followed by storage in a −80 °C freezer prior to dose solution analysis. The stability of the Cry1A.105 and BSA proteins in the TDS and TCDS, respectively, was assessed by determining the purity of the solutions. Purity was assessed using densitometric analysis of pre-dose and post-dose TDS and TCDS samples that were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resulting gels stained with colloidal Brilliant Blue G. In addition, functional activity was used to evaluate stability of the Cry1A.105 protein in the TDS. The functional activity of the Cry1A.105 protein was assessed using a corn earworm (CEW, *Helicoverpa zea*) diet-incorporation insect bioassay, where biological activity was measured as an EC_{50} value.

The purity of the Cry1A.105 protein in pre- and post-dose TDS samples differed by only 1.1% (94% vs. 93% for the pre- and post-dose TDS samples, respectively). The functional activity (EC_{50} value) of the Cry1A.105 protein for the pre- and post-dose TDS samples was 0.011 and 0.0088 µg Cry1A.105 protein per ml of diet, respectively. The purity of the BSA protein in pre- and post-dose TCDS samples differed by only 2.2% (92% vs. 90% for the pre- and post-dose TCDS samples, respectively). These results indicate that the Cry1A.105 and BSA proteins were stable in both the TDS and the TCDS.

The total protein concentrations of the TDS and TCDS were determined using amino acid analysis. The concentration of the Cry1A.105 and BSA proteins in dose solutions were determined based on total protein concentration values corrected for purity. The

concentration of the Cry1A.105 protein in the pre- and post-dose TDS samples was 32.1 mg/ml and 30.1 mg/ml, respectively. The TDS was determined to be a homogeneous suspension. The concentration of the BSA protein in the pre- and post-dose TCDS samples was 30.2 mg/ml and 29.9 mg/ml, respectively.

Based on the average pre- and post-dose purity corrected protein concentration of the TDS (31.1 mg/ml), the Cry1A.105 dose level in TDS was 2072 mg/kg body weight (BW). Using the average pre- and post-dose purity corrected protein concentration of the TCDS (30.0 mg/ml), the BSA dose level was 1998 mg/kg BW.

These data establish the dose levels and stability of the test and control article doses used in the mouse oral acute toxicity study.

2.0 Introduction

This sub-report describes the dose formulation and confirmation used in the mouse acute oral toxicity study for the Cry1A.105 protein. The *E. coli*-produced Cry1A.105 protein was characterized extensively before the initiation of the study. This sub-report includes descriptions of the formulation and analyses of the test and test control article dose solutions. Analyses included protein concentration and purity for the TDS and TCDS, and functional activity for the TDS only, both before and after the dosing of mice. These procedures were performed to evaluate administered dose concentrations and to assess if any changes in the test or test control article occurred during the performance of the acute oral toxicity study.

3.0 Purpose

The purpose of this analytical sub-report was to describe the procedure for the formulation and confirmation of the test, test control, and vehicle dose solutions used in the mouse acute oral toxicity study for the *E. coli*-produced Cry1A.105 protein, performed at Charles River Laboratories Inc. (Charles River study number EUF00081; Spencerville, OH).

4.0 Materials

4.1 Test Article

The test article was the *E. coli*-produced Cry1A.105 protein. The Cry1A.105 protein (Analytical Protein Standard lot 20-100073) was isolated from a fermentation batch of *E. coli* containing an expression plasmid. The identity, concentration, purity, stability, and functional activity of the Cry1A.105 protein (lot 20-100073) were previously determined under characterization plan 20-100073 and are described in the Certificate of Analysis (COA). A copy of the COA is archived with this study file. Lot 20-100073 has a total protein concentration of 1.2 mg/ml and a purity of 92%. Activity was confirmed using an insect bioassay with the larvae of a sensitive pest, CEW. The EC_{50} value was

5.8 ng Cry1A.105 protein per ml of diet. Prior to use, the test article was stored in a -80 °C freezer in a buffer solution containing 25 mM CAPS, pH ~10.3, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT.

4.2 Test Control Article

The test control article was bovine serum albumin (BSA) protein (lot B59628) purchased from Calbiochem (catalog # 126609). The vendor's COA is archived with this study file. According to the vendor's characterization, the protein has a purity of 98%. The relative amount of protein in the solid BSA powder was determined by amino acid analysis as 85%.

4.3 Vehicle Control

The Vehicle Control (VC) was 25.8 mM carbonate-bicarbonate, pH 10.28, and 3.1 mM reduced glutathione (lot 7622132-A). This solution was used to formulate the TCDS, and was designed to be comparable to the final buffer composition of the formulated test article.

4.4 Assay Controls

Protein molecular weight markers (Bio-Rad Broad Range, Hercules, CA) were used to calibrate SDS-polyacrylamide gels. NIST amino acid calibration control standard (National Institutes of Standards and Technology - NIST, Gaithersburg, MD) was used to calibrate the amino acid analyzer and NIST BSA was used as a hydrolysis control. Norvaline (Sigma, St. Louis) was used as an internal standard for amino acid analysis. BSA (NIST, Gaithersburg, MD) was used to generate the standard curve for the Bio-Rad protein assay.

5.0 Dose Preparation

5.1 Calculation of the Test and Test Control Article Dosing Solution Concentrations

The theoretical protein dose solution concentrations for formulation of the TDS and TCDS were calculated assuming the following:

a) The minimal acceptable dose level for each mouse is at least 1000 mg/kg BW.

b) The average mouse body weight (BW) is 0.030 kg.

c) Doses are administered at 33.3 ml/kg BW, ~1 ml/dose, twice daily:

$$0.030\ kg\ BW \times 33.3\ ml/kg \cong 1\ ml$$

For an overall dose solution concentration of 2000 mg/kg BW, the total protein concentration required is 30 mg/ml:

$$\frac{2000\ mg/kg\ BW}{33.3\ ml/kg\ BW} \cong 60\ mg/ml\ dose\ (30\ mg/ml \times 2\ doses)$$

d) The minimum total volume of sample required is 40 ml:

$$20\ mice \times 2\ doses \times 1\ ml = 40\ ml$$

The estimated purity of the Cry1A.105 protein is 92%, therefore, the TDS solution should contain approximately 33 mg/ml of total protein:

$$\frac{30\ mg/ml}{0.92} \cong 33\ mg/ml$$

The estimated purity of the BSA protein is 98% and protein content is 85%, therefore, the TCDS solution should contain approximately 36 mg/ml of BSA powder:

$$\frac{30\ mg/ml}{0.85 \times 0.98} \cong 36\ mg/ml$$

Dose levels for the Cry1A.105 and BSA proteins in the TDS and TCDS, respectively, were calculated based on the average of the experimentally determined pre- and post-dose solution concentration and purity values.

5.2 Formulation of the Test Article in the Test Dosing Solution (TDS)

The Cry1A.105 protein (lot 20-100073, ~2100 ml) was thawed and transferred to two large dialysis bags (Spectra Por®, Gardena, CA, Cat. No. 132682, 12-14 kDa MWCO). Dialysis was conducted at 4 °C against 3 x 50 L of 1 mM carbonate-bicarbonate buffer, pH 10.15-10.32, and 0.125 mM reduced glutathione. The total dialysis time was 68.5 h, and the final recovered volume was ~2300 ml. The dialyzed protein solution was quick-frozen in a dry ice/methanol bath in the bottom of six rectangular polypropylene instrument/pipette sterilizing trays (Nalgene, Cat. No. 6910-0618, ~380 ml/tray). A series of holes were drilled in the lids of each tray to allow for the passage of water vapor, and a sheet of miracloth (Calbiochem, Cat. No. 475855) was placed between the lids and bottoms of each tray to prevent losses of the final lyophilized protein powder. The trays of frozen protein solution were placed on the temperature controlled shelves of a VirTis 24 x 48 general purpose freeze dryer and lyophilized over the course of approximately 1 week. A total of 4.93 g of lyophilized powder containing ~2318 mg of Cry1A.105 protein was recovered. To prepare the TDS,

a total of 4.21 g of the lyophilized powder containing ~1979 mg of the Cry1A.105 protein was first placed in a 400 ml tared polypropylene beaker. Then a total of 57 ml of 1 mM carbonate-bicarbonate buffer, pH 10.26 (lot no. 7622026-A) was added to the powder to slowly dissolve/suspend the protein. The protein solution/suspension was stored for 3 days in a 4 °C cold room at which point a uniform protein solution/suspension was observed. Samples of the formulated protein (total of 250 µL) were taken for estimation of total protein concentration by Bio-Rad protein assays and for analysis by SDS-PAGE to assess the suitability of the TDS. Following one additional day of storage at 4 °C, the test article dosing solution (~60 ml) was transferred to a wide mouth container with a Teflon stir bar. The TDS was assigned lot 20-100073-C (Testing Facility Identification number S05.024.EUF) and shipped on wet ice to the testing facility.

5.3 Formulation of the Protein Control in the Test Control Dosing Solution (TCDS)

BSA powder (2160 mg, Calbiochem Cat. No. 126609, lot B59628) was placed in a 250 ml polypropylene beaker and brought to ~60 ml total volume by the addition of 58 ml of 25.8 mM carbonate-bicarbonate, pH 10.28, and 3.1 mM reduced glutathione (lot 7622132-A). The solution was transferred (~60 ml) to a wide mouth container with a Teflon stir bar. The TDS was assigned lot B59628-C (Testing Facility Identification number S05.023.EUF), and shipped on dry ice to the testing facility.

5.4 Formulation of the Vehicle Control

A separate wide mouth container with a Teflon stir bar was filled with 75 ml of 25.8 mM carbonate-bicarbonate, pH 10.28, and 3.1 mM reduced glutathione (lot 7622132-A, Testing Facility Identification number V05.002.EUF). The exact millimolar concentrations of the carbonate-bicarbonate salts and reduced glutathione in the vehicle were calculated following the formulation of the test article. The correct composition of the vehicle control took into account the initial salt composition of the dialysis buffer, the concentration factor for these salts, and the buffer composition of the resuspension buffer (see Section 5.2). The vehicle control was shipped on dry ice to the testing facility on the same day as its formulation.

5.5 Samples Received from the Testing Facility.

The following samples of the TDS and TCDS were received from the Testing Facility (samples were returned on dry ice) for analysis. All samples were stored in a –80 °C freezer prior to analysis.

Samples for Analysis	Amount (µL)
TDS pre-dose	500
TDS post-dose	500
TDS homogeneity (top)	250
TDS homogeneity (middle)	250
TDS homogeneity (bottom)	250
TCDS pre-dose	500
TCDS post-dose	500

6.0 Methods

A list of applicable SOPs for the methods described below is shown in Appendix 1.

6.1 Amino Acid Analysis

The total protein concentration of the pre- and post-dose TDS and TCDS samples and the TDS homogeneity samples was analyzed by amino acid analysis. Aliquots of the dosing solution samples were diluted with reagent grade water to 1-2 mg/ml prior to analysis. Total protein concentration was estimated by amino acid analysis using a Hitachi L-8800 Amino Acid Analyzer with AAA System Manager Software. Test samples, NIST BSA (used as a system suitability standard), and a NIST amino acid calibration control standard (Gaithersburg, MD), were spiked with an internal reference (norvaline, Sigma Chemical Co.) and dried in a Savant SpeedVac concentrator (Holbrook, NY). Vapor phase acid hydrolysis [6 N HCl containing 1% (v/v) phenol] was performed at 149-151°C for 90-120 min. Cooled samples were again evaporated, reconstituted in protein hydrolyzate buffer (PH-1, Hitachi Instruments) and loaded onto the instrument. Amino acids were detected using ninhydrin derivatization. Amino acid analysis was also used to determine the protein content of the lyophilized BSA protein powder (Test Control Article).

6.2 Bio-Rad Protein Assay

The Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) was performed using 96-well microtiter plates. This colorimetric dye-binding assay is based on the method of Bradford (1976). Total protein concentration of the TDS was determined (during assessment of the suitability of the TDS for dosing) as the mean of three dilutions, each dilution assayed in triplicate. A standard curve ranging from 0.05 to 0.6 mg/ml BSA was prepared in duplicate. All standards and samples were diluted with 1 mM carbonate-bicarbonate buffer, pH 10.26 (lot 7622026-A). Plates were read at 595 nm using a PowerWave X_i microplate reader (Biotek Instruments, Winooski, VT) interfaced with KC4 software (version 3.3 Rev 10).

6.3 SDS-PAGE and Purity Analysis

Aliquots of the TDS and TCDS were subjected to electrophoresis on pre-cast tris-glycine 4-20% polyacrylamide gradient mini-gels (Invitrogen, Carlsbad, CA) under reducing and denaturing conditions. For TDS suitability evaluation, aliquots of the TDS and Test Article (APS lot 20-100073) were analyzed at 0.5, 1.0, and 1.5 µg total protein per lane. For TDS and TCDS purity evaluation, protein samples were loaded at ~1, 2, and 3 µg total protein per lane. Broad range molecular weight markers (Bio-Rad, Cat. No. 161-0317, Hercules, CA) were used to estimate molecular weights. All samples were diluted in 10 mM DTT, mixed with 5X loading buffer, heated at 100.4-100.8 °C for 3 min, and then applied to a 10-well mini-gel. Electrophoresis was performed at constant voltage (114-120 V for 10-20 min followed by 180-184V for 70-76 min). Proteins were fixed with 40% (v/v) methanol, 7 % (v/v) acetic acid, stained (14-17 h) by gentle shaking with Brilliant Blue G colloidal stain (Sigma, Chemical Co., St. Louis, MO), and destained for 30s in a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid followed by further destaining in a solution of 25% methanol for 6.65-8 h. Purity and molecular weight were evaluated using a Bio-Rad GS-800 calibrated densitometer equipped with Quantity One® software (version 4.4.0 build 036). Molecular weight was evaluated for positional reference only. The purity was calculated as the mean of all three lane loads. Purity for the test article (Cry1A.105) was based on a summation of stained bands from the full length Cry1A.105 protein (~130 kDa) down to and including the trypsin-resistant core protein (~56 kDa), whereas the purity of the test control article (BSA) was based on the target band of ~66 kDa. Dosing solutions were considered to be stable for the duration of the dosing period if a ≤10% change in protein purity between the pre- and post-dose samples was observed. For the TDS suitability evaluation conducted prior to shipping the TDS to the testing facility, the banding pattern for the TDS and test article (APS lot 20-100073) were compared visually.

6.4 Functional Activity Analysis

Aliquots of the TDS from both pre- and post-dose were analyzed for the functional activity of the Cry1A.105 protein. TDS was considered to be stable for the duration of the dosing period if activity was demonstrated in both the pre- and post-dose samples. Aliquots of the pre- and post-dose samples were used to estimate the effective protein concentration necessary to inhibit the growth of the target insect (CEW) by 50% (bioactivity measured as an EC_{50} value) as described in Appendix 2.

6.5 Statistical Methods

EC_{50} determination was performed with a three-parameter logistic model using the PROC NLIN procedure in SAS (version 9.1). A full description of the model and the results of the analysis are summarized in the bioassay sub-report

(Appendix 2). SD and % CV values for TDS and TCDS concentration and purity data were calculated using Microsoft® Excel 2000 software (version 9.0.7616 SP-3).

7.0 Control of Bias and Quality Measures

Appropriate sets of dosing solution samples were analyzed concurrently on SDS-polyacrylamide gels to eliminate any run-to-run variability. Multiple dilutions were utilized for the Bio-Rad protein and functional assays to ensure that data were collected within operational ranges of the assay.

8.0 Changes to the Study-Specific Work Procedure

There were two amendments made to the Study-Specific Work Procedure. Prior to formulation of the TCDS, it was discovered that the lot number for the BSA was incorrectly specified in the Study-Specific Work Procedure. The correct lot number for the Calbiochem BSA (Cat. No. 126609) was B59628, whereas the lot number specified in the Study Specific Work Procedure was lot no. B54774. The wrong lot number specified for the BSA protein was due to an inadvertent word processing carry-over from a template document and was corrected by the Amendment No. 1. A second Amendment was filed to correct the lot number specified for the TDS in the title for Section 3.3. In the original Study-Specific Work Procedure, the TDS lot number of the Section 3.3 title was specified as lot 20-100071-C, instead of 20-100073-C. This was also due to an inadvertent word processing carry-over from a template document. There was no impact on the quality of the study in either case.

There was one deviation recorded to the Study-Specific Work Procedure. On 6/28/05, dosing solutions were shipped from the Sponsor (Monsanto) and arrived at the Testing Facility (Charles River Laboratories, Inc.) on 6/29/05. The TDS was shipped on wet ice, whereas the TCDS and vehicle control were shipped on dry ice. According Section 3.6.1 of the Study-Specific Work Procedure, the dosing solutions were to be stored in a refrigerator (~4 °C) or on wet ice until use. The TDS was placed, as expected, in a refrigerator. The TCDS and vehicle control, however, were placed in a freezer (−70 °C) upon arrival on 6/29/05. The TCDS and vehicle were subsequently thawed on 6/30/05 (the day of dosing). The storage of the TCDS and vehicle in the freezer, instead of in a refrigerator or wet ice on 6/29/05 was due to confusion about the storage conditions. There was no impact of the deviation on the study. Storage at −70 °C on 6/29/05, rather than ~4 °C or wet ice, prior to thawing on the day of dosing would have no impact on the stability of the vehicle control or the TCDS (contains BSA protein). Additionally, the expected concentration and purity values for the TCDS (BSA protein) in dosing samples, were ultimately confirmed by the Sponsor.

9.0 Data Rejected

The Bio-Rad total protein assay was conducted twice. The first assay was rejected because the CV of duplicate wells for the 0.1 and 0.2 mg/ml standards did not meet assay criteria.

10.0 Results and Discussion

10.1 Dosing Solution Formulation and Suitability Assessment

Dosing solutions containing the Cry1A.105 and BSA proteins were prepared based on the calculations and assumptions described in Section 5.1. The composition of the vehicle control was determined following formulation of the TDS (see discussion below). In order for the TDS to be judged suitable for dosing, it had to meet several criteria. The criteria were: 1) pass through an 18-gauge needle; 2) have a total protein concentration ≥ 16.3 mg/ml using a Bio-Rad colorimetric protein assay; and 3) demonstrate that the procedures used to produce the TDS did not impact the quality of the test article, based on the protein banding pattern using SDS-PAGE of the TDS compared to the test article. The TDS passed all of these criteria. The TDS readily passed through an 18-gauge needle. The total protein concentration was 27.3 mg/ml (Bio-Rad assay), and the banding pattern, using SDS-PAGE analysis, was comparable for the TDS and test article. The comparable banding pattern demonstrated that the procedures used to produce the TDS (i.e. dialysis, lyophilization and re-suspension) did not impact the physical integrity of the Cry1A.105 protein (Figure 1).

The exact millimolar concentrations of the carbonate-bicarbonate salts and reduced glutathione in the vehicle were calculated following formulation of the test article. The test article was exhaustively dialyzed in 1 mM carbonate-bicarbonate buffer, pH 10.15-10.32, and 0.125 mM reduced glutathione. The volume of the sample increased from ~2100 ml to ~2300 ml. The initial total protein concentration of the test article (APS lot # 20-100073) was 1.2 mg/ml. Following dialysis, the total protein concentration of the solution was:

$$\frac{2100\ ml}{2300\ ml} \times 1.2\ mg/ml \cong 1.1\ mg/ml$$

The dialyzed material was subsequently lyophilized (no loss of buffer salts occurs) and re-suspended in 1 mM carbonate-bicarbonate buffer, pH 10.26. The total protein concentration for the TDS was estimated at 27.3 mg/ml, based on the Bio-Rad assay. The actual concentration factor for the buffer salts could therefore be estimated by the protein concentration factor:

$$\frac{27.3\ mg/ml}{1.1\ mg/ml} \cong 24.8\ fold$$

The final buffer salt composition calculated for the vehicle control was therefore:

$$1\ mM\ carb-bicarb \times 24.8 \cong 24.8\ mM$$
$$1\ mM\ carb-bicarb\ in\ the\ resuspension\ buffer$$
$$0.125\ mM\ reduced\ glutathione \times 24.8 \cong 3.1\ mM$$
$$\sum = 25.8\ mM\ carb-bicarb, 3.1\ mM\ reduced\ glutathione$$

The final total protein concentration of the TDS (avg. of pre- and post-dose samples) was 33.3 mg/ml based on amino acid analysis. This compares well with the 27.3 mg/ml that was estimated by the Bio-Rad assay method. This indicates that the buffer salt composition of the vehicle was slightly underestimated (by ~18%).

10.2 Protein Concentration of the TDS and TCDS

Total protein concentration was determined using amino acid analysis on samples taken before and after administration of doses (Tables 1 and 2). The total protein concentrations for the pre- and post-dose TDS samples were determined as 34.1 mg/ml and 32.4 mg/ml, respectively (Table 1). The average concentration of the TDS samples was calculated as 33.3 mg/ml.

The total protein concentrations for the pre- and post-dose TCDS samples were determined as 32.8 mg/ml and 33.2 mg/ml, respectively (Table 2). The average concentration of the TDS samples was calculated as 33.0 mg/ml.

10.3 Evaluation of TDS Homogeneity

TDS homogeneity was evaluated as the percent coefficient of variance (%CV) of the average concentration of the top, middle and bottom homogeneity samples (Table 3). The average concentration was 31.9 mg/ml with a %CV of 2.67%. The criterion for homogeneity was a %CV ≤15% as specified by the Study Specific Work Procedure. The TDS was therefore judged to be homogenous.

10.4 Purity of the Cry1A.105 and BSA Proteins in the TDS and TCDS

To assess the stability of the Cry1A.105 and BSA proteins in the TDS and TCDS, respectively, dose samples taken before (pre-dose) and after (post-dose) administration to mice were analyzed using densitometry of colloidal Brilliant Blue G stained SDS-polyacrylamide gels (Figures 2 and 3). Each dosing sample was analyzed at 1, 2 and 3 µg total protein. Purity for the test article (Cry1A.105) was based on a summation of stained bands from the full length Cry1A.105 protein (~130 kDa) down to and including the trypsin-resistant core protein (~56 kDa), whereas the purity of the test control article (BSA) was based on the

target band of ~66 kDa. The percent optical density of the target bands (Cry1A.105 or BSA), relative to all bands detected in the lane, was used for the purity estimate. Purity data for the Cry1A.105 and BSA proteins are summarized in Table 4.

The average percent purity of the Cry1A.105 protein in the pre and post-dose TDS sample was 94 and 93%, respectively. The percent change in purity was 1.1% (Table 4). Therefore, the TDS was considered to be stable for the duration of the dosing period.

The average purity of the TCDS protein was 92% and 90% in pre- and post-dose TCDS samples, respectively. The percent change in purity was 2.2% (Table 4). Therefore, the TCDS was considered to be stable for the duration of the dosing period.

10.5 Functional Activity of the Cry1A.105 Protein in the TDS

Functional activity of the TDS was evaluated using a CEW diet incorporation assay and the data are summarized in Appendix 2. EC_{50} values for the pre- and post-dose TDS samples were nearly identical and determined to be 0.011 and 0.0088 µg Cry1A.105 protein/ml of diet (Table 5 and Appendix 2), respectively. Thus, the TDS was considered to be biologically active and stable throughout the period of dosing.

10.6 Calculation of the TDS and TCDS Dose Levels

The final dose levels were calculated using concentration and purity values determined for the TDS and TCDS as described in sections 10.2 and 10.4. The data for purity corrected protein concentration and final dose level are shown in Table 6.

The dose level for the TDS was determined as 2072 $\frac{mg}{kg\,BW}$:

a) Concentration corrected for purity: $33.3\frac{mg}{ml}\times 0.935 = 31.1\frac{mg}{ml}$

b) Protein level per dose: $31.1\frac{mg}{ml}\times 33.3\frac{ml}{kgBW} = 1036\frac{mg}{kgBW}$

c) Final dose level: $1036\frac{mg}{kgBW}\times 2\ doses = 2072\frac{mg}{kgBW}$

The dose level for the TCDS was determined as 1998 $\frac{mg}{kg\,BW}$:

a) Concentration corrected for purity: $33.0\,\frac{mg}{ml} \times 0.91 = 30.0\,\frac{mg}{ml}$

b) Protein level per dose: $30.0\,\frac{mg}{ml} \times 33.3\,\frac{ml}{kgBW} = 999\,\frac{mg}{kgBW}$

c) Final dose level: $999\,\frac{mg}{kgBW} \times 2\ doses = 1998\,\frac{mg}{kgBW}$

11.0 Conclusions

These results determined the dose level of the *E. coli*-produced Cry1A.105 protein (lot 20-100073) in the test dosing solution (TDS) and the dose level of the BSA protein in the test control dosing solution (TCDS). The TDS was determined to be homogenous. Stability of the Cry1A.105 protein in the TDS was established by evaluating purity and functional activity. Stability of the BSA protein in the TCDS was established by evaluating purity. Both the TDS and TCDS were determined to be stable throughout the period of dosing. The administered Cry1A.105 (TDS) dose level was calculated to be 2072 mg/kg BW. The TCDS protein was administered at a dose level of 1998 mg/kg BW.

12.0 References

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* 72: 248-254.

Table 1. Observed Total Protein Concentrations of the TDS Samples Based on Amino Acid Analysis

Sample	Aliquot	Observed Concentration (mg/ml)	Pre- or Post-dose Average Protein Concentration (mg/ml)	SD	%CV	Average of Pre- and Post-dose Concentration (mg/ml)
Pre-dose	1	33.48	34.1	0.55	1.61	33.3
	2	34.13				
	3	34.57				
Post-dose	1	32.22	32.4	0.22	0.68	
	2	32.66				
	3	32.44				

Table 2. Observed Total Protein Concentrations of the TCDS Samples Based on Amino Acid Analysis

Sample	Aliquot	Observed Concentration (mg/ml)	Pre- or Post-Dose Average Protein Concentration (mg/ml)	SD	%CV	Average of Pre- and Post-dose Concentration (mg/ml)
Pre-dose	1	33.36	32.8	0.56	1.70	33.0
	2	32.92				
	3	32.25				
Post-dose	1	33.46	33.2	0.22	0.65	
	2	33.03				
	3	33.25				

Table 3. Homogeneity of the TDS Based on Total Protein Concentration by Amino Acid Analysis.

Sample	Aliquot[1]	Observed Concentration (mg/ml)	Average Protein Concentration (mg/ml)	Overall Mean (mg/ml)	SD	%CV
Top	1	32.58	32.7	31.9	0.85	2.67
	2	32.58				
	3	33.04				
Middle	1	31.73	31.9			
	2	31.73				
	3	32.18				
Bottom	1	31.06	31.0			
	2	30.66				
	3	31.26				

[1]Homogeneity samples were taken immediately prior to dosing.

Table 4. Observed Protein Purities of the TDS and TCDS Samples

Dose Identification	Sample	Observed Purity (%)[1]	Percent Change[2]	Purity (%) Used in Dose Calculation[3]
TDS	Pre-dose	94[4]	1.1	93.5
	Post-dose	93[4]		
TCDS	Pre-dose	92	2.2	91.0
	Post-dose	90		

[1]Each value represents the mean of three purity values estimated from loadings of 1, 2, and 3 µg total protein.

[2]Calculated as follows: $\left|\frac{(\text{Pre-dose}) - (\text{Post-dose})}{(\text{Pre-dose})}\right| \times 100\%$

[3]Calculated as the mean of the pre-dose and post-dose protein purity values for each protein.

[4]Purity based on the summation of stained bands from full length Cry1A.105 protein (~130 kDa) down to and including the trypsin-resistant core protein (~56 kDa).

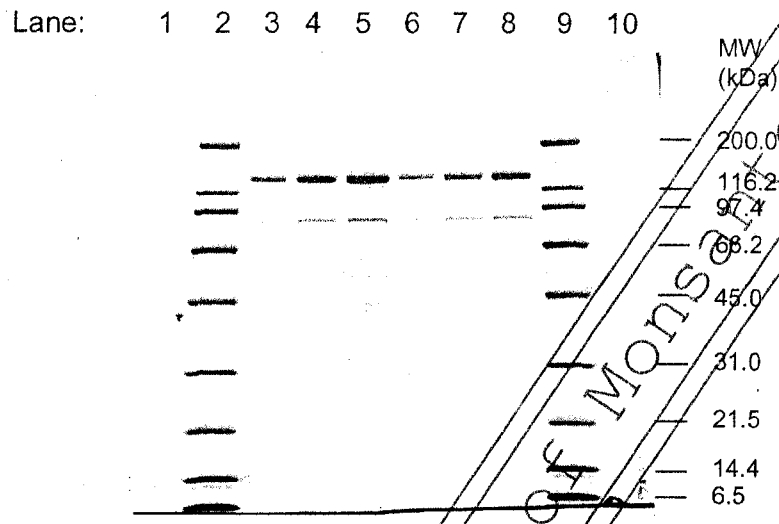
Table 5. EC_{50} Estimates for the Pre- and Post-dose TDS Samples

Sample	EC_{50} (µg Cry1A.105/ml diet)	Standard Error (µg/ml diet)
Pre-dose	0.011	0.0022
Post-dose	0.0088	0.0020

Table 6. Experimentally Determined Dose Levels

Dose Identification	Purity Corrected Concentration (mg/ml)	Dose Level (mg/kg BW)
Avg. Dose TDS[1]	31.1	2072
Avg. Dose TCDS[2]	30.0	1998

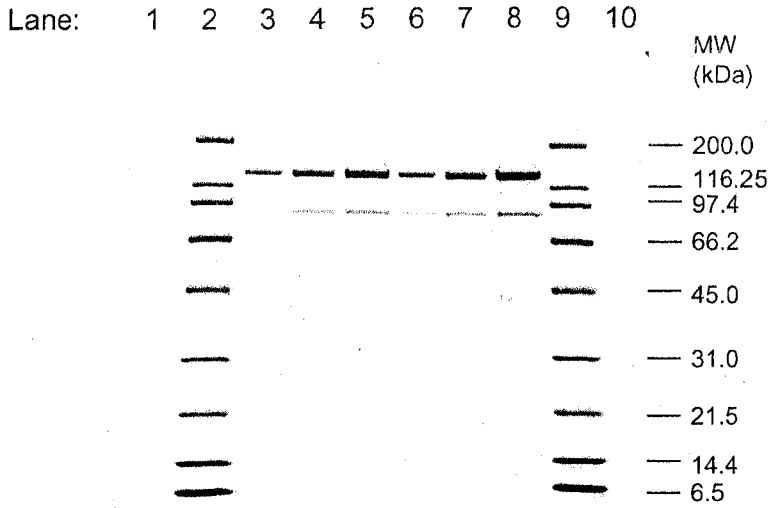
[1]TDS and TCDS dose levels were calculated as described in Section 10.6



Lane	Description	Amount (µg)
1	Empty Lane	-
2	Bio-Rad Broad Range MW markers	9
3	Test Article APS lot 20-100073	0.5
4	Test Article APS lot 20-100073	1.0
5	Test Article APS lot 20-100073	1.5
6	TDS	0.5
7	TDS	1.0
8	TDS	1.5
9	Bio-Rad Broad Range MW markers	9
10	Empty Lane	-

Figure 1. Suitability Analysis of the TDS by SDS-PAGE.

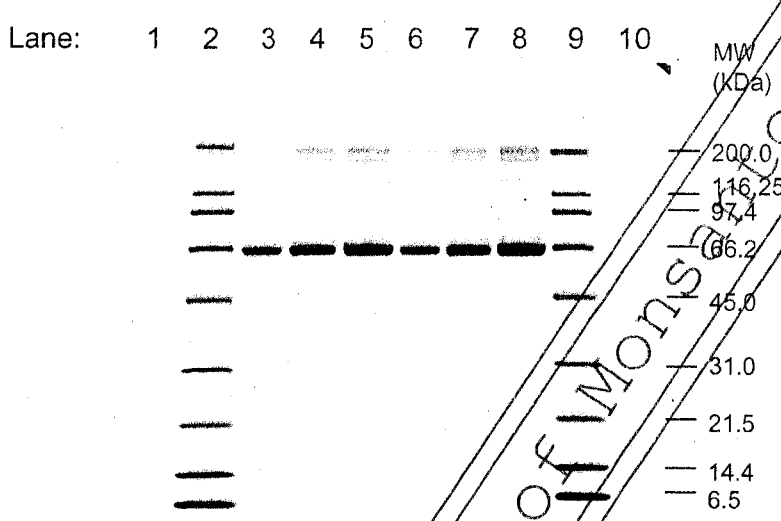
Samples of the TDS and Test Article (APS lot 20-100073) were analyzed on 4-20% polyacrylamide gradient mini-gels under denaturing and reducing conditions. The gel was stained with colloidal Brilliant Blue G. The size of the molecular weight markers is shown on the right.



Lane	Description	Amount (µg)
1	Blank Lane, 1 X loading buffer	-
2	Bio-Rad Broad Range MW markers	9
3	TDS pre-dose sample	1
4	TDS pre-dose sample	2
5	TDS pre-dose sample	3
6	TDS post-dose sample	1
7	TDS post-dose sample	2
8	TDS post-dose sample	3
9	Bio-Rad Broad Range MW markers	9
10	Blank Lane, 1 X loading buffer	-

Figure 2. SDS-PAGE Analysis of the Cry1A.105 Protein in the Pre- and Post-dose TDS Samples

Samples of the pre- and post-dose TDS samples were analyzed on 4-20% polyacrylamide gradient mini-gels under denaturing and reducing conditions. The gel was stained with colloidal Brilliant Blue G. The size of the molecular weight markers is shown on the right.



Lane	Description	Amount (µg)
1	Blank Lane, 1 X loading buffer	-
2	Bio-Rad Broad Range MW markers	9
3	TCDS pre-dose sample	1
4	TCDS pre-dose sample	2
5	TCDS pre-dose sample	3
6	TCDS post-dose sample	1
7	TCDS post-dose sample	2
8	TCDS post-dose sample	3
9	Bio-Rad Broad Range MW markers	9
10	Blank Lane, 1 X loading buffer	-

Figure 3. SDS-PAGE Analysis of BSA Protein in the Pre- and Post-dose TCDS Samples

Samples of the pre- and post-dose TDS samples were analyzed on 4-20% polyacrylamide gradient mini-gels under denaturing and reducing conditions. The gel was stained with colloidal Brilliant Blue G. The size of the molecular weight markers is shown on the right.

Appendix 1. List of Applicable SOPs

BR-EQ-0376-02	Hitachi L-8800 Amino Acid Analysis System
BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0525-01	Bio-Rad Protein Assay
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-EQ-0599-02	Bio-Rad GS-710 and GS-800 Densitometers
BR-ME-0956-02	Protein Percent Purity and Apparent Molecular Weight Determination
BR-ME-0973-01	Drying of Polyacrylamide Mini-Gels Using Invitrogen Gel Drying System (Adaptation of Invitrogen Gel Drying Procedure)
BR-ME-0044-03	Diet Incorporation Insect Bioassay for Use in Determining Biological Activity
BR-ME-0990-01	Vapor Phase Acid Hydrolysis Using 6N HCl and Subsequent Amino Acid Analysis

Appendix 2. Insect Bioassay Summary

Insect Bioassay Summary:

Study Specific Work Procedure for the Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with Cry1A.105 Protein

Study No.: CRO-2005-050

Purpose:

An insect bioassay was performed to verify the biological activity for the Cry1A.105 protein in samples collected before (pre-dose) and after dosing (post-dose) during Monsanto study SB-2005-050 in a corn earworm (CEW), *Helicoverpa zea*, diet-incorporation insect bioassay. Biological activity was measured as an EC50 value, the level of Cry1A.105 protein that produces a 50% growth inhibition.

Materials & Methods:

Pre-dose and post-dose samples prepared from the pre-dose and post-dose solutions were received from the Monsanto Product Characterization Center (PCC), identified as pre-dose TDS sample (lot #: 20-100073-C) and post-dose TDS sample (lot#: 20-100073-C). Information from the PCC indicated the purity corrected concentration for the pre-dose and post-dose samples to be 0.50 mg Cry1A.105 protein/mL. Both samples were received on wet ice suspended in 25.8 mM carbonate/bicarbonate, 3.1 mM reduced glutathione, pH 10.28. The buffer used in the study was a liquid identified as 25.8 mM carbonate/bicarbonate, 3.1 mM reduced glutathione, pH 10.28 [Lot#: 7622132-A]. Protein samples and the buffer were stored at 4° C.

Insects. CEW were obtained from Benzon Research Inc. Insect eggs were incubated at temperatures ranging from 10° C to 27° C, to achieve the desired hatch time.

Bioassays. CEW were used to measure activity of the pre-dose and post-dose samples in accordance with the Monsanto SOP BR-ME-0044-03. The pre-dose and post-dose samples were run in parallel and shared a control that contained buffer of the same composition that was used to store the proteins. The level of buffer in the control was equivalent to the highest dose level. The Cry1A.105 protein dose solutions were prepared by diluting the pre-dose and post-dose samples with purified water and incorporating the dilution into an agar-based insect diet (Southland). This dose series in diet was chosen to adequately characterize the dose-effect relationship on CEW weight gain for the proteins from both sources. The diet mixture was then dispensed in 1 mL aliquots into a 128-well tray (#BIO-BA-128, CD International, Pitman, NJ). Insect larvae were placed on these diets using a fine, soft bristle paintbrush, with a target number of 16 insects per treatment. The infested wells were covered by a ventilated adhesive cover (#BIO-CV-16, CD

International, Pitman, NJ) and the insects were allowed to feed for a period of approximately seven days in an environmental chamber programmed at 27° C, ambient relative humidity and a lighting regime of 14h:10h, light:dark. The combined weight of the surviving insects at each dose level for each source of protein was recorded at the end of the 7-day incubation period.

Dose Response Modeling and Results:

The following three-parameter logistic model was used to fit the data for each dose-response curve:

$$Wt = \frac{W_0}{1+\left(\frac{DietDose}{EC50}\right)^B} + e$$

where *Wt* is the average larvae weight in mg and *DietDose* is the Cry1A.105 protein diet dose level (µg/mL diet). The three parameters included in the model are as follows: W_0 represents the weight in mg at *DietDose* = 0.0 µg/mL diet, *EC50* represents the effective dose to inhibit the growth of the target insect by 50%, and *B* reflects the rate of weight loss as *DietDose* increases, and *e* denotes the residual (error). SAS (version 9.1) procedure PROC NLIN was used for EC50 estimation. Both samples produced a similar dose dependent response in CEW mean individual body mass (Figure 1). Results from this analysis are listed in Table 1. During a quality check of the data, it was determined that a second purity correction was inadvertently made during the preparation of the doing solutions for the pre- and post-dose solutions. Since this additional purity correction affected each dose level equally, a correction factor was used to adjust EC50 values and their respective standard errors.

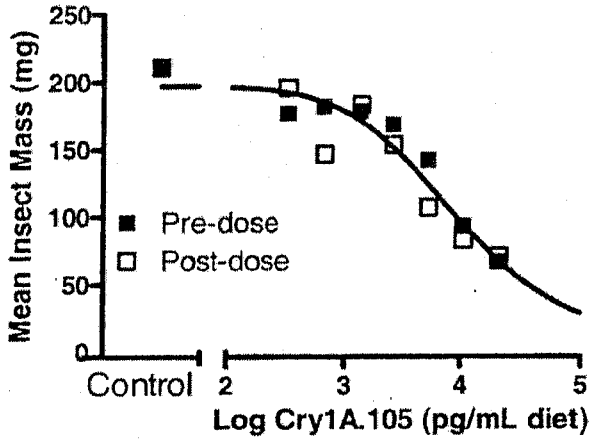


Figure 1. Corn earworm dose-response relationships for Cry1A.105 for pre-dose and post-dose samples in a diet-incorporation bioassay (prepared with GraphPad Prism software v.4.02).

Table 1. EC50 estimates for the Cry1A.105 protein pre-dose and post-dose samples

Cry1A.105 Sample	EC50 (µg/mL diet)	Standard Error (µg/mL diet)
Pre-dose	0.011	0.0022
Post-dose	0.0088	0.0020

Conclusion:

The pre-dose and post-dose samples were demonstrated to be biologically active, with EC50 values determined to be 0.011 and 0.0088 µg Cry1A.105 protein/mL diet, respectively.

Prepared by Joshua Uffman, Steven L. Levine, Ph.D, and Changjian Jiang, Ph.D:

10/31/05
Ecological Technology Center author Date

10/31/05
Ecological Technology Center author Date

10/31/05
Statistical Technology Center author Date

Appendix 3. Detailed Clinical Observation Parameters

Detailed Clinical Observation Parameters

Cage-side Observations	Recorded As
Abnormal movements or behavior	See Categorical
Resistance to removal from cage	Score

Hand-held Observations	Recorded As
Palpebral closure	Score
Lacrimation (non-colored periocular wetness)	Score
Pupil Size	Score
Salivation (non-colored perioral wetness)	Score
Muscle tone	Score
Extensor-thrust response	Score
Reactivity to handling	Score

Open-field observations	Recorded As
Responsiveness to touch	Score
Gait evaluation	Score

Categorical observations (anytime during the DCO)	Recorded As
Abnormal behavior	Description
Abnormalities of the eye	Description
Abnormal urine or feces	Description
Abnormalities of the gastrointestinal (GI) tract	Description
Injury	Description
Missing extremity	Description
Abnormal muscle movements	Description
Palpable mass/swellings	Description
Abnormal posture	Description
Abnormalities of the reproductive system	Description
Abnormal respiration	Description
Abnormal skin or hair-coat/mucous membranes	Description
Excessive soiling	Description
General abnormalities	Description

Explicitly Defined Scales for DCOs

DCO Examination Conduct

The clinical examination is conducted in a careful and systematic format. The examination begins at the head of the animal and gradually works towards the tail as outlined below.

Cage-side observations are made first.

Categorical observations include: Unusual body movements (e.g., tremors, convulsions), abnormal behaviors (e.g., circling, stereotypy) and changes in posture (e.g., arched back, splayed stance).

Resistance to Removal: The degree to which the animal attempts to escape capture is scored. The observer will slowly present a gloved hand into the cage and will grasp the animal over the shoulder area or by the tail.

1 = Decrease – clearly less resistance to capture than typical
2 = Typical – minimally to actively avoids capture and may be mildly aggressive
3 = Increase – clearly more resistance to capture than typical and is very aggressive (attempts to bite).

Eye observations: Eyes are bilaterally examined for these effects; however, if a unilateral observation is made, a concurrent observation is not made for the other eye if it is within normal limits.

Palpebral closure:

1 = Closed (50% to completely closed)
2 = Open
3 = Protruding eyes

Pupil size (aided by penlight): Under typical examination conditions (white light), the typical appearance of the pupils in albino animals is complete constriction. Therefore a decrease in pupil size cannot be observed.

0 = Unable to evaluate
1 = Decrease – clearly decreased pupil size compared to typical
2 = Typical – completely constricted pupils
3 = Increase – clearly increased pupil size compared to typical

Lacrimation (clear wetness): Under typical examination conditions, corneal dryness is not observed in rodents, nor is the eyelids excessively wet.

1 = Decrease – extremely dry appearance of cornea
2 = Typical – glistening cornea (moderate dryness or wetness)
3 = Increase – extensive wetness around the eyes

Degree of salivation: Under typical examination conditions, dryness of the oral cavity is not observed in rodents.

1 = Decrease – oral dryness
2 = Typical – limited to moderate perioral wetness, but lips and chin are dry
3 = Increase – extensive wetness around the mouth and lips

Muscle tone: An assessment of muscle tone at the time of the hand-held observations.

1 = Decrease – clearly less muscle tone than typical
2 = Typical – animal is neither very relaxed nor very tense
3 = Increase – clearly more muscle tone than typical

Extensor-thrust response: Extent of reflex response to brisk pushes (by finger) on the plantar surface of the hindfeet.

1 = Decrease – clearly less response than typical
2 = Typical – clearly detectable extensor-thrust response
3 = Increase – clearly more response than typical

Reactivity to handling: The degree to which an animal struggles to get free from hand-held restraint is ranked.

1 = Decrease – very slight or no struggling
2 = Typical – mild to moderate struggling, animal may vocalize
3 = Increase – aggressive escape behavior, may try to bite observer and usually vocalizes

Observations made in the open-field.

Responsiveness to touch: The ventral aspect of the tail is lightly stroked using a finger. Typically, the animal will lift its tail and wrap it around the finger when lightly touched.

1 = Decrease – does not lift tail, but may briefly hold tail in the air when manually lifted; no response to touch
2 = Typical – lifts tail when touched
3 = Increase – lifts tail and acts startled, may turn towards finger in an attack response

Gait evaluation: Open-field observations are used for gait evaluation. If the animal remains motionless in the open-field, it may be forced to walk on its forelegs while the hindlegs are held off the floor of the observation box ("the wheel-barrow test").

1 = Unable to walk
2 = Clear knuckling, stumbling and poor coordination, may include falling and/or dragging of one or more limbs
3 = Typical – smooth and coordinated gait

Categorical Observations: These observations can be made at anytime during the DCOs. For the categories listed below, the observer directly records the positive observation.

1.	Abnormal behavior	Description
2.	Abnormalities of the eye	Description
3.	Abnormal urine or feces	Description
4.	Abnormalities of the gastrointestinal tract	Description
5.	Injury	Description
6.	Missing extremity	Description
7.	Abnormal muscle movements	Description
8.	Palpable mass/swellings	Description
9.	Abnormal posture	Description
10.	Abnormalities of the reproductive system	Description
11.	Abnormal respiration	Description
12.	Abnormal skin or hair coat/mucous membranes	Description
13.	Excessive soiling	Description
14.	General abnormalities	Description

Appendix 4. Individual Survival and Clinical Observations

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES VEHICLE CONTROL (0 MG/KG) 30-JUN-05 to 14-JUL-05

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
A1868	DEAD	14	14-JUL-05	10:45	SCHEDULED EUTHANASIA
A1869	DEAD	14	14-JUL-05	10:45	SCHEDULED EUTHANASIA
A1874	DEAD	14	14-JUL-05	10:45	SCHEDULED EUTHANASIA
A1880	DEAD	14	14-JUL-05	10:45	SCHEDULED EUTHANASIA
A1884	DEAD	14	14-JUL-05	10:45	SCHEDULED EUTHANASIA
A1891	DEAD	14	14-JUL-05	10:46	SCHEDULED EUTHANASIA
	FIRST DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1895	DEAD	14	14-JUL-05	10:46	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1902	DEAD	14	14-JUL-05	10:46	SCHEDULED EUTHANASIA
A1903	DEAD	14	14-JUL-05	10:46	SCHEDULED EUTHANASIA
A1905	DEAD	14	14-JUL-05	10:46	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES PROTEIN CONTROL - BSA (1000 MG/KG) 30-JUN-05 to 14-JUL-05

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
A1870	DEAD	14	14-JUL-05	10:47	SCHEDULED EUTHANASIA
A1886	DEAD	14	14-JUL-05	10:47	SCHEDULED EUTHANASIA
	FIRST DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1876	DEAD	14	14-JUL-05	10:47	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1878	DEAD	14	14-JUL-05	10:47	SCHEDULED EUTHANASIA
A1879	BODY	8	8-JUL-05	13:11	RAISED AREA - UROGENITAL REGION APPROXIMATELY 5MM IN DIAMETER
	BODY	9	9-JUL-05	06:39	RAISED AREA - UROGENITAL REGION APPROXIMATELY 5MM IN DIAMETER
	BODY	10	10-JUL-05	11:06	RAISED AREA - UROGENITAL REGION APPROXIMATELY 5MM IN DIAMETER
	BODY	11	11-JUL-05	09:59	RAISED AREA - UROGENITAL REGION APPROXIMATELY 10MM IN DIAMETER
	BODY	12	12-JUL-05	12:30	RAISED AREA - UROGENITAL REGION APPROXIMATELY 10MM IN DIAMETER
	BODY	13	13-JUL-05	09:43	RAISED AREA - UROGENITAL REGION APPROXIMATELY 5MM IN DIAMETER
	BODY	14	14-JUL-05	09:47	RAISED AREA - UROGENITAL REGION APPROXIMATELY 5MM IN DIAMETER
	DEAD	14	14-JUL-05	10:47	SCHEDULED EUTHANASIA
A1890	DEAD	14	14-JUL-05	10:47	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES PROTEIN CONTROL - ESA (1000 MG/KG)

30-JUN-05 to 14-JUL-05

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
A1890	(continued)				
	FIRST DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1893	DEAD	14	14-JUL-05	10:47	SCHEDULED EUTHANASIA
A1897	DEAD	14	14-JUL-05	10:47	SCHEDULED EUTHANASIA
A1901	DEAD	14	14-JUL-05	10:47	SCHEDULED EUTHANASIA
A1907	DEAD	14	14-JUL-05	10:47	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES TEST PROTEIN - Cry1A.105 (1000 MG/KG) 30-JUN-05 to 14-JUL-05

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
A1873	DEAD	14	14-JUL-05	00:00	SCHEDULED EUTHANASIA
A1875	DEAD	14	14-JUL-05	00:00	SCHEDULED EUTHANASIA
A1885	ACTIVITY	1	1-JUL-05	08:00	WOBBLY GAIT
	RESPIRATION	1	1-JUL-05	08:00	LABORED BREATHING
	EXCRETA/EMESIS	1	1-JUL-05	08:00	FEW FECES
	BODY	1	1-JUL-05	08:01	COOL TO TOUCH
	ACTIVITY	1	1-JUL-05	12:40	WOBBLY GAIT
	RESPIRATION	1	1-JUL-05	12:40	LABORED BREATHING
	EXCRETA/EMESIS	1	1-JUL-05	12:40	FEW FECES
	BODY	1	1-JUL-05	12:40	COOL TO TOUCH
	EYE(S)	1	1-JUL-05	12:41	EYE(S) APPEAR PALE
	DEAD	1	1-JUL-05	13:08	EUTHANIZED MORIBUND PER STUDY DIRECTOR DUE TO CLINICAL OBS
	ACTIVITY	1	1-JUL-05	07:59	REACTIVITY TO HANDLING - DECREASED
	ACTIVITY	1	1-JUL-05	12:40	REACTIVITY TO HANDLING - DECREASED
	EYE(S)	1	1-JUL-05	08:00	LACRIMATION - INCREASED
	EYE(S)	1	1-JUL-05	12:41	LACRIMATION - INCREASED
	BODY	1	1-JUL-05	12:41	URINE STAIN - UROGENITAL REGION
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1888	DEAD	14	14-JUL-05	00:00	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1892	DEAD	14	14-JUL-05	00:00	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1894	DEAD	14	14-JUL-05	00:00	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES TEST PROTEIN - Cry1A.105 (1000 MG/KG) 30-JUN-05 to 14-JUL-05

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
A1894	(continued)				
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1898	EXCRETA/EMESIS	2	2-JUL-05	08:48	FEW FECES
	EXCRETA/EMESIS	3	3-JUL-05	10:07	FEW FECES
	BODY	13	13-JUL-05	09:58	RAISED AREA - UROGENITAL REGION APPROXIMATELY 5MM IN DIAMETER
	BODY	14	14-JUL-05	10:01	RAISED AREA - UROGENITAL REGION APPROXIMATELY 5MM IN DIAMETER
	DEAD	14	14-JUL-05	00:00	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1899	DEAD	14	14-JUL-05	00:00	SCHEDULED EUTHANASIA
A1904	DEAD	14	14-JUL-05	00:00	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1906	DEAD	14	14-JUL-05	00:00	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES VEHICLE CONTROL (0 MG/KG) 30-JUN-05 to 14-JUL-05

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
A1910	EXCRETA/EMESIS	2	2-JUL-05	08:26	FEW FECES
	EXCRETA/EMESIS	2	2-JUL-05	08:26	FECES SMALL IN SIZE
	EXCRETA/EMESIS	3	3-JUL-05	09:51	FEW FECES
	DEAD	14	14-JUL-05	13:28	SCHEDULED EUTHANASIA
A1913	DEAD	14	14-JUL-05	13:28	SCHEDULED EUTHANASIA
A1918	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1924	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1927	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1928	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
	FIRST DOSING OBS	0	30-JUN-05	00:00	ANIMAL STRUGGLED DURING DOSING
A1930	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1932	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1933	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1944	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES PROTEIN CONTROL - BSA (1000 MG/KG)

30-JUN-05 to 14-JUL-05

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
A1915	EXCRETA/EMESIS	2	2-JUL-05	08:38	FEW FECES
	EXCRETA/EMESIS	3	3-JUL-05	10:00	FEW FECES
	EXCRETA/EMESIS	4	4-JUL-05	08:45	FEW FECES
	EXCRETA/EMESIS	5	5-JUL-05	09:57	FEW FECES
	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1919	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1920	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1923	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1925	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1934	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1935	EXCRETA/EMESIS	2	2-JUL-05	08:42	FEW FECES
	EXCRETA/EMESIS	3	3-JUL-05	10:03	FEW FECES
	EXCRETA/EMESIS	4	4-JUL-05	08:47	FEW FECES
	EXCRETA/EMESIS	5	5-JUL-05	09:59	FEW FECES
	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1937	EXCRETA/EMESIS	2	2-JUL-05	08:43	FEW FECES
	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1938	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA
A1945	DEAD	14	14-JUL-05	13:29	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES TEST PROTEIN - Cry1A.105 (1000 MG/KG) 30-JUN-05 to 14-JUL-05

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
A1909	DEAD	14	14-JUL-05	13:30	SCHEDULED EUTHANASIA
A1912	DEAD	14	14-JUL-05	13:30	SCHEDULED EUTHANASIA
A1914	DEAD	14	14-JUL-05	13:30	SCHEDULED EUTHANASIA
A1916	DEAD	14	14-JUL-05	13:30	SCHEDULED EUTHANASIA
A1922	DEAD	14	14-JUL-05	13:30	SCHEDULED EUTHANASIA
A1926	DEAD	14	14-JUL-05	13:30	SCHEDULED EUTHANASIA
A1929	DEAD	14	14-JUL-05	13:30	SCHEDULED EUTHANASIA
A1936	DEAD	14	14-JUL-05	13:30	SCHEDULED EUTHANASIA
A1942	EXCRETA/EMESIS	3	3-JUL-05	10:12	FEW FECES
	DEAD	14	14-JUL-05	13:30	SCHEDULED EUTHANASIA
A1948	DEAD	14	14-JUL-05	13:30	SCHEDULED EUTHANASIA

Appendix 5. Individual Body Weight Data

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES VEHICLE CONTROL (0 MG/KG)

	DAY OF STUDY				
ANIMAL#	0-PRE-FAST	0-FASTED	1	7	14
A1868	29.2	27.8	N/A	30.0	31.3
A1869	29.3	28.3	N/A	29.0	30.2
A1874	30.2	28.6	N/A	31.0	32.0
A1880	28.2	26.8	N/A	28.0	30.6
A1884	27.8	26.5	N/A	30.0	30.1
A1891	26.7	25.2	N/A	28.0	28.2
A1895	28.3	26.8	N/A	30.0	31.9
A1902	29.3	27.3	N/A	29.0	29.9
A1903	27.8	26.0	N/A	29.0	31.2
A1905	28.6	27.1	N/A	29.0	31.7
MEAN	28.5	27.0	N/A	29.3	30.7
S.D.	1.00	1.03	N/A	0.95	1.17
N	10	10	N/A	10	10

N/A = NOT APPLICABLE

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES - PROTEIN CONTROL - BSA (1000 MG/KG)

	DAY OF STUDY				
ANIMAL#	0-PRE-FAST	0-FASTED	1	7	14
A1870	28.0	26.7	N/A	28.0	29.6
A1886	30.5	29.4	N/A	32.0	34.0
A1876	28.9	27.5	N/A	30.0	30.4
A1878	29.8	28.5	N/A	31.0	28.1
A1879	28.6	27.1	N/A	27.0	24.7
A1890	29.5	27.6	N/A	30.0	32.6
A1893	27.5	26.4	N/A	29.0	30.1
A1897	29.1	27.7	N/A	29.0	31.7
A1901	27.8	26.4	N/A	29.0	29.5
A1907	28.1	26.5	N/A	29.0	30.6
MEAN	28.8	27.4	N/A	29.4	30.1
S.D.	0.96	0.99	N/A	1.43	2.54
N	10	10	N/A	10	10

N/A = NOT APPLICABLE

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES TEST PROTEIN - Cry1A.105 (1000 MG/KG)

ANIMAL#	DAY OF STUDY 0-PRE-FAST	0-FASTED	1	7	14
A1873	28.2	26.6	N/A	28.0	29.0
A1875	30.5	28.7	N/A	31.0	32.1
A1885	27.7	26.3	24.8		
A1888	26.7	26.0	N/A	28.0	30.0
A1892	29.3	27.8	N/A	29.0	30.9
A1894	29.1	27.7	N/A	29.0	30.4
A1898	29.5	28.2	N/A	25.0	25.8
A1899	28.1	26.6	N/A	28.0	29.8
A1904	29.0	27.8	N/A	30.0	32.2
A1906	28.2	27.0	N/A	28.0	27.3
MEAN	28.6	27.3	24.8	28.4	29.7
S.D.	1.07	0.89	0.00	1.67	2.11
N	10	10	1	9	9

N/A = NOT APPLICABLE

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES VEHICLE CONTROL (0 MG/KG)

ANIMAL#	DAY OF STUDY 0-PRE-FAST	0-FASTED	1	7	14
A1910	26.0	25.2	N/A	27.0	28.8
A1913	27.9	26.5	N/A	29.0	30.5
A1918	26.4	24.9	N/A	27.0	27.8
A1924	24.6	23.2	N/A	25.0	25.3
A1927	25.0	23.9	N/A	26.0	27.1
A1928	26.7	25.0	N/A	26.0	26.6
A1930	26.5	24.4	N/A	27.0	26.6
A1932	24.6	23.5	N/A	25.0	25.4
A1933	27.0	25.4	N/A	27.0	27.1
A1944	25.7	24.3	N/A	26.0	25.7
MEAN	26.0	24.6	N/A	26.5	27.1
S.D.	1.08	0.98	N/A	1.18	1.62
N	10	10	N/A	10	10

N/A = NOT APPLICABLE

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES PROTEIN CONTROL - BSA (1000 MG/KG)

	DAY OF STUDY				
ANIMAL#	0-PRE-FAST	0-FASTED	1	7	14
A1915	26.8	24.7	N/A	23.0	24.9
A1919	27.3	26.2	N/A	28.0	29.3
A1920	24.7	23.7	N/A	25.0	24.7
A1923	25.5	25.0	N/A	25.0	25.2
A1925	25.5	23.9	N/A	26.0	26.4
A1934	26.5	24.7	N/A	26.0	26.6
A1935	24.4	23.3	N/A	25.0	25.4
A1937	26.3	25.0	N/A	28.0	26.3
A1938	26.0	24.6	N/A	26.0	27.0
A1945	27.0	25.4	N/A	27.0	28.2
MEAN	26.0	24.6	N/A	25.9	26.4
S.D.	0.97	0.85	N/A	1.52	1.48
N	10	10	N/A	10	10

N/A = NOT APPLICABLE

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES / TEST PROTEIN - Cry1A.105 (1000 MG/KG)

	DAY OF STUDY				
ANIMAL#	0-PRE-FAST	0-FASTED	1	7	14
A1909	25.8	24.8	N/A	27.0	27.2
A1912	27.8	25.9	N/A	28.0	28.4
A1914	26.9	25.3	N/A	25.0	25.2
A1916	24.5	23.4	N/A	24.0	24.0
A1922	27.1	26.1	N/A	27.0	28.7
A1926	26.1	24.9	N/A	25.0	25.7
A1929	25.9	25.0	N/A	27.0	26.9
A1936	26.5	25.1	N/A	26.0	27.1
A1942	25.2	23.9	N/A	24.0	24.4
A1948	24.9	23.5	N/A	24.0	25.5
MEAN	26.1	24.8	N/A	25.7	26.8
S.D.	1.03	0.93	N/A	1.49	1.60
N	10	10	N/A	10	10

N/A = NOT APPLICABLE

Appendix 6. Individual Body Weight Changes

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES VEHICLE CONTROL (0 MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1868	2.2	1.3
A1869	0.7	1.2
A1874	2.4	1.0
A1880	1.2	2.6
A1884	3.5	0.1
A1891	2.8	0.2
A1895	3.2	1.9
A1902	1.7	0.9
A1903	3.0	2.2
A1905	1.9	2.7
MEAN	2.3	1.4
S.D.	0.90	0.92
N	10	10

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES PROTEIN CONTROL - BSA (1000 MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1870	1.3	1.6
A1886	2.6	2.0
A1876	2.5	0.4
A1878	2.5	-2.9
A1879	-0.1	-2.3
A1890	2.4	2.6
A1893	2.6	1.1
A1897	1.3	2.7
A1901	2.6	0.5
A1907	2.5	1.6
MEAN	2.0	0.7
S.D.	0.91	1.92
N	10	10

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES TEST PROTEIN - Cry1A.105 (1000 MG/KG)

	DAY OF STUDY	
ANIMAL#	0-7	7-14
A1873	1.4	1.0
A1875	2.3	1.1
A1888	2.0	2.0
A1892	1.2	1.9
A1894	1.3	1.4
A1898	-3.2	0.8
A1899	1.4	1.8
A1904	2.2	2.2
A1906	1.0	-0.7
MEAN	1.1	1.3
S.D.	1.67	0.89
N	9	9

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES VEHICLE CONTROL (0 MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1910	1.8	1.8
A1913	2.5	1.5
A1918	2.1	0.8
A1924	1.8	0.3
A1927	2.1	1.1
A1928	1.0	0.6
A1930	2.6	-0.4
A1932	1.5	0.4
A1933	1.6	0.1
A1944	1.7	-0.3
MEAN	1.9	0.6
S.D.	0.48	0.72
N	10	10

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES / PROTEIN CONTROL - BSA (1000 MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1915	-1.7	1.9
A1919	1.8	1.3
A1920	1.3	-0.3
A1923	0.0	0.2
A1925	2.1	0.4
A1934	1.3	0.6
A1935	1.7	0.4
A1937	3.0	-1.7
A1938	1.4	1.0
A1945	1.6	1.2
MEAN	1.2	0.5
S.D.	1.28	1.00
N	10	10

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES TEST PROTEIN - Cry1A.105 (1000 MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1909	2.2	0.2
A1912	2.1	0.4
A1914	-0.3	0.2
A1916	0.6	0.0
A1922	0.9	1.7
A1926	0.1	0.7
A1929	2.0	-0.1
A1936	0.9	1.1
A1942	0.1	0.4
A1948	0.5	1.5
MEAN	0.9	0.6
S.D.	0.90	0.63
N	10	10

Appendix 7. Individual Food Consumption Data (Grams/Animal/Day)

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES VEHICLE CONTROL (0 MG/KG)

	DAY OF STUDY	
ANIMAL#	0-7	7-14
A1868	4.6	4.5
A1869	4.3	4.2
A1874	4.5	4.6
A1880	4.4	4.4
A1884	4.3	4.1
A1891	4.5	4.0
A1895	5.0	5.1
A1902	4.5	4.1
A1903	4.5	4.7
A1905	4.7	4.7
MEAN	4.5	4.4
S.D.	0.20	0.35
N	10	10

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES PROTEIN CONTROL - BSA (1000 MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1870	4.4	4.4
A1886	4.7	6.1
A1876	4.3	4.5
A1878	4.6	4.3
A1879	3.4	4.2
A1890	4.0	5.1
A1893	4.4	4.3
A1897	5.1	7.1
A1901	4.0	3.9
A1907	7.1	4.7
MEAN	4.6	4.9
S.D.	1.01	0.98
N	10	10

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES TEST PROTEIN - Cry1A.105 (1000 MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1873	5.4	5.6
A1875	4.3	4.6
A1888	4.6	4.9
A1892	4.2	4.9
A1894	4.6	6.1
A1898	4.2	8.9
A1899	4.3	4.3
A1904	5.1	5.1
A1906	4.2	3.9
MEAN	4.6	5.4
S.D.	0.43	1.47
N	9	9

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES / VEHICLE CONTROL (0 MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1910	5.2	5.3
A1913	5.1	4.9
A1918	4.3	4.1
A1924	3.7	4.4
A1927	5.2	5.4
A1928	4.0	4.2
A1930	5.1	6.8
A1932	4.3	4.6
A1933	3.9	4.3
A1944	4.5	5.1
MEAN	4.5	4.9
S.D.	0.58	0.81
N	10	10

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES PROTEIN CONTROL - BSA (1000 MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1915	3.3	4.4
A1919	4.2	4.9
A1920	3.9	5.3
A1923	3.9	4.0
A1925	4.4	4.9
A1934	4.3	4.5
A1935	3.9	4.9
A1937	4.6	4.6
A1938	7.7	4.6
A1945	5.8	7.6
MEAN	4.6	5.0
S.D.	1.25	0.99
N	10	10

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES / TEST PROTEIN - Cry1A.105 (1000 MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1909	4.4	5.6
A1912	6.1	9.5
A1914	3.6	4.1
A1916	3.9	5.0
A1922	4.6	6.1
A1926	4.2	4.6
A1929	5.1	6.0
A1936	5.4	5.5
A1942	3.0	4.5
A1948	4.5	5.0
MEAN	4.5	5.6
S.D.	0.91	1.51
N	10	10

Appendix 8. Individual Gross Necropsy Data

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES VEHICLE CONTROL (0 MG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1868	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1869	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1874	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1880	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1884	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1891	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1895	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1902	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1903	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1905	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES PROTEIN CONTROL - BSA (1000 MG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1870	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1886	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1876	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1878	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1879	14-JUL-05	14	SKIN: SCABBING; PRESENT OVERLYING LEFT PREPUTIAL GLAND, ONE, 0.4 CM DIAMETER	SCHEDULED EUTHANASIA
A1890	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1893	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1897	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1901	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1907	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES TEST PROTEIN - Cry1A.105 (1000 MG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1873	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1875	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1885	1-JUL-05	1	HAIRCOAT: WET MATTING; PRESENT AROUND EYES; CLEAR COLORLESS ESOPHAGUS: PERFORATION; PRESENT ADJACENT TO HEART; WHITE GELATINOUS MATERIAL SURROUNDS LUNGS	EUTHANIZED MORIBUND
A1888	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1892	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1894	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1898	14-JUL-05	14	GENERAL COMMENT: FINAL CLINICAL OBSERVATION NOT APPARENT POSTMORTEM RAISED AREA, UROGENITAL REGION	SCHEDULED EUTHANASIA
A1899	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1904	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1906	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES VEHICLE CONTROL (0 MG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1910	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1913	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1918	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1924	14-JUL-05	14	OVARY: PERIOVARIAN CYST(S); PRESENT RIGHT, 0.2 CM DIAMETER, CLEAR FLUID FILLED	SCHEDULED EUTHANASIA
A1927	14-JUL-05	14	LYMPH NODE, MANDIBULAR: ENLARGED; PRESENT RIGHT, ONE, 0.6 X 0.5 X 0.2 CM	SCHEDULED EUTHANASIA
A1928	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1930	14-JUL-05	14	OVARY: PERIOVARIAN CYST(S); PRESENT LEFT, ONE, 0.4 CM DIAMETER, CLEAR FLUID FILLED	SCHEDULED EUTHANASIA
A1932	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1933	14-JUL-05	14	OVARY: PERIOVARIAN CYST(S); PRESENT RIGHT, 0.3 CM DIAMETER, CLEAR FLUID FILLED	SCHEDULED EUTHANASIA
A1944	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES / PROTEIN CONTROL - BSA (1000 MG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1915	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1919	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1920	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1923	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1925	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1934	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1935	14-JUL-05	14	LYMPH NODE, MANDIBULAR: ENLARGED; PRESENT RIGHT, ONE, APPROXIMATELY 0.5 X 0.5 X 0.3 CM	SCHEDULED EUTHANASIA
A1937	14-JUL-05	14	STOMACH: FOCI; PRESENT GLANDULAR MUCOSA, THREE, EACH PINPOINT, BROWN	SCHEDULED EUTHANASIA
A1938	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1945	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES TEST PROTEIN - Cry1A.105 (1000 MG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1909	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1912	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1914	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1916	14-JUL-05	14	OVARY: PERIOVARIAN CYST(S); PRESENT LEFT, ONE, 0.4 CM DIAMETER, LIGHT RED FLUID FILLED	SCHEDULED EUTHANASIA
A1922	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1926	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1929	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1936	14-JUL-05	14	LIVER: DARK RED LOBE(S); PRESENT INFERIOR CAUDATE LOBE, FIRM OVARY: PERIOVARIAN CYST(S); PRESENT BILATERAL, EACH APPROXIMATELY 0.3 CM DIAMETER, CLEAR FLUID FILLED LUNG: DARK RED AREA(S); PRESENT ENTIRE RIGHT CARDIAC LOBE AND APPROXIMATELY 50% OF RIGHT APICAL LOBE	SCHEDULED EUTHANASIA
A1942	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES TEST PROTEIN - Cry1A.105 (1000 MG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1948	14-JUL-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

Appendix 9. Personnel Responsibilities

Personnel Responsibilities

Kimberly L. Bonnette, M.S., LATG	Study Director/Director, Acute Toxicology
Jason W. Smedley, B.S.	Alternate Contact/Assistant Toxicologist
Joseph C. Siglin, Ph.D., DABT	General Manager
Rusty E. Rush, M.S., LAT, DABT	Director, Toxicology
Dawn D. Rodabaugh, B.S.	Manager, Acute Toxicology
Pamela S. Smith, ALAT	Study Supervisor, Tox I
Kari L. Long, M.S.	Primary Technician/Vivarium Technician II
Delores P. Knippen	Supervisor, Formulations
Anita M. Bosau, RQAP-GLP	Division Director, Regulatory Compliance
Cheryl A. Bellamy	Manager, Report Writing
Deanna M. Talerico, RQAP-GLP	Manager, Regulatory Compliance
William H. Baker, M.S., D.V.M., DACVP	Division Director, Senior Staff Pathologist
Kathy M. Gasser	Archivist

AN ACUTE ORAL TOXICITY STUDY IN MICE WITH Cry2Ab2 PROTEIN

FINAL REPORT

Guidelines

EPA-OPPTS (870.1100), OECD (401), EEC (B.1)

Author

Kimberly L. Bonnette, M.S., LATG

Study Completed on

February 6, 2006

Performing Laboratory

Charles River Laboratories
Preclinical Services
640 North Elizabeth Street
Spencerville, OH 45887

Study No.

EUF00080

Monsanto Study No.

CRO-2005-049

Submitted to:

Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, MO 63167

Study No. EUF00080
Monsanto Study No. CRO-2005-049

The text below applies only to use of the data by the United States Environmental Protection Agency (US EPA) in connection with the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

1. STATEMENT OF *NO* DATA CONFIDENTIALITY CLAIMS

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)(1)(A), (B), or (C).

"We submit this material to the United States Environmental Protection Agency specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by EPA strictly in accordance with FIFRA. By submitting this material to EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by the company notwithstanding this submission to EPA"

Company: Monsanto Company

Company Agent: _____

Title: _____

Signature: _____ Date: _____

Study No. EUF00080
Monsanto Study No. CRO-2005-049

2. COMPLIANCE STATEMENT

This study was conducted in compliance with the Good Laboratory Practice Standards as described by the EPA (40 CFR Part 160).

Kimberly L. Bonnette, M.S., LATG
Study Director/Author
Charles River Laboratories

Date 2/6/06

Terry A. Kaempfe, B.S.
Sponsor
Monsanto Company

Date 2/8/06

Submitter
Monsanto Company

Date

3. QUALITY ASSURANCE STATEMENT

This study has been inspected by the Quality Assurance Unit to assure conformance with the Good Laboratory Practice (GLP) regulations promulgated by the EPA (40 CFR Part 160). Reports were submitted in accordance with Standard Operating Procedures as follows:

QA INSPECTION DATES

		Dates Findings Submitted to:	
Dates of Inspection	Phase(s) Inspected	Study Director	Study Director's Management
06/03/05	Protocol Review	07/18/05	07/18/05
06/07/05	Animal Receipt	06/07/05	06/07/05
06/16/05	Clinical Observations	07/18/05	07/18/05
07/18/05	Data Audit	07/18/05	07/18/05
07/18/05	Draft Report Review	07/18/05	07/18/05
07/18/05	Protocol Amendment Review	07/18/05	07/18/05
10/03/05	Revised Draft Report Review	10/03/05	10/03/05
11/16/05	Revised Draft Report Review	11/16/05	11/16/05
12/15/05	Revised Draft Report Review	12/15/05	12/15/05
01/13/06	Revised Draft Report Review	01/13/06	01/13/06
02/03/06	Final Report Review	02/03/06	02/03/06

The final report has been reviewed to assure that it accurately describes the materials and methods, and the reported results accurately reflect the raw data.

Matthew D. Bruns Date 2/3/06

Matthew D. Bruns, B.S.
Associate Quality Assurance Auditor

4. INTELLECTUAL PROPERTY RIGHTS

5. TABLE OF CONTENTS

6. SUMMARY

The acute oral toxicity of Cry2Ab2 protein was evaluated in CD-1 mice. Three groups of animals received the vehicle control article, the protein control [bovine serum albumin (BSA)], or the test protein (Cry2Ab2 protein) by oral gavage as indicated below:

Group No.	Treatment[a]	Analytically-Determined Dose Level (mg/kg)	No. of Animals	
			Male	Female
1	Vehicle Control	--	10	10
2	Protein Control (BSA)	2424	10	10
3	*E. coli*-Produced Cry2Ab2 protein	2198	10	10

[a]A total dose volume of 66.6 mL/kg was administered on day 0 as two separate 33.3 mL/kg doses.

Following dosing, the mice were observed daily, weighed weekly, and had food consumption measured weekly. A gross necropsy examination was performed on all animals at the time of scheduled euthanasia (day 14).

No mortality was observed during the study. Clinical observations noted during the 14-day post-dosing period included a single incidence of urine stain and rough coat in one male animal in the vehicle control group.

There were no statistically significant differences in body weight, body weight changes or food consumption between the three groups during the study.

No treatment-related gross pathological findings were observed at necropsy on day 14.

Under the conditions of this test, no mortality or other toxicity was observed in the Cry2Ab2 protein group. Therefore, the acute oral LD50 of Cry2Ab2 protein in the mouse is greater than 2198 mg/kg.

7. INTRODUCTION

This study was performed to assess the potential toxicity of Cry2Ab2 protein in CD-1 mice when administered by gavage as a single oral dose. This study was performed at Charles River Laboratories, 553 North Broadway, Spencerville, Ohio. The protocol was signed by the Study Director on June 3, 2005 (GLP initiation date). The in-life phase of the study was initiated with test article administration on June 16, 2005 (day 0), and concluded with necropsy on June 30, 2005.

8. MATERIALS AND METHODS

8.1. Experimental Protocol

This study was performed in general conformance with US EPA, Health Effects Test Guidelines, OPPTS 870.1100, Acute Oral Toxicity, December 1998; the OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects, Subsection 401, February 24, 1987; and the EEC Part B: Methods for the Determination of Toxicity, B.1, No. L 383 A/110, December 29, 1992.

8.2. Test Article, Control Article and Vehicle Control

The test article, control article (protein control) and vehicle control were defined as follows:

Sponsor's Identification	Monsanto Lot Number
Vehicle Control - 2mM carbonate-bicarbonate, 2mM reduced glutathione pH 10.5 Purity: None provided	7622118-A
Test Control Article - Bovine Serum Albumin, BSA (powder) Purity: 98%	B59628
Test Article – *E. coli*-produced Cry2Ab2 protein Purity: 87%	20-100071

The dosing solutions were prepared by Monsanto Company from the above materials and identified as follows:

Sponsor's ID	Assigned Testing Facility ID	Physical Description	Receipt Date	Expiration Date
Vehicle Control - 2mM carbonate-bicarbonate, 2mM reduced glutathione pH 10.5 Monsanto Lot No.: 7622118-A	V05.001.EUF	Clear colorless liquid (solution)	06/15/05	08/05
Test Control Dosing Solution (TCDS) – Solubilized BSA Monsanto Lot No.: B59628-C	S05.021.EUF	Pale yellow liquid (solution)	06/15/05	08/05
Test Dose Solution (TDS) – Concentrated *E. coli*-Produced Cry2Ab2 protein Monsanto Lot No.: 20-100071-C	S05.022.EUF	Cloudy white suspension	06/15/05	08/05

The vehicle control and the test control dosing solutions were stored frozen in a -70°C freezer until use. The test dose solution (TDS) was stored refrigerated, as required by the Sponsor. The Sponsor was responsible for any necessary evaluations related to chemical composition, strength, purity, stability and any other data required by EPA 40 CFR 160.105. The Principal Investigator for the preparation and analysis of the dosing solutions was Andre Silvanovich. The preparation analyses of the dosing solutions were performed by Monsanto Company, 800 North Lindbergh Blvd., St. Louis, MO 63167. Certificates of Analysis for the BSA and Cry2Ab2 protein were provided by the Sponsor and are presented in Appendix 1.

Expression of the Cry2Ab2 protein in corn was targeted to the chloroplast, using a chloroplast transit peptide (CTP) that has a potential cleavage site (M) three amino acids upstream from the start of the Cry2Ab2 protein. Attempts to confirm the N-terminal sequence of the Cry2Ab2 protein in planta indicated that it is blocked and the cleavage site in the chloroplast cannot be determined. Therefore, the three additional amino acids from the CTP were incorporated at the N-terminus of the Cry2Ab2 protein. The Cry2Ab2 protein is referred to as Cry2Ab2.820 in the Certificate of Analysis.

8.3. Retention Sample

The Sponsor was responsible for maintaining a retention sample of the test article.

8.4. Dosing Solutions Disposition

All unused dosing solutions were returned frozen (on dry ice) to the Sponsor following completion of the in-life phase.

8.5. Method of Dose Preparation and Analyses

Dosing solutions were prepared and analyzed by the Sponsor according to the study specific work procedure. The dosing solutions were administered as received within one day of receipt. The TCDS and vehicle control were removed from the freezer and allowed to thaw at room temperature and then gently stirred for approximately 20 minutes prior to dosing. The TCDS and vehicle control were then stirred continuously under room temperature conditions until completion of dosing. The TDS was removed from the refrigerator for dosing and gently stirred at room temperature for approximately 20 minutes prior to dosing. The TDS was then stirred continuously under room temperature conditions until completion of dosing. The TDS was determined by visual inspection to be a suspension while the TCDS was determined to be a solution. Therefore, homogeneity samples were collected from the TDS container and no homogeneity samples were collected from the TCDS. Samples collected for analysis are summarized in the following table:

Time	Dosing Solution	Samples for Analysis	
		Concentration	Homogeneity
Pre-dose	TDS	500 µL[a]	Top – 250 µL Middle – 250 µL Bottom – 250 µL
	TCDS	500 µL[b]	-------
	Vehicle Control	Not Sampled	------
Post-dose[a]	TDS	500 µL[a]	-------
	TCDS	500 µL[b]	-------
	Vehicle Control	Not sampled	-------

[a]Approximately one hour after pre-dose sample.

8.6. Sample Shipment

All samples (dosing aliquots and remaining unused dosing solutions) were immediately frozen (maintained on dry ice in a -20°C freezer until transfer later in the day to a -70°C freezer) subsequent to sampling/dosing completion. All analytical samples were transported frozen (on dry ice) to Monsanto Company as soon as practical. In addition, the bulk dosing solution containers of each received material were returned to Monsanto Company on dry ice by overnight courier for analysis. Results of the analyses are included in the Analytical Report presented in Appendix 2.

8.7. Animals and Animal Husbandry

8.7.1. Description, Identification and Housing

Young adult, Crl: CD-1®(ICR)BR (VAF/Plus®) mice were received on June 7, 2005, at the Testing Facility from Charles River Laboratories, Inc., Stone Ridge, New York. Upon receipt, metal ear tags displaying unique identification numbers were used to individually identify the animals. Cage cards displaying the study number, animal number and sex were affixed to each cage. The animals were housed individually in suspended stainless steel cages. All housing and care were based on the standards recommended by the Guide for the Care and Use of Laboratory Animals [1].

8.7.2. Environment

The animal room temperature and relative humidity ranges were 69-73°F (21-23°C) and 42-64%, respectively. Environmental control equipment was monitored and adjusted as necessary to minimize fluctuations in the animal room environment. Light timers were set to maintain a 12-hour light/12-hour dark cycle and room ventilation was set to produce 10-15 air changes/hour. The animal room temperature and relative humidity were recorded a minimum of once daily.

8.7.3. Food

PMI Certified Rodent Meal #5002 (PMI Nutrition International) was provided *ad libitum* to the animals throughout the study (except during fasting). The lot number and expiration date of each batch of diet used during the study were recorded. The feed was analyzed and certified by the supplier for nutritional components and environmental contaminants. Dietary limitations for various environmental contaminants, including heavy metals, pesticides, polychlorinated biphenyls and total aflatoxin are set by the manufacturer. Within these limits, contaminants which may have been present were not expected to compromise the purpose of this study. Results of the dietary analyses (Certificates of Analysis) are provided by the manufacturer for each lot of diet. These are maintained by the Testing Facility.

8.7.4. Water

Municipal tap water treated by reverse osmosis was available *ad libitum* throughout the study. The purified water was supplied by an automatic watering system. Monitoring of the drinking water for contaminants is conducted by the testing laboratory and the records are available for inspection. Within generally accepted limits, contaminants that may have been present were not expected to compromise the purpose of this study. The water meets the standards specified under the EPA National Drinking Water Regulations (40 CFR Part 141).

8.7.5. Acclimation

Upon receipt, the animals were removed randomly from the shipping cartons, examined by qualified personnel, identified with metal ear tags and then acclimated to the laboratory

conditions for a minimum of five days. The animals were observed daily for overt physical or behavioral abnormalities, general health/moribundity and mortality.

8.7.6. Animal Selection

Only healthy animals were chosen for study use. On the day of dosing, prior to randomization, at least 30 animals of each sex were weighed and examined in detail for adverse clinical signs. Animals determined to be suitable as test subjects were assigned randomly to groups based on body weights. The animal numbers and the respective body weight values were entered into the computer. The criterion for acceptance of the randomization was homogeneity of groups by body weight; which was met. Disposition of animals not selected for study was documented in the study records. Females were nulliparous and nonpregnant. The male animals were approximately 8 weeks of age and weighed 27.4-31.3 g and the female animals were approximately 10 weeks of age and weighed 24.3-26.7 g prior to randomization.

9. EXPERIMENTAL PROCEDURES

9.1. Dosing

On day 0, the animals chosen for use on study were weighed and fasted approximately 2-3 hours prior to dose administration. The dosing solutions were administered orally as two doses separated by 4 hours (± 20 minutes) using a ball tipped stainless steel gavage needle attached to a syringe at the following levels:

Group No.	Treatment	Target Dose Level (mg/kg)	Dose Volume (mL/kg)	No. of Animals	
				Male	Female
1	Vehicle Control	0	66.6 (33.3/dose)	10	10
2	BSA (Protein Control)	1750 (875/dose)	66.6 (33.3/dose)	10	10
3	Cry2Ab2 protein (Test Protein)	1750 (875/dose)	66.6 (33.3/dose)	10	10

Individual doses were calculated based on the animal's nonfasted (day 0) body weight. Animals were returned to *ad libitum* feeding after dosing.

Note: Due to dosing difficulties, Animal No. A1785/M was replaced with Animal No. A1805/M. Since Animal No. A1785/M did not receive a complete test article dose, any data collected for this animal will remain in the raw data file.

9.1.1. Body Weights

Individual body weights were obtained for the animals prior to fasting (day 0), prior to dosing on day 0 and on days 7 and 14.

9.1.2. Food Consumption

Individual food consumption was measured on days 0, 7 and 14.

9.1.3. Detailed Clinical Observations

Study animals were observed for clinical abnormalities two times on study day 0 (post-dose) and daily thereafter (days 1-14). The study day 0 observations occurred approximately 30 minutes and 4 hours following dosing. Clinical observations were made according to Appendix 3 and included, but were not limited to, changes in the skin and fur, eyes and mucous membranes, respiratory system, circulatory system, autonomic and central nervous systems, including tremors and convulsions, changes in level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypies or bizarre behavior. A general health/mortality check was performed twice daily (in the morning and in the afternoon).

9.2. Scheduled Euthanasia

On day 14, all surviving animals were euthanized by carbon dioxide inhalation and necropsied. Body cavities (cranial, thoracic, abdominal and pelvic) were opened and examined. No organ weights were collected. The whole animal was retained in 10% neutral buffered formalin for possible future examination. The lungs and GI tract were flushed with 10% neutral buffered formalin prior to immersion. No tissues were removed but left intact.

9.3. Protocol Deviations

For replacement Animal No. A1805/M, a Day 0 fasted body weight was not collected. However, dose volume calculations were based on a non-fasted body weight and were, therefore, not considered to have affected the study outcome.

10. DATA ACQUISITION AND ELECTRONIC RECORDS

Electronic data were recorded on a Compaq Alpha Server DS10 utilizing the Toxicology Analysis System Customized, GenToxicology Module, Version 1.0.0 or higher. The study number assigned to this study was EUF00080. The computer study number used to collect data for the study phases was EUF80. The tables within the report display the applicable computer study number.

11. ANALYSIS OF DATA

Less than 50% mortality occurred during the study, therefore, the LD50 was estimated to be greater than the administered dose.

Inferential statistical analyses were performed by the Testing Facility's Alpha DS-10 computer system. Body weights, body weight changes, and food consumption were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for group-wise comparisons to the protein control group, when appropriate. Summary data tables display calculated group means, standard deviations (S.D.), and group sizes (N), as

appropriate. All statistical comparisons were two-tailed with a minimum level of significance of 5% ($p < 0.05$).

Body weight means and standard deviations were calculated (as appropriate).

12. MAINTENANCE OF RAW DATA AND RECORDS

All original paper data, electronic records, tissues and reports will be archived on-site at the Testing Facility for a period of three years from issuance of the final report. The Sponsor will be contacted prior to final disposition of these items. Monsanto will archive the dosing solution characterization, dosing solution preparation and analysis data and corresponding sub report in the Monsanto Regulatory Archives.

13. RESULTS

13.1. Test and Control Article Analysis

Report: Appendix 2

Group No.	Identification	Target Dose Level (mg/kg)	Analytically-Determined Dose Level (mg/kg)
1	Vehicle Control	0	--
2	BSA (Protein Control)	1750	2424
3	Cry2Ab2 protein (Test Protein)	1750	2198

13.2. Mortality

Summary Data: Table 1
Individual Data: Appendix 4

No mortality occurred during the study.

13.3. Clinical Observations

Summary Data: Table 1
Individual Data: Appendix 4

Clinical abnormalities observed included a single incidence of urine stain and rough coat in one male animal in the vehicle control group. Additionally, scabs in the area of the nose were observed in two male animals in the vehicle control group. Otherwise, all animals appeared normal throughout the study.

13.4. Body Weight Data

Summary Data: Table 2 and Table 3
Individual Data: Appendix 5 and Appendix 6

Vehicle control group: One female animal exhibited body weight loss during the study day 7 to 14 interval. All other animals from this group gained weight throughout the test period.

Protein control group: Two female animals exhibited body weight loss during the study day 7 to 14 interval, however, the final body weights exceeded the initial fasted body weights. All other animals from this group gained weight throughout the test period.

Cry2Ab2 protein group: A body weight loss was noted for one male and four female animals during the study day 7 to 14 interval; however, the final body weights for the male animal and three of the female animals exceeded the initial fasted body weights. All other animals from this group gained weight throughout the test period.

Note: For replacement Animal No. A1805/M, a Day 0 fasted body weight was not collected; however, pre-fasted body weights were required for dose volume calculation.

There were no statistically significant differences in body weight or body weight change among the three groups during the study.

13.5. Food Consumption Data

Summary Data: Table 4
Individual Data: Appendix 7

No statistical differences were observed in the food consumption data during the study.

13.6. Gross Necropsy

Summary Data: Table 5
Individual Data: Appendix 8

No significant gross internal findings were noted at necropsy on study day 14. Periovarian cyst(s) were noted on one female from the vehicle group, two females from the protein control group and three females from the Cry2Ab2 protein group. The findings of periovarian cysts are common to this strain of mouse and are not considered significant.

14. CONCLUSION

Under the conditions of this test, no mortality or other toxicity was observed in the Cry2Ab2 protein group. Therefore, the acute oral LD50 of Cry2Ab2 protein in the mouse is greater than 2198 mg/kg.

Kimberly L. Bonnette, M.S., LATG
Study Director

Date 2/6/06

15. REPORT REVIEW

Rusty E. Rush, M.S., LAT, DABT
Director, Toxicology

Date 2-3-06

16. REFERENCE

1. Guide for the Care and Use of Laboratory Animals, DHHS Publication No. (NIH) 96-03, 1996.

17. TABLES

Table 1. Summary of Survival and Clinical Observations

TABLE 1

AN ACUTE ORAL TOXICITY STUDY IN MICE

SUMMARY OF SURVIVAL AND CLINICAL OBSERVATIONS (FREQUENCY/ANIMALS)

MALES

GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry2Ab2)
LEVEL (MG/KG):	0	1750	1750
DAY 0 to 14			
NORMAL			
WITHIN NORMAL LIMITS	157/10	161/10	164/10
SCHEDULED EUTHANASIA	10/10	10/10	10/10
BODY			
SCAB(S) NOSE AREA	5/ 2	0/ 0	0/ 0
URINE STAIN	1/ 1	0/ 0	0/ 0
ROUGH COAT	1/ 1	0/ 0	0/ 0
FIRST DOSING OBS			
ANIMAL STRUGGLED DURING DOSING	3/ 3	0/ 0	0/ 0
SECOND DOSING OBS			
ANIMAL STRUGGLED DURING DOSING	7/ 7	2/ 2	2/ 2

TABLE 1

AN ACUTE ORAL TOXICITY STUDY IN MICE

FEMALES SUMMARY OF SURVIVAL AND CLINICAL OBSERVATIONS (FREQUENCY/ANIMALS)

GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry2Ab2)
LEVEL(MG/KG):	0	1750	1750
DAY 0 to 14			
NORMAL			
WITHIN NORMAL LIMITS	160/10	160/10	160/10
SCHEDULED EUTHANASIA	10/10	10/10	10/10
SECOND DOSING OBS			
ANIMAL STRUGGLED DURING DOSING	1	0/ 0	0/ 0

Table 2. Summary of Body Weight Data

TABLE 2

AN ACUTE ORAL TOXICITY STUDY IN MICE

MALES

SUMMARY OF BODY WEIGHT DATA (GRAMS)

	GROUP:		VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry2Ab2)
	LEVEL(MG/KG):		0	1750	1750
DAY 0		MEAN	27.4 t	27.4	27.4
		S.D.	0.95	0.89	0.94
		N	10	10	9
DAY 7		MEAN	29.8 t	30.3	30.2
		S.D.	1.35	0.76	1.47
		N	10	10	10
DAY 14		MEAN	31.6 t	31.9	31.5
		S.D.	1.69	0.90	1.66
		N	10	10	10

STATISTICAL KEY: t=ANOVA/TUKEY-KRAMER

TABLE 2

AN ACUTE ORAL TOXICITY STUDY IN MICE

FEMALES

SUMMARY OF BODY WEIGHT DATA (GRAMS)

GROUP:		VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry2Ab2)
LEVEL(MG/KG):		0	1750	1750
DAY 0	MEAN	24.3 t	24.1	24.3
	S.D.	0.85	0.73	0.73
	N	10	10	10
DAY 7	MEAN	25.9 t	26.3	25.8
	S.D.	0.89	0.72	0.81
	N	10	10	10
DAY 14	MEAN	27.4 t	27.2	26.5
	S.D.	1.12	0.54	1.35
	N	10	10	10

STATISTICAL KEY: t=ANOVA/TUKEY-KRAMER

Table 3. Summary of Body Weight Changes

TABLE 3

AN ACUTE ORAL TOXICITY STUDY IN MICE

SUMMARY OF BODY WEIGHT CHANGES (GRAMS)

MALES

GROUP:		VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry2Ab2)
LEVEL(MG/KG):		0	1750	1750
DAY 0 TO 7	MEAN	2.4 t	2.9	2.5
	S.D.	0.76	0.46	0.57
	N	10	10	9
DAY 7 TO 14	MEAN	1.8 t	1.7	1.3
	S.D.	0.96	0.58	0.91
	N	10	10	10

STATISTICAL KEY: t=ANOVA/TUKEY-KRAMER

TABLE 3

AN ACUTE ORAL TOXICITY STUDY IN MICE

FEMALES

SUMMARY OF BODY WEIGHT CHANGES (GRAMS)

		GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry2Ab2)
		LEVEL(MG/KG):	0	1750	1750
DAY 0 TO 7		MEAN	1.7 t	2.1	1.6
		S.D.	0.60	0.54	0.52
		N	10	10	10
DAY 7 TO 14		MEAN	1.5 t	0.9	0.6
		S.D.	0.72	0.95	1.11
		N	10	10	10

STATISTICAL KEY: t=ANOVA/TUKEY-KRAMER

Table 4. Summary of Food Consumption Data (Grams/Animal/Day)

TABLE 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

MALES SUMMARY OF FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

		GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry2Ab2)
		LEVEL(MG/KG):	0	1750	1750
DAY 0 to 7		MEAN	4.6 t	5.5	5.5
		S.D.	1.45	0.41	0.60
		N	10	10	10
DAY 7 to 14		MEAN	5.3 t	5.4	5.5
		S.D.	0.45	0.17	1.07
		N	10	10	10

Statistical key: t=ANOVA/TUKEY-KRAMER

TABLE 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

FEMALES

SUMMARY OF FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

GROUP:		VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry2Ab2)
LEVEL(MG/KG):		0	1750	1750
DAY 0 to 7	MEAN	5.2 t	5.5	5.5
	S.D.	0.65	0.85	0.80
	N	10	10	10
DAY 7 to 14	MEAN	5.7 t	5.7	6.4
	S.D.	0.82	1.28	2.00
	N	10	10	10

STATISTICAL KEY: t=ANOVA/TUKEY-KRAMER

Table 5. Summary of Gross Necropsy Observations

TABLE 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS

GROUP:	VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry2Ab2)
LEVEL(MG/KG):	0	1750	1750
MALES: TOTAL NUMBER EXAMINED	10	10	10
WITHIN NORMAL LIMITS	10	10	10

TABLE 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS

GROUP:		VEHICLE CONTROL	PROTEIN CONTROL (BSA)	TEST PROTEIN (Cry2Ab2)
LEVEL(MG/KG):		0	1750	1750
FEMALES: TOTAL NUMBER EXAMINED		10	10	10
WITHIN NORMAL LIMITS		9	8	7
OVARY	N	1	2	3
PERIOVARIAN CYST	N	1	2	3

18. APPENDICES

Appendix 1. Certificates of Analysis

Analytical Protein Standard Certificate of Analysis

MONSANTO ANALYTICAL PROTEIN STANDARDS

Sample Information:

Name of APS	APS Lot Number	Recertification Date
E. coli-produced Cry2Ab2.820 protein	20-100071	August 31, 2005
Common or Alias Name(s)	**Historical APS Lot Number(s)**	**Storage Requirements (until use)**
—	—	-80 °C
Source: Fermentation of *Escherichia coli* containing the pMON70520 expression plasmid		**Comment(s)** None
Additional Background Information: —		

Characteristic	Method	Analysis Date	Result
Concentration	Amino Acid analysis	21 January 2005	0.5 mg/mL (total protein)
Purity	SDS-PAGE/Densitometry	27 January 2005	87 %
Molecular weight	SDS-PAGE/Densitometry	27 January 2005	61.1 kDa
Molecular weight	MALDI-TOF MS	7 February 2005	Results inconclusive
Identity	Immunoblot	14 March 2005	Confirmed
Identity	N-terminal sequence	4 February 2005	Confirmed MQAMDN
Identity	MALDI-TOF MS	7 February 2005	Confirmed sequence 54.9 % coverage of expected sequence
Activity	Insect Bioassay	18 February 2005	EC_{50} = 0.25 µg Cry2Ab2.820/mL diet

Buffer composition: 50 mM CAPS, pH 11, and 2 mM DTT
Physical description: Clear colorless solution
Short-term storage stability (95 days (4C, -20°C, and -80°C)) was evaluated during the certification process. Based upon the criteria provided in Characterization Plan 20-100071, no significant degradation was observed for samples stored at 4°C, –20°C and –80°C.
Purity corrected concentration is 0.4 mg/mL (0.5 mg/mL × 0.87 ≈ 0.4 mg/mL)

Joan M. Rejda-Heath
Quality Assurance Specialist — May 6, 2005 Date

Testing Facility Management — 5/6/05 Date

Analytical Protein Standards Officer — 5/6/05 Date

A COPY
Exact Copy of Original as of JUNE 14, 2005 Date
Certified By A.S. Initials or Signature
Location of Original CRO-2005-049

Exact Copy of Original as of 5/9/05 Date
Certified By Initials or Signature

Study No. EUF00080
Monsanto Study No. CRO-2005-049

CALBIOCHEM®

Certificate of Analysis

Albumin, Bovine Serum, Fraction V, Fatty Acid-Free, Nuclease- and Protease-Free

Product Number: 126609 **Lot Number:** B59628

Molecular Weight: 66,000 **CAS Number:** 9048-46-8

TEST	RESULTS
Appearance	Off-white powder
Solubility	Clear, colorless solution (1% in H_2O)
Purity by SDS-PAGE	98%
Loss on drying	1.2%
Heavy Metals	< 0.1 ppm
pH	6.9
Sulfated ash	0.6%
Nuclease	None detected
Protease	None detected
Free Fatty Acid	0.001%

Storage and Handling	REFRIGERATOR (+4°C)

Ying Peng, Ph.D, Director, QA/QC

10-May-2004
Date

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE.

USA and Canada
Tel (800) 628-8470
technical@calbiochem.com

Germany
Tel 0800 100 3496
techservice@merckbiosciences.de

United Kingdom and Ireland
Freefone 0800 622935
Toll Free 1800 409445
customer.service
@merckbiosciences.co.uk

All Other Countries
Contact Your Local Distributor
www.calbiochem.com
technical@calbiochem.com

A Brand of EMD Biosciences, Inc., an Affiliate of Merck KGaA, Darmstadt, Germany
www.calbiochem.com

PRINTED FROM CALBIOCHEM WEB SITE JUNE 14, 2005 A.S.

Appendix 2. Analytical Chemistry Report

Analytical Sub-Report Title

Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with *E. coli*-Produced Cry2Ab2 Protein

Authors

Andre Silvanovich, Ph. D.
Jamie J. Thorp
Tallis P. Brown
Richard S. Thoma
Joshua P. Uffman
Steven L. Levine, Ph.D
Changjian Jiang, Ph.D

Analytical Sub-report Completed On

January 13, 2006

Performing Laboratory

Monsanto Company
Product Characterization Center
800 North Lindbergh Boulevard
Saint Louis, Missouri 63167

Laboratory Project ID

MSL- 19901
Charles River Study #: EUF00080
Monsanto Study #: CRO-2005-049

The text below applies only to use of the data by the United States Environmental Protection Agency (US EPA) in connection with the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)

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 the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by EPA strictly in accordance with FIFRA. By submitting this material to EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by the company notwithstanding this submission to EPA."

Company: Monsanto Company

Company Agent: _____

Title: _____

Signature: _____ Date: _____

Statement of Compliance

This study meets the US EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160.

Submitter: _____ Date: _____

Sponsor Representative: _____ Date: Jan 13, 2006
Terry A. Kaemple

Principal Investigator: _____ Date: Jan 13, 2006
Andre Silvanovich, Ph. D.

Quality Assurance Statement

Analytical Sub-Report Title: Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with *E. coli*-Produced Cry2Ab2 Protein

Charles River Study No. EUF00080
Monsanto Study No. CRO-2005-049

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

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

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
06/17/2005	Dose Preparation	07/07/2005	07/07/2005
06/29/2005	Amino Acid Analysis	07/05/2005	07/05/2005
07/08/2005	Purity Determination	07/13/2005	07/13/2005
12/08/2005	Raw Data Audit	12/16/2005	12/16/2005
12/08/2005	Draft Report Audit	12/16/2005	12/16/2005

Joan Rejda-Heath
Joan Rejda-Heath, Ph.D.
Quality Assurance Unit
Monsanto Regulatory, Monsanto Company

Jan. 13, 2006
Date

Study Information

Charles River/Monsanto Study Number:	EUF00080/CRO-2005-049
Analytical Sub-Report Title:	Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with *E. coli*-Produced Cry2Ab2 Protein
Facilities:	Monsanto Company 800 North Lindbergh Boulevard Saint Louis, Missouri 63167, USA
Study Director:	Kimberly L. Bonnette, M.S., LATG
Analytical Principal Investigator:	Andre Silvanovich, Ph.D.
Study Specific Work Procedure Initiation Date:	June 3, 2005
Analytical Sub-Report Completion Date:	January 13, 2006
Retention of Records and Retention Sample of Test Article:	All Study-Specific Work Procedure raw data, Study-Specific Work Procedure plan and amendments, final analytical sub-report, a retention sample of test article and facility records were retained at Monsanto, St. Louis.
Disposition of Remaining Dosing Solutions:	Original dose containers with unused dosing solutions were returned to Monsanto. All dosing solutions and their samples were disposed prior to the close of the study per instruction of the Sponsor.

Signature of Analytical Sub-Report Approval

The results reported in this Analytical Sub-Report accurately reflect the data generated under Study-Specific Work Procedure number CRO-2005-049.

Analytical Principal Investigator: _____ Date: Jan 13 2006

Andre Silvanovich, Ph. D.

Table of Contents

Tables

Figures

Appendices

Abbreviations [1]

APS	Analytical Protein Standard
BSA	Bovine serum albumin
BW	Body weight
CAPS	N-Cyclohexyl-3-aminopropanesulfonic acid
CEW	Corn earworm
CFR	Code of Federal Regulations
COA	Certificate of analysis
DTT	Dithiothreitol
EC50	The concentration of a test material in an insect diet that produces a 50% growth inhibition
E. coli	*Escherichia coli*
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act (U.S.)
MW	Molecular weight
MWCO	Molecular weight cutoff
NIST	National Institute of Standards
NMWL	Nominal molecular weight limit
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard operating procedure
SSWP	Study Specific Work Procedure
TDS	Test Dosing Solution
TCDS	Test Control Dosing Solution
US EPA	United States Environmental Protection Agency

[1] Standard abbreviations, e.g. units of measure, concentration, mass, time etc., are used without definition according to the format described in "Instructions to Authors" in The Journal of Biological Chemistry.

1.0 Summary

This analytical sub-report describes the formulation and characterization of the Cry2Ab2 and bovine serum albumin (BSA) proteins in the test article and test control article dosing solutions, respectively, that were used in a mouse acute oral toxicity study performed at Charles River Laboratories, Inc., Spencerville, OH (Charles River Study Number EUF00080). Both dose confirmation and dose stability were evaluated.

The Cry2Ab2 and BSA proteins were each formulated in 2 mM carbonate-bicarbonate, 2 mM reduced glutathione buffer, pH 10.5 to yield the TDS (test dosing solution) and TCDS (test control dosing solution), respectively. A vehicle control dosing solution containing 2 mM carbonate-bicarbonate, 2 mM reduced glutathione buffer, pH 10.5, was also prepared. The TDS was transported from Monsanto to the testing facility on wet ice, whereas the TCDS and vehicle control were transported from Monsanto to the testing facility on dry ice. Aliquots of the TDS, TCDS and vehicle control were returned to Monsanto on dry ice followed by storage in a −80 °C freezer prior to the initiation of analysis. Ten female and ten male mice were administered two doses on the same day. The TDS, TCDS and vehicle control were administered at a rate of 33.3 ml/kg body weight (BW). Dose samples containing the Cry2Ab2 and BSA proteins were collected prior to administration to mice and after administration of the second dose to the final animal in order to assess concentration, purity, and stability.

Total protein concentrations of the test and test control article proteins were evaluated using amino acid analysis. Homogeneity aliquots taken prior to dosing from three locations in the TDS container (top, middle and bottom), demonstrated that the Cry2Ab2 protein suspension was homogeneous. The respective concentrations for the aforementioned aliquots were 39.0, 39.7 and 39.0 mg/ml. The average concentration of the TDS in the homogeneity aliquots was 39.2 ± 0.4 mg/ml and the coefficient of variance was 0.97%. The concentrations of pre- and post-dose aliquots of the TDS yielded an average concentration of 40.2 mg/ml based upon respective pre- and-post dose concentrations of 39.3 and 41.0 mg/ml. The concentrations of pre- and post-dose aliquots of the TCDS yielded an average concentration of 42.8 mg/ml based upon the respective pre- and post-dose concentrations of 42.5 and 43.1 mg/ml.

Protein purity was assessed using densitometric analysis of the dose samples separated on SDS-polyacrylamide gels and stained with colloidal Brilliant Blue G. The purity of the Cry2Ab2 protein in the TDS was determined to be 82.9% prior to the start of dosing and 81.3% at completion of the dosing indicating that the Cry2Ab2 protein had not undergone degradation during the dosing period. Likewise, the purity of the BSA protein in the TCDS was determined to be 85.4% prior to the start of dosing and 84.8% at completion of the dosing indicating that the protein had not undergone degradation during the dosing period.

The Cry2Ab2 protein displayed EC50 values of 0.12 ± 0.03 and 0.09 ± 0.02 µg/ml of test diet for the respective pre- and post-dose TDS samples. Therefore, the Cry2Ab2 protein remained biologically active during the dosing period.

The level of the Cry2Ab2 protein in the test article dose was calculated to be 2198 mg/kg body weight, using an average of the pre- and post-dose purity, and pre- and post-dose concentration values. Using an average of the pre- and post-dose purity, and pre- and post-dose concentration values, the BSA protein in the TCDS was dosed at 2424 mg/kg body weight. These data establish the dose levels of the Cry2Ab2 and BSA proteins used in the mouse oral acute toxicity study.

2.0 Introduction

This sub-report describes the formulation, and confirmation of the dose solutions used in the mouse acute oral toxicity study for the Cry2Ab2 protein. The *E. coli*-produced Cry2Ab2 protein was characterized extensively before the initiation of the study. This sub-report includes descriptions of the formulation and analyses of the test and test control article dose solutions. Analyses included protein concentration and purity for the TDS and TCDS, and functional activity for the TDS only for samples of dosing solution taken before and after the dosing of mice. These procedures were performed to evaluate administered dose concentrations and to assess if any changes in the test or test control article occurred during the performance of the acute oral toxicity study.

3.0 Purpose

The purpose of the Study-Specific Work Procedure was to describe the procedure for the formulation of the test, test control, and vehicle dose solutions, and confirmation of the test and test control dose solutions used in the mouse acute oral toxicity study for the Cry2Ab2 protein, performed at Charles River Laboratories, Inc. (Charles River Study Number EUF00080).

4.0 Materials

4.1 Test Article

The test article was the *E. coli*-produced Cry2Ab2 protein (lot 20-100071) purified at Monsanto Company. Analyses characterizing the identity, concentration, purity, molecular weight, short term stability and functional activity of the Cry2Ab2 protein (lot 20-100071) were previously determined by Monsanto Company under characterization plan 20-100071 and are summarized on a certificate of analysis (COA). A copy of the COA is archived with the study file. Lot 20-100071 has a total protein concentration of 0.5 mg/ml and a purity of

87%. Activity was confirmed using an insect bioassay with larvae of a sensitive pest, CEW. The EC50 value was 0.25 µg/ml of diet. Prior to use, the test article was stored in a -80 °C freezer in a buffer containing 50 mM CAPS, pH 11, and 2 mM DTT.

4.2 Test Control Article

The test control article was bovine serum albumin (BSA) protein (lot B59628) purchased from Calbiochem (La Jolla, CA, catalog # 126609). The vendor's COA was archived with this study file. According to the vendor's characterization data, the BSA has a purity of 98%. The relative amount of protein in the solid BSA powder determined using a Bio-Rad protein assay prior to TCDS formulation was 84%.

4.3 Vehicle Control

The vehicle control was 2 mM carbonate-bicarbonate, 2 mM reduced glutathione buffer prepared to a pH of 10.7 (lot number 7622118-A). Due to the low ionic strength of the buffer, nine days after preparation, the buffer pH was measured and found to be 10.5. Therefore, a pH of 10.5 for the vehicle control was reported throughout this report. This buffer was also used to formulate the test control article and for the buffer exchange of the test article.

4.4 Assay Controls

Protein molecular weight markers (Bio-Rad broad range, Hercules, CA) were used to calibrate SDS-polyacrylamide gels. BSA (Calbiochem, La Jolla, CA) and Cry2Ab2 (lot 20-100071) were used to calibrate the Bio-Rad protein assay. The following standards and controls were used for amino acid analysis; NIST BSA, NIST amino acid standards, and norvaline.

5.0 Dose Preparation

5.1 Calculation of the Test and Test Control Article Dosing Solution Concentrations

The theoretical protein dose solution concentrations for formulation of the TDS and TCDS were calculated assuming the following:

a) The targeted dose level for each mouse is greater than 1750 mg/kg BW.

b) The average mouse body weight (BW) is 0.030 kg.

c) Doses are administered at 33.3 ml/kg BW, ~1 ml/dose, twice daily:

$$0.030\ kg\,BW \times 33.3\ ml/kg \cong 1\ ml$$

For an overall dose solution concentration of 1750 mg/kg BW, the Cry2Ab2 or BSA protein concentration required is 26.5 mg/ml:

$$\frac{1750\ mg/kg\,BW}{33.3\ ml/kg\,BW} \cong 53\ mg/ml\ dose\ (26.5\ mg/ml \times 2\ doses)$$

d) The minimum total volume of sample required is 40 ml:

$$20\ mice \times 2\ doses \times 1\ ml = 40\ ml$$

The estimated purity of the Cry2Ab2 protein is 87%, therefore, the TDS solution should contain approximately 31 mg/ml of total protein:

$$\frac{26.5\ mg/ml}{0.87} \cong 31\ mg/ml$$

The estimated purity of the BSA protein is 98% and protein content is 84%, therefore, the TCDS solution should contain approximately 32 mg/ml of BSA powder:

$$\frac{26.5\ mg/ml}{0.84 \times 0.98} \cong 32\ mg/ml$$

Actual dose levels for the Cry2Ab2 and BSA proteins in the TDS and TCDS, respectively, were calculated based on the average of the experimentally determined pre- and post-dose solution total concentration and purity values.

5.2 Formulation of Test Article in the Test Dosing Solution (TDS)

The Cry2Ab2 protein, lot 20-100071 was thawed and a sample (1000 µl) removed for use as a pre-concentration standard for SDS-PAGE analysis of the TDS and a standard for the Bio-Rad protein assay that was used to qualify the TDS as being suitable for dosing. Aliquots of protein were added to the reservoir of an A/G Technologies Bench Top Quixstand assembly fitted with a 1400 cm^2 30,000 NMWL diafiltration cartridge. The Quixstand apparatus was used to reduce the 7.2 liter starting volume of APS standard lot 20-100071 to 500 mls of primary concentrate. The primary concentrate was collected and 250 mls of

permeate were added to the Quixstand and permitted to recirculate and wash the lumen of the diafiltration cartridge for 30 minutes. The wash was recovered and added to the primary concentrate. The diafiltration cartridge was then filled with 100 mls of permeate and the entire Quixstand assembly was stored overnight in a 4 °C cold room. The following morning, the solution contained in the diafiltration cartridge was collected and added to the previously combined primary concentrate and wash. The Quixstand was then fitted with a 110 cm^2 30,000 NMWL diafiltration cartridge and the combined primary concentrate and washes were reduced to a final volume of 60 mls. This final concentrate was transferred into 10,000 Da MWCO dialysis tubing and dialyzed in two 4 liter volumes of vehicle control buffer. A volume of 72 mls of dialysate was recovered from the dialysis tubing and 60 mls were transferred to a container containing a Teflon stir bar for shipment to Charles River Labs. The TDS was assigned lot number 20-100071-C (Testing Facility Identification number S05.022.EUF) and shipped on wet ice to the testing facility. A 100 µl aliquot of dialysate was diluted 100 fold for analysis using a Bio-Rad protein assay and SDS-PAGE analysis.

Test article dosing solution needed to meet the following criteria for use in the study. (1) Pass through an 18 or 20 gauge needle; (2) Have a concentration of ≥30.5 mg/ml and; (3) Demonstrate a similar profile of bands protein bands to pre-concentration bands stained in SDS-PAGE analysis.

5.3 Formulation of the Protein Control in the Test Control Dosing Solution (TCDS)

BSA powder (2.85 g, Calbiochem catalog #126609, lot B59628) was weighed and brought to 60 ml with vehicle control buffer (2 mM carbonate-bicarbonate, 2 mM reduced glutathione buffer, pH 10.5, lot number 7622118-A). The TCDS was assigned lot number B59628-C (Testing Facility Identification number S05.021.EUF) and was transferred to a wide-mouth container with a Teflon stir bar prior to shipping on dry ice to the testing facility.

5.4 Formulation of the Vehicle Control

A wide-mouth container containing a Teflon stir bar was filled with 60 ml of 2 mM carbonate-bicarbonate, 2 mM reduced glutathione buffer, pH 10.5 (lot number 7622118-A, Testing Facility Identification number V05.001.EUF) and was shipped on dry ice to the testing facility.

5.5 Samples Received from the Testing Facility

The following samples of the TDS and TCDS were received from the Testing Facility (samples were returned on dry ice) for analysis. All samples were stored in a −80 °C freezer up to the time of amino acid analysis. Once thawed for amino acid analysis, the samples were stored at 4 °C.

Samples for Analysis	Amount (µl)
TDS pre-dose	500
TDS post-dose	500
TDS homogeneity (top)	250
TDS homogeneity (middle)	250
TDS homogeneity (bottom)	250
TCDS pre-dose	500
TCDS post-dose	500

6.0 Methods

6.1 Amino Acid Analysis

The total protein concentration of the pre- and post-dose TDS and TCDS samples and the TDS homogeneity samples was analyzed by amino acid analysis. Aliquots of the dosing solution samples were diluted with reagent grade water to 1-2 mg/ml prior to analysis. Total protein concentration was estimated by amino acid analysis using a Hitachi L-8800 Amino Acid Analyzer with AAA System Manager Software. Test samples, BSA (NIST, Gaithersburg, MD) and an amino acid calibration control standard (NIST, Gaithersburg, MD), were spiked with norvaline, an internal reference (Sigma Chemical Co., St. Louis, MO) and dried in a Savant SpeedVac centrifuge (Thermo Electron Co., Milford, MA). Vapor phase acid hydrolysis [6 N HCl containing 1% (v/v) phenol] was performed at 150-151 °C for 90-120 min. Cooled samples were again evaporated, reconstituted in protein hydrolyzate buffer (PH-1, Hitachi High Technologies America, Inc. Life Sciences Division, San Jose, CA) and loaded onto the instrument. Amino acids were detected using ninhydrin derivatization.

To assess the homogeneity of the Cry2Ab2 protein in the test article dosing solution, samples from the bottom, middle, and top of the dosing solution container were assayed as described above. The dosing solution was considered homogeneous if the %CV of the average protein concentration was ≤15%.

6.2 Bio-Rad Protein Assay

A Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) is a colorimetric dye-binding assay based on the method of Bradford (1976). The Bio-Rad assay was performed using 96-well microtiter plates and was used during the assessment of suitability of the TDS prior to dosing, and to determine the protein content in BSA powder prior to formulation of the TCDS. Total protein concentration of the TDS was performed in triplicate using 100× and 200× dilutions of the TDS using a Cry2Ab2 standard curve of 0.1 to 0.5 mg/ml. All Cry2Ab2 standards and TDS samples were diluted with a CAPS buffer (50 mM CAPS, 2 mM DTT, pH 11.0, lot 7622123-C). Total protein content in the BSA powder (Calbiochem, La Jolla, CA, catalog #126609, lot B59628) was determined by formulating a 0.5 mg of BSA powder per ml PBS solution and analyzing this solution using a Bio-Rad protein assay that was calibrated using a BSA standard (Calbiochem, LaJolla, CA). All BSA standards and samples were diluted with PBS lot 7622123-A. Plates for both sets of Bio-Rad assays were read at 595 nm using a PowerWave X microplate reader (Biotek Instruments, Winooski, VT) interfaced with KC4 software (version 3.3).

6.3 SDS-PAGE and Purity Analysis

Samples of the pre- and post-dose TDS and TCDS were diluted to 0.2 µg/µl with 5× sample buffer and water to a final concentration of 1× sample buffer. For the TDS, lanes containing 1, 2, and 3 µg total protein were subjected to electrophoresis at 125 V for 90 min on pre-cast Tris-glycine 4-20% gradient polyacrylamide minigels (Invitrogen, Carlsbad, CA). For the TCDS, lanes containing 1, 2, and 3 µg of total protein were subjected to electrophoresis at 125 V for 30 min followed by 150 V for 60 min. Gels were calibrated using a broad-range molecular weight protein marker (Bio-Rad Laboratories, Hercules, CA; Cat. #161-0317). Gels were fixed for 30 min with a solution containing 40% (v/v) methanol and 7 % (v/v) acetic acid. The fixed gels were stained for 16 hrs with Brilliant Blue G colloidal stain (Sigma, Chemical Co., St. Louis, MO), and destained for 30 s with a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid followed by a solution of 25% methanol for 6-8 hrs. Purity was evaluated using a Bio-Rad GS-800 calibrated densitometer and calculated as the mean of all three loads. Dosing solutions were considered to be stable for the

duration of the dosing period if a ≤10% change in protein purity between the pre- and post-dose samples was observed.

To determine the suitability for use of the concentrated Cry2Ab2 as a TDS, samples of pre-concentration APS standard 20-100071 and post-concentration dialysate were diluted to 0.1 µg/µl with 5× sample buffer and CAPS buffer (50 mM CAPS, 2 mM DTT, pH 11.0, lot 7622123-C) to a final concentration of 1× sample buffer. Duplicate lanes of 1 µg of the pre- and post-concentration solutions were electrophoresed at 125 V for 90 min on pre-cast Tris-glycine 4-20% gradient polyacrylamide minigels. The gel was fixed for 30 min with a solution containing 40% (v/v) methanol and 7% (v/v) acetic acid. The fixed gel was stained for 16 hrs with Brilliant Blue G colloidal stain (Sigma, Chemical Co., St. Louis, MO), and destained for 30 s with a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid followed by a solution of 25% methanol for 6 hrs 35 min. The gel was visually inspected to determine the suitability of the concentrated Cry2Ab2 protein for dosing.

6.4 Functional Activity Analysis

Corn earworm larvae (*Helicoverpa zea*) were used to measure activity of the pre- and post-dose samples in accordance with Monsanto SOP BR-ME-0044-03. The assay was performed using a buffer control and pre- and post-dose samples run in parallel with a dose series ranging from 0.016 – 2.0 µg Cry2Ab2 protein/ml diet (The methods and results are contained in Appendix 1). Functional activity is expressed as an EC50 value, the amount of Cry2Ab2 protein per ml of diet that reduces larval growth 50% relative to a control diet.

6.5 Statistical Methods

The arithmetic mean, standard deviation, and percent coefficient of variance were calculated to demonstrate TDS homogeneity. The arithmetic mean was used to calculate the dose. Statistical methods employed to determine the functional activity for the TDS are described in Appendix 1.

7.0 Control of Bias and Quality Measures

Appropriate sets of dosing samples were analyzed concurrently on SDS-polyacrylamide gels to eliminate any run-to-run variability. BSA and Cry2Ab2 protein standards were used to generate standard curves for the respective Bio-Rad protein assays of solutions performed prior to TCDS formulation or during the assessment of TDS suitability.

8.0 Changes to the Study-Specific Work Procedure

In section 3.2 of the SSWP, "Concentration of the Test Article" the SSWP states in reference to the concentrated test article, "A 250 µl aliquot will be taken for pre-study analyses." Upon review of the data, it was determined that a total of 350 µl was taken for pre-study analyses. This deviation had no impact on the study as sufficient sample was available to afford the removal of 350 µl of sample.

Section 3.3.1 of the SSWP was amended in title, from "Purity Analysis" to "SDS-PAGE Analysis", and to remove the requirement that the current version of SOP BR-ME-0956 be followed. The intent of the SDS-PAGE analysis in SSWP Section 3.3.1 was to qualify the TDS for use in the study. Since the gel was to be analyzed by visual inspection, it was unnecessary to follow SOP BR-ME-0956 which details the densitometric analysis of the gel, and estimation of protein molecular weight and purity. This amendment had no impact on the final outcome of the study.

In section 3.3.2 of the SSWP, "Protein Concentration Determination" the SSWP states "Three aliquots of the TDS will be removed and diluted to a concentration that falls within the range of the standard curve (0.1 to 0.5 mg/ml)." Furthermore, the SSWP stated: "The acceptance criteria for the standard curve will be a CV ≤ 15% for the blank corrected OD readings of the duplicate standard samples and an R^2 value ≥ 0.95". Upon review of the data, it was determined that only a single aliquot of the TDS was sampled and diluted for analysis by Bio-Rad Protein Assay, and that the diluted sample was analyzed in triplicate, not in duplicate. These deviations had no impact on the study since the protein concentration value determined using the Bio-Rad assay was only used to assess the suitability of the TDS for the gavage study. Concentration values used to determine the Cry2Ab2 dose in this study were obtained using amino acid analysis.

In section 3.6.1 of the SSWP, "Dose Formulation", the SSWP states "Dose solutions will then be transported on wet ice (TDS) and dry ice (TCDS) from Monsanto Company (Creve Coeur, MO) to Charles River Laboratories, Inc. (Spencerville, OH, ATTN: Kimberly Bonnette) where they will then be stored in a refrigerator (~4 °C) or on wet ice until use." Upon review of the data it was determined that the TCDS was stored in a -80 °C freezer overnight prior to thawing. This deviation had no impact on the final results of the study as the TCDS was shipped and stored in a frozen state prior to thawing.

In section 3.6.2 of the SSWP, "The analytical samples will be transported frozen (on dry ice) from Charles River Laboratories, Inc. to Monsanto Company (ATTN: Andre Silvanovich) and stored in a -80 °C freezer until analyzed or dispensed." Upon receipt of the samples, the samples were stored in a -80 °C freezer until they were initially thawed

for amino acid analysis. Once thawed, the samples were then stored at 4 °C and subsequently dispensed for protein purity and insect bioactivity analyses. The samples were not re-frozen and stored in a -80 °C freezer to avoid the potential of cryogenic protein precipitation. This deviation had no impact on the final results of the study as the TDS sample purity and bioactivity were comparable to the test article.

Section 4.0 of the SSWP, "Methods for Analytical Analysis" was amended to remove the words "and MW". Section 4.2 of the SSWP, "SDS-PAGE Analysis" was amended to include the following additional text: "All steps described under the image analysis section of BR-MR-0956 will be followed with the exception of step 15 where only the "Relative Quantity" (and not the "Molecular Weight") report will be printed." The intent of SDS-PAGE described in this section of the SSWP is to determine the purity of Cry2Ab2 in the pre- and post-dose TDS, and BSA in the pre- and post-dose TCDS. This amendment served to correct a typographical error in section 4.0 and to clarify in section 4.2 that only a Relative Quantity report and not a MW report be printed. This amendment had no impact on the final outcome of the study.

In section 4.1 of the SSWP and in reference to amino acid analysis, the SSWP states: "Three aliquots of each sample will be removed and diluted to a concentration that is compatible with amino acid analysis (0.5 to 3 mg/ml)." Rather than diluting three separate aliquots, a single aliquot of each sample was removed, diluted and analyzed in triplicate. This deviation had no impact on the final results of the study since the requisite numbers of samples were analyzed and the solutions were homogenous.

9.0 Rejected Data

The initial SDS-PAGE gel scan and its subsequent analysis by densitometry were rejected because several user defined variables in the software interfaced with the densitometer were not set correctly.

A series of printouts from the KC4 software which is interfaced with the 96 well plate reader were rejected because data related to the blank were not printed.

The first statistical analysis for the insect bioassay data was rejected due to a data entry error.

10.0 Results and Discussion

10.1 Dosing Solution Formulation and Suitability Assessment

Dosing solutions containing the Cry2Ab2 protein, BSA protein, and vehicle buffer were prepared for the mouse acute oral toxicity study. The Cry2Ab2 protein yielded a white, milk-like suspension following concentration and this

appearance was retained following exhaustive dialysis. After concentration and dialysis, several tests were performed to assess the suitability of the test article dose. The Cry2Ab2 protein suspension readily passed through a 20-gauge needle and was therefore considered capable of passing through the larger diameter 18-gauge needle. The total protein concentration was assessed using a Bio-Rad colorimetric protein assay relative to APS standard lot 20-100071, the Cry2Ab2 protein standard. Using the Bio-Rad assay, the concentration was estimated to be 45 mg/ml, well in excess of the of 30.5 mg/ml target specified in the SSWP. Samples of the Cry2Ab2 protein taken before and after concentration were compared using a colloidal coomassie blue stained SDS-PAGE gel. Visual analysis of this gel (result not shown) revealed similar banding patterns for each protein sample. Based upon these criteria, the concentrated Cry2Ab2 was deemed to be suitable as a TDS.

10.2 Protein Concentration of the TDS and TCDS

Total protein concentration was determined using amino acid analysis on samples taken before and after administration of doses (Tables 1 and 3). The average total protein concentration of the Cry2Ab2 protein in the test article dosing solution was 40.2 mg/ml. This value is in close agreement with the concentration determined from the separate homogeneity samples (Table 2). The average total protein concentration of the BSA protein in the test control article dosing solution was 42.8 mg/ml (Table 3).

10.3 Evaluation of TDS Homogeneity

Since Cry2Ab2 in the test article dosing solution displayed a milk-like appearance and was likely to be a suspension, samples were taken from three locations in the dosing container (top, middle and bottom) prior to administration of the dose. Total protein concentration was determined for each sample using amino acid analysis. To evaluate homogeneity, the percent coefficient of variance (%CV) was determined for the total protein concentration of the three collected samples. The dosing solution was considered homogeneous because the %CV of the average protein concentration was 0.97% (Table 2), below the limit of ≤15% specified in the SSWP. The BSA protein was visually observed to be a clear amber-colored solution; therefore no homogeneity samples were taken from the test control article dosing solution.

10.4 Purity of the Cry2Ab2 and BSA Proteins in the TDS and TCDS

To assess the stability of the Cry2Ab2 and BSA proteins in the TDS and TCDS, samples taken before and after administration to mice were analyzed using

colloidal Brilliant Blue G stained SDS-polyacrylamide gels followed by densitometry (Figures 1 and 2). Each dosing sample was analyzed at approximately 1, 2 and 3 µg of total protein. Purity was estimated as the percent optical density of the major band relative to all bands detected in the lane. The principal protein band detected on gels of the test article migrated with a molecular weight estimated to be 62 kDa by visual inspection (Figure 1, lanes 3-8) and consistent with 61.1 kDa, the value reported on the COA for APS lot 20-100071. The principal protein band detected on gels of the test control article migrated to ~65 kDa (Figure 2, lanes 3-8), consistent with the manufacturer's reported MW estimate of 66 kDa. Purity data for the Cry2Ab2 and BSA proteins are based upon densitometric analysis of the aforementioned principal bands and are summarized in Table 4. The average percent purity of the Cry2Ab2 protein before dosing and after dosing was 82.9% and 81.3%, respectively. The difference between the pre- and post-dose values equals 1.9% and therefore based on the criteria specified in the SSWP (a ≤ 10% decrease is considered stable), the Cry2Ab2 is stable. The average purity of the BSA protein in the TCDS for samples taken before and after dosing was 85.4% and 84.8%, respectively. Likewise, the difference between the pre- and post-dose values equals 0.7% and therefore based on the criteria specified in the SSWP (a ≤ 10% decrease is considered stable), the BSA is stable.

10.5 Functional Activity of the Cry2Ab2 Protein in the TDS

Corn earworm larvae were used to measure activity of Cry2Ab2 protein in the pre- and post-dose samples. The assay was performed using a buffer control, and pre- and post-dose samples run in parallel with a dose series ranging from 0.016 – 2.0 µg Cry2Ab2 protein/ml diet. EC50 values of 0.12 ± 0.03 and 0.09 ± 0.02 µg/ml of test diet for the respective pre- and post-dose TDS samples were obtained. Since both the pre- and post-dose samples displayed biological activity, the Cry2Ab2 protein was biologically active throughout the dosing period. Results of the insect bioassay are included in Appendix 1.

10.6 Dose Levels of the Test and Test Control Articles

The TDS was shown to be homogenous, stable, and active during the dosing period. Likewise, the TCDS was inferred to be homogenous and found to be stable during the dosing period. Using the calculations shown below, the Cry2Ab2 protein was dosed at 2198 mg/kg body weight. Similarly, using the calculations shown below, the BSA protein was dosed at 2424 mg/kg body weight. The dose levels were calculated using the following data.

- The Cry2Ab2 protein test article, the BSA protein control and vehicle control doses were administered at 33.3 ml/kg BW (~1 ml per dose per animal).
- The average total protein concentration and purity of the Cry2Ab2 protein in the TDS were 40.2 mg/ml and 82.1%, respectively.
- The total protein concentration and purity of the BSA protein in the TCDS were 42.8 mg/ml and 85.1%, respectively.

The final dose levels were calculated as follows.

For the TDS:

a) Concentration corrected for purity: $40.2\frac{mg}{ml}\times 0.821 = 33.0\frac{mg}{ml}$

b) Protein quantity per dose: $33.0\frac{mg}{ml}\times 33.3\frac{ml}{kgBW} = 1099\ \frac{mg}{kgBW}$

c) Final dose level: $1099\ \frac{mg}{kgBW}\times 2\ doses = 2198\ \frac{mg}{kgBW}$

For the TCDS:

a) Concentration corrected for purity: $42.8\frac{mg}{ml}\times 0.851 = 36.4\frac{mg}{ml}$

b) Protein quantity per dose: $36.4\frac{mg}{ml}\times 33.3\frac{ml}{kgBW} = 1212\ \frac{mg}{kgBW}$

c) Final dose level: $1212\ \frac{mg}{kgBW}\times 2\ doses = 2424\ \frac{mg}{kgBW}$

11.0 Conclusions

A panel of analytical tests was performed to characterize the dose of the Cry2Ab2 protein used in the oral acute toxicity study. These results established the dose level of the *E. coli*-produced Cry2Ab2 protein (lot 20-100071) in the TDS, and the dose level of the BSA protein in the TCDS. The Cry2Ab2 protein dosing solution was shown to be homogenous and stable. Likewise, based upon TCDS clarity and purity analysis, the BSA was deemed homogenous and stable. Based on pre- and post-dose purity and concentration averages, the Cry2Ab2 and BSA proteins dose levels were calculated to be 2198 mg/kg body weight and 2424 mg/kg body weight, respectively.

12.0 References

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* 72: 248-254.

Table 1. Observed Total Protein Concentration of the TDS Samples Based on Amino Acid Analysis

Sample	Aliquot	Observed Total Protein Concentration (mg/ml)	Pre- or Post-Dose Average Protein Concentration (mg/ml)	Average of Pre- and Post-Dose Protein Concentration (mg/ml)
Pre-Dose	1	39.50	39.3	40.2
	2	40.02		
	3	38.44		
Post-Dose	1	38.55	41.0	
	2	44.17		
	3	40.16		

Table 2. Homogeneity of the TDS Based on Total Protein Concentration by Amino Acid Analysis.

Sample	Aliquot	Total Protein Concentration (mg/ml)	Average (mg/ml)	Average Concentration (mg/ml)	Standard Deviation (mg/ml)	Coefficient of Variance (%)
Top	1	39.45	39.0	39.2	0.4	0.97
	2	39.70				
	3	37.93				
Middle	1	39.41	39.7			
	2	38.86				
	3	40.79				
Bottom	1	39.02	39.0			
	2	38.27				
	3	39.77				

Table 3. Observed Total Protein Concentration of the TCDS Samples Based on Amino Acid Analysis

Sample	Aliquot	Observed Total Protein Concentration (mg/ml)	Pre- or Post-Dose Average Protein Concentration (mg/ml)	Average of Pre- and Post-Dose Protein Concentration (mg/ml)
Pre-Dose	1	42.35	42.5	42.8
	2	42.10		
	3	43.09		
Post-Dose	1	42.82	43.1	
	2	42.82		
	3	43.56		

Table 4. Observed Protein Purities of the TDS and TCDS Samples

Dose Identification	Sample	Observed Purity %[1]	Percent Change[2]	Purity (%) Used in Dose Calculation
TDS	Pre-Dose	82.9	1.9	82.1
	Post-Dose	81.3		
TCDS	Pre-Dose	85.4	0.7	85.1
	Post-Dose	84.8		

[1] Each value represents the mean of three purity values estimated from loadings of 1, 2, and 3 µg total protein.

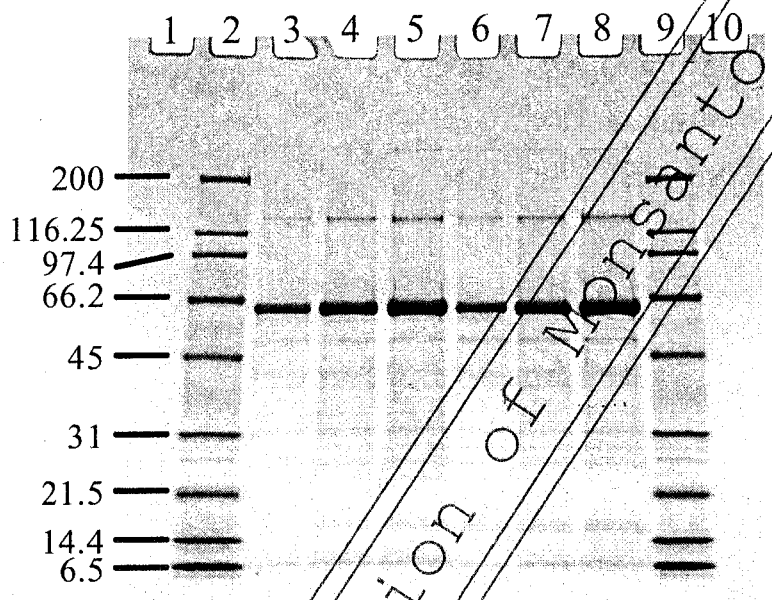
[2] Calculated as follows: $\left|\frac{(\text{Pre - Dose}) - (\text{Post - Dose})}{(\text{Pre - Dose})}\right| \times 100\%$

Table 5. EC50 Estimates for the Pre- and Post-dose TDS Samples

Sample	EC50 (µg Cry2Ab2/ml diet)	Standard Error (µg/ml diet)
Pre-dose	0.12	0.03
Post-dose	0.09	0.02

Table 6. Experimentally Determined Dose Levels

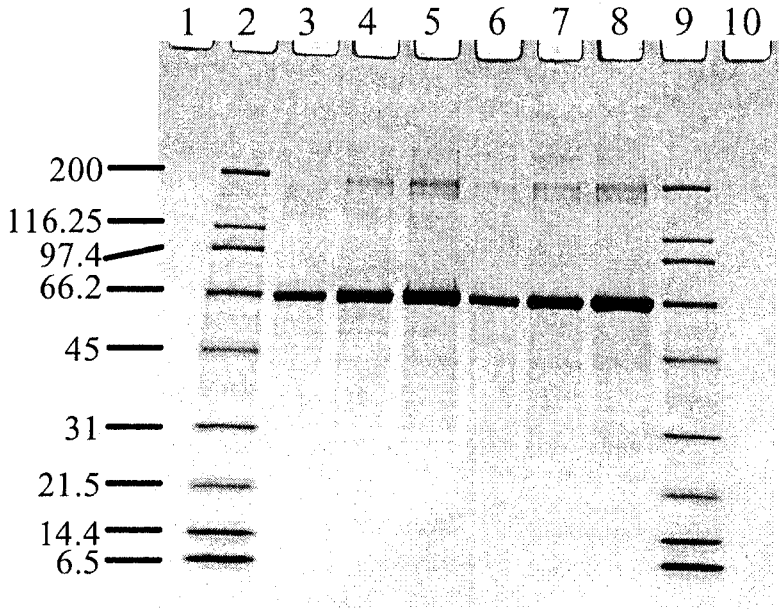
Dose Identification	Purity Corrected Dose Concentration (mg/ml)	Dose Level (mg/kg BW)
TDS	33.0	2198
TCDS	36.4	2424



Lane	Sample	Amount (µg)
1	Empty	-
2	MW marker	4.5
3	Pre-dose	1
4	Pre-dose	2
5	Pre-dose	3
6	Post-dose	1
7	Post-dose	2
8	Post-dose	3
9	MW marker	4.5
10	Empty	-

Figure 1. SDS-PAGE Analysis of the Cry2Ab2 Protein in the Test Article Dosing Solution

The gel contains samples of the test article dosing solution collected before and after dosing of the mice. Gradient 4-20% polyacrylamide minigels were stained with Brilliant Blue G colloidal stain. Amounts loaded correspond to total protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 2 and 9.



Lane	Sample	Amount (µg)
1	Empty	-
2	MW marker	4.5
3	Pre-dose	1
4	Pre-dose	2
5	Pre-dose	3
6	Post-dose	1
7	Post-dose	2
8	Post-dose	3
9	MW marker	4.5
10	Empty	-

Figure 2. SDS-PAGE Analysis of the BSA Protein in the Test Control Article Dosing Solution

The gel contains samples of the test control article dosing solution collected before and after dosing of the mice. Gradient 4-20% polyacrylamide minigels were stained with Brilliant Blue G colloidal Stain. Amounts loaded correspond to total protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 2 and 9.

Appendix 1. Insect Bioassay Summary

Insect Bioassay Summary:

Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with *E. coli*-produced Cry2Ab2 Protein

Study No.: CRO-2005-049

Purpose:

A corn earworm (CEW) diet incorporation insect bioassay was performed to verify the biological activity for the Cry2Ab2 protein in TDS dosing solution collected before (pre-dosing) and after (post-dosing) dosing of the mice during Monsanto study SB-2005-049. Biological activity was measured as an EC50 value, the level of Cry2Ab2 protein that produces a 50% growth inhibition.

Materials & Methods:

Diluted pre-dose and post-dose aliquots prepared from the pre-dose and post-dose TDS samples were received from the Product Characterization Center (PCC), identified as diluted pre-dose TDS (Lot # 20-100071-pre) and diluted post-dose TDS (Lot # 20-100071-post). Information from the PCC indicated the purity corrected concentration for the diluted pre-dose and post-dose aliquots to be 0.5 mg Cry2Ab2 protein/mL. Both aliquots were received on wet ice. A buffer control substance, a liquid identified as 2 mM carbonate-bicarbonate, 2 mM reduced glutathione, pH 10.5 [Lot#: 7622118-A] was received from the PCC. The protein aliquots were stored at approximately -80° C and the buffer control substance was stored at approximately 4° C.

Insects. CEW were obtained from Benzon Research Inc. Insect eggs were incubated at a temperature of 10° C to 27° C, to achieve the desired hatch time.

Bioassays. CEW were used to measure activity of the pre-dose and post-dose TDS samples in accordance with the Monsanto SOP BR-ME-0044-03. The pre-dose and post-dose TDS samples were run in parallel with a dose series ranging from 0.033 – 2.0 µg Cry2Ab2 protein/mL diet. The dose-response curves for the pre-dose and post-dose samples shared a control that contained buffer of the same composition that was used to store the proteins. The level of buffer in the control was equivalent to the amount present in the highest dose level. The insect bioassay dose series was prepared by further diluting the pre-dose and post-dose aliquots with purified water and incorporating the dilution into an agar-based insect diet (Southland). This dose series in diet was chosen to adequately characterize the dose-effect relationship on CEW weight gain for the protein being tested. The diet mixture was then dispensed in 1 mL aliquots into a 128-well tray (#BIO-BA-128, CD International, Pitman, NJ). Insect larvae were placed on these diets using a fine paintbrush, with a target number of 16 insects per treatment. The infested wells were

covered by a ventilated adhesive cover (#BIO-CV-16, CD International, Pitman, NJ) and the insects were allowed to feed for a period of seven days in an environmental chamber programmed at 27° C, ambient relative humidity and a lighting regime of 14 hrs light:10 hrs dark. The combined weight of the surviving insects at each dose level for each protein sample tested was recorded at the end of the 7-day incubation period.

Dose Response Modeling and Results:

The following three-parameter logistic model (equation 1) was used to fit the data for each dose-response curve:

(1) $$Wt = \frac{W_0}{1+\left(\frac{DietDose}{EC50}\right)^B} + e$$

where Wt is the average larvae weight in mg and *DietDose* is the Cry2Ab2 protein diet dose level (µg/mL diet). The three parameters included in the model are as follows: W_0 represents the weight in mg at *DietDose* = 0.0 µg/mL diet, *EC50* represents the effective dose to inhibit the growth of the target insect by 50%, and B reflects the rate of weight loss as *DietDose* increases, and e denotes the residual (error). SAS (version 9.1) procedure PROC NLIN was used to fit the data. Both samples produced a similar dose dependent response in CEW mean individual body mass (Figure 1). Results from this analysis are listed in Table 1.

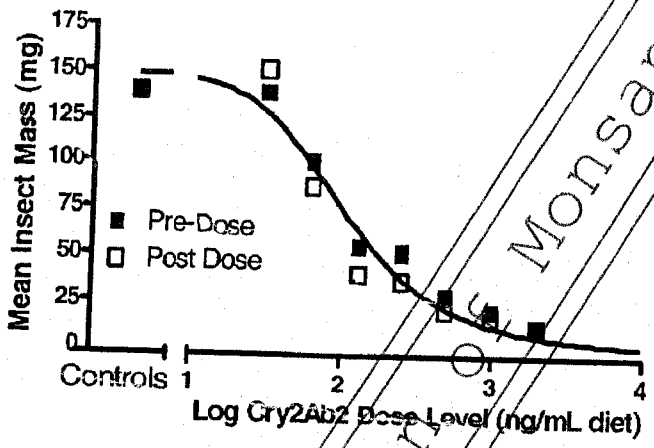


Figure 1. Corn earworm dose-response relationships for Cry2Ab2 for pre-dose and post-dose samples in a diet-incorporation bioassay (prepared with GraphPad Prism software v.4.02).

Table 1. EC_{50} estimates for the Cry2Ab2 protein pre-dose and post-dose TDS samples

Cry2Ab2 Sample	EC50 (µg/mL diet)	Standard Error (µg/mL diet)
Pre-dose	0.13	0.03
Post-dose	0.09	0.02

Conclusion:

The pre-dose and post-dose TDS samples were demonstrated to be biologically active, with EC50 values determined to be 0.12 and 0.09 µg Cry2Ab2 protein/mL diet, respectively.

Prepared by Joshua Uffman, Steven L. Levine, Ph.D, and Changjian Jiang, Ph.D:

Ecological Technology Center author — Date 1/11/2006

Ecological Technology Center author — Date 1/11/2006

Statistical Technology Center author — Date 1/11/06

Appendix 2. List of Applicable SOPs

BR-EQ-0376-02	Hitachi L-8800 Amino Acid Analysis System
BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0525-01	Bio-Rad Protein Assay
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-EQ-0599-02	Bio-Rad GS-710 and GS-800 Densitometers
BR-ME-0956-02	Protein Percent Purity and Apparent Molecular Weight Determination
BR-ME-0973-01	Drying of Polyacrylamide Mini-Gels Using Invitrogen Gel Drying System (Adaptation of Invitrogen Gel Drying Procedure)
BR-ME-0044-03	Diet Incorporation Insect Bioassay for Use in Determining Biological Activity
BR-ME-0990-01	Vapor Phase Acid Hydrolysis Using 6 N HCl and Subsequent Amino Acid Analysis
BR-EQ-0660-01	PowerWave XS Microplate Reader

Appendix 3. Detailed Clinical Observation Parameters

Detailed Clinical Observation Parameters

Cage-side Observations	Recorded As
Abnormal movements or behavior	See Categorical
Resistance to removal from cage	Score

Hand-held Observations	Recorded As
Palpebral closure	Score
Lacrimation (non-colored periocular wetness)	Score
Pupil Size	Score
Salivation (non-colored perioral wetness)	Score
Muscle tone	Score
Extensor-thrust response	Score
Reactivity to handling	Score

Open-field observations	Recorded As
Responsiveness to touch	Score
Gait evaluation	Score

Categorical observations (anytime during the DCO)	Recorded As
Abnormal behavior	Description
Abnormalities of the eye	Description
Abnormal urine or feces	Description
Abnormalities of the gastrointestinal (GI) tract	Description
Injury	Description
Missing extremity	Description
Abnormal muscle movements	Description
Palpable mass/swellings	Description
Abnormal posture	Description
Abnormalities of the reproductive system	Description
Abnormal respiration	Description
Abnormal skin or hair-coat/mucous membranes	Description
Excessive soiling	Description
General abnormalities	Description

Explicitly Defined Scales for DCOs

DCO Examination Conduct

The clinical examination is conducted in a careful and systematic format. The examination begins at the head of the animal and gradually works towards the tail as outlined below.

Cage-side observations are made first.

Categorical observations include: Unusual body movements (e.g., tremors, convulsions), abnormal behaviors (e.g., circling, stereotypy) and changes in posture (e.g., arched back, splayed stance).

Resistance to Removal: The degree to which the animal attempts to escape capture is scored. The observer will slowly present a gloved hand into the cage and will grasp the animal over the shoulder area or by the tail.

1 = Decrease – clearly less resistance to capture than typical
2 = Typical – minimally to actively avoids capture and may be mildly aggressive
3 = Increase – clearly more resistance to capture than typical and is very aggressive (attempts to bite).

Eye observations: Eyes are bilaterally examined for these effects; however, if a unilateral observation is made, a concurrent observation is not made for the other eye if it is within normal limits.

Palpebral closure:

1 = Closed (50% to completely closed)
2 = Open
3 = Protruding eyes

Pupil size (aided by penlight): Under typical examination conditions (white light), the typical appearance of the pupils in albino animals is complete constriction. Therefore a decrease in pupil size cannot be observed.

0 = Unable to evaluate
1 = Decrease – clearly decreased pupil size compared to typical
2 = Typical – completely constricted pupils
3 = Increase – clearly increased pupil size compared to typical

Lacrimation (clear wetness): Under typical examination conditions, corneal dryness is not observed in rodents, nor is the eyelids excessively wet.

1 = Decrease – extremely dry appearance of cornea
2 = Typical – glistening cornea (moderate dryness or wetness)
3 = Increase – extensive wetness around the eyes

Degree of salivation: Under typical examination conditions, dryness of the oral cavity is not observed in rodents.

1 = Decrease – oral dryness
2 = Typical – limited to moderate perioral wetness, but lips and chin are dry
3 = Increase – extensive wetness around the mouth and lips

Muscle tone: An assessment of muscle tone at the time of the hand-held observations.

1 = Decrease – clearly less muscle tone than typical
2 = Typical – animal is neither very relaxed nor very tense
3 = Increase – clearly more muscle tone than typical

Extensor-thrust response: Extent of reflex response to brisk pushes (by finger) on the plantar surface of the hindfeet.

1 = Decrease – clearly less response than typical
2 = Typical – clearly detectable extensor-thrust response
3 = Increase – clearly more response than typical

Reactivity to handling: The degree to which an animal struggles to get free from hand-held restraint is ranked.

1 = Decrease – very slight or no struggling
2 = Typical – mild to moderate struggling, animal may vocalize
3 = Increase – aggressive escape behavior, may try to bite observer and usually vocalizes

Observations made in the open-field.

Responsiveness to touch: The ventral aspect of the tail is lightly stroked using a finger. Typically, the animal will lift its tail and wrap it around the finger when lightly touched.

1 = Decrease – does not lift tail, but may briefly hold tail in the air when manually lifted; no response to touch
2 = Typical – lifts tail when touched
3 = Increase – lifts tail and acts startled, may turn towards finger in an attack response

Gait evaluation: Open-field observations are used for gait evaluation. If the animal remains motionless in the open-field, it may be forced to walk on its forelegs while the hindlegs are held off the floor of the observation box ("the wheel-barrow test").

1 = Unable to walk
2 = Clear knuckling, stumbling and poor coordination, may include falling and/or dragging of one or more limbs
3 = Typical – smooth and coordinated gait

Categorical Observations: These observations can be made at anytime during the DCOs. For the categories listed below, the observer directly records the positive observation.

1.	Abnormal behavior	Description
2.	Abnormalities of the eye	Description
3.	Abnormal urine or feces	Description
4.	Abnormalities of the gastrointestinal tract	Description
5.	Injury	Description
6.	Missing extremity	Description
7.	Abnormal muscle movements	Description
8.	Palpable mass/swellings	Description
9.	Abnormal posture	Description
10.	Abnormalities of the reproductive system	Description
11.	Abnormal respiration	Description
12.	Abnormal skin or hair coat/mucous membranes	Description
13.	Excessive soiling	Description
14.	General abnormalities	Description

Appendix 4. Individual Survival and Clinical Observations

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES VEHICLE CONTROL (OMG/KG)

ANIMAL#	CATEGORY	DAY	DATE	OBSERVATIONS
A1782	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	FIRST DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
A1788	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1791	BODY	14	30-JUN-05	URINE STAIN; SLIGHT
	BODY	14	30-JUN-05	ROUGH COAT
	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
A1793	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	FIRST DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
A1794	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
A1795	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
A1802	BODY	5	21-JUN-05	SCAB(S) NOSE AREA
	BODY	6	22-JUN-05	SCAB(S) NOSE AREA
	BODY	7	23-JUN-05	SCAB(S) NOSE AREA
	BODY	8	24-JUN-05	SCAB(S) NOSE AREA
	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1804	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES VEHICLE CONTROL (OMG/KG)

ANIMAL#	CATEGORY	DAY	DATE	OBSERVATIONS
A1807	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	FIRST DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
A1812	BODY	6	22-JUN-05	SCAB(S) NOSE AREA
	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES PROTEIN CONTROL - BSA (1750MG/KG)

ANIMAL#	CATEGORY	DAY	DATE	OBSERVATIONS
A1781	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1796	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1797	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
A1798	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1801	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1806	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
A1810	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1814	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1816	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1818	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES TEST PROTEIN - Cry2Ab2 (1750MG/KG)

ANIMAL#	CATEGORY	DAY	DATE	OBSERVATIONS
A1780	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
A1783	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1805	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1787	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1790	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1799	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
A1808	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1811	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1815	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1819	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES VEHICLE CONTROL (OMG/KG)

ANIMAL#	CATEGORY	DAY	DATE	OBSERVATIONS
A1821	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1825	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1829	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1831	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1834	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1844	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1845	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1847	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1850	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
	SECOND DOSING OBS	0	16-JUN-05	ANIMAL STRUGGLED DURING DOSING
A1856	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES PROTEIN CONTROL - BSA (1750MG/KG)

ANIMAL#	CATEGORY	DAY	DATE	OBSERVATIONS
A1823	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1827	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1830	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1837	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1848	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1851	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1853	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1857	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1858	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1859	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES, TEST PROTEIN - Cry2Ab2 (1750MG/KG)

ANIMAL#	CATEGORY	DAY	DATE	OBSERVATIONS
A1820	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1822	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1824	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1826	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1828	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1838	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1839	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1841	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1843	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA
A1854	DEAD	14	30-JUN-05	SCHEDULED EUTHANASIA

Appendix 5. Individual Body Weight Data

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES VEHICLE CONTROL (OMG/KG)

ANIMAL#	DAY OF STUDY 0-PRE-FAST	0-FASTED	7	14
A1782	29.7	28.0	29.9	32.0
A1788	27.5	26.0	28.8	31.8
A1791	28.2	26.5	29.8	31.6
A1793	29.9	28.8	32.0	32.4
A1794	28.6	27.7	29.3	30.3
A1795	28.1	27.0	28.6	29.9
A1802	27.8	27.2	29.0	29.7
A1804	27.8	26.3	27.9	30.0
A1807	30.4	28.6	31.8	34.9
A1812	29.2	27.6	30.6	33.4
MEAN	28.7	27.4	29.8	31.6
S.D.	1.01	0.95	1.35	1.69
N	10	10	10	10

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES TCDS - BSA (1750MG/KG)

ANIMAL#	DAY OF STUDY 0-PRE-FAST	0-FASTED	7	14
A1781	29.3	27.5	30.3	31.5
A1796	30.7	28.8	31.6	33.0
A1797	28.5	27.3	30.4	32.4
A1798	27.7	27.0	29.6	32.1
A1801	28.0	26.8	30.7	32.6
A1806	28.6	27.4	29.9	30.8
A1810	27.6	25.8	29.1	30.4
A1814	29.8	28.7	31.2	33.0
A1816	27.9	26.8	29.6	32.2
A1818	28.8	27.7	30.1	31.3
MEAN	28.7	27.4	30.3	31.9
S.D.	1.00	0.89	0.76	0.90
N	10	10	10	10

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES TDS TEST (175QMG/KG)

ANIMAL#	DAY OF STUDY 0-PRE-FAST	0-FASTED	7	14
A1780	27.9	26.3	28.4	29.9
A1783	29.2	28.1	30.9	30.6
A1805	31.3	-a	33.2	33.9
A1787	27.8	26.6	28.6	30.3
A1790	28.4	27.5	29.5	29.9
A1799	28.0	27.3	30.4	32.5
A1808	30.3	28.9	30.8	32.8
A1811	27.4	26.2	29.4	29.9
A1815	29.7	28.4	31.5	34.0
A1819	28.5	27.2	29.2	31.1
MEAN	28.9	27.4	30.2	31.5
S.D.	1.25	0.94	1.47	1.66
N	10	9	10	10

a FASTED BODY WEIGHT INADVERTENTLY NOT COLLECTED. HOWEVER, DOSING VOLUMES WERE BASED ON A PRE-FASTED BODY WEIGHT.

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES VEHICLE CONTROL (OMG/KG)

ANIMAL#	DAY OF STUDY 0-PRE-FAST	0-FASTED	7	14
A1821	26.4	24.6	26.6	26.3
A1825	26.7	26.3	27.5	29.6
A1829	25.9	24.8	25.6	26.7
A1831	25.5	23.8	26.5	28.6
A1834	24.9	24.2	26.6	28.0
A1844	24.4	23.4	24.5	25.8
A1845	25.9	24.2	25.5	27.5
A1847	25.3	24.0	25.9	27.1
A1850	25.0	23.4	25.1	27.0
A1856	24.7	23.8	25.3	27.0
MEAN	25.5	24.3	25.9	27.4
S.D.	0.75	0.85	0.89	1.12
N	10	10	10	10

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES TCDS - BSA (1750MG/KG)

ANIMAL#	DAY OF STUDY 0-PRE-FAST	0-FASTED	7	14
A1823	24.9	23.6	25.5	26.6
A1827	25.3	24.2	27.1	27.0
A1830	26.2	24.9	26.2	26.9
A1837	25.2	23.8	26.6	27.3
A1848	24.3	23.0	25.3	27.7
A1851	24.7	23.8	25.6	27.0
A1853	26.5	25.2	27.4	26.9
A1857	25.8	24.1	25.8	28.1
A1858	25.4	23.6	26.4	26.6
A1859	26.5	25.1	26.9	27.9
MEAN	25.5	24.1	26.3	27.2
S.D.	0.76	0.73	0.72	0.54
N	10	10	10	10

APPENDIX 5

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES TDS - TEST (1750MG/KG)

ANIMAL#	DAY OF STUDY 0-PRE-FAST	0-FASTED	7	14
A1820	25.2	23.6	25.7	26.2
A1822	25.0	23.8	25.7	25.5
A1824	24.8	23.7	24.2	23.2
A1826	26.6	25.2	27.0	26.8
A1828	25.3	24.0	25.6	26.4
A1838	25.9	25.0	25.8	28.0
A1839	26.3	25.2	26.8	26.5
A1841	25.8	24.4	26.0	27.4
A1843	25.9	24.5	26.3	27.1
A1854	24.5	23.1	25.1	27.5
MEAN	25.5	24.3	25.8	26.5
S.D.	0.68	0.73	0.81	1.35
N	10	10	10	10

Appendix 6. Individual Body Weight Changes

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES VEHICLE CONTROL (OMG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1782	1.9	2.1
A1788	2.8	3.0
A1791	3.3	1.8
A1793	3.2	0.4
A1794	1.6	1.0
A1795	1.6	1.3
A1802	1.8	0.7
A1804	1.6	2.1
A1807	3.2	3.1
A1812	3.0	2.8
MEAN	2.4	1.8
S.D.	0.76	0.96
N	10	10

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES TCDS - BSA (1750MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1781	2.8	1.2
A1796	2.8	1.4
A1797	3.1	2.0
A1798	2.6	2.5
A1801	3.9	1.9
A1806	2.5	0.9
A1810	3.3	1.3
A1814	2.5	1.8
A1816	2.8	2.6
A1818	2.4	1.2
MEAN	2.9	1.7
S.D.	0.46	0.58
N	10	10

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES TDS - TEST (1750MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1780	2.1	1.5
A1783	2.8	-0.3
A1805	a	0.7
A1787	2.0	1.7
A1790	2.0	0.4
A1799	3.1	2.1
A1808	1.9	2.0
A1811	3.2	0.5
A1815	3.1	2.5
A1819	2.0	1.9
MEAN	2.5	1.3
S.D.	0.57	0.91
N	9	10

a FASTED DAY 0 BODY WEIGHT INADVERTENTLY NOT COLLECTED. HOWEVER, DOSING VOLUMES WERE BASED ON A PRE-FASTED BODY WEIGHT.

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES VEHICLE CONTROL (0MG/KG)

	DAY OF STUDY	
ANIMAL#	0-7	7-14
A1821	2.0	-0.3
A1825	1.2	2.1
A1829	0.8	1.1
A1831	2.7	2.1
A1834	2.4	1.4
A1844	1.1	1.3
A1845	1.3	2.0
A1847	1.9	1.2
A1850	1.7	1.9
A1856	1.5	1.7
MEAN	1.7	1.5
S.D.	0.60	0.72
N	10	10

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES TCDS - BSA (1750MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1823	1.9	1.1
A1827	2.9	-0.1
A1830	1.3	0.7
A1837	2.8	0.7
A1848	2.3	2.4
A1851	1.8	1.4
A1853	2.2	-0.5
A1857	1.7	2.3
A1858	2.8	0.1
A1859	1.8	1.0
MEAN	2.1	0.9
S.D.	0.54	0.95
N	10	10

APPENDIX 6

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES TDS - TEST (1750MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1820	2.1	0.5
A1822	1.9	-0.2
A1824	0.5	-1.0
A1826	1.8	-0.2
A1828	1.6	0.8
A1838	0.8	2.2
A1839	1.6	-0.3
A1841	1.6	1.4
A1843	1.8	0.8
A1854	2.0	2.4
MEAN	1.6	0.6
S.D.	0.52	1.11
N	10	10

Appendix 7. Individual Food Consumption Data (Grams/Animal/Day)

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES VEHICLE CONTROL (OMG/KG)

	DAY OF STUDY	
ANIMAL#	0-7	7-14
A1782	4.9	5.5
A1788	5.1	5.2
A1791	5.3	5.4
A1793	5.5	5.4
A1794	4.5	4.7
A1795	4.8	5.3
A1802	5.0	4.9
A1804	4.7	5.2
A1807	5.5	6.4
A1812	0.5	5.2
MEAN	4.6	5.3
S.D.	1.45	0.45
N	10	10

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES PROTEIN CONTROL - BSA (1750MG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1781	6.4	5.3
A1796	5.2	5.0
A1797	5.3	5.2
A1798	5.6	5.6
A1801	5.4	5.4
A1806	5.8	5.6
A1810	5.2	5.5
A1814	5.4	5.4
A1816	5.0	5.4
A1818	5.2	5.4
MEAN	5.5	5.4
S.D.	0.41	0.17
N	10	10

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES TEST PROTEIN - Cry2Ab2 (1750MG/KG)

	DAY OF STUDY	
ANIMAL#	0-7	7-14
A1780	5.5	5.2
A1783	5.3	5.0
A1805	7.1	8.4
A1787	5.2	5.0
A1790	5.3	5.1
A1799	5.2	5.2
A1808	5.0	5.3
A1811	5.5	4.7
A1815	5.5	5.7
A1819	5.2	5.2
MEAN	5.5	5.5
S.D.	0.60	1.07
N	10	10

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES VEHICLE CONTROL (OMG/KG)

ANIMAL#	DAY OF STUDY 0-7	7-14
A1821	4.9	6.6
A1825	5.1	5.2
A1829	4.1	5.2
A1831	4.6	5.2
A1834	6.5	6.4
A1844	5.0	5.3
A1845	4.9	5.3
A1847	5.1	5.1
A1850	5.7	5.4
A1856	5.5	7.5
MEAN	5.2	5.7
S.D.	0.65	0.82
N	10	10

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES /PROTEIN CONTROL - BSA (1750MG/KG)

	DAY OF STUDY	
ANIMAL#	0-7	7-14
A1823	4.9	5.2
A1827	7.0	9.0
A1830	4.8	5.4
A1837	5.4	5.6
A1848	5.4	5.3
A1851	7.0	6.4
A1853	5.5	5.4
A1857	5.2	5.2
A1858	4.8	4.6
A1859	4.8	4.5
MEAN	5.5	5.7
S.D.	0.85	1.28
N	10	10

APPENDIX 7

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES TEST PROTEIN - Cry2Ab2 (1750MG/KG)

	DAY OF STUDY	
ANIMAL#	0-7	7-14
A1820	4.8	5.6
A1822	4.8	5.0
A1824	5.0	7.4
A1826	5.5	11.5
A1828	6.5	7.6
A1838	6.9	6.4
A1839	6.4	5.2
A1841	5.2	5.5
A1843	5.5	5.1
A1854	4.7	5.3
MEAN	5.5	6.4
S.D.	0.80	2.00
N	10	10

Appendix 8. Individual Gross Necropsy Data

STUDY NO.: EUF80
MONSANTO COMPANY CRO-2005-049

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES VEHICLE CONTROL (OMG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1782	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1788	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1791	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1793	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1794	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1795	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1802	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1804	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1807	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1812	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES PROTEIN CONTROL - BSA (1750MG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1781	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1796	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1797	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1798	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1801	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1806	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1810	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1814	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1816	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1818	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES TEST PROTEIN - Cry2Ab2 (1750MG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1780	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1783	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1805	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1787	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1790	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1799	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1808	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1811	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1815	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1819	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES VEHICLE CONTROL (OMG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1821	30-JUN-05	14	OVARY: PERIOVARIAN CYST; PRESENT LEFT, 0.4 CM DIAMETER, CLEAR FLUID FILLED	SCHEDULED EUTHANASIA
A1825	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1829	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1831	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1834	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1844	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1845	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1847	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1850	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1856	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES PROTEIN CONTROL - BSA (1750MG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1823	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1827	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1830	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1837	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1848	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1851	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1853	30-JUN-05	14	OVARY: PERIOVARIAN CYST; PRESENT RIGHT, 0.3 CM DIAMETER, CLEAR FLUID FILLED	SCHEDULED EUTHANASIA
A1857	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1858	30-JUN-05	14	OVARY: PERIOVARIAN CYST; PRESENT BILATERAL, EACH APPROXIMATELY 0.2 CM DIAMETER, CLEAR FLUID FILLED	SCHEDULED EUTHANASIA
A1859	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE ORAL TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES /TEST PROTEIN - Cry2Ab2 (1750MG/KG)

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
A1820	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1822	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1824	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1826	30-JUN-05	14	OVARY: PERIOVARIAN CYST; PRESENT RIGHT, 0.3 CM DIAMETER, CLEAR FLUID FILLED	SCHEDULED EUTHANASIA
A1828	30-JUN-05	14	OVARY: PERIOVARIAN CYST; PRESENT LEFT, 0.6 CM IN DIAMETER, CLEAR FLUID-FILLED	SCHEDULED EUTHANASIA
A1838	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1839	30-JUN-05	14	OVARY: PERIOVARIAN CYST; PRESENT BILATERAL, EACH APPROXIMATELY 0.3 CM DIAMETER, CLEAR FLUID FILLED	SCHEDULED EUTHANASIA
A1841	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1843	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
A1854	30-JUN-05	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

Appendix 9. Personnel Responsibilities

Personnel Responsibilities

Kimberly L. Bonnette, M.S., LATG	Study Director/Director, Acute Toxicology
Jason W. Smedley, B.S.	Alternate Contact/Assistant Toxicologist
Joseph C. Siglin, Ph.D., DABT	General Manager
Rusty E. Rush, M.S., LAT, DABT	Director, Toxicology
Dawn D. Rodabaugh, B.S.	Manager, Acute Toxicology
Pamela S. Smith, ALAT	Study Supervisor, Tox I
Heather M. Neeper, B.S.	Primary Technician/Vivarium Technician II
Delores P. Knippen	Supervisor, Formulations
Anita M. Bosau, RQAP-GLP	Division Director, Regulatory Compliance
Cheryl A. Bellamy	Manager, Report Writing
Deanna M. Talerico, RQAP-GLP	Manager, Regulatory Compliance
William H. Baker, M.S., D.V.M., DACVP	Division Director, Senior Staff Pathologist
Kathy M. Gasser	Archivist