2) Cavato, T.A. and Lirette, R.P. (2001) PCR analysis and DNA sequence of the insert in corn rootworm event MON 863. MSL-17108, an unpublished study conducted by Monsanto Company.

Study Title

PCR Analysis and DNA Sequence of the Insert in Corn Rootworm Event MON 863

Authors

Tracey A. Cavato
Ronald P. Lirette

Study Completed On

March 16, 2001

Performing Laboratory

Monsanto Company
Product Characterization Center
Biotechnology Regulatory Sciences
700 Chesterfield Parkway North
St. Louis, MO 63198

Laboratory Project ID

Study 01-01-39-02
MSL-17108

[redacted]

Information claimed confidential on the basis of its falling within the scope of FIFRA section 10 (d) (1) (A), (B), or (C) has been removed to a confidential appendix, and is cited by cross reference number in the body of the study.

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

Company: Monsanto Company

Company Agent: _____

Title: _____

Signature: _____ Date: _____

Statement of Compliance

This study meets the requirements under GLP as specified in 40 CFR Part 160 with the following exception:

Sequence information, generated by the Monsanto Genomic Sequencing Center, was not generated in compliance with the GLP regulations, however all experiments conducted to confirm sequence data within this report were performed in compliance with the GLP regulations.

Submitter — Date

Ravinds S Sidhu — March 16, 2001
Sponsor Representative — Date

Tracey A. Lavato — March 16, 2001
Study Director — Date

Quality Assurance Statement

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

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

Dates Of Inspection / Audit	Phase	Date Reported To: Study Director	Management
January 19, 2001	PCR/Sequence	February 6, 2001	February 6, 2001
March 5, 2001	Raw Data Audit	March 12, 2001	March 12, 2001
March 5, 2001	Draft Report Audit	March 16, 2001	March 16, 2001

Michelle Higgins
Quality Assurance
Monsanto Regulatory, Monsanto Company

03-16-2001
Date

Signatures of Approval

Study Number: 01-01-39-02

Title: PCR Analysis and DNA Sequence of the Insert in Corn Rootworm Event MON 863

Primary Facility: Monsanto Company
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St. Louis, MO 63198

Monsanto Genome Sequencing Center: Monsanto Company
Genomics Sequencing Center
800 North Lindbergh Boulevard
St. Louis, MO 63167

Sponsor Representative: Ravinder S. Sidhu

Study Director: Tracey A. Cavato

Study Initiation Date: January 16, 2001

Study Completion Date: March 16, 2001

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

Sample Storage: Any study samples that are to be retained will be stored at Monsanto, St. Louis.

Signatures of Approval:

Tracey A. Cavato — March 16, 2001
Study Director — Date

Ravinder S Sidhu — March 16, 2001
Sponsor Representative — Date

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Abbreviations

~	approximately
35S	cauliflower mosaic virus (CaMV) 35S promoter
4-AS1	promoter containing 4 tandem copies of the AS1 element and the 35S promoter
ble	coding region for bleomycin resistance
cry3Bb1	class III (Coleoptera-specific) crystal protein coding region
dNTP	deoxynucleotide triphosphate
LIMS	laboratory information management system
MgCl	magnesium chloride
NOS 3'	nopaline synthase 3' polyadenylation sequence
nptII	coding region for neomycin phosphotransferase II
PCR	polymerase chain reaction
ract1	intron from the rice actin gene
tahsp17 3'	3' nontranslated region of the wheat heat shock protein 17.3 containing the polyadenylation sequence
wtCAB leader	5' untranslated leader of wheat major chlorophyll a/b binding protein

I. SUMMARY

The molecular characterization of corn rootworm event MON 863 has been previously described in detail (Cavato *et al.*, 2000). This characterization, largely based on Southern blot analysis, demonstrated that one copy of the DNA fragment used for transformation was present in corn event MON 863. In this study, PCR analysis and subsequent DNA sequencing were performed on the insert in corn rootworm event MON 863. These analyses confirmed the results of the previous characterization by demonstrating the expected linkage of the elements contained within the insert in corn event MON 863. Furthermore, the DNA sequence of the entire insert in corn rootworm event MON 863 was obtained and is reported.

II. INTRODUCTION

A. Background. Corn rootworm event MON 863 was generated by transformation of corn cells using a particle acceleration method with a 4.7-Kb agarose gel-isolated *Mlu* I restriction fragment from the plasmid vector PV-ZMIR13 (Figure 1). The DNA fragment used for transformation contained two gene expression cassettes: an *nptII* selectable marker cassette containing the *nptII* coding sequence under the regulation of the cauliflower mosaic virus (CaMV) 35S promoter, and a nopaline synthase (NOS) 3' polyadenylation sequence; and a *cry3Bb1* cassette containing the *cry3Bb1* coding sequence under the regulation of the 4-AS1 plant promoter, and the wtCAB leader, rice actin intron, and tahsp17 3' polyadenylation sequence. Previous molecular characterization of the insert in event MON 863 (Cavato *et al.*, 2000) demonstrated that one copy of the DNA fragment used for transformation is present in corn event MON 863 (Figure 2). In addition, the genomic DNA sequences flanking the insert, as well as the insert-to-plant junctions, were previously identified and confirmed (Cavato and Lirette, 2000)

B. Purpose. The purpose of this study was to generate overlapping PCR products spanning the length of the single DNA insert in corn event MON 863, and to sequence those PCR products in order to obtain the sequence of the entire insert within corn rootworm event MON 863.

III. MATERIALS AND METHODS

A. Test Substance. The test substance for this study was corn rootworm event MON 863. Grain (LIMS ID 99ZMGRO01029) harvested from Production Plan # 99-01-39-08 was used in this study.

B. Control Substance. The control substance was the non-transgenic corn line

MON 846. Grain (LIMS ID 99ZMGRO01019) harvested from Production Plan # 99-01-39-08 was used in this study.

C. Reference Substances. The reference substances include the plasmid PV-ZMIR13 which was used as a positive control in the PCR analyses. Additionally, the 500 bp DNA Ladder from Gibco BRL (8.5 Kb-0.5 Kb, catalog #10594-018) was used for size estimations in the PCR analyses.

D. Test System. There was no test system. This study used analytical methods to analyze the corn event.

E. DNA Isolation. Prior to the start of the study, DNA from the test and control substances was extracted from grain tissue using Qiagen's DNeasy Plant Mini Kit (catalog # 68163) according to the manufacturer's recommended protocol with one exception. The grain was processed in a blender and not ground with a mortar and pestle with liquid nitrogen.

F. DNA Quantitation. Quantitation of the DNA samples was performed prior to the start of the study using a Hoefer DyNA Quant 200 Fluorometer (San Francisco, CA)(SOP BR-EQ-0065-01) using Boehringer Mannheim molecular size marker IX as a calibration standard when quantitating genomic DNA. The protocol originally stated that plasmid pBR322 would be used as the calibration standard when quantitating plasmid DNA, however, Boehringer Mannheim molecular size marker IX was used in the study.

G. PCR Analysis and Sequence Confirmation of the Organization of the Insert in Corn Rootworm Event MON 863. The linkage of the elements contained within the insert was confirmed by generating six overlapping PCR products which spanned the length of the insert (Products A-F, Figure 3). The PCR analyses were conducted multiple times using 40-47 ng of genomic DNA or 29 ng of PV-ZMIR13 plasmid DNA as a template in a 50 µl reaction volume containing a final concentration of 1.5 mM $MgCl_2$, 0.4 µM of each primer, 200 µM each dNTP, and 2.5 units of Red*Taq* DNA polymerase (Sigma Chemical Co.). The reactions were performed under the following cycling conditions: 94°C for 3 minutes; 38 cycles at 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1.5 minutes; 1 cycle at 72°C for 10 minutes. The PCR products were separated using 1.0 % agarose gel electrophoresis at 120-180 V for ~0.5-1.5 hours. Following electrophoresis, all six PCR products derived from corn rootworm event MON 863 were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, catalog # 28704) following the procedure supplied by the manufacturer. The purified PCR products were then sequenced with the initial PCR primers as well as primers designed internal to the amplified sequences. All sequencing was performed by the Monsanto Genomics Sequencing Center using dye-terminator chemistry.

IV. RESULTS AND DISCUSSION

A. Organization and Sequence of the Inserted DNA in Corn Rootworm Event MON 863. The organization of the elements within the insert was confirmed using PCR analysis by amplifying six overlapping regions of DNA which span the entire length of the insert. The location of the PCR products generated in relation to the insert, as well as the results of the PCR analyses, are shown in Figure 3. The control reactions containing no template DNA (lanes 4, 8, 12, 16, 20, and 23) did not generate PCR products with any of the primer sets, as expected. The MON 846 non-transgenic negative control reactions (lanes 2, 5, 9, 13, 17, and 21) did not generate PCR products with five out of the six primer sets. The primer set used to generate Product F did generate multiple faint PCR products in the MON 846 non-transgenic DNA (lane 21), but none of the products was the 2257 bp expected size product. The same faint PCR products were also generated using corn event MON 863 DNA as a template (lane 22). Since one of the primers used to generate Product F was located in the genomic DNA sequence flanking the insert, this PCR primer alone is most likely non-specifically amplifying sequences in the corn genome. The plasmid PV-ZMIR13 was used as a positive control in the four PCR analyses (Products B-E) which did not include a primer located in the genomic DNA flanking sequence. In these four analyses, corn rootworm event MON 863, as well as the plasmid PV-ZMIR13, generated the expected size PCR products of 1230 bp for Product B (lanes 6 and 7), 474 bp for Product C (lanes 10 and 11), 792 bp for Product D (lanes 14 and 15), and 2480 bp for Product E (lanes 18 and 19). In three out of the four PCR reactions containing plasmid PV-ZMIR13 DNA, faint, higher molecular weight products were visible which were most likely due to non-specific amplification (lanes 7, 15, and 19). Corn rootworm event MON 863 also generated the expected size PCR products of 1165 bp for Product A (lane 3) and 2257 bp for Product F (lane 22). The generation of the predicted size PCR products from corn event MON 863 establishes that the arrangement or linkage of elements in the insert is the same as those in plasmid PV-ZMIR13 and that the elements within the insert are arranged as depicted in the schematic of the insert in Figure 2.

The plasmid PV-ZMIR13 was originally used as a positive control in all six of the PCR analyses. Since one of the primers used to generate both PCR Products A and F is located in the genomic DNA sequence flanking the insert, the plasmid was not an appropriate control for these reactions and should not have generated a PCR product. However, in both analyses a product was generated. These products were shown to be due to non-specific amplification or the result of a single primer; therefore, the data are not reported.

All six of the PCR products generated in corn rootworm event MON 863 were subjected to DNA sequencing to further confirm the organization of the elements within the insert. The consensus sequence representing the insert in corn rootworm event MON 863, that

was generated by compiling numerous sequencing reactions performed on the six PCR products which spanned the length of the insert, is shown in Figure 4. The DNA sequence of the insert contains 4,675 base pairs beginning at base 156 of PV-ZMIR13 which is located in the polylinker sequence immediately before the 35S promoter, and ending at base 4830 in the polylinker sequence immediately after the tahsp17 3' polyadenylation sequence of PV-ZMIR13 (Figure 1).

V. CONCLUSIONS

Previous molecular analyses of corn rootworm event MON 863 demonstrated that there is one copy of the DNA fragment used for transformation in event MON 863 (Cavato *et al.*, 2000), and confirmed the genomic DNA sequences flanking the insert, as well as the insert-to-plant junctions in corn event MON 863 (Cavato and Lirette, 2000). As part of this study, PCR and DNA sequence analyses were performed which confirmed the organization of the elements within the insert, as well as the complete DNA sequence of the insert in corn rootworm event MON 863.

VI. REFERENCES

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McElwain, E. and Spiker, S. 1989. A wheat cDNA clone which is homologous to the 17 kd heat-shock protein gene family of soybean. Nucleic Acids Res. 17, 1764.

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Table 1. Summary of Genetic Elements in the Linear DNA Restriction Fragment from PV-ZMIR13 Used in the Transformation of Corn Rootworm Event MON 863.

Genetic Element	Size Kb	Bp Location in Insert (Figure 4)	Function
35S	0.32	19-336	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985)
nptII	0.97	370-1337	The coding sequence for the enzyme neomycin phosphotransferase type II from Tn5, a transposon isolated from *Escherichia coli* (Beck et al., 1982). The DNA derived from *E. coli* also includes a 153 bp segment of the gene (*ble*) encoding bleomycin binding protein
NOS 3'	0.26	1357-1612	A 3' nontranslated region of the nopaline synthase gene of *Agrobacterium tumefaciens* which terminates transcription and directs polyadenylation (Bevan *et al.*, 1983)
4-AS1	0.22	1638-1859	Promoter containing four tandem copies of the AS-1 element (Lam and Chua, 1990) and a portion of the 35S promoter of cauliflower mosaic virus (Odell *et al.*, 1985).

Table 1 Continued. Summary of Genetic Elements in the Linear DNA Restriction Fragment from PV-ZMIR13 Used in the Transformation of Corn Rootworm Event MON 863[1]

Genetic Element	Size Kb	Bp Location in Insert (Figure 4)	Function
wtCAB leader	0.06	1864-1928	5' untranslated leader of the wheat chlorophyll a/b-binding protein (Lamppa *et al.*, 1985)
ract1 intron	0.49	1942-2429	Intron from the rice actin gene (McElroy *et al.*, 1990)
cry3Bb1	1.96	2435-4396	Gene encoding a synthetic variant of the Cry3Bb1 protein of *Bacillus thuringiensis* (Donovan *et al.*, 1992)
tahsp17 3'	0.23	4411-4644	A 3' nontranslated region of the wheat heat shock protein 17.3 which terminates transcription and directs polyadenylation (McElwain and Spiker, 1989)

[1] Base pairs located between the genetic elements are plasmid linker DNA which do not encode for additional elements.

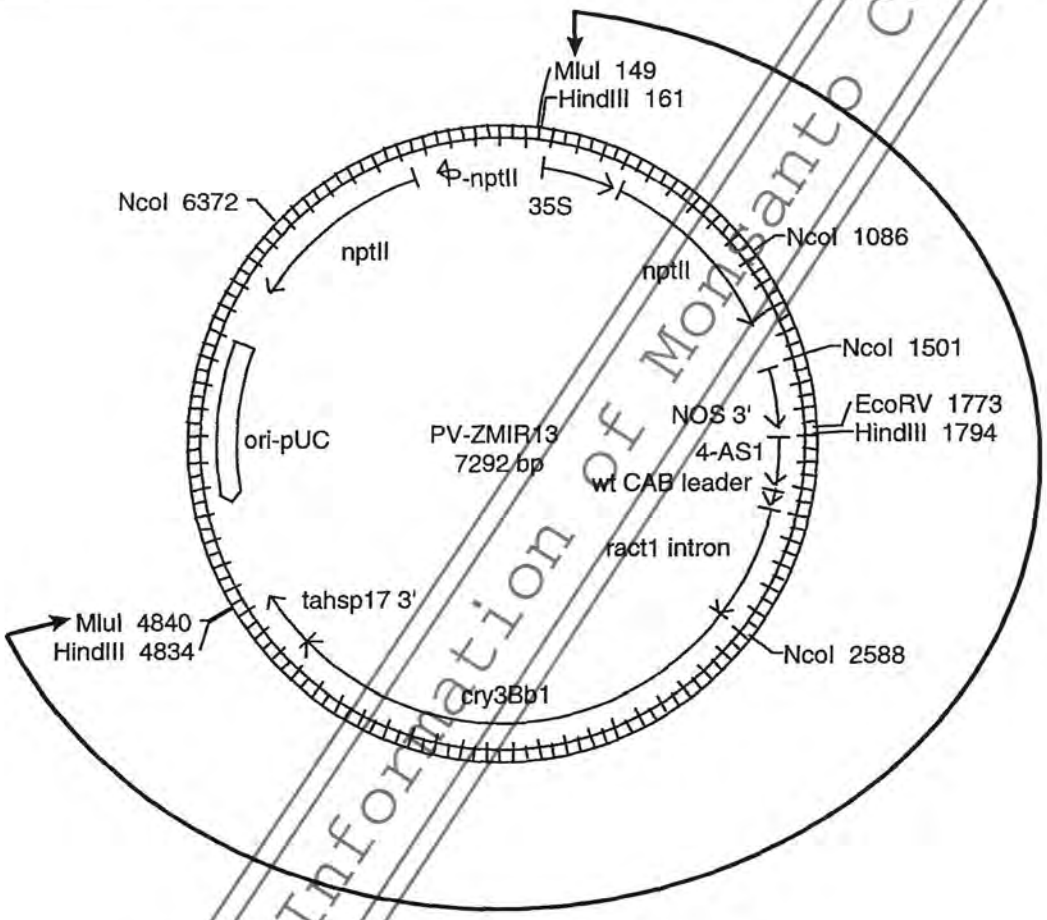


Figure 1. Plasmid Map of PV-ZMIR13. The *Mlu* I restriction fragment from plasmid PV-ZMIR13 was used to generate corn rootworm event MON 863.

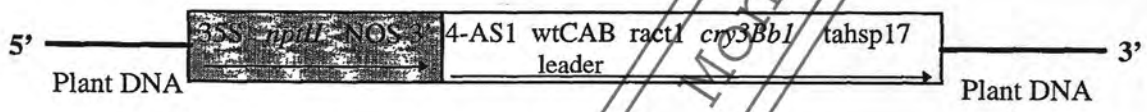
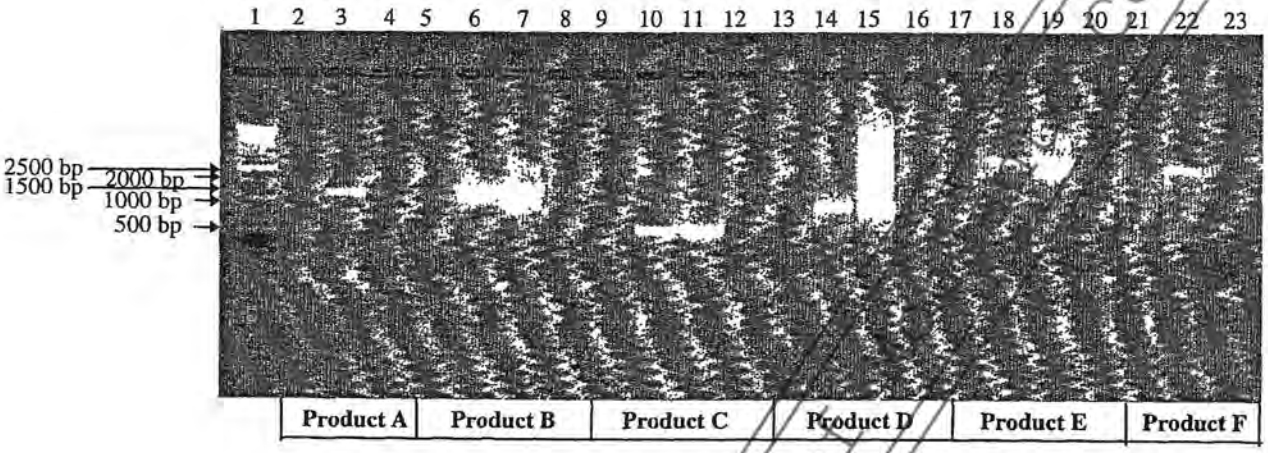


Figure 2. Schematic Representation of the Insert in Corn Rootworm Event MON 863. This figure depicts the predicted insert for event MON 863 as presented in Cavato *et al.* (2000). There is one copy of the DNA fragment used for transformation.



DNA Insert in Corn Rootworm Event MON 863

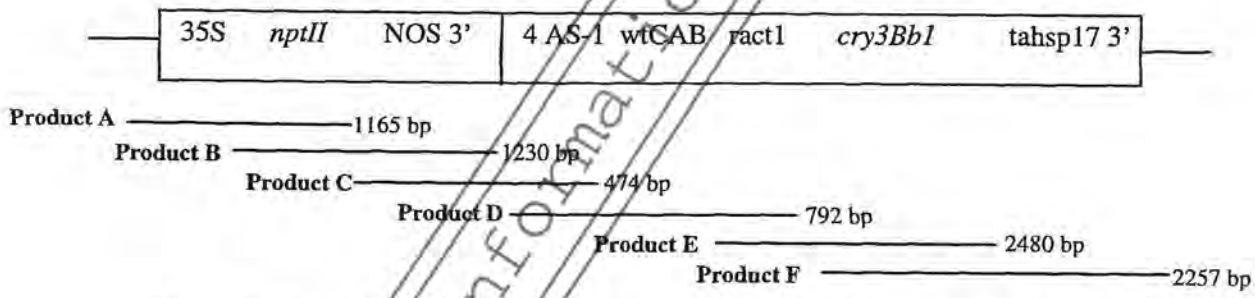


Figure 3. Overlapping PCR Analysis Demonstrating the Organization of the Inserted DNA in Corn Rootworm Event MON 863. PCR analyses which generated six overlapping products (A-F) demonstrating the linkage of the individual elements within the insert were performed on PV-ZMIR13 plasmid DNA (lanes 7, 11, 15, and 19) and corn event MON 863 genomic DNA extracted from grain tissue (lanes 3, 6, 10, 14, 18, and 22). Lanes 2, 5, 9, 13, 17, and 21 contain MON 846 non-transgenic DNA extracted from grain tissue while lanes 4, 8, 12, 16, 20, and 23 are control reactions containing no template DNA. Lane 1 contains Gibco BRL 500 bp DNA ladder. Ten microliters of each of the PCR products were loaded on the gel.

→ Symbol denotes sizes obtained from MW markers on ethidium stained gel.

[CBI CROSS REFERENCE 1]

[CBI CROSS REFERENCE 1]

[CBI CROSS REFERENCE 1]

Appendix 1

Study Protocol

Monsanto Study #: **01-01-39-02**

Study Title: PCR Analysis and DNA Sequence of the Insert in Corn Rootworm Event MON 863

Sponsor:
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Biotechnology Regulatory Sciences
700 Chesterfield Parkway North
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Quality Assurance Specialist
Monsanto Company
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Date

[redacted]

Confidentiality

All information regarding the identity of the test substance(s), associated samples, and data must be kept strictly confidential. No raw data, worksheets, observations, data or information summaries, reports or other information related to this study may be revealed or released to any third party without prior notification and authorization of Monsanto Company.

1.0 Regulatory Compliance

1.1 GLP Compliance

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

2.0 Purpose

The purpose of this study is perform PCR analysis across the insert and sequence the DNA insert in corn rootworm event MON 863

3.0 Timelines

3.1	Proposed Experimental Start Date:	January 17, 2001
3.2	Proposed Experimental Termination Date:	January 31, 2001

4.0 Test, Control and Reference Substances

4.1 Test Substance

The test substance is corn rootworm event MON 863. Grain harvested from Production Plan # 99-01-39-08 (LIMS ID 99ZMGRO01029) will be used.

4.2 Control Substance

The control substance is the non-transgenic corn line MON 846. Grain harvested from Production Plan # 99-01-39-08 (LIMS ID 99ZMGRO01019) will be used.

4.3 Reference Substances

The reference substances will include the plasmid PV-ZMIR13 (used to create corn rootworm event MON 863) which will be used as a positive control in the PCR analysis. The 500 bp DNA Ladder from Gibco BRL will be used to estimate band sizes in the PCR analyses.

[redacted]

4.4 Characterization of Test, Control and Reference Substances

The identity of the test and control substances was confirmed prior to the start of the study and is archived with Study # 99-01-39-22. The chain of custody for the test and control samples will be confirmed by the study director.

5.0 Description of Experimental Design

Overlapping PCR products will be generated across the entire length of the insert to demonstrate the linkage of the genetic elements within the insert. These PCR products will be purified and then sent for sequencing to the Monsanto Genomics Sequencing Center (a non-GLP facility) in order to determine the sequence of the entire insert in corn rootworm event MON 863.

5.1 Analytical Methods

All methods will be conducted as described below or by other appropriate methods approved by the Study Director and documented in the raw data.

5.1.1 DNA Extraction

DNA for both the test and control substance was extracted from grain tissue prior to the start of the study. The raw data detailing the extraction will be archived with this study. All previously extracted DNAs have been stored in a 4°C refrigerator. If necessary, additional DNA will be extracted from leaf or seed tissue under this protocol using methods approved by the study director and documented in the raw data.

5.1.2 DNA Quantitation

Any needed DNA quantitation will be conducted using Hoefer's DyNA Quant 200 Fluorometer according to SOP # BR-EQ-0065-01 using Roche's Molecular Weight Marker IX or pBR322 when calibrating genomic DNA or plasmid DNA, respectively.

5.1.3 Polymerase Chain Reaction

PCR amplification will be performed using genomic DNA template from both the test and control substances following standard PCR methodologies which will be documented in the raw data associated with this study. Plasmid PV-ZMIR13 will be used as a positive control in all PCR reactions. Various primers homologous to the inserted DNA and the flanking genomic DNA will be used in the PCR analyses.

5.1.3.1 Agarose Gel Electrophoresis for PCRs

PCR products will be separated on an agarose gel according to SOP # GEN-PRO-003-01. After electrophoresis, the DNA from the test substance amplifications will be purified from the agarose

matrix using an extraction kit following the procedure supplied by the manufacturer.

5.1.3.2 Sequencing of Purified PCR Products

Purified PCR products will be mixed with appropriate primers and submitted to the Monsanto Genomics Sequencing Center for sequencing.

6.0 Control of Bias

A PCR containing no template DNA will be prepared with each primer set to serve as a negative control. In addition, the plasmid PV-ZMIR13 will serve as a positive control in PCR analyses.

7.0 Records to be Maintained

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

8.0 Changes to the Protocol

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

[redacted]

ATTACHMENT