3) Cavato, T. A., E. C. Rigden, D. W. Mittanck and R. P. Lirette (2001). Amended report for MSL-16505: Molecular analysis of corn event MON 863. MSL-17152, an unpublished study conducted by Monsanto Company.

Study Title

Amended Report For MSL-16505: Molecular Analysis of Corn Event MON 863

Authors

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Study Completed On

Amendment 1
April 10, 2001

Performing Laboratory

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Laboratory Project ID

Study 99-01-39-27
MSL-17152

Statement of No Data Confidentiality Claim

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

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

Company: Monsanto Company

Company Agent: Dennis P. Ward

Title: Regulatory Affairs Manager

Signature: _____ Date: 7 May 2001

Statement of Compliance

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

Sequence information used in this study was generated by the Monsanto Sequencing Center, which does not generate its data in compliance with the GLP regulations.

Submitter — Date: 7 May 2001

Sponsor — Date: April 10, 2001

Study Director — Date: April 10, 2001

Quality Assurance Statement

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

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

Dates Of Inspection / Audit	Phase	Date Reported To: Study Director	Management
9/24/1999	Protocol Review	9/24/1999	9/24/1999
10/20/1999	PCR	10/25/1999	10/25/1999
3/02/2000	Raw Data/Draft Report	3/02/2000	3/02/2000
4/4/01	Amended Report Audit	4/9/01	4/9/01

Michelle R. Higgins
Michelle R. Higgins
Quality Assurance Specialist
Monsanto Regulatory, Monsanto Company

04-10-01
Date

Signatures of Approval

Study Number:	99-01-39-27
Title:	Amended Report For MSL-16505: Molecular Analysis of Corn Event MON 863
Facility:	Monsanto Company 700 Chesterfield Parkway North St. Louis, Missouri 63198
Sponsor:	Patrick T. Weston
Study Director:	Tracey A. Cavato
Contributors:	Ellen C. Rigden, Donald W. Mittanck, and Ronald P. Lirette
Study Initiation Date:	September 21, 1999
Original Study Completion Date:	March 3, 2000
Amended Report Completion Date:	April 10, 2001
Records Retention:	All study specific raw data, protocols, final reports and facility records will be retained at Monsanto, St. Louis.
Sample Storage:	Any study samples that are to be retained will be stored at Monsanto, St. Louis.

Signatures of Approval (con't)

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

MSL-17152 Amended Report	MSL-16505 Original Report	Amendments
1. Title Page (Pg 1)	1. Title Page (Pg 1)	a. Added "Amended Report For MSL-16505" in front of the study title b. Added a new line "Amendment 1" after "Study Completed on" c. Added new report completion date
2. QA Statement - (Pg 4)	2. QA Statement - (Pg 4)	a. Changed date of inspection for PCR phase b. Added a new line "Amended Report Audit" to the list of phases
3. Signatures of Approval - (Pg 5)	3. Signatures of Approval - (Pg 5)	a. Added "Amended Report For MSL-16505" in front of the study title b. Added the word "Original" to the Study Completion Date and immediately following, inserted a new line "Amended Report Completion Date"
4. Page 6	4. Not Included	Added "Amendments to Report" section
5. Pages 7-9	5. Pages 6-8	Table of Contents-changed pagination
6. Page 12	6. Page 11	Reworded the background paragraph to reflect new protein information
7. Page 23	7. Page 22	Inserted a new reference: Hileman, R. E. and Astwood, J. D., 2001.
8. Table 1, Page 27	8. Table 1, Page 26	a. Added the name "Chua" to the 4-AS1 reference b. Removed the MCS genetic element from the table c. Added the name "Spiker" in place of et. al in the tahsp 17 3' reference

Tracey A. Cavato — April 10, 2001
Study Director — Date

Sponsor — April 10, 2001
Date

Table of Contents

Table of Contents (cont'd.)

Table of Contents (cont'd.)

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	gene for bleomycin resistance
B.t.	*Bacillus thuringiensis*
CRW	corn rootworm
cry3Bb1	class III (Coleoptera-specific) crystal protein gene
Cry3Bb1	class III (Coleoptera-specific) crystal protein
CTAB	cetyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
HCl	hydrochloric acid
MCS	multiple cloning site
MW	molecular weight
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
Na_2HPO_4	sodium phosphate
NOS 3'	nopaline synthase 3' polyadenylation sequence
nptII	gene for neomycin phosphotransferase II
NPTII	neomycin phosphotransferase II protein
PCR	polymerase chain reaction
PVP	polyvinylpyrolidine
ract1	intron from the rice actin gene
SDS	sodium dodecyl sulfate
SSC	20X is 3 M sodium chloride, 0.3 M sodium citrate
SSU CTP	maize small subunit chloroplast transit peptide
Tris	tris(hydroxymethyl)-aminomethane
tRNA	transfer RNA
tahsp17	3' nontranslated region of the wheat heat shock protein 17.3 containing the polyadenylation sequence
TE buffer	Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA)
UV	ultraviolet
wtCAB	5' untranslated leader of wheat major chlorophyll a/b binding protein

I. SUMMARY

Corn plants *(Zea mays* L.) have been modified to produce a *Bacillus thuringiensis* (*B.t.*) Cry3Bb1 insecticidal protein which protects against corn rootworm (CRW, *Diabrotica*), a major North American corn pest. The genetically modified corn event, MON 863, was produced by particle acceleration technology using the *Mlu*I restriction fragment of plasmid PV-ZMIR13. The DNA fragment used for corn transformation contained two expression cassettes: 1) the *cry3Bb1* coding region regulated by the 4-AS1 plant promoter, and the wheat major chlorophyll a/b-binding protein (wtCAB) mRNA leader sequence, rice actin intron (ract1), and the wheat heat shock protein 17.3 (tahsp17) 3' polyadenylation sequence; and 2) the *nptII* coding region regulated by the 35S promoter, and the NOS 3' polyadenylation sequence. The enzyme neomycin phosphotransferase II, NPTII, (Beck et al., 1982) was used as a selectable marker (Fraley et al., 1983) during development of the genetically modified corn plant.

The purpose of this study was to perform molecular analyses to characterize the integrated DNA in transformation event MON 863. Specifically, the insert number (number of integration sites within the corn genome), the copy number (the number of copies of the DNA fragment used for transformation within one locus), the integrity of the inserted cassettes, and confirmation of the absence of backbone sequences were assessed by Southern blot analyses. Probes included the whole plasmid, the linear DNA fragment used in transformation, both intact coding regions and their respective promoters, introns, and polyadenylation sequences, and the plasmid backbone. The data show that event MON 863 contains a single DNA insertion with one copy of both the *cry3Bb1* and the *nptII* cassettes. No additional elements from the DNA fragment used in transformation, linked or unlinked to intact cassettes, were detected in the genome of event MON 863. PCR analysis and DNA sequencing were also used to verify the 5' and 3' junction sequences of the insert with the plant genome, as well as the intactness of the 5' and 3' ends of the inserted cassettes. Data also show that this event does not contain any detectable backbone sequence from plasmid PV-ZMIR13, including *ori*-pUC or the *nptII* coding region regulated by a bacterial promoter. Therefore, these data support that only the two expected full length proteins, Cry3Bb1 and NPTII, should be encoded by the insert in event MON 863. In addition, the genetic stability of the inserted DNA was demonstrated by Southern blot analysis on genomic DNA from the F2 generation (self cross of first R0 cross) and another F2 generation (two generations removed from the first F2 generation).

II. INTRODUCTION

A. Background. *Bacillus thuringiensis* (*B.t.*) is a gram-positive bacterium commonly present in soil. Many different strains of *B.t.* have been shown to produce crystal proteins or inclusion bodies which are specifically effective in controlling certain orders and species

of insect pests. *B.t.* based products have been widely used as pesticides since 1961 (McClintock et al., 1995). Pesticides based on microbes have been commercially available and used as environmentally acceptable insecticides because they are specific for the targeted insect pests and are typically harmless to plants and other non-targeted organisms. *B.t.* proteins have been generally classified based on their insecticidal activity (*e.g.*, Cry1, Cry2, Cry3, and Cry4 proteins are toxic to lepidopteran, lepidopteran/dipteran, coleopteran, and dipteran pests, respectively) (Bravo, 1997; Hofte and Whiteley, 1989). The Cry3 class protein, Cry3Bb1, has natural insecticidal activity against the coleopteran pest, corn rootworm, *Diabrotica* (Von Tersch et al., 1994). The Cry3Bb1 protein was previously referred to as CryIIIB2 (or Cry3B2) as well as Cry3Bb or CryIIIC. This protein should be referred to as Cry3Bb1 protein according to the most recent and accepted nomenclature (Crickmore et al., 1998). A variant of the wild type *cry3Bb1* coding sequence (GenBank Accession No. M89794) was designed to encode a protein with enhanced insecticidal activity against corn rootworm. This *cry3Bb1* coding sequence variant was used to create recombinant *B.t.* strain EG11098. Expression of this gene in *Bacillus* results in the production of a protein, Cry3Bb1.11098, which contains a total of five amino acid differences from the wild type Cry3Bb1 protein sequence. This coding sequence was further manipulated to enhance expression in plants and placed into a vector used for the transformation of corn (*Zea mays*). Transformation event MON 863 produces a variant of the Cry3Bb1.11098 protein that differs from the wild type protein sequence by seven amino acids and from the *Bacillus*-produced Cry3Bb1.11098 protein sequence by two amino acids. The protein produced in MON 863 corn is hereafter referred to simply as a Cry3Bb1 variant protein (Hileman and Astwood, 2001). In addition to the *cry3Bb1* coding region, the neomycin phosphostransferase II (*nptII*) coding region is present in these genetically modified plants as the selectable marker, enabling selection of transgenic plants in tissue culture that contain the *nptII* coding region.

Target plant cells were transformed using particle acceleration technology with a linear DNA fragment derived from plasmid PV-ZMIR13 (Figure 1). The linear DNA fragment was prepared by digestion of the plasmid with the restriction enzyme *Mlu*I, separation of the fragments by agarose gel electrophoresis and isolation of the DNA fragment containing the *cry3Bb1* and *nptII* expression cassettes (PV-ZMIR13L shown in Figure 2). The *MluI* fragment should not contain any plasmid backbone DNA sequence, except for residual DNA derived from multiple cloning sites. An expression cassette, or cassette, is defined in this report as including a promoter, a coding region, and a polyadenylation sequence. The expression cassettes for this study consist of 1) the *cry3Bb1* coding region regulated by the 4-AS1 plant promoter, and the wtCAB leader, rice actin intron, and tahsp17 3' polyadenylation sequence; and 2) the *nptII* coding region regulated by the 35S promoter, and the NOS 3' polyadenylation sequence. Descriptions of each of these elements are found in Table 1.

B. Purpose. The purpose of this study was to characterize the inserted DNA in the corn

rootworm-protected event MON 863. Genomic DNA was analyzed using Southern blot analysis for the number of insertion events, the copy number of the inserted DNA, the integrity of the inserted cassettes, and the presence or absence of plasmid backbone sequence. Polymerase chain reactions (PCR) and DNA sequencing were performed to verify the 5' and 3' insert-to-plant junctions, as well as determine whether the 5' and 3' ends of the insert were intact. In addition, the stability of the inserted DNA was assessed by Southern blot fingerprint analysis on genomic DNA from the F2 generation (self cross of first R0 cross) and another F2 generation (two generations removed from the first F2 generation).

III. MATERIALS AND METHODS

A. Test substance. The test substance for this study was the corn rootworm event MON 863.

B. Control substance. The control substance was the non-transgenic corn line MON 846 (A1xA634).

C. Reference substances. The reference substances include the plasmid PV-ZMIR13 from which the DNA fragment used in the transformation of the corn line was purified. For insert number, copy number, and stability analyses, DNA from the control line was mixed with the plasmid, then digested and separated by electrophoresis on agarose gels. For all other analyses, DNA from the control line was digested, mixed with the plasmid *Hind*III restriction fragment representing the cassette of interest, and separated by electrophoresis on agarose gels. The mixed DNA samples provided an accurate size marker for the expected fragments of the plasmid. The plasmid DNA also served as a positive hybridization control and was spiked into control line DNA at concentrations representing approximately 0.5 and 1 copy of the element being analyzed to demonstrate the sensitivity of the Southern blotting method. Additionally, molecular size markers from Boehringer Mannheim [molecular size markers II (23.1 Kb-0.6 Kb) and IX (1.4 Kb-0.072 Kb), catalog #236 250 and #1449 460, respectively] and Gibco BRL [High Molecular Weight DNA Marker (48.5 Kb-8.3 Kb) and 1 Kb ladder (12.2 Kb-0.075 Kb), catalog #15618-010 and #15615-024, respectively] were used for size estimations on Southern blots. Gibco BRL 100 bp DNA Ladder (2.1 Kb-0.1 Kb), catalog #15628-050 was used for size estimations for the PCR analysis.

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

E. DNA isolation. DNA extracted from leaf tissue was used to obtain the 5' and 3' insert-to-plant junction sequences for event MON 863. DNA extracted from grain was

used for the other analyses in this report, with the exception of the stability portion of the study which also utilized DNA extracted from seed. Grain samples, harvested from individual plots across four different field sites, were blended separately to a fine powder using a commercially available blender, and stored dry at -80°C prior to use. Approximately 6 g of the processed grain was transferred to a prechilled 30-35 ml centrifuge tube, and ~16 ml of CTAB extraction buffer [1.5% (w:w) CTAB, 75 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 1.05 M NaCl, and 0.75% (w:w) PVP (MW 40,000)] was added to the processed grain. The samples were mixed, incubated at 65°C for approximately 30 minutes with intermittent mixing, and then allowed to cool to room temperature. An equal volume (~16 ml) of room temperature chloroform:isoamyl alcohol (24:1) was added to the samples. The suspension was mixed by inversion, and the two phases separated by centrifugation at 13,800 x g. The aqueous (top) layer was removed using a transfer pipet and placed into a clean 30-35 ml centrifuge tube. Approximately 1/10 volume (~1.6 ml) of 10% CTAB buffer [10% (w:w) CTAB and 0.7 M NaCl] and an equal volume (~16 ml) of chloroform:isoamyl alcohol (24:1) was added to each sample, which was then mixed by inversion. The samples were centrifuged at 13,800 x g to separate the phases. The aqueous (upper) phase was removed, mixed with an equal volume (~15 ml) of CTAB precipitation buffer [1% (w:w) CTAB, 50 mM Tris pH 8.0, and 10 mM EDTA pH 8.0] and allowed to stand at room temperature for approximately 1 hour. The samples were centrifuged at 8,800 x g to pellet the DNA, the supernatant was removed using a transfer pipet, and the pellet was re-dissolved in approximately 2 ml of High Salt TE [10mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, and 1 M NaCl] by incubating at 37°C with gentle swirling for approximately 2 hours. Centrifugation was performed at 20,000 x g to pellet any remaining impurities. The supernatant was removed, placed into a clean 13 ml tube, and ~150 µl of 3M NaOAc and 2 volumes (~4 ml relative to the supernatant) of chilled 100% ethanol were added to precipitate the DNA. The precipitated DNA was subsequently removed using a sealed and hooked glass Pasteur pipet and washed with approximately 1 ml of 75% ethanol. The DNA was pelleted by centrifugation at 14,000 x g, vacuum dried, and re-dissolved in TE, pH 8.0, at 4°C overnight.

F. DNA quantitation and restriction enzyme digestion. Quantitation of the DNA samples was performed using a Hoefer DyNA Quant 200 Fluorometer (San Francisco, CA)(SOP BR-EQ-0065-01) using Boehringer Mannheim molecular size marker IX or plasmid pBR322 as a calibration standard when quantitating genomic or plasmid DNA, respectively. Approximately 10 µg of genomic DNA from the control line and 20 µg from the test event was used for the restriction enzyme digests. The 10 µg of digested control line DNA was loaded in one well of an agarose gel, while the 20 µg of test event DNA was split evenly between two wells of an agarose gel. Overnight digests were performed at 37°C according to SOP GEN-PRO-010-01 in a total volume of 500 µl using 100 units of restriction enzyme. All restriction enzymes were purchased from

Boehringer Mannheim (Indianapolis, IN). After digestion, the samples were precipitated by adding 1/10 volume (~50 µl) of 3M NaOAc and 2 volumes (~1 ml relative to the original digest volume) of 100% ethanol, followed by incubation at -20°C for at least one hour. The digested DNA was pelleted by centrifugation at 14,000 x g, washed with 75% ethanol, vacuum dried for approximately 5 minutes, and re-dissolved at room temperature in TE, pH 8.0.

G. DNA probe preparation. Plasmid DNA was isolated from *E. coli* cultures grown overnight. Most probe templates were prepared by PCR using plasmid PV-ZMIR12 as a template. PV-ZMIR12 is identical in its sequence to PV-ZMIR13 except that it contains the SSU CTP immediately adjacent to the 5' end of the *cry3Bb1* coding region. Linearized whole PV-ZMIR13 plasmid and PV-ZMIR13L (the DNA fragment used for transformation) were also used as probe templates. Approximately 25 ng of each probe template (except the NOS 3' polyadenylation sequence) were labeled with ^{32}P-dCTP (6000 Ci/mmol) using the random priming method (RadPrime DNA Labeling System, Gibco BRL, Gaithersburg MD). The NOS 3' polyadenylation sequence was labeled using PCR with NOS 3' template (5 ng), NOS 3' specific primers (0.25 µM each), 1.5 mM $MgCl_2$, 3 µM dATP, dGTP, and dTTP, 100 µCi of ^{32}P-dCTP (6000 Ci/mmol), and 2.5 Units of *Taq* DNA polymerase in a final volume of 20 µl. The cycling conditions were as follows: 1 cycle at 94°C for 3 minutes; 5 cycles at 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 1 minute; 1 cycle at 72°C for 10 minutes. All radiolabeled probes were purified using a Sephadex G-50 column (Boehringer Mannheim).

H. Southern blot analysis. Southern blot analyses (Southern, 1975) were performed according to SOP GEN-PRO-025-02. The samples of DNA digested with restriction enzymes were separated, based on size, using 0.6% agarose gel electrophoresis according to SOP GEN-PRO-003-01. A 'long run' and a 'short run' were performed for most gels. The long run allowed for greater separation of higher molecular weight DNAs while the short run allowed smaller molecular weight DNAs (<700 bp) to be retained on the gel. The long run samples were loaded onto the gel and typically electrophoresed for ~15 hours at 25-35 volts. The short run samples were then loaded in adjacent lanes on the same gel and typically the gel was electrophoresed for 4-6 additional hours at 75-85 volts.

After photographing the gel, it was placed in a depurination solution (0.125 N HCl) for approximately 10 minutes followed by a denaturing solution (0.5 M NaOH, 1.5 M NaCl) for ~30 minutes and then a neutralizing solution (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) for ~30 minutes. The DNA from the agarose gels was transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL) using a Turboblotter (Schleicher & Schuell, Keene, NH). The DNA was allowed to transfer for approximately 18 hours (using 20X SSC as the transfer buffer) and covalently cross-linked to the membrane with a UV Stratalinker 1800 (Stratagene, La Jolla, CA) using the auto crosslink setting. The blots were prehybridized for 2-72 hours in an aqueous solution of 500 mM

$Na_2HPO_4 \bullet 7H_2O$, 7% SDS, and 0.1 mg/ml tRNA. Hybridization with the radiolabeled probe was performed in fresh prehybridization solution for 8-24 hours at approximately 65°C. Membranes were washed in an aqueous solution of 0.1% SDS and 0.1X SSC for two ~15 minute periods followed by two ~20 minute periods at approximately 65°C using fresh solution for each of the four washes. Multiple exposures of blots were generated at approximately -80°C using Kodak Biomax MS™ film in conjunction with one Kodak Biomax MS™ intensifying screen.

I. Verification of 5' and 3' genomic flanking sequences. The 5' and 3' insert-to-plant genomic DNA junctions were verified using PCR followed by DNA sequencing. The 5' junction was verified using one primer designed to the 5' genomic flanking sequence, paired with a second primer in the 35S promoter (primers A and B, Figure 14). This primer pair covered approximately 312 bp. The 3' junction was verified using a primer designed to the 3' genomic flanking sequence with a second primer located in the tahsp17 polyadenylation sequence (primers C and D, Figure 14). The amplified region was approximately 363 bp long. The PCR reactions were conducted using 100 ng of genomic DNA as a template in a 50 µl reaction volume containing a final concentration of 2.5 mM Mg^{2+}, 0.4 µM of each primer, 200 µM each dNTP, and 2.5 units of *Taq* DNA polymerase. The amplification of the reactions was performed under the following cycling conditions: 94°C for 1 minute; 38 cycles at 96°C for 30 seconds, 70°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 approximately 45 volts for two hours, and subjected to DNA sequencing by the Monsanto Genomic Sequencing Center to further confirm the junction sequences.

IV. RESULTS AND DISCUSSION

A. Southern blot strategy.

Genomic DNA from corn rootworm-protected event MON 863 was digested with a variety of restriction enzymes and subjected to Southern blot hybridization analysis to characterize the DNA that was integrated into the genome. In every case, control genomic DNA was digested with the same restriction enzymes as the test sample. A map of the linear DNA fragment, PV-ZMIR13L, that was used to generate the MON 863 transgenic corn event, along with the locations of the restriction sites utilized for Southern analysis, is shown in Figure 2. The Southern blot figures presented in this report are representative of the data generated in the study.

1. Insert number. The insert number (the number of integration sites of transgenic DNA in the corn genome) was evaluated by digesting the test and control DNAs with the restriction enzyme *Nde*I, which does not cleave within the DNA fragment used for transformation. This enzyme should release a fragment containing the inserted DNA and

adjacent plant genomic DNA. The blot was probed with PV-ZMIR13L, the linear DNA fragment used for transformation (Figure 2). The number of fragments detected indicates the number of inserts present.

2. Copy number. The number of copies of the transformation cassette inserted into one locus was determined by digesting the genomic DNA with the restriction enzyme *Eco*RV, an enzyme that cuts only once in the linear DNA fragment used to generate the event. The blot was probed with the entire plasmid from which the DNA used to transform the line was generated. If the event contains one copy of the transformation cassette, two bands should be produced, representing two border fragments. Each of these border fragments would contain a portion of the transformation cassette and flanking corn genomic DNA. Therefore, from this analysis it was possible to determine if the event contains one or multiple copies of the inserted DNA.

3. *cry3Bb1* cassette intactness. The integrity of the *cry3Bb1* cassette was evaluated by digestion with the restriction enzyme *Hind*III, which cleaves at the 5' and 3' ends of the *cry3Bb1* cassette. Individual Southern blots were probed with the 4-AS1 promoter-wt CAB leader-ract1 intron, the *cry3Bb1* coding region, or the tahsp17 3' polyadenylation sequence. The presence of a band representing the expected size of the *cry3Bb1* cassette indicates that the cassette and each of its elements are intact.

4. *nptII* cassette intactness. The integrity of the *nptII* cassette was evaluated by digestion with the restriction enzyme *Hind*III, which cleaves at the 5' and 3' ends of the *nptII* cassette. Individual Southern blots were probed with the 35S promoter, the *nptII* coding region, or the NOS 3' polyadenylation sequence. The presence of a band representing the expected size of the *nptII* cassette indicates that the cassette and each of its elements are intact.

5. Analysis for backbone. The backbone region of the plasmid consists of the *Mlu*I-*Mlu*I restriction fragment that was not used to transform the plant. The backbone (Figure 1) consists of *ori*-pUC and the *nptII* coding region regulated by a bacterial promoter. Genomic DNA was digested with the restriction enzyme *Hind*III and probed with two PCR-generated probes to confirm the absence of backbone. One probe covered the unique backbone sequence located 5' of the *nptII* coding region, while the second probe covered the remaining unique backbone sequence located 3' of the *nptII* coding region. Together, these two probes encompassed the entire backbone sequence except for the *nptII* coding region. The linear DNA used to transform the plants also contained the *nptII* coding region, and, as a result, this sequence could not be used as a backbone probe. However, the *nptII* probe used to evaluate *nptII* coding region intactness would have shown backbone sequence containing that element had it been present in the event. The

absence of any hybridization bands indicates the absence of detectable backbone sequence in the event.

6. Stability of inserted DNA. The restriction enzyme *Nco*I generates a unique Southern blot banding pattern fingerprint for event MON 863 when probed with the *nptII* coding region. Genomic DNA from the F2 generation (self cross of first R0 cross) and another F2 generation (two generations removed from the first F2 generation) was digested, blotted and probed with the entire *nptII* coding region to assess the stability of the inserted DNA over time and breeding generations.

B. Analysis of event MON 863

1. Insert number. Test and control DNA were digested with *Nde*I. Plasmid PV-ZMIR13 plus MON 846 control DNA was digested with *Nde*I and *Eco*RV. Since *Nde*I does not cleave within the plasmid, a second enzyme, *Eco*RV, was added to linearize the plasmid to facilitate its migration through the gel so as to serve as an accurate size estimator. The blot was probed with radiolabeled PV-ZMIR13L DNA (Figure 2), the linear DNA fragment used in the transformation. The results are shown in Figure 3. MON 846 control DNA (lane 1) produced no detectable bands, as expected for the negative control. Plasmid PV-ZMIR13 DNA mixed with MON 846 DNA (lanes 3 and 4) produced the expected size band at approximately 7.3 Kb, the size of the entire plasmid. MON 863 DNA (lanes 2 and 5) produced one band at approximately 5.0 Kb. The linear DNA fragment used for transformation was approximately 4.7 Kb (Figure 2). Therefore, this result establishes that MON 863 contains one insert located on an approximately 5.0 Kb *Nde*I restriction fragment.

2. Copy number. Test DNA, control DNA, and control DNA mixed with plasmid PV-ZMIR13 DNA were digested with *Eco*RV. The blot was probed with PV-ZMIR13 (Figure 1), the source plasmid for the linear DNA fragment used in transformation. The results are shown in Figure 4. MON 846 control DNA (lane 1) showed a faint band at 9.3 Kb which is likely due to non-specific hybridization since it is also present in the MON 863 lanes (lanes 2 and 5). MON 846 DNA mixed with PV-ZMIR13 DNA (lanes 3 and 4) produced the expected size band at approximately 7.3 Kb, representing the linearized plasmid. The band at 9.3 Kb due to non-specific hybridization cannot be seen in lanes 3 and 4 due to the plasmid band. MON 863 DNA (lanes 2 and 5) produced two unique bands at approximately 3.7 Kb and 9.6 Kb. The restriction enzyme, *Eco*RV, used in this analysis cleaves only once within the PV-ZMIR13L fragment used in transformation (Figure 2). Thus, for an event containing only one copy of the inserted DNA, two border fragments should be produced. If an event contains more than two bands, this would suggest more than one copy of the DNA fragment used in transformation. Since only two

bands were produced in this analysis, this result establishes that MON 863 contains only one copy of the DNA fragment used for transformation at the locus of integration.

3. *cry3Bb1* cassette intactness. Test and control DNA were digested with *Hind*III to release the *cry3Bb1* cassette. Digested control DNA was spiked with the 3.0 Kb *Hind*III restriction fragment from PV-ZMIR13 containing the *cry3Bb1* gene cassette. Individual Southern blots were probed with the 4-AS1 promoter-wtCAB leader-ract1 intron, the full-length *cry3Bb1* coding region, or the tahsp17 3' polyadenylation sequence.

a. 4-AS1 promoter-wt CAB leader-ract1 intron (Figure 5). MON 846 control DNA (lane 1) showed no detectable hybridization bands, as expected for the negative control. PV-ZMIR13 *cry3Bb1* cassette DNA mixed with MON 846 DNA (lanes 3 and 4) produced the expected size band for the *cry3Bb1* cassette at approximately 3.0 Kb (Figure 1). MON 863 DNA (lanes 2 and 5) produced a band at approximately 3.2 Kb. This band is slightly larger than the expected size band. Genomic flanking sequence data indicates that approximately 10 bp, including the *Hind*III site and *Mlu*I half site, is missing at the 3' end of PV-ZMIR13L; however, there is a genomic *Hind*III site approximately 175 bp from the 3' end of the insert (Figure 14). Therefore, the expected size band for the inserted *cry3Bb1* cassette is approximately 3.2 Kb. No unexpected bands were detected, indicating that event MON 863 does not contain any additional, detectable 4-AS1 promoter, wtCAB leader, or ract1 element other than that associated with the intact *cry3Bb1* cassette.

b. *cry3Bb1* coding region (Figure 6). MON 846 control DNA (lane 1) showed no detectable hybridization bands as expected for the negative control. PV-ZMIR13 *cry3Bb1* cassette DNA mixed with MON 846 DNA (lanes 3 and 4) produced the expected size band for the *cry3Bb1* cassette at approximately 3.0 Kb (Figure 1). MON 863 DNA (lanes 2 and 5) produced a band at approximately 3.2 Kb, corresponding to the correct size of an intact *cry3Bb1* cassette due to the missing *Hind*III site at the 3' end of PV-ZMIR13L [see Section 3 (a)]. No unexpected bands were detected, indicating that event MON 863 does not contain any additional, detectable *cry3Bb1* coding region other than that associated with the intact *cry3Bb1* cassette.

c. tahsp17 3' polyadenylation sequence (Figure 7). MON 846 control DNA (lane 1) showed no detectable hybridization bands, as expected for the negative control. PV-ZMIR13 *cry3Bb1* cassette DNA mixed with MON 846 control DNA (lanes 3 and 4) produced the expected size band at approximately 3.0 Kb, corresponding to an intact *cry3Bb1* gene cassette (Figure 1). MON 863 DNA (lanes 2 and 5) produced an approximately 3.2 Kb band, corresponding to the correct size of an intact *cry3Bb1* cassette [see Section 3(a) above]. Genomic flanking sequence

indicates that while the *Hind*III site at the 3' end of PV-ZMIR13L is missing, the entire tahsp17 3' polyadenylation sequence is present in event MON 863. No unexpected bands were detected, indicating that event MON 863 does not contain any additional, detectable tahsp17 3' polyadenylation sequence element other than that associated with the intact *cry3Bb1* cassette.

The expected size band was produced when assessing *cry3Bb1* cassette intactness using the 4-AS1 promoter-wt CAB leader-ract1 intron, the *cry3Bb1* coding region, and the tahsp17 3' polyadenylation sequence as probes. Genomic flanking sequence data indicates that approximately 10 bp, including the *Hind*III site and *Mlu*I half site, is missing from the 3' end of PV-ZMIR13L, however, the tahsp17 3' polyadenylation sequence is intact. These data support the conclusion that event MON 863 contains a single, intact *cry3Bb1* cassette without any additional, detectable *cry3Bb1* cassette elements located in the genome.

4. *nptII* cassette intactness. Test DNA and control DNA were digested with *Hind*III to release the *nptII* cassette. Digested control DNA was spiked with the 1.6 Kb *Hind*III restriction fragment from PV-ZMIR13 containing the *nptII* cassette. Individual Southern blots were probed with the 35S promoter, the entire *nptII* coding region, or the NOS 3' polyadenylation sequence.

a. 35S promoter (Figure 8). MON 846 control DNA (lane 1) showed no detectable hybridization bands, as expected for the negative control. Plasmid PV-ZMIR13 *nptII* cassette DNA mixed with MON 846 control DNA (lanes 3 and 4) produced the expected size band for the intact *nptII* cassette at approximately 1.6 Kb (Figure 1). MON 863 DNA (lanes 2 and 5) also produced an approximately 1.6 Kb band, corresponding to the correct size of an intact *nptII* cassette. No unexpected bands were detected, indicating that event MON 863 does not contain any additional, detectable 35S promoter element other than that associated with the intact *nptII* cassette.

b. *nptII* coding region (Figure 9). MON 846 control DNA (lane 1) showed no detectable hybridization bands, as expected for the negative control. Plasmid PV-ZMIR13 *nptII* cassette DNA mixed with MON 846 control DNA (lanes 3 and 4) produced one band of the expected size for the intact *nptII* cassette at approximately 1.6 Kb (Figure 1). MON 863 DNA (lanes 2 and 5) also produced an approximately 1.6 Kb band, corresponding to the correct size of an intact *nptII* cassette. No unexpected bands were detected, indicating that event MON 863 does not contain any additional, detectable *nptII* coding region other than that associated with the intact *nptII* cassette.

c. NOS 3' polyadenylation sequence (Figure 10). MON 846 DNA (lane 1) showed no detectable hybridization bands, as expected for the negative control. Plasmid PV-ZMIR13 *nptII* cassette DNA mixed with MON 846 control DNA (lanes 3 and 4) produced one band of the expected size for the intact *nptII* cassette at approximately 1.6 Kb (Figure 1). MON 863 DNA (lanes 2 and 5) also produced an approximately 1.6 Kb band. Non-specific hybridization occurred between lanes 4 and 5 near the bottom of the blot. This non-specific hybridization does not affect the interpretation of the results from this blot. No unexpected bands were detected, indicating that event MON 863 does not contain any additional, detectable NOS 3' polyadenylation sequence element other than that associated with the intact *nptII* cassette.

The expected size bands were produced when assessing *nptII* cassette intactness using the 35S promoter, the *nptII* coding region, and the NOS 3' polyadenylation sequence as probes. These data support the conclusion that event MON 863 contains a single, intact *nptII* cassette without any additional, detectable *nptII* cassette elements located in the genome

5. Analysis for backbone. Test DNA and control DNA were digested with *Hind*III. Digested control DNA was spiked with the 2.6 Kb *Hind*III restriction fragment from PV-ZMIR13 containing the entire backbone. The blot was probed with the entire backbone sequence except for the *nptII* coding region (Figure 11). MON 846 control DNA (lane 1) showed no detectable hybridization bands, as expected for the negative control. There is an area of faint non-specific hybridization at approximately 2.4 Kb spanning into a portion of lane 1, which does not affect the interpretation of the results from this blot. Plasmid PV-ZMIR13 backbone DNA mixed with MON 846 control DNA (lanes 3 and 4) produced one band at the expected size for the entire backbone at approximately 2.6 Kb (Figure 1). MON 863 (lanes 2 and 5) showed no detectable hybridization bands. This result, in conjunction with the Southern blot analysis for the *nptII* coding region (Analysis of event MON 863, Section 4(b), Figure 9), establishes that event MON 863 does not contain any detectable plasmid backbone sequences including *ori*-pUC or the *nptII* coding region regulated by a bacterial promoter.

6. Stability of the inserted DNA. Control DNA, control DNA mixed with PV-ZMIR13 DNA, and test DNA from the F2 generation (self cross of first R0 cross) and another F2 generation (two generations removed from the first F2 generation) were digested with *Nco*I. The blot was probed with the entire *nptII* coding region (Figure 12). MON 846 control DNA (lane 1) showed no detectable hybridization bands, as expected for the negative control. Plasmid PV-ZMIR13 DNA mixed with MON 846 control DNA (lane 2) produced three bands at the expected sizes of 0.4, 2.0, and 3.7 Kb (Figure 1), along with two additional faint background bands at approximately 6.4 Kb and 2.4 Kb which

are most likely due to partial digestion. MON 863 F2 generation (self cross of first R0 cross) and MON 863 F2 generation (two generations removed from the first F2 generation, lanes 3 and 4) produced the expected fingerprint bands at 0.4 Kb and 8.0 Kb. The 0.4 Kb band represents an internal segment of the insert while the 8.0 Kb band represents a border fragment off of the 5' end of the insert (Figure 2). No differences in banding pattern were observed between DNA extracted from the first F2 generation and that from the second F2 generation. This demonstrates the stability of the inserted DNA in samples spanning three generations.

7. Genomic flanking sequence. PCR and DNA sequencing of the products were performed on genomic DNA to confirm the unique junction sequences at the 5' and 3' ends of the MON 863 insert. The results of these PCR reactions are shown in Figure 13. The negative controls of MON 846, distilled water, and an unrelated transgenic corn line did not yield a PCR product when either the 5' or 3' primer set was used (lanes 1, 3, 4, 6, 8, and 9). The MON 863 genomic DNA yielded the correct size products of 312 bp for the 5' PCR (lane 2) and 363 bp for the 3' PCR (lane 7) as predicted by MON 863 sequence analysis. This demonstrates the specificity of the primer pairs to MON 863. The sequences of these PCR products were compared to previously obtained sequence data for MON 863 and confirmed. Therefore, this PCR analysis verified the 5' and 3' border sequences of event MON 863.

V. CONCLUSIONS

The corn rootworm-protected event MON 863 was produced by particle acceleration technology using a *Mlu*I DNA restriction fragment from plasmid PV-ZMIR13 containing the *nptII* and *cry3Bb1* cassettes. The MON 863 event contains one DNA insert located on a 5.0 Kb *Nde*I fragment. This insert contains one copy of the fragment used in transformation. No additional elements from the DNA fragment used in transformation, linked or unlinked to intact cassettes, were detected in the genome. PCR and DNA sequencing were used to verify the 5' and 3' junction sequences of the insert with the plant genome, as well as the intactness of the 5' and 3' ends of the insert. Approximately 10 bp from the 3' end of PV-ZMIR13L, including the *Hind*III restriction site, is missing; however, the tahsp17 3' polyadeylation sequence is intact. Additionally, event MON 863 does not contain any detectable plasmid backbone sequence including *ori*-pUC or the *nptII* coding region regulated by a bacterial promoter. These data support the conclusion that only the two expected full length proteins, Cry3Bb1 and NPTII, should be encoded by the insert in event MON 863. In addition, the genetic stability of the inserted DNA was demonstrated by Southern blot analysis on genomic DNA from the F2 generation (self cross of first R0 cross) and another F2 generation (two generations removed from the first F2 generation) of corn event MON 863.

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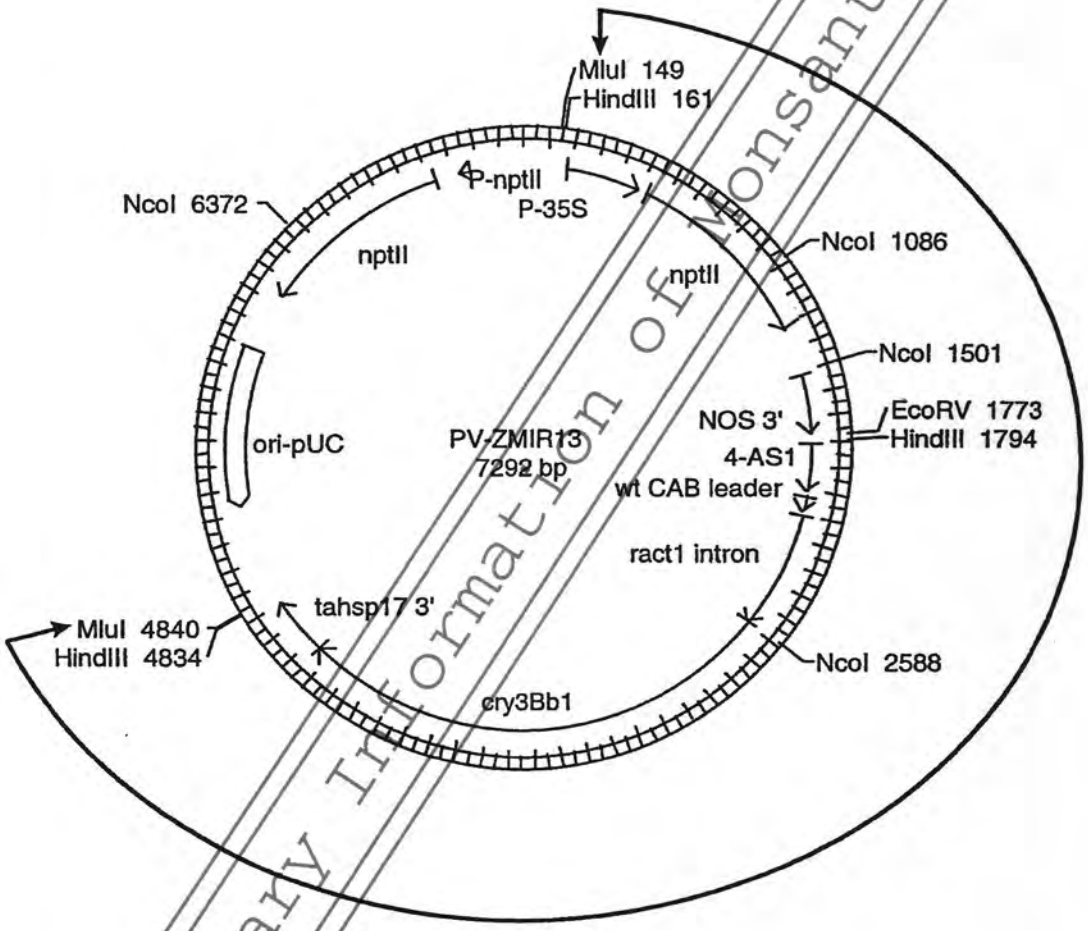
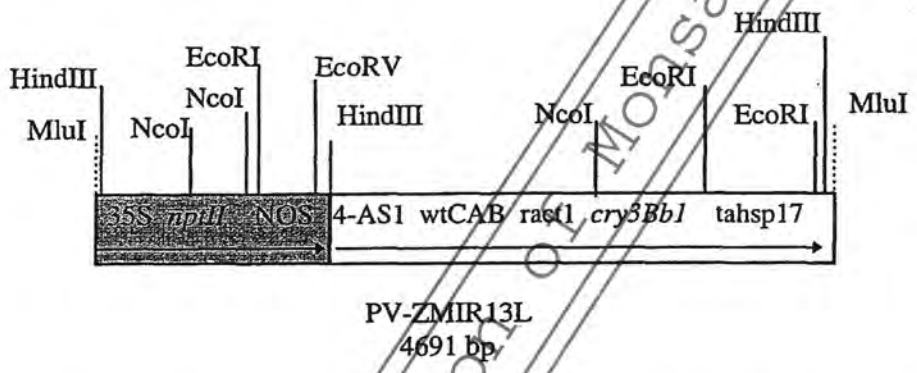


Figure 1. Plasmid Map of PV-ZMIR13. The *Mlu*I fragment of PV-ZMIR13 plasmid was used to generate corn rootworm-protected corn event MON 863.



PV-ZMIR13L
4691 bp

Figure 2. Map of DNA fragment PV-ZMIR13L. The DNA fragment PV-ZMIR13L was used to generate corn rootworm-protected event MON 863 by particle acceleration technology. The dashed lines represent the remaining *Mlu*I half sites following digestion of PV-ZMIR13.

Table 1. Summary of genetic elements in linear DNA fragment PV-ZMIR13L used for transformation of event MON 863

Genetic Element	Size Kb	Function
***Cry3Bb1* cassette:**		
4-AS1	0.22	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).
wt CAB	0.06	5' untranslated leader of the wheat chlorophyll a/b-binding protein (Lamppa et al., 1985)
ract1 intron	0.49	Intron from the rice actin gene (McElroy et al., 1990)
cry3Bb1	1.96	Gene encoding a synthetic variant of the Cry3Bb1 protein of *Bacillus thuringiensis* (Donovan et al., 1992)
tahsp 17 3'	0.23	A 3' nontranslated region of the wheat heat shock protein 17.3 which terminates transcription and directs polyadenylation (McElwain and Spiker, 1989)

Table 1 Continued. Summary of genetic elements in linear DNA fragment PV-ZMIR13L used in transformation of event MON 863

Genetic Element	Size Kb	Function
Selectable marker elements:		
35S	0.35	The cauliflower mosaic virus (CaMV) promoter (Odell et al., 1985)
nptII	0.97	The gene 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	A 3' nontranslated region of the nopaline synthase gene of *Agrobacterium tumefaciens* T-DNA which terminates transcription and directs polyadenylation (Bevan et al., 1983)

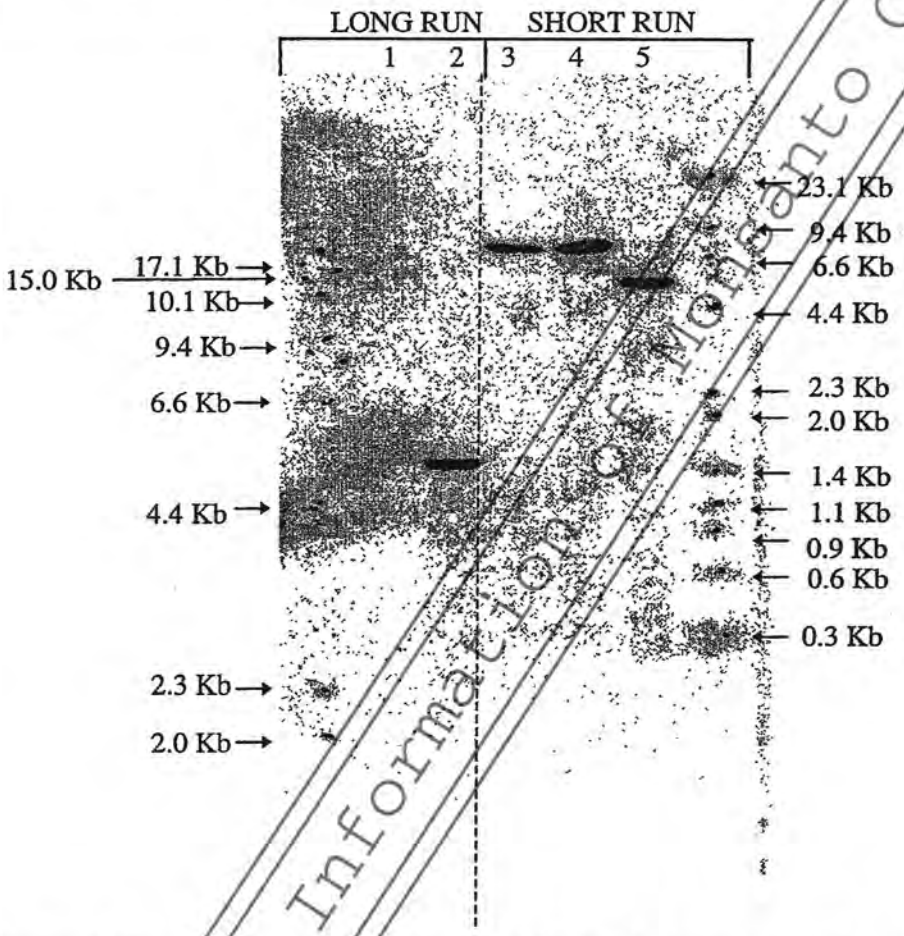


Figure 3. Southern blot analysis of event MON 863: insert number analysis.
Ten micrograms of MON 846 (control) and twenty micrograms of MON 863 (test) genomic DNA extracted from grain were digested with *Nde*I. Plasmid PV-ZMIR13 DNA mixed with MON 846 DNA was digested with *Nde*I and *Eco*RV. The blot was probed with ^{32}P-labeled PV-ZMIR13L. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 (1.0 copy)
5: MON 863 [10 µg]

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

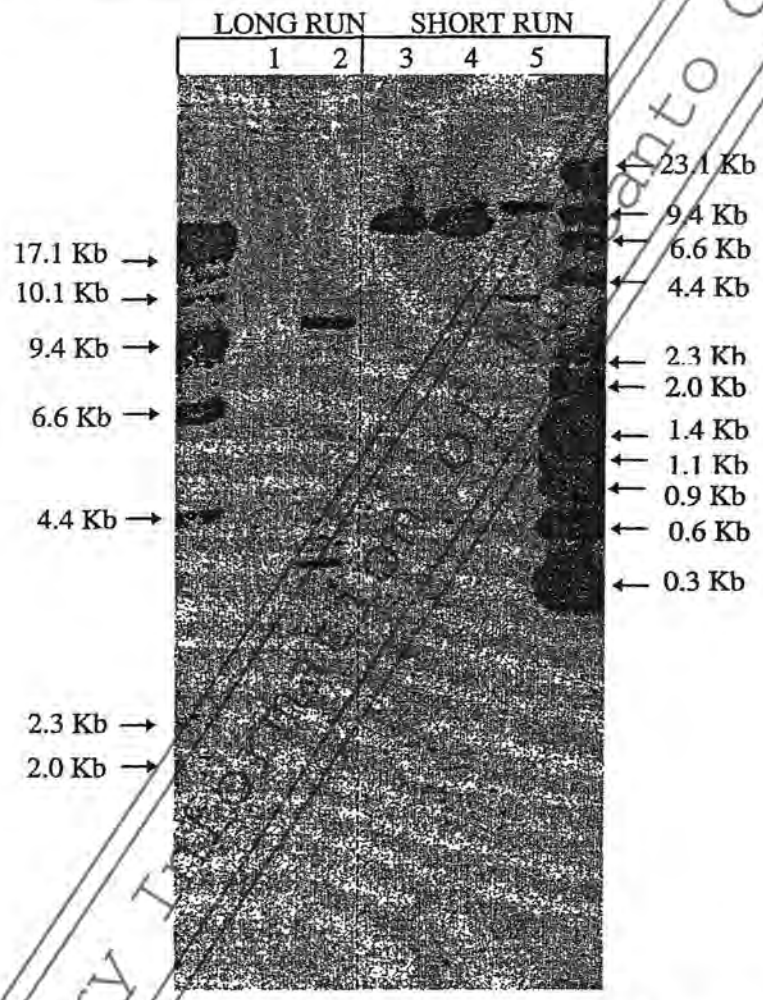


Figure 4. Southern blot analysis of event MON 863: copy number analysis.
Ten micrograms of MON 846 (control) and twenty micrograms of MON863 (test) genomic DNA extracted from grain were digested with *Eco*RV. The blot was probed with ^{32}P-labeled PV-ZMIR13. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg] (Long Run)
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 (1.0 copy)
5: MON 863 [10 µg]

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

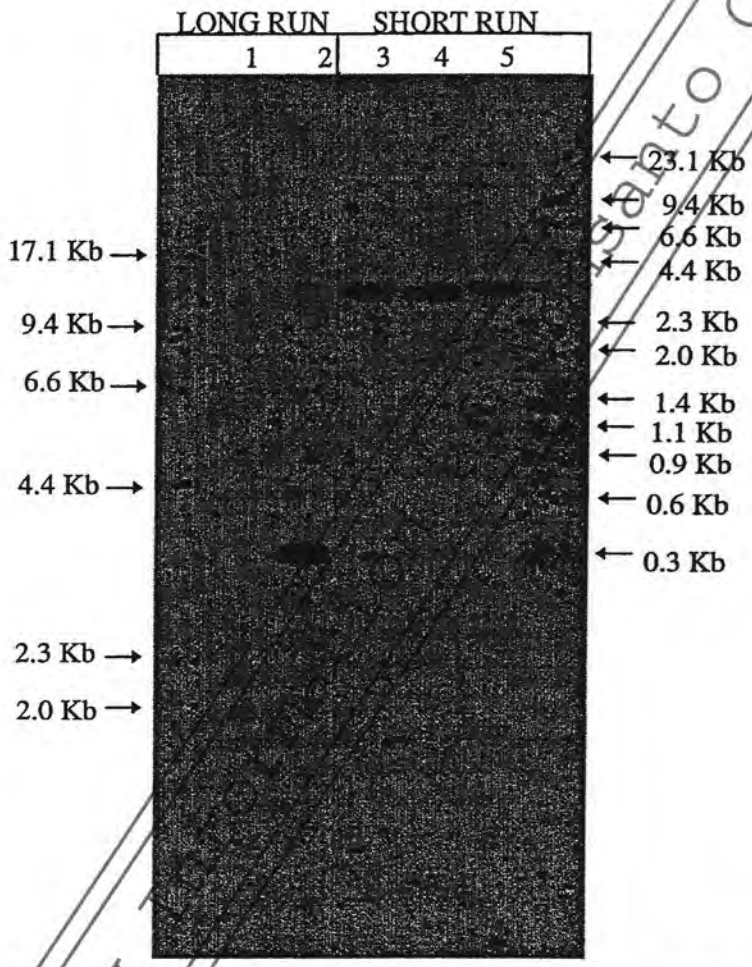


Figure 5. Southern blot analysis of event MON 863: *cry3Bb1* cassette intactness probed with the 4-AS1 promoter-wt CAB leader-ract1 intron. Ten micrograms of MON 846 (control) and twenty micrograms of MON 863 (test) genomic DNA extracted from grain were digested with *Hind*III. The blot was probed with the ^{32}P-labeled full length 4-AS1 promoter-wt CAB leader-ract1 intron . Lane designations are as follows:
Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *cry3Bb1* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *cry3Bb1* cassette (1.0 copy)
5: MON 863 [10 µg]

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

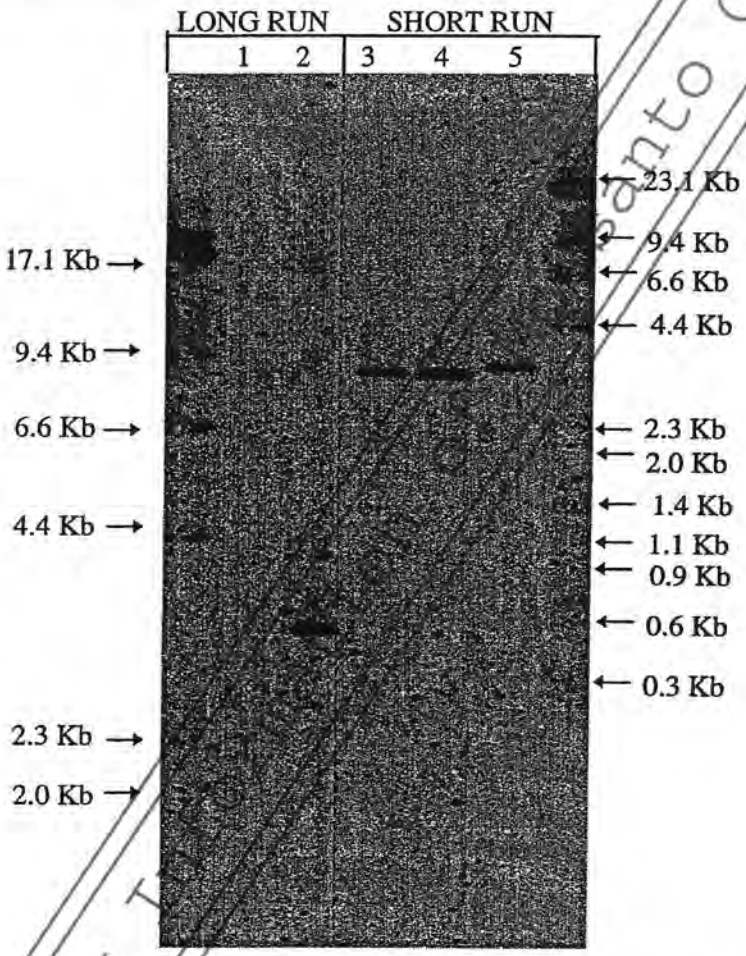


Figure 6. Southern blot analysis of event MON 863: *cry3Bb1* cassette intactness probed with the *cry3Bb1* coding region . Ten micrograms of MON 846 (control) and twenty micrograms of MON 863 (test) genomic DNA extracted from grain were digested with *Hind*III. The blot was probed with the ^{32}P-labeled full length *cry3Bb1* coding region. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *cry3Bb1* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *cry3Bb1* cassette (1.0 copy)
5: MON 863 [10 µg]

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

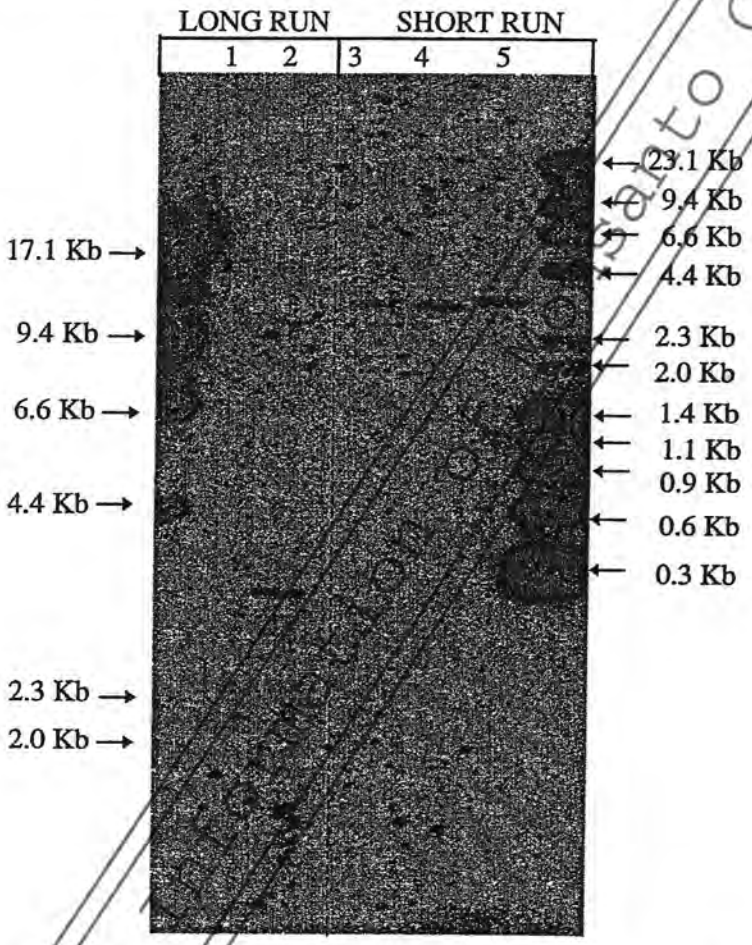


Figure 7. Southern blot analysis of event MON 863: *cry3Bb1* cassette intactness probed with the tahsp17 3' polyadenylation sequence. Ten micrograms of MON 846 (control) and twenty micrograms of MON 863 (test) genomic DNA extracted from grain were digested with *Hind*III. The blot was probed with the ^{32}P-labeled full length tahsp17 3' polyadenylation sequence. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *cry3Bb1* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *cry3Bb1* cassette (1.0 copy)
5: MON 863 [10 µg]

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

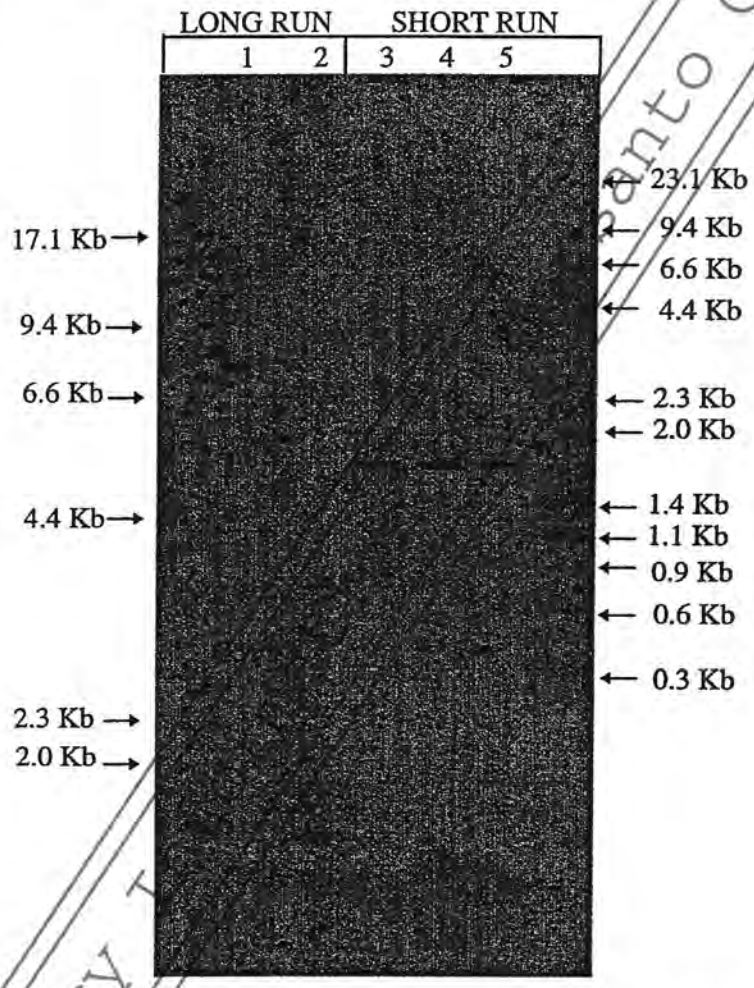


Figure 8. Southern blot analysis of event MON 863: *nptII* cassette intactness probed with the 35S promoter. Ten micrograms of MON 846 (control) and twenty micrograms of MON 863 (test) genomic DNA extracted from grain were digested with *Hind*III. The blot was probed with the ^{32}P-labeled full length 35S promoter. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *nptII* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *nptII* cassette (1.0 copy)
5: MON 863 [10 µg]

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

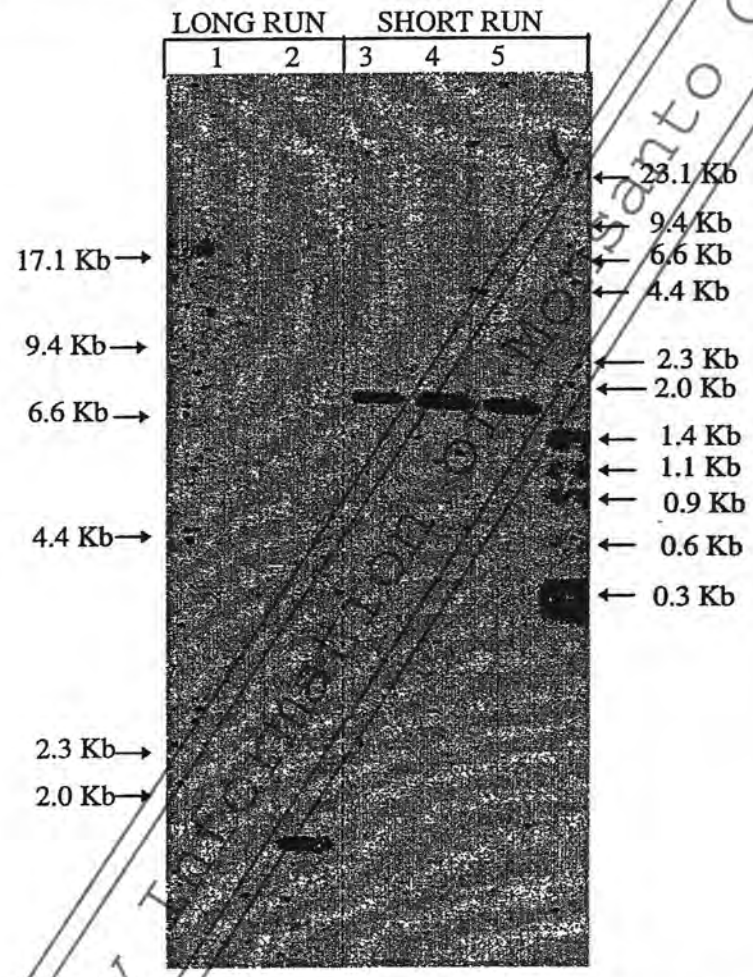


Figure 9. Southern blot analysis of event MON 863: *nptII* cassette intactness probed with the *nptII* coding region. Ten micrograms of MON 846 (control) and twenty micrograms of MON 863 (test) genomic DNA extracted from grain were digested with *Hind*III. The blot was probed with a ^{32}P-labeled full length *nptII* coding region. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *nptII* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *nptII* cassette (1.0 copy)
5: MON 863 [10 µg]

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

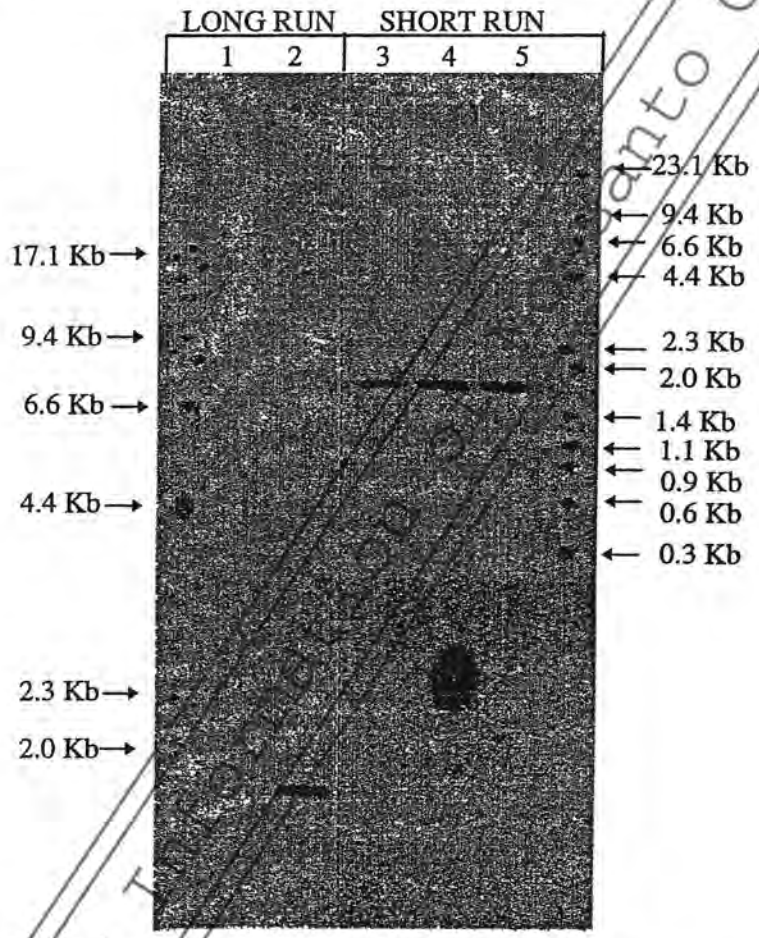


Figure 10. Southern blot analysis of event MON 863: *nptII* cassette intactness probed with the NOS 3' polyadenylation sequence. Ten micrograms of MON 846 (control) and twenty micrograms of MON 863 (test) genomic DNA extracted from grain were digested with *Hind*III. The blot was probed with a ^{32}P-labeled full length NOS 3' polyadenylation sequence. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *nptII* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *nptII* cassette (1.0 copy)
5: MON 863[10 µg]

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

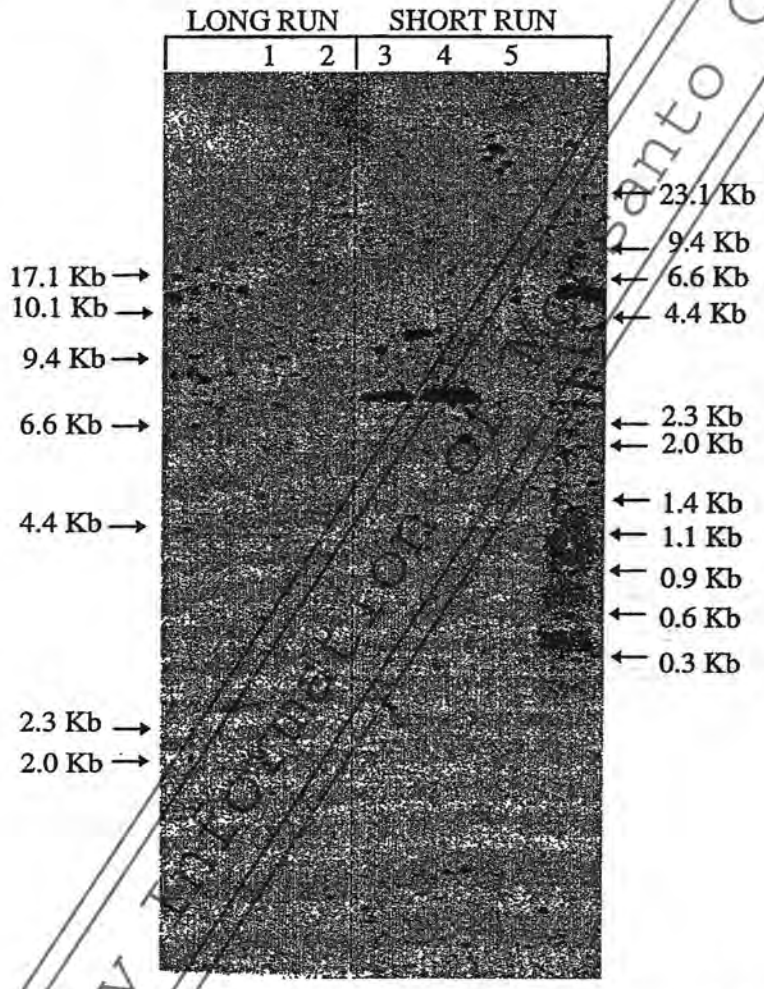


Figure 11. Southern blot analysis of event MON 863: backbone analysis.
Ten micrograms of MON 846 (control) and twenty micrograms of MON 863 (test) genomic DNA extracted from grain were digested with *Hind*III. The blot was probed with two ^{32}P-labeled backbone probes encompassing the entire backbone except for the *nptII* coding region. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 backbone region (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 backbone region (1.0 copy)
5: MON 863 [10 µg]

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

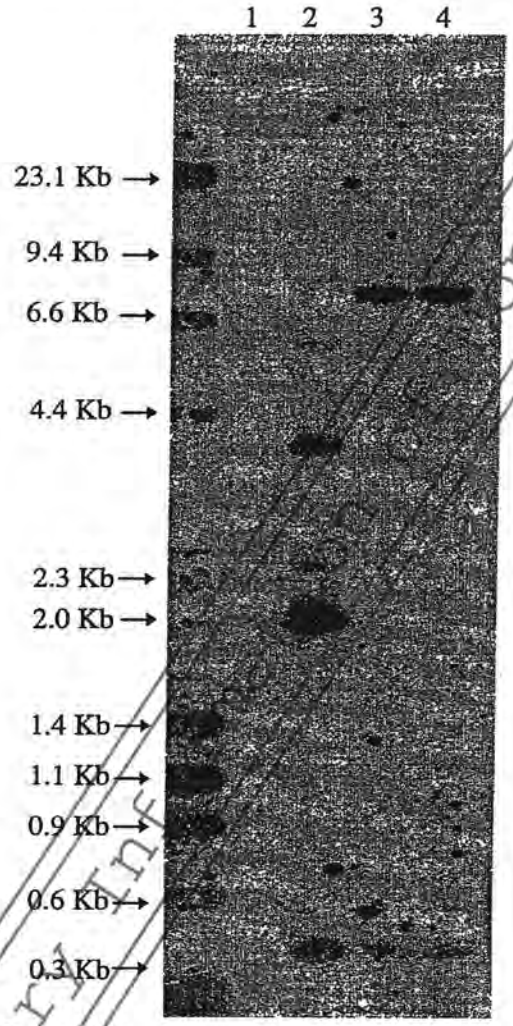


Figure 12. Southern blot analysis of event MON 863: stability of inserted DNA
Ten micrograms of MON 846 (control) and MON 863 (test) genomic DNA extracted from grain and seed were digested with *Nco*I. The blot was probed with a ^{32}P-labeled full length *nptII* coding region. Lane designations are as follows:
Lane 1: MON 846 [10 µg] grain DNA
2: MON 846 [10 µg] grain DNA spiked with ~19 pg PV-ZMIR13 (1.0 copy)
3: MON 863 F2 (self cross of first R0 cross) grain DNA [10 µg]
4: MON 863 F2 (two generations removed from the first F2) seed DNA [10 µg]

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

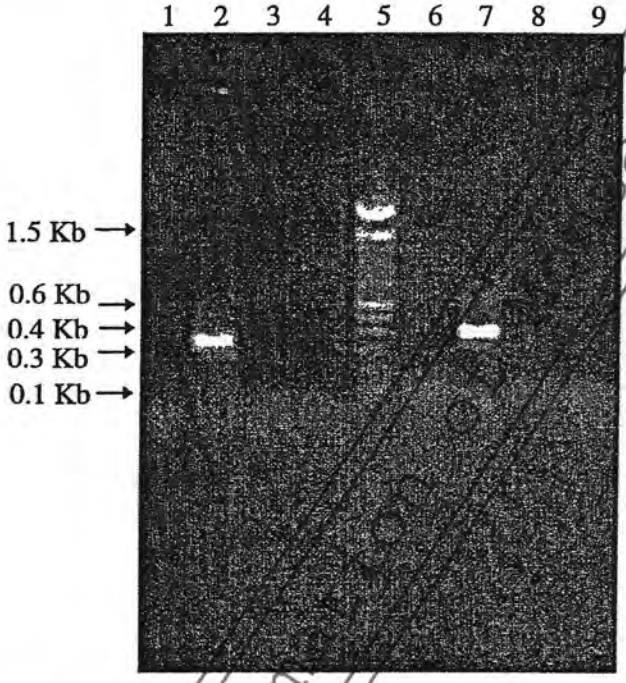


Figure 13. PCR verification of the 5' and 3' border sequences of the MON 863 insert. PCR was performed using primers specific to the 5' and 3' border sequences for MON 863 on genomic DNA extracted from grain from MON 846 (non-transgenic control), an unrelated transgenic corn line, and MON 863. DNAs were amplified with primers A and B from the 5' end of MON 863, amplifying a 312 bp product, and primers C and D from the 3' end of MON 863, amplifying a 363 bp product (Figure 14). Lane designations are as follows:

Lane 1: 10 µl of 5' PCR, MON 846 (non-transgenic)
2: 10 µl of 5' PCR, MON 863
3: 10 µl of 5' PCR, an unrelated transgenic corn line
4: 10 µl of 5' PCR, no template control
5: Gibco BRL 100 bp DNA Ladder
6: 10 µl of 3' PCR, MON 846 (non-transgenic)
7: 10 µl of 3' PCR, MON 863
8: 10 µl of 3' PCR, an unrelated transgenic corn line
9: 10 µl of 3' PCR, no template control

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

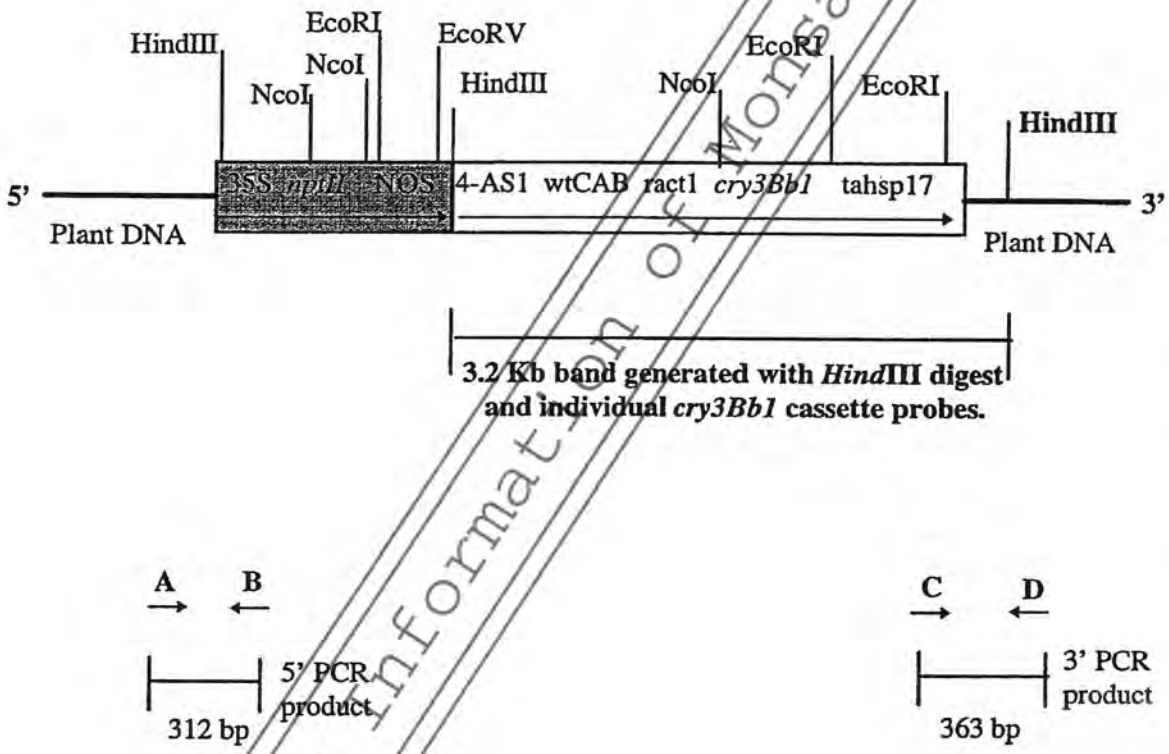


Figure 14. Schematic representation of the MON 863 insert. This figure depicts the predicted insert in corn event MON 863 based on data from Southern blot analyses and PCR confirmation of the sequences at the 5' and 3' ends of the insert. There is one copy of the PV-ZMIR13L fragment that was used to generate corn rootworm event MON 863. Genomic flanking sequence on the 3' end indicates that approximately 10 bp, including the *Hind*III site and *Mlu*I half site, are missing. However, the tahsp17 3' polyadenylation sequence is intact.

Appendix 1

Standard Operating Procedures

BR-EQ-0065-01	DyNA Quant 200 Fluorometer
GEN-PRO-010-01	Procedure for Restriction Enzyme Digestion of DNA
GEN-PRO-003-01	Procedure for Agarose Gel Electrophoresis
GEN-PRO-025-02	Procedure for Southern Blot Analysis

Appendix 2

Study Protocol and Protocol Amendments

Study #: 99-01-39-27

Study Title: Molecular Analysis of Corn Event MON 863 Containing the *cry3Bb1* gene

Sponsor:
Patricia R. Sanders
Monsanto Company
700 Chesterfield Parkway North BB5F
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Phone (636) 737-6412
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Primary Testing Facility:
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Study Director:
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Technical Center Leader:
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[redacted]

Approved By:

Testing Facility Management Representative:

Date: 9/21/99

Patrick T. Weston
Monsanto Company
BB5F, (636) 737-5407

Sponsor:

Patricia R Sanders Date: 9-21-99

Patricia R. Sanders
Monsanto Company
BB5F, (636) 737-6412

Technical Center Leader:

Date: 9-21-99

Ronald P. Lirette
Monsanto Company
BB5F, (636) 737-5603

Study Director:

Tracey A. Cavato Date: 9/21/99

Tracey A. Cavato
Monsanto Company
BB5K, (636) 737-5154

[redacted]

1.0 Purpose

The purpose of this study is to characterize the transgene insert in corn event MON 863. Corn has been modified to express the protein 11098 from *Bacillus thuringiensis* strain EG11098 (hereafter referred to as Cry3Bb1) which has insecticidal activity against corn rootworm. In addition to the *cry3Bb1* gene, the neomycin phosphostransferase II (*nptII*) gene is present as the selectable marker, enabling selection of cells that contain the *cry3Bb1* gene in tissue culture. The control line has background genetics representative of the test event, but has not been genetically modified and therefore, does not contain the *cry3Bb1* or *nptII* genes. The control line provides a background matrix used in the analysis of banding patterns on Southern blots.

The genomic DNA of corn event MON 863 will be evaluated using Southern blot analysis. The following will be determined:

1) number of inserts;
2) intactness of the *cry3Bb1* coding region;
3) intactness of the *cry3Bb1* gene cassette;
4) intactness of the *nptII* gene cassette;
5) presence or absence of plasmid backbone sequence; and
6) number of copies of the *cry3Bb1/nptII* transformation cassette per insert

In addition, the 5' and 3' insert-to-plant junctions will be confirmed by performing PCR at both borders using primer sets composed of a primer specific to the flanking sequence (previously obtained by the Insect Control Product Team) and a primer specific to the insert. The PCR products resulting from both ends will be sequenced by the Monsanto Genomic Sequencing Center (a non-GLP facility) to confirm the 5' and 3' insert-to-plant junctions.

2.0 Timelines

2.1 Proposed experimental start date: September, 1999
2.2 Proposed experimental termination date: April, 2000

3.0 Experimental design

3.1 Test Substance

The test substance is the corn event MON 863. Grain and leaf tissue were collected from plants grown as part of the 1999 U.S. field trials (Plan # 99-01-39-08). These samples were processed and stored as part of Plan # 99-01-39-08. Additional plant material of this event may be obtained from greenhouse grown plants. This protocol will be amended to include any greenhouse production, if needed.

[redacted]

3.2 Control Substance

The control substance is the non-transgenic corn line MON 847. Grain and leaf tissue were collected from plants grown as part of the 1999 U.S. field trials (Plan # 99-01-39-08). These samples were processed and stored as part of Plan # 99-01-39-08. Additional plant material of this same line may be obtained from greenhouse grown plants. This protocol will be amended to include any greenhouse production, if needed.

3.3 Reference Substances

The reference substance is the plasmid PV-ZMIR13 from which the linear DNA fragment was purified for transformation. The plasmid will be used as a size indicator and positive hybridization control. Additionally, molecular size markers from Boehringer Mannheim (DNA Molecular Weight Markers II and IX, catalog # 236 250 and # 1449 460, respectively) and Life Technologies (High Molecular Weight DNA Markers, catalog # 15618-010) will be used for size estimations.

3.4 Test and Control Substance Characterization

This is a characterization study. The identity of the test and control substances was confirmed in Study # 99-01-39-22.

3.5 Test System

There is no test system. This study will use Southern blot analysis (Southern, 1975) and other appropriate techniques to characterize corn event MON 863 containing the *cry3Bb1* gene.

3.6 Justification of Test System

There is no test system. Southern blot analysis is a common and widely accepted tool of molecular biology.

3.7 Procedure for Identification of Test System

There is no test system. The nylon membranes used for Southern blotting will be appropriately labeled. Autoradiographic films and any photographs of agarose gels will be labeled in indelible ink. Labels will include the study number, initials (or signature) of the individual responsible for generating the data, the date, and a unique identifier consisting of the study number followed by a sequential number linking related photographs, membranes, and films to each other and the study.

3.8 Description of Experiment Design

Genomic DNA from the test substance will be analyzed by Southern blotting for the following: 1) number of inserts in the corn genome; 2) intactness of the *cry3Bb1* coding region; 3) intactness of the *cry3Bb1* gene cassette; 4) intactness of the *nptII* gene cassette; 5) presence or absence of any plasmid backbone sequence;

[redacted]

and 6) number of copies of the *cry3Bb1/nptII* transformation cassette per insert. Genomic DNA will be quantitated, digested with appropriate restriction enzymes, separated by gel electrophoresis, and blotted. The Southern blots will be probed with either the entire source plasmid or a portion of the plasmid deemed appropriate by the researcher.

In addition, the 5' and 3' insert-to-plant junctions will be confirmed by performing PCR at both borders using primer sets composed of a primer specific to the flanking sequence (previously obtained by the Insect Control Product Team) and a primer specific to the insert. The PCR products resulting from both ends will be sequenced by the Monsanto Genomic Sequencing Center (a non-GLP facility) to confirm the 5' and 3' insert-to-plant junctions.

Additional analytical tools and techniques may be used to characterize the transgenic DNA if deemed necessary. These will be documented by the researcher and approved by the study director.

4.0 Analytical Methods

4.1 DNA Extraction

DNA will be isolated from grain and/or leaf tissue. DNA will be isolated from grain using the procedure of Rogers and Bendich (1985) while DNA will be isolated from leaf according to SOP # GEN-PRO-006-00. Alternatively, other methods may be used if they are found to yield better quality DNA, are documented by the researcher, and approved by the study director. The extracted DNA will be stored in a 4°C refrigerator.

4.2 DNA Quantitation

Extracted DNA will be quantitated using Hoefer's DyNA Quant 200 Fluorometer according to SOP # BR-EQ-0065-01 or by following another appropriate quantitation procedure to be documented.

4.3 Restriction Enzyme Digestion

Ten micrograms of extracted genomic DNA (spiked with plasmid DNA where appropriate) will be digested according to SOP # GEN-PRO-010-01. The amount of plasmid DNA added to each control digest will be calculated so as to approximate 0.5 and 1 copy of the target sequence on a Southern blot. Restriction enzymes will be documented in the study data.

4.3.1 Insert Number

All DNA preparations will be digested with a restriction enzyme that does not cleave within the inserted DNA.

[redacted]

4.3.2 *cry3Bb1* Coding Region Intactness

All DNA preparations will be digested with a restriction enzyme(s) that cuts at both ends of the *cry3Bb1* coding region.

4.3.3 *cry3Bb1* Gene Cassette Intactness

All DNA preparations will be digested with a restriction enzyme(s) that cuts at both ends of the *cry3Bb1* gene cassette.

4.3.4 *nptII* Gene Cassette Intactness

All DNA preparations will be digested with a restriction enzyme(s) that cuts at both ends of the *nptII* gene cassette.

4.3.5 Backbone Analysis

All DNA preparations will be digested with a restriction enzyme(s) that removes the entire backbone sequence from the source plasmid.

4.3.6 Copy Number

All DNA preparations will be digested with a restriction enzyme that cuts only once in the DNA fragment used for transformation.

4.4 Agarose Gel Electrophoresis

Digested DNAs will be resolved on agarose gels according to SOP # GEN-PRO-003-01. All samples will be "double loaded" in an effort to provide better resolution of larger DNA fragments, yet retain any smaller DNA fragments on the gel. The "double load" will consist of duplicate digestions of DNA run on the same gel, with one set of samples run for a longer period of time than the second set allowing for a long and short run on the same gel.

4.5 Southern Blot Analysis

DNA will be transferred from agarose gels onto nylon membranes and the blots probed with radiolabeled DNA according to SOP # GEN-PRO-025-02 or other appropriate blotting procedure to be documented.

4.5.1 Probe Table

^{32}P-labeled probes will be prepared by polymerase chain reaction or according to instructions provided with a suitable labeling kit. Probe templates will either be generated in this study or used from Study 98-01-39-17 if available. The following table lists the DNAs that will be used as probes along with the corresponding analysis (see section 4.3).

[redacted]

Analysis	DNA Probe
insert number	PV-ZMIR13 plasmid
cry3Bb1 coding region intactness	*cry3Bb1* coding region
cry3Bb1 gene cassette intactness	*cry3Bb1* coding region 4-AS1 promoter tahsp17 terminator
nptII gene cassette intactness	*nptII* coding region 35S promoter NOS 3' terminator
backbone detection	entire backbone sequence minus the *nptII* coding region
copy number	PV-ZMIR13 plasmid

Other techniques may be used to answer critical questions about the inserted DNA. These techniques will be approved by the study director and documented in the study data.

4.6 Proposed Statistical Method

A statistical method is not appropriate for this analysis.

4.7 Control of Bias

Proper positive and negative controls will be included on all agarose gels and Southern blots.

5.0 Records to be Maintained

All raw data, including photographs of agarose gels, autoradiographs, and computer printouts, shall be archived upon completion of the study. Excess study material such as purified genomic DNA will be stored in a 4°C refrigerator while plasmid DNA and probe templates will be retained in a -20°C freezer until notified of final disposition by the Sponsor. Records will be retained of all sampling and observational raw data, the protocol and all deviations and amendments thereto, and copies of all letters, memoranda, and other correspondence related to this study. Upon completion of the study, raw data will be transferred to the archives of the Sponsor.

6.0 Study Conduct Statement

This experiment shall be conducted in accordance with the protocol. Any change, revision, or deviation from this protocol should be documented promptly according to the current version of SOP # GEN-POL-005 and communicated to the Study Director. All data and information will be recorded directly and promptly in indelible ink. The exceptions are electronically captured data, for which a printout will be generated and included with other study data. All entries will be dated on

[redacted]

the day of entry and signed or initialed by the person entering the information. Computer printouts will be dated and initialed by the person responsible for their generation. All data sheets must contain the Study Number. Any change in entries will be made so as not to obscure the original entry, must indicate the reason for the change, and must be dated and signed (or initialed) at the time of the change.

7.0 Confidentiality

No raw data, worksheets, summaries, reports, or other information related to this study may be revealed or released to any third party without prior authorization of Monsanto.

8.0 GLP Compliance

This experiment will be conducted in compliance with the United States EPA FIFRA Good Laboratory Practice Regulations (40 CFR Part 160).

9.0 References

Rogers, S.O. and Bendich, A.J. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol.* 5:69-76.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.

[redacted]

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

Study #: 99-01-39-27 Amendment #: 1

1) Date Change Implemented: 10/18/99

Page No/s. &/or Section/s: Pg. 1, Title Page

Protocol originally stated:
Pg. 1, Title Page: Sponsor: Patricia R. Sanders
Monsanto Company
700 Chesterfield Parkway North BB5F
St. Louis, MO 63198
Phone (636) 737-6412
FAX (636) 737-6189

Amended as Follows:
Sponsor: Patrick T. Weston
Monsanto Company
700 Chesterfield Parkway North BB5F
St. Louis, MO 63198
Phone (636) 737-5407
FAX (636) 737-6189

Reason for Amendment and what impact will result from this change: The original sponsor of the study, Patricia R. Sanders, left the company and has been replaced by Patrick Weston. There will be no impact to the study due to this change.

2) Date Change Implemented: 10/18/99
Page No/s. &/or Section/s: Pg. 3, Section 1.0; Pg. 4, Section 3.8; Pg. 5, Section 4.3.2; Pg. 7, Section 4.5.1

Protocol originally stated:
Pg.3, Section 1.0: The genomic DNA of corn event MON 863 will be evaluated using Southern blot analysis. The following will be determined:

1) number of inserts
2) intactness of the *cry3Bb1* coding region
3) intactness of the *cry3Bb1* gene cassette
4) intactness of the *nptII* gene cassette
5) presence or absence of plasmid backbone sequence; and
6) number of copies of the *cry3Bb1/nptII* transformation cassette per insert

Amended as Follows:
The genomic DNA of corn event MON 863 will be evaluated using Southern blot analysis. The following will be determined:

1) number of inserts

2) intactness of the *cry3Bb1* gene cassette
3) intactness of the nptII coding region
4) presence or absence of plasmid backbone sequence; and
5) number of copies of the *cry3Bb1/nptII* tranformation cassette per insert

Protocol originally stated:
Pg. 4, Section 3.8: Genomic DNA from the test substance will be analyzed by Southern blotting for the following: 1) number of inserts in the corn genome; 2) intactness of the *cry3Bb1* coding region; 3) intactness of the *cry3Bb1* gene cassette; 4) intactness of the *nptII* gene cassette; 5) presence or absence of any plasmid backbone sequence; and 6) number of copies of the *cry3Bb1/nptII* transformation cassette per insert. Genomic DNA will be quantitated, digested with appropriate restriction enzymes, separated by gel electrophoresis, and blotted. The Southern blots will be probed with either the entire PV-ZMIR13 plasmid or a portion of the plasmid deemed appropriate by the researcher.

Amended as Follows:
Genomic DNA from the test substance will be analyzed by Southern blotting for the following: 1) number of inserts in the corn genome; 2) intactness of the *cry3Bb1* gene cassette; 3) intactness of the *nptII* gene cassette; 4) presence or absence of any plasmid backbone sequence; and 5) number of copies of the *cry3Bb1/nptII* transformation cassette per insert. Genomic DNA will be quantitated, digested with appropriate restriction enzymes, separated by gel electrophoresis, and blotted. The Southern blots will be probed with either the entire PV-ZMIR13 plasmid or a portion of the plasmid deemed appropriate by the researcher.

Protocol originally stated:
Pg. 6, Section 4.3.2: All DNA preparations will be digested with a restriction enzyme(s) that cuts at both ends of the *cry3Bb1* coding region.

Amended as Follows:
The contents of this section are deleted and all section numbers remain the same.

Protocol originally stated:
Pg. 6, Section 4.5.1:

Analysis	DNA Probe
insert number	PV-ZMIR 13 plasmid
cry3Bb1 coding region intactness	*cry3Bb1* coding region
cry3Bb1 gene cassette intactness	*cry3Bb1* coding region 4-AS1 promoter tahsp17 terminator
nptII gene cassette intactness	*nptII* coding region 35S promoter NOS 3' terminator
backbone detection	entire backbone sequence minus the *nptII* coding region
copy number	PV-ZMIR13 plasmid

Amended as Follows:

Analysis	DNA Probe
insert number	PV-ZMIR 13 plasmid
cry3Bb1 gene cassette intactness	*cry3Bb1* coding region 4-AS1 promoter tahsp17 terminator
nptII gene cassette intactness	*nptII* coding region 35S promoter NOS 3' terminator
backbone detection	entire backbone sequence minus the *nptII* coding region
copy number	PV-ZMIR13 plasmid

Reason for Amendment and what impact will result from this change: The intactness of the *cry3Bb1* coding region is evaluated through *cry3Bb1* gene cassette intactness. The intactness of the coding region can be inferred from the gene cassette intactness analysis, therefore there will be not be an impact on the study or its results.

3) Date Change Implemented: 10/18/99 and 11/2/99
Page No/s. &/or Section/s: Pg. 4, Section 3.3; Pg. 5, Section 4.3

Protocol originally stated:
Pg. 4, Section 3.3: The reference substance is the plasmid PV-ZMIR13 from which the linear DNA fragment was purified for transformation. The plasmid will be used as a size indicator and positive hybridization control.

Amended as Follows:
The reference substance is the plasmid PV-ZMIR13 from which the linear DNA fragment was purified for transformation. The plasmid or fragments resulting from digestion of the

plasmid will be used as size indicators and positive hybridization controls on Southern blots.

Protocol originally stated:
Pg. 5, Section 4.3: Ten micrograms of extracted genomic DNA (spiked with plasmid DNA where appropriate) will be digested according to SOP # GEN-PRO-010-01. The amount of plasmid DNA added to each control digest will be calculated so as to approximate 0.5 and 1 copy of the target sequence on a Southern blot.

Amended as Follows:
Ten micrograms of extracted control genomic DNA (spiked with plasmid DNA for insert and copy number analyses, sections 4.3.1 and 4.3.6, respectively) and twenty micrograms of extracted test genomic DNA will be digested according to SOP # GEN-PRO-010-01. In all analyses except insert and copy number, an appropriate restriction fragment of plasmid DNA (i.e., *nptII* gene cassette, *cry3Bb1* gene cassette, or backbone) will be spiked into two control DNA digests prior to loading on the agarose gel. The amount of plasmid fragment DNA or whole plasmid DNA will be calculated so as to approximate 0.5 and 1 copy of the target sequence on a Southern blot. The twenty micrograms of test DNA will be split equally between two lanes on an agarose gel and subsequent Southern blot so that ten micrograms are run in each lane.

Reason for Amendment and what impact will result from this change: Plasmid DNA was digested with the same enzyme as the genomic DNA allowing it to serve as a size indicator and positive hybridization control. However, this can lead to overdigestion of the plasmid resulting in extra bands on Southern blots. To avoid extra bands due to overdigestion of plasmid DNA, the plasmid DNA will be digested in a separate reaction to release certain restriction fragments of the plasmid such as the *nptII* gene cassette, *cry3Bb1* gene cassette, and backbone region. These fragments will be spiked into control digests prior to gel loading and will serve as appropriate size indicators and positive hybridization controls, which is the purpose of the plasmid spike. This change will impact the study in a positive way by minimizing the possibility of extra bands appearing in the plasmid spike lanes on Southern blots. Also, performing one test DNA digest consisting of 20 ug of DNA and then splitting the digest between two lanes will provide more consistency in the results obtained on Southern blots for test samples. This will have a positive impact on the study by ensuring consistency between test samples located on the same Southern blot.

4) Date Change Implemented: 10/18/99
Page No/s. &/or Section/s: Pg. 6, Section 4.5.1

Protocol originally stated:

Pg.7, Section 4.5.1

Analysis	DNA Probe
insert number	PV-ZMIR 13 plasmid
cry3Bb1 gene cassette intactness	*cry3Bb1* coding region 4-AS1 promoter tahsp17 terminator
nptII gene cassette intactness	*nptII* coding region 35S promoter NOS 3' terminator
backbone detection	entire backbone sequence minus the *nptII* coding region
copy number	PV-ZMIR13 plasmid

Amended as Follows:

Analysis	DNA Probe
insert number	PV-ZMIR 13 plasmid
cry3Bb1 gene cassette intactness	*cry3Bb1* coding region 4-AS1 promoter + Wt CAB leader + ract1 intron tahsp17 terminator
nptII gene cassette intactness	*nptII* coding region 35S promoter NOS 3' terminator
backbone detection	entire backbone sequence minus the *nptII* coding region
copy number	PV-ZMIR13 plasmid

Reason for Amendment and what impact will result from this change: In order to characterize the event completely, the wt CAB leader and ract1 intron should be used as probe with the 4-AS1 probe. It will be included per the above table. This will positively impact the study by more completely characterizing the insertion event.

5) **Date Change Implemented:** 10/18/99
Page No/s. &/or Section/s: Experiment not in original protocol; Amend Pg. 3, Section 1.0, Section 3.1; Pg. 5, Section 3.8, Section 4.3; Pg. 7, Section 4.5.1

Add to Protocol:
Pg. 3, Section 1.0; Pg. 5, Section 3.8

Add to list of Southern blot analyses,
6) stability of inserted DNA across generations.

Add to Protocol:
Pg. 3, Section 3.1
In addition, MON 863 seed will be obtained from Tim Coombe for use in analyzing the stability of the inserted DNA across generations. Appropriate chain of custody documentation will be supplied with the seed.

Add to Protocol:
Pg. 5, Section 4.3
4.3.7 Stability Analysis
All DNA preparations will be digested with the restriction enzyme *Nco*I.

Add to Protocol:
Pg. 7, Section 4.5.1

Analysis	DNA Probe
insert number	PV-ZMIR13 plasmid
cry3Bb1 gene cassette intactness	*cry3Bb1* coding region 4-AS1 promoter + ract1 intron tahsp17 terminator
nptII gene cassette intactness	*nptII* coding region 35S promoter NOS 3' terminator
backbone detection	entire backbone sequence minus the *nptII* coding region
copy number	PV-ZMIR13 plasmid
stability analysis	*nptII* coding region

Reason for Amendment and what impact will result from this change: Demonstrating the stability of the insert is a regulatory requirement by some foreign government agencies. This analysis was not originally included in the study but will be performed to satisfy the submission requirement.

Signatures of Approval

Study Director:

Tracy Cavato Date: 12/3/99

Sponsor/Testing Facilities Management Representative:

Date: 12/3/99

Technical Center Leader: :

Date: 12-3-99

Monsanto Company
Biotechnology Regulatory Sciences

Protocol
Amendment Form
SOP Ref.: GEN-POL-005

Study #: 99-01-39-27

Amendment #: 2

1) Date Change Implemented: 12/6/99

Page No/s. &/or Section/s: Pg. 4, Section 3.2

Protocol originally stated:
The control substance is the non-transgenic corn line MON 847.

Amended as Follows:
The control substance is the non-transgenic corn line MON 846.

Reason for Amendment and what impact will result from this change: The non-transgenic control was incorrectly identified in the original protocol.

Signatures of Approval

* Technical Center Leader:
~~Study Director:~~

Date: 1-4-00

Sponsor/Testing Facilities Management Representative:

Date: 1/3/2000

* Study Director:
~~Technical Center Leader~~

Tracey A. Cuenco

Date: 1/4/00

* Signed in the wrong place. TAC 1/4/00

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

Study #: 99-01-39-27 Amendment #: 3

1) Date Change Implemented: 1/10/00

Page No/s. &/or Section/s: Pg. 6, Section 4.5.1

Protocol originally stated:

Analysis	DNA Probe
insert number	PV-ZMIR 13 plasmid
cry3Bb1 gene cassette intactness	*cry3Bb1* coding region 4-AS1 promoter + wt CAB leader +ract1 intron tahsp17 terminator
nptII gene cassette intactness	*nptII* coding region 35S promoter NOS 3' terminator
backbone detection	entire backbone sequence minus the *nptII* coding region
copy number	PV-ZMIR13 plasmid
stability analysis	*nptII* coding region

Amended as Follows:

Analysis	DNA Probe
insert number	PV-ZMIR 13L (DNA fragment used in transformation)
cry3Bb1 gene cassette intactness	*cry3Bb1* coding region 4-AS1 promoter + wt CAB leader +ract1 intron tahsp17 terminator
nptII gene cassette intactness	*nptII* coding region 35S promoter NOS 3' terminator
backbone detection	entire backbone sequence minus the *nptII* coding region
copy number	PV-ZMIR13 plasmid
stability analysis	*nptII* coding region

Reason for Amendment and what impact will result from this change: The backbone of plasmids tends to lead to a higher amount of background on Southern blots when used as a probe. Since the backbone of PV-ZMIR13 is used as a probe independently, it does not need to be included in the probe when analyzing MON 863 for insert number. Therefore, to reduce the background on the Southern blot for insert number, the fragment used for transformation, PV-ZMIR13L, will be used as a probe. The impact from this change

should be a insert number Southern blot with a reduced level of background.

Signatures of Approval

Study Director:

Stacy Cavato Date: 1/24/00

Sponsor/Testing Facilities Management Representative:

Date: Jan 21, 2000

Technical Center Leader

Date: 1-24-00