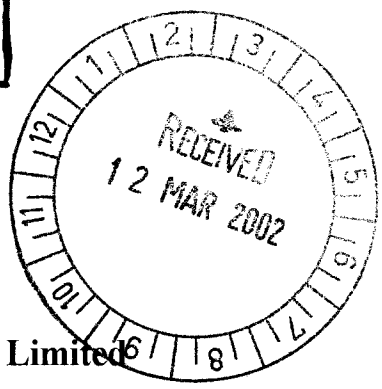
MONSANTO



APPLICANT: **Monsanto Australia Limited**

A416
CP4 ESPS gene in Roundup Ready® Corn Line NK603

SUBMISSION: Application to Australia New Zealand Food Authority for the inclusion of corn containing the CP4 EPSPS gene by Monsanto in Standard A18 - Food Derived From Gene Technology

VOLUME: 1 of 1

SUPPORTING INFORMATION

DATE: 08 March 2002

PREPARED BY: Megan Shaw
Regulatory Product Manager

Study Title

Amended Report for MSL-16857: Confirmation of the Genomic DNA Sequences Flanking the 5' and 3' Ends of the Insert in Roundup Ready® Corn Event NK603

Authors

Tracey A. Cavato
Ming Y. Deng
Ronald P. Lirette

Report Completed

Amendment 1
December 19, 2001

Performing Laboratory

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

Laboratory Project ID

Study 00-01-46-30
MSL-17617

Statement of Data Confidentiality Claims

A 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).

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 performed to confirm sequence data within this report were performed in compliance with the GLP regulations.

Submitter	Date
Linda K. Lahman	19 December 2001
Sponsor	Date
Tracey A. Cavato	December 19, 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
August 14, 2000	PCR	August 14, 2000	August 14, 2000
August 14, 2000	Agarose Gel Electrophoresis	August 14, 2000	August 14, 2000
August 30, 2000	Raw Data Audit	August 30, 2000	August 30, 2000
October 12, 2000	Raw Data/Draft Report Audit	October 12, 2000	October 12, 2000
December 19, 2001	Raw Data Audit	December 19, 2001	December 19, 2001
December 19, 2001	Draft Report Amendment Audit	December 19, 2001	December 19, 2001

C. Marie Braton

C. Marie Braton
Quality Assurance Specialist
Monsanto Regulatory, Monsanto Company

December 19, 2001
Date

Signatures of Approval

Study Title:	Amended Report for MSL-16857: Confirmation of the Genomic DNA Sequences Flanking the 5' and 3' Ends of the Insert in Roundup Ready® Corn Event NK603
Primary Facility:	Monsanto Company 700 Chesterfield Parkway North St. Louis, MO 63198
Monsanto Genome Sequencing Center:	Monsanto Company Genomics Sequencing Center 800 North Lindbergh Boulevard St. Louis, MO 63167
Sponsor Representative:	Linda K. Lahman
Study Director:	Tracey A. Cavato
Contributors:	Ming Y. Deng, Jinsong You, Gregory Heck, and Ronald P. Lirette
Study Initiation Date:	August 8, 2000
Original Report Completion Date:	October 12, 2001
Amended Report Completion Date:	December 19, 2001
Records Retention:	All study specific raw data, protocols, and final reports and facility records will be retained at Monsanto, St. Louis.

Amendments to Report:

MSL- Amended Report	MSL-16857 Original Report	Amendments
1. Title Page (Pg 1)	1. Title Page (Pg 1)	a. Added "Amended Report For MSL-16857" in front of the study title b. Added a new line "Amendment 1" after "Report Completed on" c. Added new report completion date
2. QA Statement - (Pg 4)	2. QA Statement - (Pg 4)	a. Added "Raw Data Audit" and "Draft Report Amendment Review" with appropriate dates b. Changed "Joan Rejda-Heath" to "C. Marie Braton" as Quality Assurance Specialist
3. Signatures of Approval - (Pg 5)	3. Signatures of Approval - (Pg 5)	a. Added "Amended Report For MSL- 16857" in front of the study title b. Added the word "Original" to the Report 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 7-8	5. Pages 6-7	Table of Contents-changed pagination
6. Page 10	6. Page 9	Summary: Changed "308 bp" to "307 bp"
7. Page 12	7. Page 11	Section G: Changed "498 bp" to "500 bp"
8. Page 13	8. Page 12	Section A: Changed "498 bp" to "500 bp"
9. Page 13	9. Page 12	Section B: Changed "308 bp" to "307 bp" Section B: Changed "...190 bp at the 5' end of the rice actin promoter" to, "193 bp at the 5' end of the insert containing 50 bp of polylinker sequence and 143 bp of the rice actin promoter."
10. Page 14	10. Page 12	Conclusion: Changed "308 bp" to "307 bp"
11. Page 15	11. Page 14	Changed "498 bp" to "500 bp"
12. Page 17	12. Page 16	Changed first 2 sentences of figure legend to "Bases 1-307 represent flanking corn genomic DNA. The italicized underlined bases 308-357 represent 50 bp of polylinker sequence, while the underlined bases 358-500 represent the 5' portion of the rice actin promoter."

Signatures of Approval:

Tracey A. Cavato — December 19, 2001
Study Director — Date

Linda K. Lahman — 19 December 2001
Sponsor Representative — Date

Table of Contents

Table of Contents (cont'd)

Abbreviations

~	approximately
CaMV	cauliflower mosaic virus
CP4 EPSPS	enzyme 5-enolpyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium* sp. strain CP4
CTAB	cetyltrimethylammonium bromide
CTP2	chloroplast transit peptide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
e35S	cauliflower mosaic virus promoter with the duplicated enhancer region
EDTA	ethylenediaminetetraacetic acid
Mg^{2+}	magnesium
MW	molecular weight
NaCl	sodium chloride
NOS 3'	nopaline synthase 3' polyadenylation sequence
PCR	polymerase chain reaction
P-ract1	rice actin promoter
ract1 intron	rice actin intron
PVP	polyvinylpyrrolidone
TE buffer	Tris-EDTA buffer (10mM Tris, pH 8.0, 1mM EDTA)
Tris	tris(hydroxymethyl)aminomethane
*Zm*HSP70	maize (*Zea mays*) *hsp*70 gene (heat-shock protein)

I. SUMMARY

The molecular characterization of Roundup Ready® corn event NK603 has been previously described in detail (Deng *et al.*, 1999). This characterization demonstrated that one complete copy of the DNA fragment used for transformation was present in the genome of corn event NK603, along with a 217-bp segment containing a portion of the enhancer region of the rice actin promoter inversely linked to the 3' end of the inserted transformation cassette. PCR analysis was also performed as part of that characterization to verify the maize genomic sequences flanking the 5' and 3' ends of the insert. In order to further confirm the 5' and 3' genomic DNA sequences flanking the 5' and 3' ends of the NK603 insert, PCR and subsequent DNA sequence analysis of the PCR products were performed in the current study. DNA sequences of the 5' and 3' ends of the insert, as well as the corn genomic DNA sequences flanking the 5' end (307 bp) and 3' end (497 bp) of the insert in event NK603 are described in this report.

II. INTRODUCTION

A. Background.

Roundup Ready corn event NK603 was generated through particle acceleration using a 6.7-Kb agarose gel-isolated *Mlu* I restriction fragment from the plasmid vector PV-ZMGT32. The DNA fragment used for transformation contained two gene expression cassettes: an EPSPS cassette containing the CP4 EPSPS coding sequence under the regulation of the rice actin promoter (P-ract1), a rice actin (ract1) intron, a chloroplast transit peptide (CTP2) sequence, and a nopaline synthase (NOS) 3' polyadenylation sequence; and a second EPSPS cassette containing the CP4 EPSPS coding sequence under the regulation of the cauliflower mosaic virus (CaMV) enhanced 35S plant promoter (e35S), a maize heat-shock protein 70 (*Zm*HSP70) intron, CTP2, and the NOS 3' polyadenylation sequence. Previous molecular characterization of the insert in event NK603 (Deng *et al.*, 1999) demonstrated that one complete copy of the DNA fragment used for transformation is present in the genome of corn event NK603. In addition to the one complete copy, a 217-bp segment containing a portion of the enhancer region of the rice actin promoter is inversely linked to the 3' end of the inserted transformation cassette in Roundup Ready event NK603 (Figure 1A). This portion of the enhancer region of the rice actin promoter is highly unlikely to act as a promoter (Deng *et al.*, 1999).

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B. Purpose.

The objective of this study was to further confirm the DNA sequences flanking the 5'and 3' ends of the insert in Roundup Ready corn event NK603.

III. MATERIALS AND METHODS

A. Test Substance. The test substance for this study was the Roundup Ready corn event NK603. DNA extracted in Study # 99-01-46-26 was used in this analysis.

B. Control Substance. The control substance was the non-transgenic corn line B73. DNA extracted in Study # 99-01-46-26 was used in this analysis.

C. Reference Substance. The reference substance was the 100 bp DNA Ladder (2.1 Kb-0.1 Kb) molecular size marker from Gibco BRL (Gaithersburg, MD).

D. Test System. There was no test system. Analytical methods were used in this study.

E. DNA Isolation. Corn leaf tissue (7.8-9.5 g) was ground to a fine powder using a pre-cooled mortar and pestle and transferred to a 35-ml centrifuge tube. Sixteen milliliters 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 each tube; the tubes were then incubated at 60°C for 60 min and then allowed to cool at room temperature for 5-10 min. An equal volume (~16 ml) of chloroform:isoamyl alcohol (24:1) was added to each sample. The suspension was mixed by inversion of the tube several times and centrifuged for 10 min at approximately 8,800 x *g* at 20°C. The upper aqueous phase was transferred to a clean 35-ml centrifuge tube and re-extracted with chloroform:isoamyl alcohol. The upper aqueous phase was transferred to a new tube, approximately 10 ml of isopropanol was added to each tube, and the contents of each tube were mixed by inversion. The samples were kept in a -20°C freezer for at least 30 min. The samples were centrifuged at 14,000 x *g* for 20 min at 4°C to pellet the DNA, and the supernatant was discarded. The pellet was re-dissolved in 2 ml of TE [10mM Tris-HCl pH 8.0, 1 mM EDTA] and transferred to a 13-ml tube. Approximately 20 µl of 10 mg/ml DNase-free RNase was added to each sample and the tubes were incubated at 37°C for 30 min. Following the incubation, two milliliters of chloroform:isoamyl alcohol (24:1) was added to each sample. The suspension was mixed by inversion and centrifuged for 10 min at 8,000 x *g* at room temperature. The upper aqueous phase was transferred to a new tube and 1 ml of 7.5 M ammonium acetate was added to each tube and the contents were gently mixed. Approximately 2 volumes (5 ml) of 100% ethanol was added to each tube and the tubes were kept in a -20°C freezer for 2h to overnight. The DNA was pelleted by centrifugation at 14,000 x *g* for 20 min at 4°C, the pellet was washed with 70% ethanol,

vacuum dried for 10 min , re-dissolved in 0.5 ml TE, pH 8.0, and stored in a 4°C refrigerator.

F. DNA Quantitation. DNA quantitation was conducted using a Hoefer DyNA Quant 200 Fluorometer using Boehringer Mannheim (Indianapolis, IN) molecular size marker IX as a DNA calibration standard.

G. PCR Analyses of the Genomic Sequences Flanking the 5' and 3' Ends of the Insert in Corn Event NK603. PCR analysis of the genomic sequences flanking the 5' end of the insert in NK603 was performed using one primer derived from the 5' genomic flanking sequence paired with a second primer located in the rice actin promoter near the 5' end of the inserted DNA, covering a 500-bp region (Primers 1 and 2, Figure 1A). The PCR analysis for genomic sequences flanking the 3' end of the NK603 insert was conducted using one primer located in the NOS 3' polyadenylation sequence near the 3' end of the insert paired with a second primer derived from the 3' genomic flanking sequence spanning a 1183-bp region (Primers 3 and 4, Figure 1A). The PCR analyses were conducted using 50 ng of event NK603 genomic DNA or B73 non-transgenic genomic DNA template in a 50 µl reaction volume containing a final concentration of 1.5 mM Mg^{2+}, 0.4 µM of each primer, 200 µM each dNTP, and 2.5 units of *Taq* DNA polymerase. The reactions were performed under the following cycling conditions: 1 cycle at 94°C for 3 minutes; 38 cycles of 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 1.5 % agarose gel electrophoresis at 75 V for ~1 hour and visualized by ethidium bromide staining.

H. Isolation and Sequencing of the 5' and 3' PCR Products. PCR products of the expected sizes representing the 5' and 3' flanking sequences generated with numerous primer pairs were isolated by electrophoretic separation of 10-20 µl of the PCR products on 2.0% agarose gels. The PCR fragments representing the 5' and 3' flanking sequences 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 5' PCR products were then sequenced with the initial 5' PCR primers. Due to the length of the 3' PCR products, sequencing was performed with both the initial 3' PCR primers as well as primers designed internal to the amplified sequence. All sequencing was performed by the Monsanto Genomics Sequencing Center using dye-terminator chemistry.

IV. RESULTS AND DISCUSSION

A. PCR Analyses of the DNA Sequences Flanking the 5' and 3' Ends of the Insert in Corn Event NK603. PCR analyses were performed on genomic DNA extracted from Roundup Ready corn event NK603 and non-transgenic corn line B73 to verify the DNA sequence flanking the 5' and 3' ends of the insert in corn event NK603. The positions of the PCR primers, as well as the results of the PCR analyses are presented in Figures 1A and 1B, respectively. The control reactions containing no template (Lanes 4 and 8) as well as the reactions containing B73 non-transgenic corn DNA (Lanes 2 and 6) did not generate a PCR product with either primer set, as expected. PCR on the Roundup Ready corn event NK603 DNA (Lanes 3 and 7) generated the expected size products of 500 bp representing the 5' flanking sequence and 1183 bp representing the 3' flanking sequence. These results demonstrate that a predicted size PCR product is generated from both ends of the insert in Roundup Ready corn event NK603.

B. DNA Sequence Verification of the Genomic Sequences Flanking the 5' and 3' Ends of the Insert in Corn Event NK603. The consensus sequence of numerous PCR products representing the genomic sequence flanking the 5' end of the insert is presented in Figure 2A. Sequence data indicate that the amplicon depicted in Figure 1B consists of 307 bp of corn genomic DNA flanking the insert followed by 193 bp at the 5' end of the insert containing 50 bp of polylinker sequence and 143 bp of the rice actin promoter. The consensus sequence of a number of PCR products representing the DNA flanking the 3' end of the insert is presented in Figure 2B. The amplicon in Figure 1B consists of 164 bp of the NOS 3' polyadenylation sequence which defines the 3' end of the insert, immediately followed by a 217-bp segment of DNA derived from the 5' end of the transformation cassette positioned in the inverse orientation. This 217-bp segment includes plasmid PV-ZMGT32 polylinker sequence (50 bp) and the first 167 bp of the enhancer region of the rice actin promoter, position -835 to -669 from the start of transcription as defined by McElroy *et al.* (1990). This 217-bp segment is followed by 305 bp of DNA with homology to chloroplast DNA. Immediately adjacent to this DNA is 497 bp of corn genomic DNA. These data delineate the 5' and 3' ends of the insert in NK603 and show the DNA which immediately flanks the insert on both ends.

V. CONCLUSIONS

Previous molecular analyses of Roundup Ready corn event NK603 (Deng *et al.*,1999) demonstrated that there is one complete copy of the DNA fragment used for transformation in event NK603, along with a 217-bp segment containing a portion of the enhancer region of the rice actin promoter inversely linked to the 3' end of the inserted transformation cassette. As part of this study, PCR and sequence analyses were performed which confirmed the 5' and 3' ends of the insert in corn event NK603, and

verified the genomic sequence flanking the 5' end (307 bp) and 3' end (497 bp) of the insert.

VI. REFERENCES

Deng, M.Y., Lirette, R.P., Cavato, T.A., and Sidhu, R.S. 1999. Molecular Characterization of Roundup Ready® (CP4 EPSPS) Corn Line NK603. MSL-16214, an unpublished study by Monsanto.

McElroy, D., Zhang, W., Cao, J., and Wu, R. 1990. Isolation of an efficient actin promoter for use in rice transformation. Plant Cell. 2, 163-171.

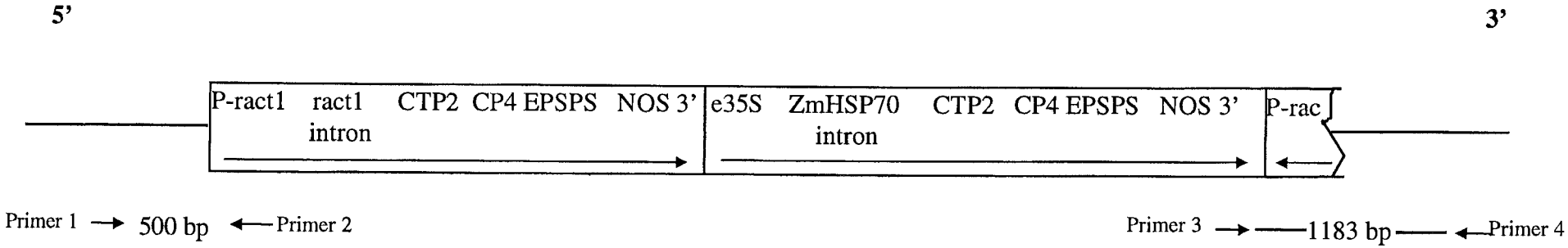

Figure 1A. Schematic Representation of the Insert in Roundup Ready Corn Event NK603. This figure depicts the predicted insert for event NK603 as presented in Deng *et al.* (1999). There is one complete copy of the DNA fragment used for transformation containing two CP4 EPSPS cassettes. Immediately 3' of the second CP4 EPSPS cassette there is a 217-bp segment of the transformation cassette containing a portion of the enhancer region of the rice actin promoter positioned in the inverse orientation. The primers used for the PCR analyses are illustrated, Primer 1 sits in the 5' flanking sequence of NK603 while Primer 2 sits in the rice actin promoter, and Primer 3 sits in the NOS 3' polyadenylation sequence while Primer 4 sits in the 3' flanking sequence of NK603.

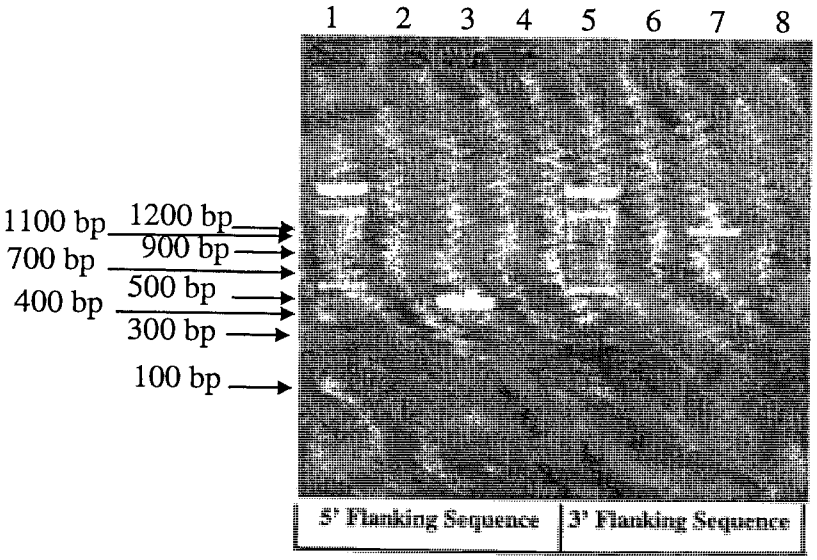

Figure 1B. PCR Analyses of the 5' and 3' Flanking Genomic DNA Sequences in Corn Event NK603. PCR analyses were performed using Primers 1 and 2 to confirm the 5' flanking sequence and Primers 3 and 4 to confirm the 3' flanking sequence on DNA extracted from leaf tissue of event NK603 (Lanes 3 and 7) and B73 non-transgenic DNA (Lanes 2 and 6). Lanes 1 and 5 contain Gibco BRL 100 bp DNA Ladder for use as a size indicator and Lanes 4 and 8 were no template control PCR reactions. Approximately 10 µl of each PCR reaction were loaded on a 2.0% agarose gel stained with ethidium bromide.

→ Symbol denotes sizes obtained from the Gibco BRL 100bp DNA Ladder.

[CBI CROSS REFERENCE 1]

[CBI CROSS REFERENCE 2]

Appendix 1

Study Protocol

Monsanto Study #: **00-01-46-30**

Study Title: Confirmation of the genomic DNA sequences flanking the 5' and 3' ends of the insert in Roundup Ready® Corn Line NK603.

Sponsor:
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St. Louis, MO 63198

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Additional Testing Facility:
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Genomics Sequencing Center
800 North Lindbergh
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Study Director:
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[redacted]

Approved By:

Aug 8, 2000
Date

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Linda K. Lahman
August 8, 2000
Date

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Reviewed By:

Joan M. Rojda-Heath
August 8, 2000
Date

Quality Assurance Specialist
Monsanto Company
Monsanto Regulatory

[redacted]

[redacted]

1.0 Regulatory Compliance

1.1 GLP Compliance

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

2.0 Purpose

The purpose of this study is to confirm the genomic DNA sequence flanking the 5' and 3' ends of the insert in Roundup Ready corn line NK603.

3.0 Timelines

3.1	Proposed Experimental Start Date:	August 14, 2000
3.2	Proposed Experimental Termination Date:	August 30, 2000

4.0 Test, Control and Reference Substances

4.1 Test Substance

The test substance is the Roundup Ready corn line NK603.

4.2 Control Substance

The control substance is the non-transgenic corn line B73.

4.3 Reference Substance

The reference substance will be the molecular size markers from Gibco BRL (100 bp ladder).

4.4 Characterization of Test, Control and Reference Substances

The identity of the test and control substances was confirmed and is archived in the Monsanto Regulatory archives under Study # 99-01-46-26.

5.0 Description of Experimental Design

Genomic DNA from the test substance will be subjected to PCR for the amplification of the DNA sequences containing the 5' and 3' ends of the insert, the insert-to-plant junctions, as well as portions of the plant DNA flanking the

[redacted]

insert. The amplicons produced will be purified and sequenced by the Monsanto Genomics Sequencing Center (a non-GLP facility).

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

The DNA from both the test and control substances was previously isolated from leaf tissue under Study # 99-01-46-26. The raw data, or copy of the raw data, detailing the extraction will be archived with this study. All previously extracted DNAs have been stored at 2-8°C. If necessary, additional DNA will be extracted under this protocol using methods approved by the study director.

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.

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. The 5' end will be amplified using one primer designed to the 5' genomic flanking sequence paired with a second primer located in the insert. The 3' sequence will be amplified using a primer designed to the 3' genomic flanking sequence with a second primer located in the insert.

5.1.4 Agarose Gel Electrophoresis

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.

5.1.5 Sequencing of Purified 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 control substance DNA will serve as a negative control for PCR.

[redacted]

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]

Protocol Amendment Form **Amendment #: 1**

Monsanto Study #: **00-01-46-30**

Date changes implemented: December 17, 2001

Protocol amended as follows Reopened on December 17, 2001

Reason for the amendment and what impact will result from this change:
The sequence reported for the 5' end of the insert is missing two bases which upon manual review of the sequence data are actually present in the raw data. The raw data will be amended to reflect the presence of these two bases. In addition, the beginning of the rice actin promoter in the figures reporting the 5' and 3' flanking sequences is inconsistent and will be reconciled. The final report will then be amended to reflect these changes. This will have a positive impact on the study.

Approved By:

Patrick T. Weston — Dec 17, 2001
Testing Facility Management Representative Date

Tracey A. Cavato — December 17 2001
Study Director Date

Linda K. Lahman — 17 December 2001
Sponsor Representative Date

Reviewed By:

Quality Assurance Specialist — Dec. 17, 2001
Date

[redacted]