Molecular analysis to determine the genetic stability of Roundup Ready ® corn event NK603 across additional generations

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Molecular Analysis to Determine the Genetic Stability of Roundup Ready® Corn Event NK603 Across Additional Generations

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This study meets the requirements under GLP as specified in 40 CFR Part 160. Formal documentation of approval of the protocol by the Sponsor occurred four days after study initiation and three days after experimental start.

Submitter — Date

12/21/00

Sponsor Representative — Date

12/21/00

Study Director — Date

QUALITY ASSURANCE UNIT STATEMENT

Study Title: Molecular Analysis to Determine the Genetic Stability of Roundup Ready® Corn Event NK603 Across Additional Generations

Study Number: 00-01-46-23

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	Date Reported To: Management
May 8, 2000	Agarose Gel Electrophoresis	May 16, 2000	May 16, 2000
November 30, 2000	Restriction Enzyme Digestion	December 8, 2000	December 8, 2000
December 13, 2000	Raw Data Audit	December 20, 2000	December 20, 2000
December 19, 2000	Draft Report Audit	December 20, 2000	December 20, 2000

Michelle Higgins
Michelle Higgins
Quality Assurance
Monsanto Regulatory, Monsanto Company

12-20-00
Date

Signatures of Approval

Study Number: 00-01-46-23

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Study Initiation Date: May 5, 2000

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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.

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Abbreviations

~	approximately
Ci, μCi	curie, microcurie
CP4 EPSPS	EPSPS from *Agrobacterium* sp. strain CP4
CTAB	cetyltrimethylammonium bromide
CTP2	chloroplast transit peptide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EPSPS	enzyme 5-enolpyruvylshikimate-3-phosphate synthase
e35S	cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region
HCl	hydrochloric acid
MW	molecular weight
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
Na_2HPO_4	sodium phosphate dibasic
NOS 3'	nopaline synthase 3' polyadenylation sequence
PCR	polymerase chain reaction
P-ract1/ract1 intron	rice actin promoter and intron
PVP	polyvinylpyrrolidone
SDS	sodium dodecyl sulfate
SSC	Saline-sodium citrate buffer. 20X SSC is 3 M sodium chloride, 0.3 M sodium citrate
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TE buffer	Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA)
UV	ultraviolet
V	volts
*Zm*HSP70	maize (*Zea mays*) hsp70 gene (heat-shock protein)

[Standard abbreviations, e.g., units of measure, according to format described in 'Instructions to Authors' in the Journal of Biological Chemistry]

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. The purpose of this study was to perform Southern blot fingerprint analysis by digesting genomic DNA from seven generations containing Roundup Ready corn event NK603 with the restriction enzyme *Eco*R V and probing with the CTP2-CP4-EPSPS coding region to assess the genetic stability of the integrated DNA across all five branches of the breeding tree. The data show that all of the generations analyzed yielded the expected size bands and no differences in banding pattern were observed among DNA extracted from any of the seven generations. These results demonstrate the stability of the inserted DNA in seven generations containing Roundup Ready corn event NK603 which represent all five branches of the breeding tree.

II. INTRODUCTION

A. Background. Corn event NK603 was produced by transformation of corn tissue with a 6.7-kb linear DNA fragment PV-ZMGT32L (Figure 1) derived from the plasmid vector PV-ZMGT32 (Figure 2), using particle acceleration technology. The DNA fragment used for corn transformation contained two gene expression cassettes: the first EPSPS gene cassette, containing the CP4 EPSPS coding sequence under the regulation of the rice actin promoter, a rice actin intron, a chloroplast transit peptide (CTP2) sequence, and a nopaline synthase (NOS) 3' polyadenylation sequence; and the second EPSPS gene 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. A description of the elements in the linear DNA fragment PV-ZMGT32L is given in Table 1. Previous molecular characterization of the insert in Roundup Ready corn 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 corn event NK603 (Figure 3). This portion of the enhancer region of the rice actin promoter is highly unlikely to act as a promoter (Deng *et al.,* 1999).

B. Purpose. The purpose of this study was to use Southern blot analysis to assess the genetic stability of the integrated DNA in corn event NK603 across seven generations: LH82xNK603+/B73BC5F1, LH59/LH51xNK603+/B73BC4F4, LH82xB73BC1,

B73BC1F1, LH82xB73BC2F3, A1BC2F1, 23CDC1/5727xA1BC3F3 and LH59/LH51xB73BC4F4 (Figure 4).

III. MATERIALS AND METHODS

A. Test Substances. The test substance for this study was the corn event NK603. The seven generations containing the NK603 event that were used in the study are as follows: LH82xNK603+/B73BC5F1 (Lot # TRO-0005-10287-I), LH59/LH51xNK603+/B73BC4F4 (Lot # TRO-0005-10290-I), LH82xB73BC1 (Lot # TRO-0008-10540-S), B73BC1F1 (Lot # TRO-0008-10536-S), LH82xB73BC2F3 (Lot # TRO-0008-10541-S), A1BC2F1 (Lot # TRO-0008-10538-S), LH59/LH51xB73BC4F4 (Lot # TRO-0008-10542-S) and 23CDC1/5727xA1BC3F3 (Lot # TRO-0007-10485-S).

B. Control Substances. The control substance for this study were the non-transgenic corn lines B73xLH82 (Lot # TCP-0005-10289-I), LH59/LH51xB73 (Lot # TCP-0005-10292-I), A1 (Lot # TPC-0011-10751-S) and B73 (Lot # TPC-0011-10750-S).

C. Reference Substances. The reference substances included the plasmid PV-ZMGT32 from which the DNA fragment used in the transformation of corn event NK603 was purified. DNA from the A1 non-transgenic control corn line was mixed with the plasmid, digested, and separated by electrophoresis on agarose gels. The plasmid DNA served as a positive hybridization control and it was spiked into the control line DNA at a concentration of approximately 1 copy of the plasmid DNA per copy of the genomic DNA to demonstrate the sensitivity of the Southern blotting method. The generation B73BC1F1 was used as a reference substance since it represents the generation of corn event NK603 which was originally characterized in Deng *et al.,* 1999. Additional reference substances were MW size markers from Boehringer Mannheim (Indianapolis, IN) (MW Markers II (23.1 -0.1 kb) and IX (1.4 -0.072 kb), catalog #236 250 and #1449 460, respectively).

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

E. Identity of Test and Control Substances. The presence or absence of corn event NK603 was confirmed by NK603 event specific PCR on the control substances. The identity of the test substances were confirmed through the analysis performed in the study. The sources of the test and control substances were verified after the start of this study and documented as such.

F. DNA extraction. The control substances, B73 and A1, were extracted from leaf tissue using the Qiagen DNeasy Plant Maxi Kit (Valencia, CA, Cat. # 68163) as per the manufacturer's instructions. The generations representing the test substances were extracted from grain tissue using one of the two following methods.

Method 1. Corn grain tissue (~6 g) was ground to a fine powder using a blender and transferred to a 50-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)] were added to each tube and the tubes were incubated at 65°C for 30 min and then allowed to cool at room temperature for approximately 5 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 5 min at approximately 13,800 x *g* at room temperature. The upper aqueous phase was transferred to a clean 50-ml centrifuge tube, 1/10 the volume (~1.5 ml) of CTAB buffer was added, and the tubes were mixed by inversion several times. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the tubes were mixed by inversion for approximately 5 min and centrifugation was performed at approximately 13,800 x *g* at room temperature for approximately 5 min. The upper aqueous phase was transferred to a clean 50-ml centrifuge tube and an equal volume (~15 ml) of CTAB precipitation buffer [1% w/w CTAB, 50mM Tris pH 8.0, and 10mM EDTA pH 8.0] was added. The tubes were incubated at room temperature for 60 min and then centrifuged at approximately 8,800 x *g* at room temperature for 10 minutes. The upper aqueous phase was transferred to a new tube and the pellet was re-dissolved in 2 ml of high salt TE [10mM Tris-HCl pH 8.0, 1 mM EDTA and 1M NaCl]. The tubes were incubated with gentle swirling at 37°C for 2 h or at 4°C overnight. The samples were centrifuged at approximately 20,000 x *g* for approximately 2 min at room temperature. The supernatant was collected using a pipet and 150 µl of 3M sodium acetate and 2 volumes (~4 ml) of 100% ethanol were added. The tubes were mixed by inversion to precipitate the DNA which was then hooked out using a glass Pasteur pipet and placed in a microcentrifuge tube containing 70% ethanol. The tubes were centrifuged at approximately 16,000 x *g* for approximately 5 min. The supernatant was discarded and the pellets were vacuum dried for approximately 4 min. The pellet was resuspended in TE buffer, pH 8.0 and stored in a 4°C refrigerator.

Method 2. Corn grain tissue (4-5 g) was ground to a fine powder using a blender and transferred to a 50-ml centrifuge tube. Twenty 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)] were added to each tube and the tubes were incubated at 55°C for 60 min and then allowed to cool at room temperature for approximately 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 13,800 x *g* at room temperature. The upper aqueous phase

was transferred to a clean 50-ml centrifuge tube and the chloroform extraction was repeated two additional times as above. The upper aqueous phase was transferred to a clean 50-ml centrifuge tube and 2/3 volume (~13 ml) of isopropanol was added. The tubes were inverted several times to mix. The DNA was precipitated at approximately -20°C overnight. The tubes were then centrifuged at approximately 13,800 x *g* at 4°C for 20 min. The pellet was re-dissolved in 4 ml of TE buffer, pH 8.0 and transferred to a 13 ml tube. Approximately 40 µl of 10 mg/ml RNase were added and the tubes incubated at 37°C for 30 min. An equal volume (~4 ml) of chloroform:isoamyl alcohol (24:1) was added to each sample. The suspension was mixed several times and centrifuged for 10 min at approximately 13,800 x *g* at room temperature. The upper aqueous phase was transferred to a clean 50-ml centrifuge tube and the chloroform extraction was repeated once more as above. Approximately one half volume (2 ml) of 7.5 M ammonium acetate was added and the tubes were gently mixed. Two to two and one half volumes of 100% ethanol (~8ml) were added and the tubes were mixed and placed in a -20°C freezer for 2 h. The DNA was pelleted by centrifugation at approximately 13,800 x *g* at 4°C for 20 min. The DNA was washed with 70% ethanol, vacuum dried, re-dissolved in 1 ml TE, pH 8.0, and stored in a 4°C refrigerator.

G. 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) with Boehringer Mannheim molecular size marker IX used as a calibration standard for quantitating genomic DNA. Approximately 10 µg of genomic DNA from the test and control lines were used for the restriction enzyme digests. 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/Roche. After digestion, the samples were precipitated by adding 1/10 volume (~50 µl) of 3 M NaOAc and 2 volumes (~1 ml relative to the original digest volume) of 100% ethanol, followed by incubation in a -20°C freezer for at least 1 h. The digested DNA was pelleted by centrifugation, washed with 70% ethanol, vacuum dried for approximately 4-10 min, and re-dissolved at room temperature in TE, pH 8.0.

H. Preparation of DNA probes. Plasmid DNA was isolated from *E. coli* cultures. The probe template for the CTP2-CP4 EPSPS coding region was prepared by PCR amplifying a fragment of DNA which contained the CTP2-CP4 EPSPS coding region. The CTP2-CP4 EPSPS fragment was obtained by digestion of plasmid PV-ZMGT32 with the restriction enzymes *Eco*R I and *Nco* I followed by gel purification. Approximately 25 ng of the probe template were labeled with ^{32}P using the random priming method (RadPrime DNA Labeling System, Gibco BRL).

I. Southern blot analysis. Southern blot analyses were performed to assess the stability of the DNA that was integrated into the corn genome in event NK603 across multiple generations. These analyses were performed according to SOP# GEN-PRO-025-02. DNA samples digested with the restriction enzyme *Eco*R V were separated, based on size, using 0.6% agarose gel electrophoresis according to SOP# GEN-PRO-003-01. The samples were loaded onto the gel and electrophoresed for 15-16 h at 35 V. After photographing, the gel was placed in a depurination solution (0.125 N HCl) for approximately 10 min followed by a denaturing solution (0.5 M NaOH, 1.5 M NaCl) for ~30 min and then a neutralizing solution (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) for ~30 min. After neutralizing the gel was placed in 20X SSC for 30 minutes while the transfer apparatus was assembled. 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 19-22 h (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 pre-hybridized for at least 4 h in an aqueous solution containing 500 mM $Na_2HPO_4 \bullet 7H_2O$, 7% SDS, and 0.1 mg/ml *Escherichia coli* (*E. coli*) tRNA. Hybridization with the radiolabeled probe was performed in fresh prehybridization solution for 15-20 h 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 the blot were generated using Kodak Biomax MS film (Eastman Kodak, Rochester, NY) in conjunction with a Kodak Biomax MS intensifying screen.

IV. RESULTS AND DISCUSSION

A. Southern blot strategy. Genomic DNA from seven generations containing Roundup Ready corn event NK603 was digested with the restriction enzyme *Eco*R V and subjected to Southern blot hybridization analysis to assess the stability of the DNA that was integrated into the genome. The restriction enzyme *Eco*R V generates a unique Southern blot banding pattern fingerprint for Roundup Ready corn event NK603 when probed with the CTP2-CP4 EPSPS coding region consisting of 2 bands at ~3.8kb and ~2.8kb (Deng *et al.,* 1999). Control non-transgenic corn genomic DNA was digested with the same restriction enzyme as the test samples. The stability analysis was conducted in two separate Southern blot experiments since the generations tested were available at different times during the study. A map of the DNA fragment used for transformation of corn event in NK603, along with the locations of the *Eco*R V restriction sites utilized for Southern blot analysis is shown in Figure 1.

B. Stability of the inserted DNA in NK603.

1. Stability of the inserted DNA in the LH82xNK603+/B73BC5F1 and LH59/LH51xNK603+/B73BC4F4 generations. Control DNA, control DNA mixed with PV-ZMGT32 and test DNA from the LH82xNK603+/B73BC5F1 and LH59/LH51xNK603+/B73BC4F4 generations were digested with *Eco*R V. The blot was probed with the full-length CTP2-CP4 EPSPS coding region (Figure 5). The control DNA (lanes 3 and 5) showed no hybridization signals as expected. The NK603 reference substance, B73BC1F1 (lane 6) produced the expected bands of ~3.8 and ~ 2.8 kb. Plasmid PV-ZMGT32 DNA mixed with the control DNA (lanes 2 and 4), LH82xNK603+/B73BC5F1 generation (lane 7) and the LH59/LH51xNK603+/B73BC4F4 generation (lane 8) all produced the expected bands of ~3.8 kb and ~2.8 kb. No differences in banding pattern were observed between DNA extracted from the LH82xNK603+/B73BC5F1 generation or the LH59/LH51xNK603+/B73BC4F4 generation. This demonstrates the stability of the inserted DNA in samples spanning across two branches of the breeding tree (Figure 4).

2. Stability of the inserted DNA in the A1BC2F1, 23CDC1/5727xA1BC3F3, LH82xB73BC1, B73BC1F1, LH82xB73BC2F3 and LH59/LH51xB73BC4F4 generations. Control DNA, control DNA mixed with PV-ZMGT32 and test DNA from the A1BC2F1, 23CDC1/5727xA1BC3F3, LH82xB73BC1, B73BC1F1, LH82xB73BC2F3 and LH59/LH51xB73BC4F4 generations were digested with *Eco*R V. The blot was probed with the full-length CTP2-CP4 EPSPS coding region (Figure 6). The B73 non-transgenic control DNA (lane 5) showed no hybridization signals. Plasmid PV-ZMGT32 DNA mixed with the A1 non-transgenic control DNA (lane 2), A1BC2F1 (lane 3), 23CDC1/5727xA1BC3F3 (lane 4), LH82xB73BC1(lane 6), B73BC1F1 (lane 7), LH82xB73BC2F3 (lane 8), and LH59/LH51xB73BC4F4 (lane 9) all produced the expected bands of ~3.8 kb and ~2.8 kb. No differences in banding pattern were observed between DNA extracted from any of the six generations. This demonstrates the stability of the inserted DNA in samples spanning across all five branches of the breeding tree (Figure 4).

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. The genetic stability of the inserted DNA was analyzed by Southern blot analysis on genomic DNA extracted from seven generations including the B73BC1F1 generation used in the original molecular characterization of Roundup Ready

corn event NK603, demonstrating the stability of the inserted DNA in all seven generations which represent all five branches of the breeding tree.

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 conducted by Monsanto Company.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-17.

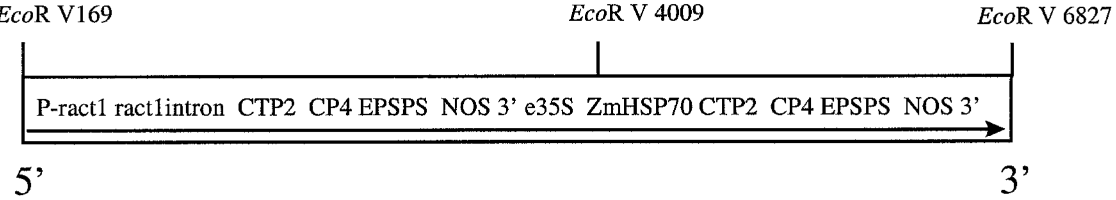

Figure 1. Linear map of PV-ZMGT32L. The DNA fragment PV-ZMGT32L was used to generate Roundup Ready corn event NK603 by particle acceleration technology. The *Eco*R V restriction sites used in Southern blot analysis are shown here.

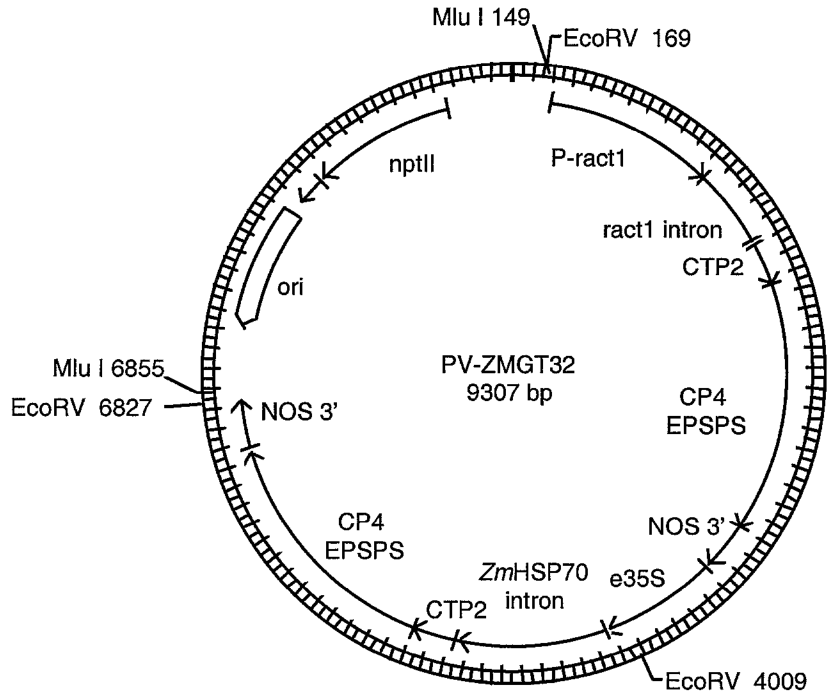

Figure 2. Plasmid map of PV-ZMGT32. The *Mlu* I fragment (bp 149-6855) of plasmid PV-ZMGT32 was used to generate corn event NK603.

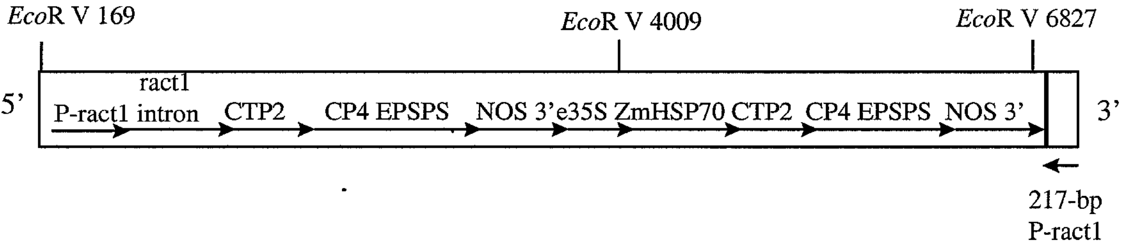

Figure 3. Schematic representation of the corn event NK603 insert. This figure depicts the predicted insert in corn event NK603. There is one complete copy of the PV-ZMGT32L fragment that was used in transformation to generate the NK603 event. At the 3' end of the insert there is a 217-bp fragment located in the inverse orientation containing 167-bp of the enhancer region of the rice actin promoter and 50-bp of plasmid PV-ZMGT32 polylinker sequence.

Table 1. Summary of genetic elements in linear DNA fragment PV-ZMGT32L used for transformation of corn line NK603

Genetic Element	Size (kb)	Function
The first EPSPS gene cassette:		
P-ract1/ ract1 intron	1.4	5' region of rice (*Oryzae sativa*) actin 1 gene containing the promoter, transcription start site and first intron.
CTP2	0.2	DNA sequence for chloroplast transit peptide, isolated from *Arabidopsis thaliana* EPSPS; transit peptide directs the CP4-EPSPS protein to the chloroplast, the site of aromatic amino acid synthesis.
CP4 EPSPS	1.4	The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) sequence isolated from *Agrobacterium* sp. strain CP4 which imparts tolerance to glyphosate.
NOS 3'	0.3	A 3' nontranslated region of the nopaline synthase gene from *Agrobacterium tumefaciens* T-DNA which ends transcription and directs polyadenylation of the mRNA.
The second EPSPS gene cassette:		
e35S	0.6	The cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region.
*Zm*HSP70	0.8	Intron from the maize (*Zea mays*) *hsp*70 gene (heat-shock protein) present to stabilize the level of gene transcription.
CTP2	0.2	DNA sequence for chloroplast transit peptide, isolated from *Arabidopsis thaliana* EPSPS, present to direct the CP4 EPSPS protein to the chloroplast, the site of aromatic amino acid synthesis.
CP4 EPSPS	1.4	The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) sequence isolated from *Agrobacterium* sp. strain CP4 which imparts tolerance to glyphosate.
NOS 3'	0.3	A 3' nontranslated region of the nopaline synthase gene from *Agrobacterium tumefaciens* T-DNA which ends transcription and directs polyadenylation of the mRNA.

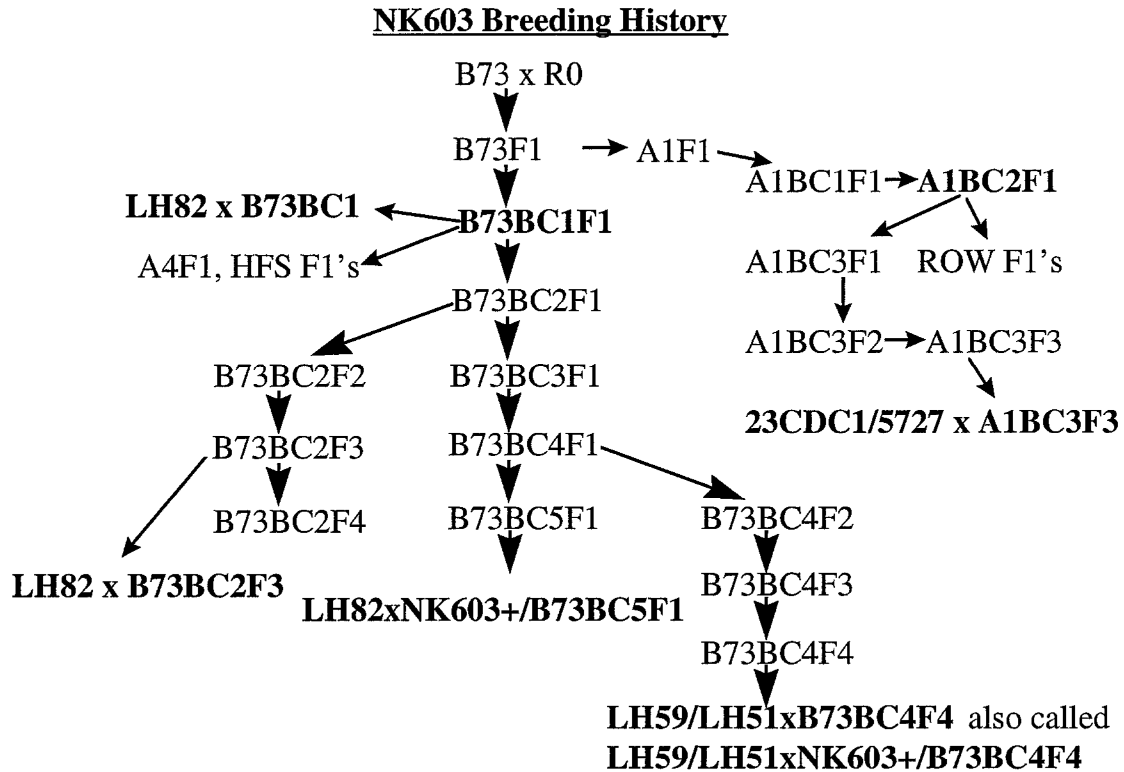

Figure 4. Corn event NK603 breeding history. The generations in bold print are those included in Southern blot analyses to assess the molecular genetic stability of the NK603 insert.

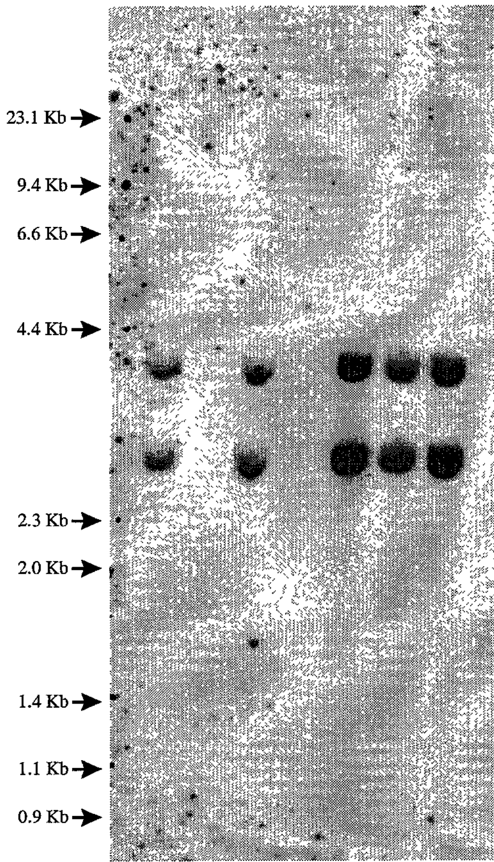

Figure 5. Corn NK603 event specific Southern blot fingerprint. Ten micrograms of genomic DNA extracted from corn grain were digested with *Eco*RV. The DNA samples were then blotted and probed with the full length ^{32}P-labeled CTP2-CP4 EPSPS coding sequence. Lane designations are as follows:

Lane 1: MW Marker II and MW Marker IX (Boehringer Mannheim)
2: B73xLH82 non-transgenic control spiked with 29 pg of plasmid PV-ZMGT32
3: B73xLH82 non-transgenic control
4: LH59/LH51xB73 non-transgenic control spiked with 29 pg of plasmid PV-ZMGT32
5: LH59/LH51xB73 non-transgenic control
6: B73BC1F1
7: LH82xNK603+/B73BC5F1
8: LH59/LH51xNK603+/B73BC4F4

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

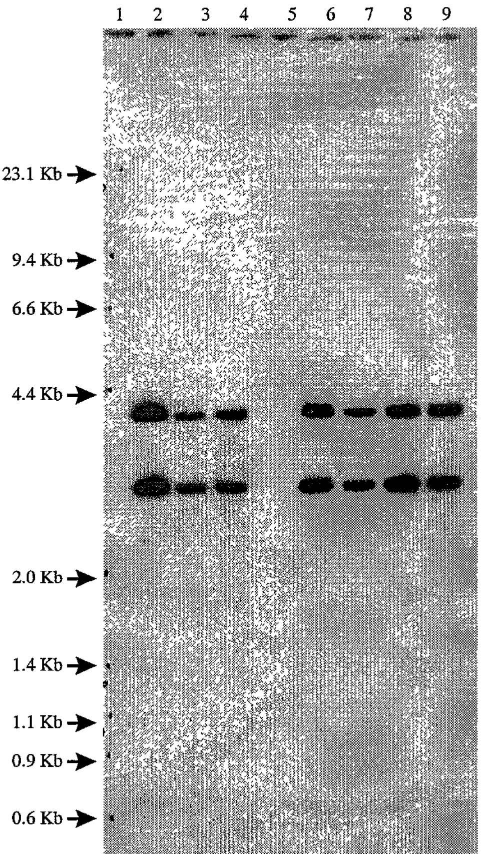

Figure 6. Corn NK603 event specific Southern blot fingerprint of additional generations. Ten micrograms of NK603 genomic DNA extracted from corn grain of seven different generations were digested with *Eco*R V. Ten micrograms of genomic DNA was extracted from leaf tissue of the non-transgenic control substances A1 and B73 were also digested with *Eco*R V. The DNA samples were blotted and probed with the full length ^{32}P-labeled CTP2-CP4 EPSPS coding sequence. Lane designations are as follows:

Lane 1: MW Marker II and MW Marker IX (Boehringer Mannheim)
2: A1 non-transgenic control spiked with 29 pg of plasmid PV-ZMGT32
3: A1BC2F1
4: 23CDC1/5727xA1BC3F3
5: B73 non-transgenic control
6: LH82xB73BC1
7: B73BC1F1
8: LH82xB73BC2F3
9: LH59/LH51xB73BC4F4

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

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

Monsanto Study #: **00-01-46-23**

Study Title: Additional molecular analysis to determine the genetic stability of corn event NK603

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

Primary Testing Facility: Monsanto Company
Biotechnology Regulatory Sciences
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Monsanto Company Confidential

Approved By:

Date May 5, 2000

Patrick T. Weston
Testing Facility Management Representative
Monsanto Company
Biotechnology Regulatory Sciences

Date May 9, 2000

Linda K. Lahman
Sponsor Representative
Monsanto Company
Biotechnology Regulatory Affairs
Phone: 636-737-7653

Date 5/5/00

Ming Y. Deng
Study Director
Monsanto Company
Biotechnology Regulatory Sciences

Reviewed By:

Date May 5, 2000

Quality Assurance Specialist
Monsanto Company
Monsanto Regulatory

Date May 5, 2000

Ronald P. Lirette
Technical Center Leader
Monsanto Company
Biotechnology Regulatory Sciences

Confidentiality

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

1.0 Regulatory Compliance

1.1 GLP Compliance

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

2.0 Purpose

The purpose of this study is to analyze the genetic stability of the inserted DNA in corn event NK603 by Southern blot. Event NK603 was created by modifying a corn line to express the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein from *Agrobacterium* sp. strain CP4(CP4 EPSPS) which confers tolerance to glyphosate, the active ingredient in Roundup® herbicide. The control lines have background genetics representative of the test event, but have not been genetically modified and therefore, do not contain the CP4 EPSPS coding region. The control lines provide a background matrix used in the analysis of banding patterns on Southern blots. An event specific Southern blot fingerprint was developed for NK603 and will be used in this study.

3.0 Timelines

3.1	Proposed Experimental Start Date:	May 5, 2000
3.2	Proposed Experimental Termination Date:	October 31, 2000

4.0 Test, Control and Reference Substances

4.1 Test Substance(s)

The test substance is corn event NK603. Lot numbers, TRO-0005-10287-I and TRO-0005-10290-I representing generations LH82xNK603+/B73BC5F1 and LH59/LH51xNK603+/B73BC4F4 of NK603 respectively, will be tested. Additional generations may be tested and will be added by amending the protocol.

4.2 Control Substance(s)

The control substance is the non-transgenic parental control B73 x LH82 (Lot number TCP-0005-10289-I) for generation LH82xNK603+/B73BC5F1 and the non-transgenic parental control LH59/LH51 x B73 (Lot number TCP-0005-10292-I) for generation LH59/LH51xNK603+/B73BC4F4.

4.3 Reference Substance(s)

The reference substances include the plasmid (PV-ZMGT32) from which the DNA fragment used in the transformation of the corn line was obtained. The plasmid DNA will be used as a size marker and positive hybridization control by digesting with *Eco*RV. NK603 DNA extracted as part of Study 99-01-46-26 will also be used as a reference substance. Additionally, molecular size markers from Boehringer Mannheim (molecular size markers II and IX, catalog #236 250 and #1449 460, respectively) will also be used for band size estimations.

4.4 Characterization of Test, Control and Reference Substances

The Study Director determined the identity of the test, control and reference substances by verifying the chain-of-custody documentation supplied with the samples. The Southern blot analysis performed in this study will confirm the identity of the test and control substances.

5.0 Description of Experimental Design

Genomic DNA from the test substance will be analyzed by Southern blot for the stability of the inserted DNA. The genomic DNA will be digested with the restriction enzyme *Eco*RV and the blot probed with the CTP2-CP4 EPSPS coding region. Chain of custody for the reference substance originating in previous studies will be documented.

5.1 Analytical Methods

5.1.1 DNA Extraction

DNA was extracted from seed prior to the start of the study. A CTAB-based method was used and 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 from seed using methods approved by the study director. Following extraction, the isolated DNA will be stored at 2-8 °C.

5.1.2 Restriction Enzyme Digestion

Ten micrograms of extracted genomic DNA and approximately 1 copy number equivalent of plasmid DNA (spiked into 10 ug of

non-transgenic genomic DNA) will be digested with *Eco*RV following SOP # GEN-PRO-010-01.

5.1.3 DNA Quantitation

Any DNA extracted in this study will be quantitated using Hoefer's DyNA Quant 200 Fluorometer according to SOP # BR-EQ-0065-01.

5.1.4 Agarose Gel Electrophoresis:

The digested DNA will be electrophoresed on agarose gels according to SOP # GEN-PRO-003-01.

5.1.5 Southern Blot Analysis:

The agarose gels containing digested DNA will be blotted to nylon membranes and probed with the full-length radiolabeled CTP2-CP4 EPSPS coding region according to SOP # GEN-PRO-025-02.

6.0 Control of Bias

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

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 Biotech Regulatory Science 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.

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Protocol Amendment Form **Amendment #: 1**

Monsanto Study #: 00-01-46-23

Date changes implemented: November 29, 2000

Page number(s) and section(s): Page 4, Section 5.1.1 "DNA Extraction"

Protocol originally stated: DNA was extracted from seed prior to the start of the study. A CTAB-based method was used and 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 from seed using methods approved by the study director. Following extraction, the isolated DNA will be stored at 2-8°C.

Protocol amended as follows: DNA was extracted from seed prior to the start of the study. Additional generations were extracted from seed prior to Amendment 1 (see Test Substances section of Amendment 1). A CTAB-based method was used and the raw data detailing the extraction will be archived with this study. If necessary, additional DNA will be extracted under this protocol from seed using methods approved by the study director. Additional control substances B73, lot number TPC-0011-10750-S, and A1, lot number TPC-0011-10751-S will be extracted from leaf tissue using the Qiagen DNeasy Plant Maxi Kit. All extracted DNA was/will be stored at 2-8°C.

Reason for the amendment and what impact will result from this change:
Additional generations needed to be tested by Southern blot analysis to analyze the genetic stability of the inserted DNA in corn event NK603. There was little seed available for the two control substances, therefore the DNA will be extracted from leaf tissue. Since additional generations will be analyzed resulting in the generation of additional data supporting the stability of the insert, there will be a positive impact on the study.

Protocol Amendment Form **Amendment #: 1**

Page number(s) and section(s): Page 3, Section 4.1. "Test Substance(s)"

Protocol originally stated: The test substance is corn event NK603. Lot numbers, TRO-0005-10287-I and TRO-0005-10290-I representing generations LH82xNK603+/B73BC5F1 and LH59/LH51xNK603+/B73BC4F4 of NK603 respectively, will be tested. Additional generations may be tested and will be added by amending the protocol.

Protocol amended as follows: The test substance is corn event NK603. Additional generations may be tested and will be added by amending the protocol. The generations of NK603 with their corresponding lot numbers which will be analyzed in this study are in the table below.

NK603 Generation	Lot Number
LH82xNK603+/B73BC5F1	TRO-0005-10287-I
LH59/LH51xNK603+/B73BC4F4	TRO-0005-10290-I
LH82xB73BC1	TRO-0008-10540-S
B73BC1F1	TRO-0008-10536-S
LH82xB73BC2F3	TRO-0008-10541-S
A1BC2F1	TRO-0008-10538-S
23CDC1/5727xA1BC3F3	TRO-0007-10485-S
LH59/LH51xB73BC4F4	TRO-0008-10542-S

Reason for the amendment and what impact will result from this change:
Additional generations of NK603 need to be tested by Southern blot to analyze the genetic stability of the inserted DNA. Since additional generations will be analyzed resulting in the generation of additional data supporting the stability of the insert, there will be a positive impact on the study.

Protocol Amendment Form **Amendment #: 1**

Page number(s) and section(s): Page 4, Section 4.2 "Control Substance(s)"

Protocol originally stated: The control substance is the non-transgenic parental control B73 x LH82 (Lot number TCP-0005-10289-I) for generation LH82xNK603+/B73BC5F1 and the non-transgenic parental control LH59/LH51 x B73 (Lot number TCP-0005-10292-I) for generation LH59/LH51xNK603+/B73BC4F4.

Protocol amended as follows: The non-transgenic corn lines which will serve as control substances for the additional test generations are shown in the table below along with their corresponding generation representing test substances.

Control Substance	Lot Number	Corresponding Test Substance(s)
B73 x LH82	TCP-0005-10289-I	LH82xNK603+/B73BC5F1
LH59/LH51 x B73	TCP-0005-10292-I	LH59/51xNK603+/B73BC4F4
B73	TPC-0011-10750-S	LH82xB73BC1 B73BC1F1 LH82xB73BC2F3 LH59/LH51xB73BC4F4
A1	TPC-0011-10750-S	A1BC2F1 23CDC1/5727xA1BC3F3

Reason for the amendment and what impact will result from this change:
Additional generations of NK603 will be tested by Southern blot analysis to analyze the genetic stability of the inserted DNA and the appropriate non-transgenic controls are needed for these generations. There is no impact on the study.

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Protocol Amendment Form

Amendment #: 1

Approved By:

Patrick T. Weston
Testing Facility Management Representative

Nov 29, 2000
Date

Ming Y. Deng
Study Director

Nov. 29, 2000
Date

Linda K. Lahman
Sponsor Representative

November 29, 2000
Date

Reviewed By:

Quality Assurance Specialist

Nov. 29, 2000
Date

Protocol Amendment Form

Amendment #: 2

Monsanto Study #: 00-01-46-23

Date changes implemented: November 29, 2000

Page number(s) and section(s) of Amendment 1:
Page 3, Section 4.2 “Control Substance(s)”

Amendment 1 originally stated:

Control Substance	Lot Number	Corresponding Test Substance(s)
B73 x LH82	TCP-0005-10289-I	LH82xNK603+/B73BC5F1
LH59/LH51 x B73	TCP-0005-10292-I	LH59/51xNK603+/B73BC4F4
B73	TPC-0011-10750-S	LH82xB73BC1 B73BC1F1 LH82xB73BC2F3 LH59/LH51xB73BC4F4
A1	TPC-0011-10750-S	A1BC2F1 23CDC1/5727xA1BC3F3

Protocol amended as follows:

Control Substance	Lot Number	Corresponding Test Substance(s)
B73 x LH82	TCP-0005-10289-I	LH82xNK603+/B73BC5F1
LH59/LH51 x B73	TCP-0005-10292-I	LH59/LH51xNK603+/B73BC4F4
B73	TPC-0011-10750-S	LH82xB73BC1 B73BC1F1 LH82xB73BC2F3 LH59/LH51xB73BC4F4
A1	TPC-0011-10751-S	A1BC2F1 23CDC1/5727xA1BC3F3

Reason for the amendment and what impact will result from this change:
Two typographical errors were made in the original table. There is no impact on the study.

Protocol Amendment Form

Amendment #: 2

Approved By:

Patrick T. Weston
Testing Facility Management Representative

Date: Nov 29, 2000

Ming Y. Deng
Study Director

Date: Nov. 29 2000

Linda K. Lahman
Sponsor Representative

Date: November 29, 2000

Reviewed By:

Quality Assurance Specialist

Date: Nov. 29, 2000

Protocol Amendment Form

Amendment #: 3

Monsanto Study #: 00-01-46-23

Date changes implemented: December 19, 2000

Page number and sections: Page 2, "Approved By"

Protocol originally stated: Linda K. Lahman, Sponsor Representative, Monsanto Company, Biotechnology Regulatory Affairs, Phone: 636-737-7653

Protocol amended as follows: Kent A. Croon, Sponsor Representative, Monsanto Company, Biotechnology Regulatory Affairs, Phone: 636-737-7488

Reason for the amendment and what impact will result from this change:
Sponsor representative unavailable for signing of the final document.

Approved By:

Patrick T. Weston
Testing Facility Management Representative

Dec 20, 2000
Date

Ming Y. Deng
Study Director

Dec. 20, 2000
Date

Kent A. Croon
Sponsor Representative

12/20/2000
Date

Reviewed By:

Quality Assurance Specialist

12-20-00
Date (TE) MRH 12/20/00