

**Immuno-detectability of CP4EPSPS and CP4EPSPS L214P  
proteins in the grain of Roundup Ready ® Corn event  
NK603 after heat treatment.**

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**TITLE:** Immuno-detectability of CP4 EPSPS and CP4 EPSPS L214P Proteins in the Grain of Roundup Ready® Corn Event NK603 After Heat Treatment

**AUTHORS:** Gyula Holleschak, Jamie J. Thorp, Joan L. Lee, Cherian George and James D. Astwood

**ABSTRACT:** Monsanto Company has developed Roundup Ready corn event NK603 that is tolerant to glyphosate, the active ingredient in the Roundup family of agricultural herbicides. Roundup Ready corn event NK603 contains two *cp4 epsps* coding sequences. The first CP4 EPSPS is the same as the one found in Roundup Ready soybeans, canola, cotton, and sugar beet. The second CP4 EPSPS coding sequence has two nucleotide changes, one which is silent, and one which results in an amino acid substitution of proline for leucine at amino acid position 214 (L214P).

The purpose of this study was to assess the effect of heat treatment on the immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214P proteins in the grain of Roundup Ready corn event NK603. Standard curves generated from *E. coli*-produced CP4 EPSPS and CP4 EPSPS L214P proteins were used to estimate the levels of the CP4 EPSPS proteins in heated and unheated NK603 grain extracts. The immuno-detectability of both the CP4 EPSPS and CP4 EPSPS L214P proteins are similar when detected with antibodies raised against the CP4 EPSPS protein (Lee *et al.*, 2002).

The results of this study demonstrated that heating lowered the immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214P proteins in Roundup Ready corn event NK603 by ~90%. It was therefore concluded that the heating conditions associated with typical corn processing will eliminate the immuno-detectability of CP4 EPSPS and CP4 EPSPS L214P proteins present in the grain of Roundup Ready corn event NK603.



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Study Title

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Study Director

Date: *Sept 18, 2002*

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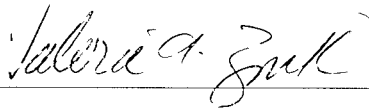
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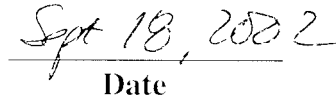
**Immuno-detectability of CP4 EPSPS and CP4 EPSPS L214P Proteins in the Grain of Roundup Ready® Corn Event NK603 After Heat Treatment**

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

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

<b>Dates Of Inspection / Audit</b>	<b>Phase</b>	<b>Date Reported To:</b>	
		<b>Study Director</b>	<b>Management</b>
04/15/2002	Protein Extraction and/or Quantitation	04/19/2002	04/19/2002
07/17/2002	Raw Data Audit	07/31/2002	07/31/2002
08/26/2002	Draft Report Review	08/29/2002	08/29/2002





**Date**

**Quality Assurance Specialist  
Monsanto Regulatory, Monsanto Company**

### Study Information

**Study Number:** 02-01-46-14

**Title:** Immuno-detectability of CP4 EPSPS and CP4 EPSPS L214P Proteins in the Grain of Roundup Ready® Corn Event NK603 After Heat Treatment

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**Completion Date:** September 18, 2002

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### Abbreviations

~	Approximately
ECL	Enhanced chemiluminescence
IPC	Insect protected corn
IgG	Immunoglobulin G
kDa	Kilodalton
MW	Molecular weight
NFDM	Non-fat dried milk
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate buffered saline containing Tween 20
PVDF	Polyvinylidene difluoride membrane
SDS	Sodium dodecylsulfate
SOP	Standard operating procedure

## 1.0 Summary

Monsanto Company has developed Roundup Ready<sup>1</sup> corn event NK603 that is tolerant to glyphosate, the active ingredient in the Roundup<sup>1</sup> family of agricultural herbicides. Roundup Ready corn event NK603 contains two *cp4 epsps* coding sequences. The first CP4 EPSPS is the same as the one found in Roundup Ready soybeans, canola, cotton, and sugar beet. The second CP4 EPSPS coding sequence has two nucleotide changes, one which is silent, and which results in an amino acid substitution of proline for leucine at amino acid position 214 (L214P).

The purpose of this study was to assess the effect of heat treatment on the immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214P proteins in the grain of Roundup Ready corn event NK603. Standard curves generated from *E. coli*-produced CP4 EPSPS and CP4 EPSPS L214P proteins were used to estimate the levels of the CP4 EPSPS proteins in heated and unheated NK603 grain extracts. The immuno-detectibility of both the CP4 EPSPS and CP4 EPSPS L214P proteins are similar when detected with antibodies raised against the CP4 EPSPS protein (Lee *et al.*, 2002).

The results of this study demonstrated that heating lowered the immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214P proteins in Roundup Ready corn event NK603 by ~90%. It was therefore concluded that the heating conditions associated with typical corn processing will eliminate the immuno-detectability of CP4 EPSPS and CP4 EPSPS L214P proteins present in the grain of Roundup Ready corn event NK603.

## 2.0 Introduction

Monsanto Company has developed Roundup Ready corn event NK603 that is tolerant to glyphosate, the active ingredient in the Roundup family of agricultural herbicides, by insertion of the *cp4 epsps* coding sequence from *Agrobacterium sp.* strain CP4 into corn. Studies have shown that glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in the shikimate pathway (Franz *et al.*, 1997). The CP4 EPSPS protein is functionally similar to plant EPSPS enzymes but has a much reduced affinity for glyphosate (Padgett *et al.*, 1993). In conventional plants, glyphosate binds to the plant EPSPS enzyme and blocks the biosynthesis of aromatic amino acids, thereby starving plants of these essential nutrients (Steinrücken and Amrhein, 1980; Haslam, 1993). In Roundup Ready plants, the nutritional requirements for growth and development are met by the continued action of the glyphosate-tolerant CP4 EPSPS enzyme in the presence of glyphosate (Padgett *et al.*, 1996). A comprehensive safety assessment of the CP4 EPSPS protein has been described in the literature (Harrison *et al.*, 1996).

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The *cp4 epsps* gene from *Agrobacterium ssp.* strain CP4 has been sequenced and it encodes a 47.6 kDa CP4 EPSPS protein consisting of a single polypeptide of 455 amino acids (Padgett *et al.*, 1993). Roundup Ready corn event NK603 contains two *cp4 epsps* coding sequences. The first CP4 EPSPS is the same as the one found in Roundup Ready soybeans, canola, cotton, and sugar beet. The second CP4 EPSPS coding sequence has two nucleotide changes, one of which is silent, and one which results in an amino acid substitution of proline for leucine at amino acid position 214 (L214P) (Astwood *et al.*, 2001).

### **3.0 Purpose**

The purpose of this study was to assess the effect of heat treatment on the immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214P proteins in the grain of Roundup Ready corn event NK603.

### **4.0 Test, Control and Reference Substance Characterization**

**4.1 Test Substance.** The test substance was Roundup Ready corn event NK603 (LIMS 00ZMGRO02886, lot SCP71A9C1T). Due to a transcriptional error, the lot number listed in the protocol was incorrect. This deviation from the protocol had no impact on the quality of the study. Grain from Roundup Ready corn event NK603, which contains both the CP4 EPSPS and CP4 EPSPS L214P proteins, was grown according to Production Plan 00-01-46-18. The identity of corn event NK603 grain was confirmed by line specific polymerase chain reaction (PCR) prior to initiation of this study. A copy of the certificate of analysis for the test substance is included with the study data. The test substance was used to assess the effect of heating on the immuno-detectability of CP4 EPSPS and CP4 EPSPS L214P proteins produced in the grain of corn event NK603. Because of the short-term nature of the study, stability of the test substance was not assessed.

**4.2 Control Substance.** The control substance was corn line RX670 (LIMS 00ZMGRO02889, lot 1746LDRP) that was also produced according to Production Plan 00-01-46-18. The absence of the NK603 event has been confirmed by PCR. A copy of the certificate of analysis is archived with the study data. Because of the short-term nature of the study, stability of the control substance was not assessed.

**4.3 Reference Substances.** Two reference substances were used in this study: (i) *E. coli*-produced CP4 EPSPS protein (lot 5192245) and (ii) *E. coli*-produced CP4 EPSPS L214P protein (lot MON-0124-PS). Due to a typographical error, a deviation from the protocol was written to correct the concentration of KCl in the storage buffer of the CP4 EPSPS protein. This deviation from the protocol had no impact on the quality of the study. Both reference substances were stored in a -80 °C freezer. Copies of the

certificates of analysis are archived with the study data. Summaries of the characterization data for the reference substances are described in a table below.

<b>Characteristic</b>	<b>CP4 EPSPS (lot 5192245)</b>	<b>CP4 EPSPSL214P (lot MON-0124-PS)</b>
Purity	90.0 %	95.2%
Concentration	3.96 mg.mL <sup>-1</sup>	2.4 mg.mL <sup>-1</sup>
Composition	50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 50 % (v/v) glycerol	50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 25 % (v/v) glycerol

## 5.0 Analytical Methods

The temperature selected (204 °C) for this study simulated the temperature used for preparation of corn products for human consumption. For the production of corn flakes (the most popular ready-to-eat breakfast cereals in the United States), flakes are toasted in a gas-fired oven for 50 sec at 302 °C or 2-3 min at 288 °C (Rooney and Serna-Saldivar, 1994). Snack food products such as corn and tortilla chips are deep-fat fried during production at a recommended temperature of 188-210 °C (Rooney and Serna-Saldivar, 1994). The temperature chosen, 204 °C, is consistent with the temperatures used for the commercial preparation of corn food products. Water was added at 25% (v/w) to the samples prior to application of the heat treatment to simulate the effect of manufacturing of corn products. The method selected to detect the presence of CP4 EPSPS and CP4 EPSPS L214P proteins in corn grain is highly specific and sensitive and is ideally suited for the detection of CP4 EPSPS and CP4 EPSPS L214P proteins in complex matrices such as corn grain extracts.

**5.1 Heating.** The test and control substances were ground to a fine powder under study 02-01-46-15. A portion of each of the ground grain was heated at ~204 °C for 15 minutes in a programmable electric furnace. The protocol described heating the samples for 30 minutes at ~ 204°C, but heating the grain samples for 30 minutes resulted in charring. Therefore, the protocol was amended to change the heating time to 10-35 minutes instead of 30 minutes.

**5.2 Extraction of CP4 EPSPS and CP4 EPSPS L214P Proteins from NK603 Grain.** Untreated and heat treated samples of NK603 and control grain were homogenized separately in plastic centrifuge tubes in PBST and 1× Laemmli buffer. Phosphate buffered saline containing Tween [PBST, 1 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 137 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub> and 0.05% (v/v) Tween-20] was used to represent a relatively mild aqueous extraction buffer with physiologic ionic strength and pH, whereas 1× Laemmli buffer [31.3 mM TRIS pH 6.8, 1% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, 12.5% (v/v) glycerol, and 0.005% (w/v) Bromophenol Blue] was used to represent a denaturing and

reducing extraction buffer. The tissue-to-buffer volume ratio was 1 g tissue to 10 mL of extraction buffer. Samples were homogenized with a hand held homogenizer (Ultra-Turrax T8, Germany) at setting 5 for ~ 20 seconds. Extracts were centrifuged for ~ 20 minutes and ~ 450  $\mu$ L of the supernatants were transferred to a fresh vial. Extracts prepared in PBST were diluted with 2 $\times$  Laemmli buffer. All samples were stored in a - 80  $^{\circ}$ C freezer until analyzed.

**5.3** *SDS-PAGE and Immunoblotting.* Samples were heated for 3 minutes in a heating block set to a temperature of ~102  $^{\circ}$ C and SDS-PAGE was performed using 4 $\rightarrow$ 20% polyacrylamide gradient gels according to SOP BR-ME-0388-01 using the mini gel system (NOVEX, San Diego, CA). Electrophoresis was conducted at 100 V for 30 minutes followed by an increase to 125 V for 30 minutes and a finally to 150 V for 30 minutes (until the dye front reached the bottom of the gel). Proteins separated by SDS-PAGE were electrophoretically transferred to PVDF membranes (0.45  $\mu$ m, Invitrogen) at 200 mA constant current for 90 minutes. Non-specific sites on the membranes were blocked with 5% (w/v) NFDN in PBST for 90 minutes. The blot was probed with a goat antiserum (Lot JB6313149) raised against the CP4 EPSPS protein at a 1:2000 dilution in PBST that contained 1% (w/v) NFDN. After an overnight incubation at 4  $^{\circ}$ C, excess goat antibody was removed by three washes with PBST for 5-10 minutes each. Biotinylated protein G (Pierce, P/N 29988, lot 7072032, Rockford, IL) at a 1:2000 dilution in PBST that contained 1% (w/v) NFDN was used to bind to the goat antibody already bound to the test protein on the blot. After a one hour incubation at room temperature, unbound protein G was removed by three washes with PBST for 5-10 minutes each. Finally, to probe for the bound protein G, NeutrAvidin conjugated to horseradish peroxidase (Pierce, P/N 31001, lot 7072031, Rockford IL) was used at a 1:10000 dilution in PBST that contained 1% (w/v) NFDN. Because of the lack of available reagents, different lot numbers than originally specified in the protocol for both the Biotinylated protein G and NeutrAvidin conjugated to horseradish peroxidase were used in the study. Furthermore, the concentration of NFDN used in the dilution buffer for the primary antibody, protein G, and NeutrAvidin HRP was also changed. These changes were documented with a protocol deviation without any impact on the quality of the study. After incubation for one hour at room temperature, unbound NeutrAvidin was removed by three washes with PBST for 5-15 minutes each. Amersham High Range color molecular weight marker (RPN 756) was analyzed on each gel. Immunoreactive bands were detected using enhanced chemiluminescent (ECL) reagents (Amersham, P/N RPN 2106, Buckinghamshire, UK) and Hyperfilm<sup>1M</sup> (Amersham, P/N RPN 3114K, Buckinghamshire, UK).

**5.4** *Image analysis.* Image analysis was performed using a BioRad model GS-710 calibrated imaging densitometer (Hercules, CA) supplied with Quantity One software (version 4.3.0) according to SOP BR-EQ-0599-01. The protocol initially described that version 4.0.3 of the Quantity Software would be used for densitometric analysis.

however, the software was later upgraded to version 4.3.0. This deviation from the protocol has no impact on the quality of the study. The CP4 EPSPS and CP4 EPSPS L214P protein levels were estimated by comparing the contour quantities (the product of the average band OD multiplied by the band area in mm<sup>2</sup>) of the heated and unheated samples to a standard curve.

## 6.0 Control of Bias and Quality Control Measures

Appropriate controls and standards were included with each analysis to calibrate the immunoblots and confirm transfer to the nitrocellulose membranes. Appropriate sets of samples were analyzed concurrently so that any run-to-run variation may be eliminated. Both reference substances were spiked into control matrix to account for any possible matrix effects.

## 7.0 Results and Discussion

Heated and unheated corn grain powders were extracted separately with PBST or 1× Laemmli buffer. PBST represented a relatively mild extraction buffer having physiological ionic strength and pH, whereas 1× Laemmli buffer represented a stringent extraction buffer that contained detergent [SDS, 1% (w/v)] and reducing agent [2-mercaptoethanol, 2.5% (v/v)].

The amounts of CP4 EPSPS and CP4 EPSPS L214 protein standards detected in corn samples before and after heating are summarized in Table 1. Although the levels of the CP4 EPSPS proteins in non-heated grain extracts were above the highest data point on the standard curve, the equation derived from the standard curve regressions was still used to estimate the values of CP4 EPSPS proteins. This resulted in an under estimation of the true levels contained in these samples. While the estimated levels of CP4 EPSPS proteins before heating were extrapolated values, the data clearly indicates a loss of immunoreactivity after heat treatment. Figures 1-4 show the effect of heating and extraction on the immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214 proteins in corn matrix.

Varying amounts of the reference substances spiked into control grain extracts were loaded on all four immunoblots to generate a standard curve. *E. coli*-produced CP4 EPSPS and CP4 EPSPS L214P proteins were spiked into control grain extracted with PBST (Figures 1 and 3, lanes 2, 3, 4, 5 and 6) or 1× Laemmli buffer (Figures 2 and 4, lanes 2, 3, 4, 5 and 6). Extracts from unheated and heated samples of control grain were loaded in lanes 7 and 9 of each gel (Figures 1-4), respectively. Extracts from unheated and heated samples of grain from event NK603 were loaded in lanes 8 and 10 (Figures 1-4), respectively.

The data demonstrate that heating significantly decreased the immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214P proteins. The reduction in detectability was much greater for extracts prepared in PBST than for the extracts prepared with 1× Laemmli buffer. In the case of the PBST extraction, heating of the powdered grain reduced the immuno-detectability of CP4 EPSPS and CP4 EPSPS L214P proteins to non-detectable levels. In the case of the 1× Laemmli buffer extraction, heating of the powdered grain reduced the immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214P proteins by at least ~ 90%.

The polyclonal antibody used in this study identified both the CP4 EPSPS and CP4 EPSPS L214P proteins. However, other minor cross-reactive bands were observed on the blots. The band migrating just above the CP4 EPSPS band (Figures 1-4; lanes 2-6) is one such example. These cross-reactive bands in no way interfered with data interpretation. The reduced immuno-reactivity of the CP4 EPSPS and CP4 EPSPS L214P proteins due to heating is very dramatic and the visual loss in signal is clearly observed in the results obtained.

## **8.0 Conclusions**

Corn is treated at high temperatures during commercial processing. The temperature selected (204 °C) for this study simulated the temperature used for the commercial production of corn products for human consumption. The results from the PBST extraction experiments demonstrated that heat treatment at 204 °C for 15 min resulted in the complete loss of the immuno-detectability of CP4 EPSPS and CP4 EPSPS L214P proteins present in Roundup Ready corn event NK603 (Figures 1 and 3). For samples extracted with 1× Laemmli buffer, the levels of the CP4 EPSPS proteins declined from a range of approximately 1.7 – 1.5 ng to 0.11 – 0.16 ng upon heat treatment (Figures 2 and 4, lane 8 versus lane 10). It was therefore concluded that heating conditions associated with typical corn processing will significantly reduce the immuno-detectability of the CP4 EPSPS proteins when present in corn grain matrix.

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**Table 1. Levels of CP4 EPSPS and CP4 EPSPS L214P proteins detected in grain samples extracted using PBST and 1x Laemmli buffer before and after heating.**

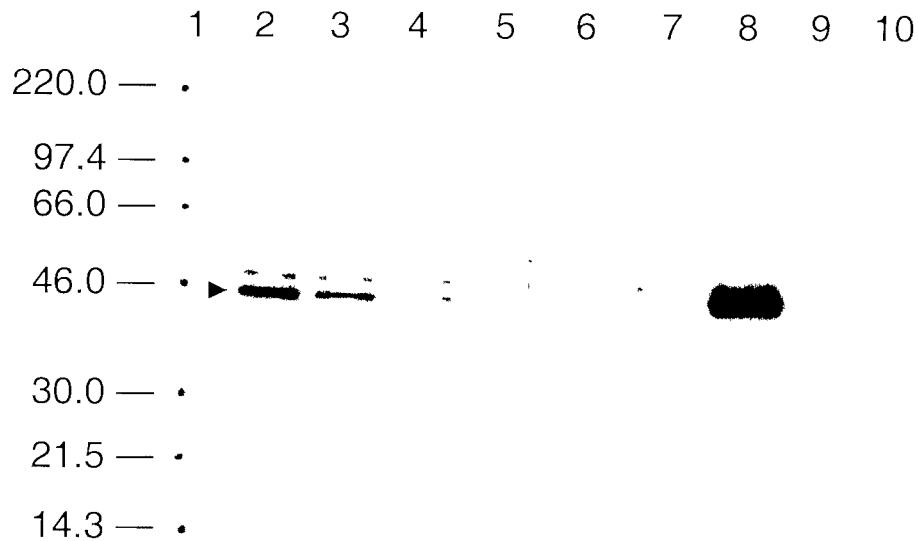
Extraction Buffer	Estimated levels of the CP4 EPSPS proteins observed in grain extracts before heating <sup>a</sup>	Estimated levels of the CP4 EPSPS proteins observed in grain extracts after heating <sup>a</sup>	CP4 EPSPS or CP4 EPSPS L214P Standard curve used to estimate protein levels in heated and unheated corn samples	Estimated loss of immuno-reactivity <sup>b</sup>	R <sup>2</sup> values for the standard curve linear regression fit <sup>c</sup>	Limit of detection <sup>d</sup>
PBST	1.6 ng <sup>e</sup>	Not observed on blot	CP4 EPSPS (Figure 1)	≥ 99% <sup>b</sup>	0.999	0.09 ppm
1x Laemmli	1.6 ng <sup>e</sup>	0.11 ng	CP4 EPSPS (Figure 2)	93%	0.989	0.05 ppm
PBST	1.7 ng <sup>e</sup>	Not observed on blot	CP4 EPSPS L214P (Figure 3)	≥ 99% <sup>f</sup>	0.986	0.10 ppm
1x Laemmli	1.5 ng <sup>e</sup>	0.16 ng	CP4 EPSPS L214P (Figure 4)	89%	0.996	0.05 ppm

<sup>a</sup>Quantitation by image analysis of blots using BioRad Quantity One software (Version 4.3.0) and Model GS 710 calibrated imaging densitometer. The levels were estimated using Excel 2000 software version 9.0.4402 SR-1, by comparing the contour quantity to a linear regression equation derived from the standard curve.

<sup>b</sup>% loss immuno-reactivity = 100 - ((lowest observed CP4 EPSPS protein standard / estimated CP4 EPSPS protein level before heating) × 100)

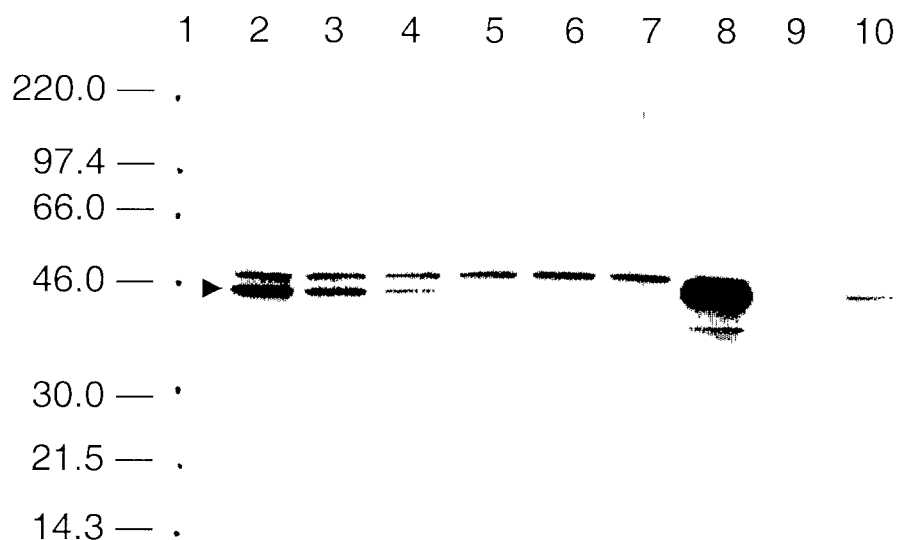
<sup>c</sup>The linear regression equation and the R<sup>2</sup> values were calculated using SigmaPlot 2001® software version 7.101

<sup>d</sup>The estimated values of the CP4 EPSPS proteins in non-heated grain samples were deduced from values above the highest standard curve point. The limit of detection for each immunoblot was defined by the amount of control extract matrix into which the protein standard was spiked



**Figure 1. Immunoblot analysis showing the effect of heating on the detectability of CP4 EPSPS proteins extracted from NK603 grain with PBST using a CP4 EPSPS protein standard curve.** Proteins were separated by SDS-PAGE; electro-blotted to PVDF membrane, detected with CP4 EPSPS antisera followed by protein G and NeutrAvidin HRP<sup>TM</sup> binding. The immunoblot was developed using the ECL system. Lane 1 corresponds to Amersham rainbow high-range MW markers (kDa) shown on the left. Lanes 2-6 correspond to the CP4 EPSPS protein standard. The arrow (►) indicates the position of the CP4 EPSPS protein. The size of the image has been adjusted to allow for presentation in figure format.

Lane	Sample	Volume Loaded	Amount Loaded
1	Protein MW Markers	20 $\mu$ L	1 $\mu$ g/band
2	CP4 EPSPS from <i>E. coli</i> control grain (RX670) PBST extract	6 $\mu$ L	~ 0.45 ng (CP4 EPSPS)
3	CP4 EPSPS from <i>E. coli</i> control grain (RX670) PBST extract	6 $\mu$ L	~ 0.18 ng (CP4 EPSPS)
4	CP4 EPSPS from <i>E. coli</i> + control grain (RX670) PBST extract	6 $\mu$ L	~ 0.09 ng (CP4 EPSPS)
5	CP4 EPSPS from <i>E. coli</i> - control grain (RX670) PBST extract	6 $\mu$ L	~ 0.05 ng (CP4 EPSPS)
6	CP4 EPSPS from <i>E. coli</i> control grain (RX670) PBST extract	6 $\mu$ L	~ 0.02 ng (CP4 EPSPS)
7	PBST extract from <b>unheated</b> RX670 control grain	10 $\mu$ L	-
8	PBST extract from <b>unheated</b> NK603 grain	10 $\mu$ L	-
9	PBST extract from <b>heated</b> RX670 control grain	10 $\mu$ L	-
10	PBST extract from <b>heated</b> NK603 grain	10 $\mu$ L	-



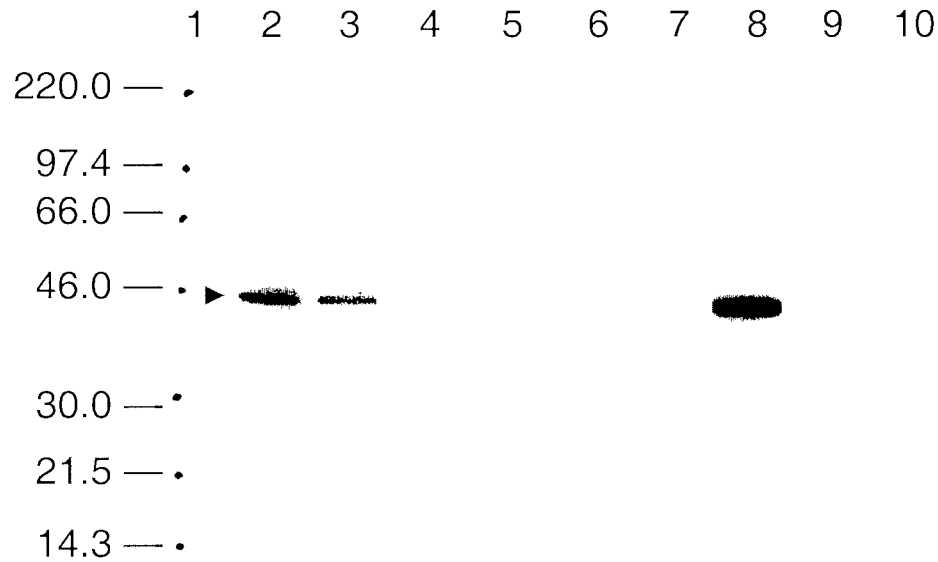
**Figure 2. Immunoblot analysis showing the effect of heating on the detectability of CP4 EPSPS proteins extracted from NK603 grain with 1× Laemmli buffer using a CP4 EPSPS protein standard curve.** Proteins were separated by SDS-PAGE; electroblotted to PVDF membrane, detected with polyclonal CP4 EPSPS antisera followed by protein G and NeutrAvidin HRP™ binding. The immunoblot was developed using the ECL system. Lane 1 corresponds to Amersham rainbow high-range MW markers (kDa) shown on the left. Lanes 2-6 correspond to the CP4 EPSPS protein standard. The arrow (►) indicates the position of the CP4 EPSPS protein. The size of the image has been adjusted to allow for presentation in figure format.

Lane	Sample	Volume Loaded	Amount Loaded
1	Protein MW Markers	20 µL	1 µg/band
2	CP4 EPSPS from <i>E coli</i> control grain (RX670) 1× Laemmli extract	6 µL	~ 0.45 ng (CP4 EPSPS)
3	CP4 EPSPS from <i>E coli</i> - control grain (RX670) 1× Laemmli extract	6 µL	~ 0.18 ng (CP4 EPSPS)
4	CP4 EPSPS from <i>E coli</i> + control grain (RX670) 1× Laemmli extract	6 µL	~ 0.09 ng (CP4 EPSPS)
5	CP4 EPSPS from <i>E coli</i> · control grain (RX670) 1× Laemmli extract	6 µL	~ 0.05 ng (CP4 EPSPS)
6	CP4 EPSPS from <i>E coli</i> control grain (RX670) 1× Laemmli extract	6 µL	~ 0.02 ng (CP4 EPSPS)
7	1× Laemmli extract from <b>unheated</b> RX670 control grain	5 µL	-
8	1× Laemmli extract from <b>unheated</b> NK603 grain	5 µL	-
9	1× Laemmli extract from <b>heated</b> RX670 control grain	5 µL	-
10	1× Laemmli extract from <b>heated</b> NK603 grain	5 µL	-



**Figure 3. Immunoblot analysis showing the effect of heating on the detectability of CP4 EPSPS proteins extracted from NK603 grain with PBST using a CP4 EPSPS L214P protein standard curve.** Proteins were separated by SDS-PAGE; electro-blotted to PVDF membrane, detected with polyclonal CP4 EPSPS antisera followed by protein G and NeutrAvidin HRP<sup>TM</sup> binding. The immunoblot was developed using the ECL system. Lane 1 corresponds to Amersham rainbow high-range MW markers (kDa) shown on the left. Lanes 2-6 correspond to the CP4 EPSPS L214P protein standard. The arrow (►) indicates the position of the CP4 EPSPS L214P protein. The size of the image has been adjusted to allow for presentation in figure format.

Lane	Sample	Volume Loaded	Amount Loaded
1	Protein MW Markers	20 µL	1 µg/band
2	CP4 EPSPS L214P from <i>E. coli</i> control grain (RX670) PBST extract	6 µL	~ 0.5 ng (CP4 EPSPS L214P)
3	CP4 EPSPS L214P from <i>E. coli</i> control grain (RX670) PBST extract	6 µL	~ 0.2 ng (CP4 EPSPS L214P)
4	CP4 EPSPS L214P from <i>E. coli</i> - control grain (RX670) PBST extract	6 µL	~ 0.1 ng (CP4 EPSPS L214P)
5	CP4 EPSPS L214P from <i>E. coli</i> - control grain (RX670) PBST extract	6 µL	~ 0.05 ng (CP4 EPSPS L214P)
6	CP4 EPSPS L214P from <i>E. coli</i> - control grain (RX670) PBST extract	6 µL	~ 0.025 ng (CP4 EPSPS L214P)
7	PBST extract from <b>unheated</b> RX670 control grain	10 µL	-
8	PBST extract from <b>unheated</b> NK603 grain	10 µL	-
9	PBST extract from <b>heated</b> RX670 control grain	10 µL	-
10	PBST extract from <b>heated</b> NK603 grain	10 µL	-



**Figure 4. Immunoblot analysis showing the effect of heating on the detectability of CP4 EPSPS proteins extracted from NK603 grain with 1× Laemmli buffer using CP4 EPSPS L214P standard curve.** Proteins were separated by SDS-PAGE: electroblotted to PVDF membrane, detected with polyclonal CP4 EPSPS antisera followed by protein G and NeutrAvidin HRP<sup>TM</sup> binding. The immunoblot was developed using the ECL system. Lane 1 corresponds to Amersham rainbow high-range MW markers (kDa) shown on the left. Lanes 2-6 correspond to the CP4 EPSPS L214P protein standard. The arrow (▶) indicates the position of the CP4 EPSPS L214P protein. The size of the image has been adjusted to allow for presentation in figure format.

Lane	Sample	Volume Loaded	Amount Loaded
1	Protein MW Markers	20 µL	1 µg/band
2	CP4 EPSPS L214P from <i>E. coli</i> + control grain (RX670) 1× Laemmli extract	6 µL	~ 0.5 ng (CP4 EPSPS L214P)
3	CP4 EPSPS L214P from <i>E. coli</i> + control grain (RX670) 1× Laemmli extract	6 µL	~ 0.2 ng (CP4 EPSPS L214P)
4	CP4 EPSPS L214P from <i>E. coli</i> + control grain (RX670) 1× Laemmli extract	6 µL	~ 0.1 ng (CP4 EPSPS L214P)
5	CP4 EPSPS L214P from <i>E. coli</i> + control grain (RX670) 1× Laemmli extract	6 µL	~ 0.05 ng (CP4 EPSPS L214P)
6	CP4 EPSPS L214P from <i>E. coli</i> + control grain (RX670) 1× Laemmli extract	6 µL	~ 0.025 ng (CP4 EPSPS L214P)
7	1× Laemmli extract from <b>unheated</b> RX670 control grain	5 µL	–
8	1× Laemmli extract from <b>unheated</b> NK603 grain	5 µL	–
9	1× Laemmli extract from <b>heated</b> RX670 control grain	5 µL	–
10	1× Laemmli extract from <b>heated</b> NK603 grain	5 µL	–

**Appendix 1**  
**List of Applicable Method SOPs**

<b><u>SOP Number</u></b>	<b><u>SOP Title</u></b>
BR-ME-0388-01	SDS Polyacrylamide Gel Electrophoresis
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)
BR-IQ-0599-01	Bio-Rad GS-710 Densitometer

**Appendix 2**  
**Protocol and Amendment**

**Monsanto Study Number:** 02-01-46-14

**Study Title:** Immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214P Proteins in Heat-treated Grain of Roundup Ready<sup>®</sup> Corn Event NK603

**Sponsor:** Monsanto Company  
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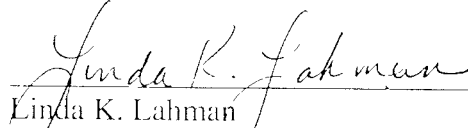
**Principal Investigators:** Joan L. Lee  
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Approved By:



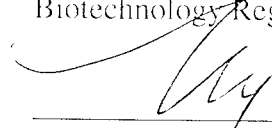
Patrick T. Weston  
**Testing Facility Management Representative**  
Monsanto Company  
Biotechnology Regulatory Sciences

April 10, 2002  
Date



Linda K. Lahman  
**Sponsor Representative**  
Monsanto Company  
Biotechnology Regulatory Affairs

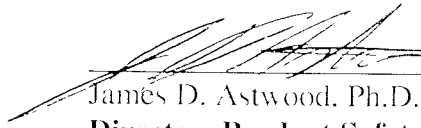
10 April 2002  
Date



Gyula Holleschak  
**Study Director**  
Monsanto Company  
Product Safety Center

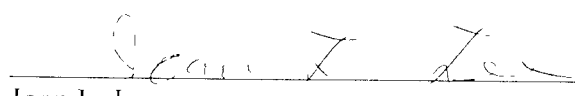
April 10, 2002  
Date

Reviewed By:



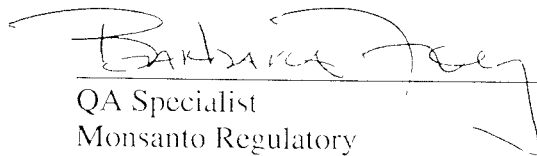
James D. Astwood, Ph.D.  
**Director, Product Safety Center**  
Monsanto Company  
Product Safety Center

April 10, 2002  
Date



Joan L. Lee  
**Principal Investigator**

4/10/02  
Date



Barbara J. Jey  
QA Specialist  
Monsanto Regulatory

10 April 2002  
Date

## 1.0 Regulatory Compliance

- 1.1** *GLP Compliance.* This is a product characterization study as defined by section §160.135(b) of the United States Environmental Protection Agency (EPA) 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

Monsanto Company has developed Roundup Ready<sup>®</sup> corn event NK603 that is tolerant to glyphosate (the active ingredient in Roundup<sup>®</sup> family of agricultural herbicides) at the whole plant level. The corn event NK603 was produced via stable insertion of the *cp4 epsps* coding region from vector PV-ZMGT32L into the genome of corn. The inserted DNA in corn event NK603 has been sequenced. Roundup Ready corn event NK603 contains two *cp4 epsps* coding regions, which differ by two nucleotides, one of which results in an amino acid substitution of proline for leucine at amino acid 214 (L214P) in the encoded protein. Both *cp4 epsps* coding regions were present in the first (F1) and the second (BC1F1) generations after transformation. Thus, the corn event NK603 produces both the CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium* sp. strain CP4) and CP4 EPSPS L214P proteins, each of which contain 455 amino acids.

The purpose of this study was to assess the effect of heat treatment on the immuno-detectability of the CP4 EPSPS L214P and CP4 EPSPS proteins produced in the grain of Roundup Ready corn event NK603.

## 3.0 Timelines

Proposed experimental start date: April 2002  
Proposed experimental termination date: May 2002

## 4.0 Test, Control and Reference Substances

- 4.1** *Test Substance.* The test substance is Roundup Ready corn event NK603 (LIMS 00ZMGRO02886, lot SCP71A9CIT). Grain from Roundup Ready corn event NK603, which contains both the CP4 EPSPS and CP4 EPSPS L214P proteins, was grown according to Production Plan 00-01-46-18. The test substance will be used to assess the effect of heating on the immuno-detectability of CP4 EPSPS and CP4 EPSPS L214P proteins contained in the grain of NK603 corn. Because of the short-term nature of the study, stability of the test substance will not be assessed.

- 4.2** *Control Substance.* The control substance is corn line RX670 (LIMS 00ZMGRO02889, lot 1746LDRP). The control substance was also produced according to Production Plan 00-01-46-18. The absence of the *cp4 epsps* coding region has been confirmed by PCR. A certificate of analysis verifying the lack of contamination of the control substance with transgenic corn events will be archived with the study data.
- 4.3** *Reference Substance.* *L. coli*-produced CP4 EPSPS protein (lot 5192245) will be used as a reference standard for immunoblots. The reference substance will be stored in a  $-80^{\circ}\text{C}$  freezer.
- 4.4** *Test and reference substance characterization.* The identity of the test corn event has been confirmed by line specific PCR (Production Plan 00-01-46-18). A certificate of analysis confirming the results of the PCR characterization analyses will be archived with the study data.

The CP4 EPSPS protein reference substance was initially characterized by Harrison *et al.* (1993). The reference substance is stored in a  $-80^{\circ}\text{C}$  freezer in a buffer solution containing 50 mM Tris-Cl, pH 7.5, 150 mM KCl, 2 mM DTT and 50% (v/v) glycerol at 3.96 mg/mL total protein. The purity of the reference substance is  $>90\%$ . The initial characterization data for the reference substance was confirmed in August of 2000. A certificate of analysis for the CP4 EPSPS standard will be archived with the study file.

## **5.0 Description of Experimental Design**

The effect of heat treatment on the immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214P contained in the grain of Roundup Ready corn event NK603 will be assessed using western blot analysis. Aliquots of the ground corn grain containing event NK603 will be heated for 30-35 minutes at  $202-260^{\circ}\text{C}$ , extracted, and analyzed using the western blot analysis. Heated control samples and unheated samples of the test and control line will also be prepared. Moisture may be added to the samples prior to application of the heat treatments. Heated and unheated samples will be extracted in a physiologically relevant buffer system such as phosphate buffered saline with 0.05% Tween-20 enriched with a cocktail of protease inhibitors. Samples will be loaded onto SDS-PAGE gels, transblotted to either a nitrocellulose or PVDF membrane and the CP4 EPSPS and CP4 EPSPS L214P proteins detected on blots using a series of appropriate blocking solutions, washes, antibodies and detection reagents. Image analysis of blot films will be ultimately be conducted to assess the effect of heating on the immuno-detection of the combination of the CP4 EPSPS and CP4 EPSPS L214P proteins.

## 5.1 Analytical Methods and Procedures

**5.1.1** *Justification of the Analytical Methods.* The western blot analytical procedure is a highly specific and sensitive immunological method to detect cross-reactive protein bands in complex mixtures such as protein extracts prepared from corn grain (Deucher, 1990).

**5.1.2** *Extraction of samples.* Unbaked samples will be used to enable the comparison of heating on the extraction of the CP4 EPSPS and CP4 EPSPS L214P proteins. Extraction will be performed using two separate buffers: Phosphate buffered saline containing Tween 20 (PBS-T) represents a relatively mild aqueous extraction buffer with physiologic ionic strength and pH, whereas Laemmli buffer represents a denaturing and reducing extraction buffer. The baked and unbaked samples will be extracted in PBS-T and Laemmli buffer. Extracts prepared in PBS-T will be diluted with an equal volume of 2× Laemmli buffer and heated for 3-5 min at ~100 °C. Extracts prepared in Laemmli buffer will also be heated for 3-5 min at ~100 °C. Extracts prepared in both PBS-T and Laemmli buffer will be clarified by centrifugation and subjected to SDS-PAGE and immunoblot analysis. Both PBS-T and Laemmli corn grain extracts may be stored at -80 °C.

**5.1.3** *SDS-PAGE/Immunoblot analysis.* Extracts prepared from heated and unheated ground corn grain samples in Laemmli buffer (1970) will be separated by SDS-PAGE gradient gels under reducing condition using a NOVEX 4→20% polyacrylamide gradient mini-gel according to SOP BR-ME-0388-01. Separated proteins will be electrophoretically transferred to either a nitrocellulose or PVDF membrane and immunoblotting subsequently conducted according to SOP GEN-PRO-002-03. Non-specific sites on blots will be blocked using 5% non-fat dry milk (NFDM) in 1× PBST [1 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, 0.05% (v/v) Tween 20, pH 7.4]. Blots will be probed for the presence of the CP4 EPSPS and L214P CP4 EPSPS proteins using a 1:5000 dilution of goat anti-CP4 EPSPS polyclonal antibody (lot # JB6313149) in 1× PBST with 5% (w/v) NFDM at room temperature. Characterization of this antibody has been previously described (Bhakta *et al.*, 2001). Unbound polyclonal antibody will be rinsed away with washes of 1× PBST. Polyclonal antibody bound to the membrane will be probed with a 1:2000 dilution of biotinylated protein G (Pierce, Rockford, IL, lot 7019603) in 5% NFDM at room temperature. Unbound secondary antibody will be rinsed away with washes of 1× PBST. The membrane will be washed with 1× PBST followed by the addition of a 1:10000 dilution of NeutrAvidin<sup>TM</sup> (HRP; Pierce, Rockford IL, lot 7019602) in 5% NFDM at room temperature. Immunoreactive bands will ultimately be visualized on X-ray film, using the ECL kit of Amersham (Cat. No. RPN 2106) and the instructions provided by the manufacturer. Pre-stained molecular weight markers will be used.

to verify electrotransfer of proteins. Films will be developed using an automated film processor.

**5.1.4 Optional Analysis.** Should immunoreactive bands be observed in lanes containing extracts of backed grain samples, image analysis of immunoreactive bands on blot films may be conducted using a BioRad model GS-710 calibrated imaging densitometer (Hercules, CA) supplied with Quantity One software (version 4.0.3) according to SOP BR-IEQ-0599-01. The level of signal for the combination of (CP4 EPSPS L214P + CP4 EPSPS) will be determined as band contour quantity (avg. band OD  $\times$  band area in mm<sup>2</sup>). An equation (linear or quadratic) will be fitted to the contour quantity data obtained for multiple loadings of the *E. coli*-produced CP4 EPSPS protein reference standard spiked into control matrix and analyzed concurrently to afford a quantitative analysis of the immuno-reactive level observed for these protein in the unheated and heated corn grain samples. The limit of detection for the CP4 EPSPS reference protein in parts per million (ppm) will be defined by the level of the lowest visible standard in nanograms on each blot divided by the amount of control matrix analyzed in milligrams.

## **6.0 Proposed Statistical Methods.**

No statistical functions such as ANOVA or t-test will be employed to compare means in this study. Curve fitting functions in Excel and other instrumentation may be employed as required for the generation of best-fit equations to standard curve data.

## **7.0 Control of Bias/Quality Measures**

For immunoblot analysis, the *E. coli*-produced protein standards will be spiked into control matrix to correct for any effect of the matrix on the migration of these proteins. Pre-stained molecular weight markers will be used to verify electrotransfer of the proteins to PVDF membrane. Appropriate sets of samples will be analyzed concurrently so that any run-to-run variation is eliminated. All assay tubes will be appropriately labeled so that no mix-up of sample identity can occur.

## **8.0 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 at Monsanto Company.

## 9.0 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.

## 10.0 References

Bhakta, N. S., Watson, J. A. and Lirette, R.P. 2001. The Development and Validation of an Enzyme-Linked Immunosorbent Assay for Quantitation of CP4 5-enolpyruvyl shikimate-3-phosphate synthase (CP4 EPSPS) Protein Levels in Soybean Tissues. Monsanto Technical Report, St. Louis, MSL-16508

Deutcher, M. P. 1990. Guide to Protein Purification, in: *Methods in Enzymology* 182. Academic Press, Inc., Harcourt Brace Javanovich, Publishers, New York.

Harrison, L. A., Bailey, M. R., Leimgruber, R. M., Smith, C. E., Nida, D. L., Taylor, M. L., Gustafson, M., Geeren, B. and Padgett, S.R. 1993. Characterization of Microbially-Expressed Protein: CP4 EPSPS. Monsanto Technical Report, St. Louis, MSL-12901

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*: **227**, 680-685.

**Attachment: SOP List**

<b><u>SOP Number</u></b>	<b><u>SOP Title</u></b>
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)
BR-ME-0388-01	SDS Polyacrylamide Gel Electrophoresis

**Protocol Amendment Form**

**Amendment #: 1**

**Monsanto Study #:** 02-01-46-14

**Date changes implemented:** April 15, 2002

**Page number(s) and section(s):** Page 4, section 5.0

**Protocol originally stated:**

**5.0 Description of Experimental Design**

The effect of heat treatment on the immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214P contained in the grain of Roundup Ready corn event NK603 will be assessed using western blot analysis. Aliquots of the ground corn grain containing event NK603 will be heated for **30**-35 minutes at 202-260 °C, extracted, and analyzed using the western blot analysis. Heated control samples and unheated samples of the test and control line will also be prepared. Moisture may be added to the samples prior to application of the heat treatments. Heated and unheated samples will be extracted in a physiologically relevant buffer system such as phosphate buffered saline with 0.05% Tween-20 enriched with a cocktail of protease inhibitors. Samples will be loaded onto SDS-PAGE gels, transblotted to either a nitrocellulose or PVDF membrane and the CP4 EPSPS and CP4 EPSPS L214P proteins detected on blots using a series of appropriate blocking solutions, washes, antibodies and detection reagents. Image analysis of blot films will be ultimately be conducted to assess the effect of heating on the immuno-detection of the combination of the CP4 EPSPS and CP4 EPSPS L214P proteins.

**Protocol amended as follows:**

**5.0 Description of Experimental Design**

The effect of heat treatment on the immuno-detectability of the CP4 EPSPS and CP4 EPSPS L214P contained in the grain of Roundup Ready corn event NK603 will be assessed using western blot analysis. Aliquots of the ground corn grain containing event NK603 will be heated for **10**-35 minutes at 202-260 °C, extracted, and analyzed using the western blot analysis. Heated control samples and unheated samples of the test and control line will also be prepared. Moisture may be added to the samples prior to application of the heat treatments. Heated and unheated samples will be extracted in a physiologically relevant buffer system such as phosphate buffered saline with 0.05% Tween-20 enriched with a cocktail of protease inhibitors. Samples will be loaded onto SDS-PAGE gels, transblotted to either a nitrocellulose or PVDF membrane and the CP4 EPSPS and CP4 EPSPS L214P proteins detected on blots using a series of appropriate blocking solutions, washes, antibodies and detection reagents. Image analysis of blot

**Protocol Amendment Form**

**Amendment #: 1**

films will be ultimately be conducted to assess the effect of heating on the immuno-detection of the combination of the CP4 EPSPS and CP4 EPSPS L214P proteins.

**Reason for the amendment and what impact will result from this change.** During the study, both the test and reference substances were heated at ~204°C for 30 minutes. Heating for 30 minutes resulted in the complete charring of the corn samples and the experiment was repeated. This amendment describes the change in heating time from 30 minute to 15 minutes. The effect of heating samples for 15 minutes did not negatively impact the study.

**Date changes implemented:** May 1, 2002

**Page number(s) and section(s):** Page 4, section 4.3 and 4.4

**Protocol originally stated:**

**4.3 Reference Substances.** *E. coli*-produced CP4 EPSPS protein (lot 5192245) will be used as a reference standard for immunoblots. The reference substance will be stored in a -80 °C freezer.

**Protocol amended as follows:**

**4.3 Reference Substances.** Two different *E. coli*-produced CP4 EPSPS reference substances will be used during the study. The first reference substance is CP4 EPSPS protein (lot 5192245), and the second reference substance is CP4 EPSPS L214P protein (lot MON-0124-PS).

**\*The following sentence will be added to section 4.4 to support the characterization of the CP4 EPSPS L214P protein.**

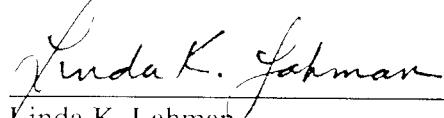
The CP4 EPSPS L214P protein will be characterized under study 02-01-46-15. The reference substances will be stored in a -80 °C freezer.

**Reason for the amendment and what impact will result from this change.** The CP4 EPSPS L214P protein will be used as an additional reference substance for the immunoblot analysis. The addition of a second reference substance will improve the quality of the study and serve as an appropriate reference for the expressed CP4 EPSPS L214P protein in NK603 corn grain. This amendment will not impact the study negatively.

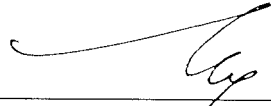
**Protocol Amendment Form**

**Amendment #: 1**

**Approved By:**

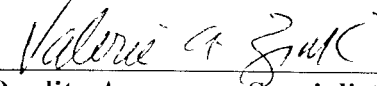
  
\_\_\_\_\_  
Linda K. Lahman  
Sponsor Representative

1 May 2002  
Date

  
\_\_\_\_\_  
Gyula Holleschak  
Study Director

May 1, 2002  
Date

**Reviewed By:**

  
\_\_\_\_\_  
Valerie A. Zink  
Quality Assurance Specialist

August 29, 2002  
Date