Title

Assessment of Human IgE Binding to SDA Soy, Control, and Reference Soy Extracts

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Study Completed On

September 4, 2007

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Laboratory Project ID

MSL-20553

Study Plan 05-01-83-03

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Statement of Compliance

This study does not meet the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160. Measures taken to ensure study quality have been included in the Quality Measures section of the report.

Submitter: _____   Date: _____

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Monsanto Company
Final Report
Product Characterization Center

Study Plan: 05-01-83-03
MSL: 20553
Page 4 of 42

Summary of Quality Control Review

This report was reviewed to ensure that it accurately reflects the data provided for the study.

Reviewed by:

_____   _____

Quality Assurance Specialist   Date   Aug. 27, 2007
Monsanto Regulatory QAU
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Study Certification Page

This report is an accurate and complete representation of the study activities.

Approved By:

Elena A. Rice   _Sept. 04, 2007_

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Reviewed By:

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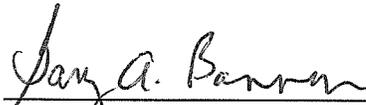
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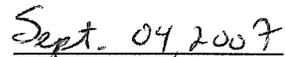
Study Plan Information Page

Study Plan Number: 05-01-83-03

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Records Retention: Study plan, amendments, deviations, final report, and originals or copies of the data will be retained at Monsanto, St. Louis.

Table of Contents

List of Tables

List of Figures

List of Appendices

Abbreviations[1]

CAP-FEIA	Capsulated Hydrolic Carrier Polymer-FluoroEnzyme ImmunoAssay
COC	Chain Of Custody
CV	Coefficient of Variation
DBPCFC	Double-Blind Placebo Controlled Food Challenge
ELISA	Enzyme-Linked Immunosorbent Assay
IgE	Immunoglobulin E
LOD	Limit of Detection
NA serum pool	Non-Allergic serum pool
n/d	Not detected
NSB	Non-Specific Binding
OD	Optical Density
PBST	Phosphate Buffered Saline containing Tween-20
SD	Standard Deviation
SDA	Stearidonic Acid

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[1] Standard abbreviations, e.g. units of measure, concentration, mass, time, etc., are used without definition according to the format described in "Instructions to Authors" in The Journal of Biological Chemistry.

1.0   Summary

A study was conducted to determine the binding levels of IgE antibody from clinically documented, soy allergic patients to protein extracts prepared from three stearidonic acid (SDA)-containing soybean varieties (MON 87769, MON 87736, and MON 87714) and 24 commercial soybean varieties which served to establish a range in IgE binding. The level of IgE binding provides an estimate of the amount of endogenous soybean allergens present in the seed. The commercially available soybean reference varieties included Roundup Ready®, conventional, high protein, high oil, and food-grade (tofu) lines already on the market and used for human consumption.

Sera from 16 clinically documented, soy allergic patients and 6 non-allergic patients were used to assess the range of IgE binding to each soybean extract. Only soy allergic patients with a documented history of anaphylactic reactions to soybean and a positive Double-Blind Placebo Controlled Food Challenge (DBPCFC) were included in this study.

Aqueous extracts were prepared from the ground seed of three SDA soybeans, control (A3525), and the commercial reference soybean varieties and analyzed by a validated enzyme linked immunosorbent assay (ELISA) for IgE binding. Each soybean extract was tested in triplicate at protein concentrations of 10 µg of total seed protein/ml of extract. Soy-specific IgE was quantified by use of a soy-specific IgE standard curve and expressed as ng of IgE/ml of serum. The standard curve was created by loading serial dilutions of human serum PEI 163 containing a known amount of soy-specific IgE into wells coated with internal reference soybean extract. The bound soy-specific IgE was detected using biotin conjugated anti-human IgE polyclonal antibody from goat.

The IgE binding values obtained for reference soybean extracts were used to calculate a 99% tolerance interval for each patient serum. The 99% tolerance interval represents the range of IgE binding for each patient serum to the extracts prepared from the commercial soybean varieties such that 99% of the IgE binding values are expected to fall within this range with 95% confidence. The IgE binding levels obtained for protein extracts prepared from SDA soybean varieties were compared to the calculated tolerance intervals. For all of the SDA and control soybeans, IgE values fall within the established tolerance intervals obtained for each serum (Figure 1 and Appendix 2) with three exceptions. The IgE binding values for serum # 6 to extracts from MON 87769 and for serum #10 to extracts from MON 87769 and MON 87736 were below the assay's limit of detection. None of the tested soybean varieties showed IgE binding to sera from non-allergic patients.

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The results of this study demonstrate that the levels of endogenous soybean allergens in MON 87769, MON 87736 and MON 87714 and non-transgenic control, A3525, are comparable to the levels of endogenous soybean allergens in the soybean varieties that are currently on the market.

2.0   Introduction

Monsanto has developed genetically modified soybeans to produce stearidonic acid (SDA) in soy oil by introducing two desaturase genes that encode for the membrane localized proteins, *Primula juliae* Δ6 desaturase (PjΔ6D) and *Neurospora crassa* Δ15 desaturase (NcΔ15D). SDA is an omega-3 fatty acid which is the metabolic precursor to long chain omega-3 fatty acids. Long chain omega-3 fatty acids are known to reduce the risk of heart attacks and enhance heart health in humans.

Food crops that have been developed through agricultural biotechnology for commercial use are thoroughly assessed for their safety. One of the key elements in the safety assessment of the genetically improved crop is an evaluation of potential changes in their allergenic properties. Allergenic properties of the crop can potentially be altered if a known allergen or a protein that has high potential to become an allergen is introduced. In addition, the level of expression of endogenous allergens might be altered as a result of transformation and insertion of the new gene into the plant genome (König et al., 2004).

Soybean is one of eight allergenic foods that are responsible for approximately 90% of all food allergies (FAO, 1995). Soybean is less allergenic than other foods in this group and rarely responsible for severe, life-threatening reactions (Cordle, 2004). Allergy to soybean is more prevalent in children than adults and is considered a transient allergy of infancy/childhood (Sicherer et al., 2000). Since soybeans are a known allergenic food crop, there is a need to ensure that the introduction of the genes and production of the PjΔ6D and NcΔ15D proteins in soybean did not cause an unintended change in the levels of endogenous allergenic proteins. This question can be addressed by comparing levels of soy-specific IgE binding observed in the biotechnology-derived soybean varieties to the set of binding values observed in reference soybean varieties that are already on the market. Determining the levels of direct IgE binding using an enzyme linked immunosorbent assay (ELISA) has been shown to be an appropriate method to perform such comparisons (Sten et al., 2004), especially when the assay is validated and calibrated prior to the production of data (Ahlstedt et al., 2003).

Validated and calibrated ELISA assays were utilized in this study to compare the levels of endogenous soybean allergens in MON 87769, MON 87736, MON 87714 and A3525 to the levels of allergens in 24 commercial soybean varieties that are currently on the market.

3.0   Purpose

The purpose of this study was to determine the levels of binding of soy-specific IgE antibody from soy-allergic patients to protein extracts prepared from seeds of three SDA soybean varieties, a parental non-transgenic control soybean , and 24 commercial soybean varieties grown in the United States.

4.0   Materials

Monsanto supplied the test, control, and reference substances described below. Soybean seeds were coarsely ground and shipped on dry ice from the Monsanto Company (Creve Coeur, MO) to the Principal Investigator at Paul-Ehrlich-Institut, where they were stored in a -20 ºC freezer until they were ground to a fine powder. The fine powder material was stored in a -70 ºC freezer.

4.1   Test Substances

The test substances are three SDA soybean varieties (see Table 1 for sample identifiers).

4.2   Control Substance

The control substance is the parental non-transgenic soybean A3525 (seed lot REF-0409-15502-S).

4.3   Reference Substances

The reference substances are 24 commercially available soybean varieties (see Table 1 for sample identifiers).

4.4   Characterization of Test, Control, and Reference Substances

The identity of the soybean test substances was confirmed by event specific polymerase chain reaction (PCR). A copy of the analysis results for the test substances will be archived with this study.

Table 1 contains seed lot numbers of the control and reference soybean varieties used in this study. Chain of custody (COC) records served as characterization data for the control substance and reference materials. No additional characterizations of the control and reference materials were conducted.

5.0   Experimental Design

Aqueous extracts prepared from test, control, and reference substances were analyzed by ELISA for soy-specific IgE binding using sera of soybean allergic and non-allergic

patients. The bound soy-specific IgEs were detected using biotin conjugated anti-human IgE polyclonal antibody from goat (KPL, Gaithersburg, Maryland, USA).

6.0 Analytical Methods

6.1 Grinding of Soybean Seed

Samples of test, control, and reference substance seeds were coarsely ground at Monsanto and transported on dry ice to Prof. Dr. Stefan Vieths at Paul-Ehrlich-Institut. At Paul-Ehrlich-Institute, coarsely ground seeds were stored in a -20 ºC freezer until they were re-ground to a fine powder. Fine powder of test, control, and reference substances was stored in a -70 ºC freezer until extraction. After thawing, the fine powder was maintained on wet ice prior to extraction.

6.2 Preparation of Soy Extracts

Aqueous extracts were prepared at the Paul-Ehrlich-Institut according to the following methodology. Finely ground soybean powders were extracted by shaking in $1\times$ PBST (1 g tissue / 10mL PBST) at 4-8 ºC for 4-5 hours. Two independent extracts were prepared for each sample. The variability (%CV) in the total protein content between independent extractions using this method was demonstrated to be <10%, hence the two extracts were pooled together, clarified by centrifugation at ~13, 000 $\times$ g and passed through a 0.22 μm cellulose acetate filter. The clarified extracts were divided into 10 equal volumes of 750 μl and stored at -70 °C until used. Once thawed, the extracts were maintained on wet ice and used within 6 hours. The protein concentrations of the clarified extracts were determined using a commercially available ready-to-use Bradford reagent according to the manufacturer's instructions (Roti® Nanoquant, Carl Roth GmbH, Germany). The stability of the proteins in aqueous soybean extracts stored at -70°C has not been determined.

Each pooled soybean extract, diluted with Coating buffer to a total protein concentration of 10 μg/ml, was added to a 96-well plate at 100 μl/well and each extract was tested in triplicate.

6.3 Serum

Sera from soy-allergic patients were collected by the Principal Investigator, Dr. Stefan Vieths, with the assistance of clinical partners. Patients were diagnosed as soy allergic on the basis of:

1) a documented case history of anaphylactic reactions to soybean;
2) a positive Double-Blind Placebo Controlled Food Challenge (DBPCFC) to soybean.

A brief summary of the serum collection, preparation, and measurement of soybean specific IgE from each patient is archived with the study 04-01-83-05 file. A total of 26 sera from soy-allergic patients and six sera from non-allergic individuals were collected. Sera from soy-allergic patients were coded numerically from #1 through #26. Sera from non-allergic patients were coded numerically from #27 through #32. The level of total soy-specific IgE was measured using CAP-FEIA (Phadia, Uppsala, Sweden).

6.4 ELISA

The Paul-Ehrlich-Institut laboratory developed and performed the human IgE immunoassays for this study. A Study Specific Work Procedure (Appendix 1) describes a validated ELISA protocol that was followed for testing sera from soybean allergic and non-allergic patients for IgE binding to extracts from test and reference substances.

6.4.1 ELISA Plate Layout

Each 96-well microtiter plate contained soybean extract to create a standard curve, an internal reference soybean extract (Yellow soybean "Hensel – GMO-free", W. Schoenenberger GmbH & Co. KG, Magstadt, Germany), human serum PEI 46-4 (PEI is the designation given to all sera collected at Paul-Ehrlich-Institut) containing soy-specific IgE, which served as a positive control for inter-assay precision, and test, control, or reference soybean sample extracts. Controls utilized for data reduction included non-specific reagent binding (NSB) and non-allergic (NA) serum pool binding (mixture of equal volumes of six sera from non-allergic subjects, PEI 19, 208, 230, 231, 233, and 245).

6.4.2 Generation of a Standard Curve

Soy-specific IgE binding was quantified by use of a soy-specific IgE standard curve and expressed as ng/ml of serum. The standard curve was created by loading serial dilutions of human serum PEI 163 that contained a known amount of soy-specific IgE into wells coated with internal reference soybean extract. The concentration of soy-specific IgE in serum PEI 163 was 36 kU/l as measured by CAP-FEIA. Conversion of IgE concentration expressed as U/ml into ng/ml was done according to the following conversion ratio: 2.4 ng/ml IgE = 1 U/ml. Standard curves were generated with serial 4-fold dilutions of human serum PEI 163 in an incubation buffer and then loading the following concentrations of soy-specific IgE: 21.6, 5.4, 1.35, 0.34, 0.080, and 0.021 ng/ml.

6.4.3 Data Reduction and IgE Binding Quantifications

Plates were read bichromatically at 450 nm with a 630 nm reference wavelength. Optical density (OD) values recorded at 630 nm were

subtracted from OD values recorded at 450 nm for each well to produce reduced OD values using SoftmaxPro software (Molecular Devices), version 2.4.1. Mean values of triplicate ODs from each sample were calculated.

To calculate a limit of detection (LOD) for the standard curve (LOD1), the mean OD values for non-specific binding reagent control (NSB1) added to the wells coated with internal non-transgenic reference soybean extract were subtracted from the OD values obtained for the non-allergic serum pool added to the wells coated with internal non-transgenic reference soybean extract (designated as NA1). For NA1, the standard deviation (SD) of the calculated mean OD values was determined. The LOD1 was calculated as follows: $LOD1 = [Mean\ OD\ (NA1) + 3 \times SD\ (NA1)] - Mean\ OD\ (NSB1)$. The obtained OD values were interpolated versus the standard curve and expressed as ng/ml of IgE.

For each test, control, and reference substance extracts, a specific LOD was calculated (LOD2) as follows. The mean OD values for non-specific binding reagent control added to the wells coated with tested soybean extracts (designated as NSB2) were subtracted from the mean OD values obtained for non-allergic serum pool added to the wells coated with tested soybean extracts (designated as NA2). For NA2, the SD of the calculated mean OD values was determined. The LOD2 was calculated as follows: $LOD2 = [OD\ (NA\ 2) + 3 \times SD\ (NA\ 2)] - OD\ (NSB\ 2)$. The obtained OD values were interpolated versus the standard curve and expressed as ng/ml of IgE.

All data were normalized for non-specific binding reagent control and for non-allergic serum pool.

6.4.4 ELISA Acceptance Criteria

The following criteria were applied to ELISA performance and used to determine if the assay was generating acceptable data:
a) Standard curve: maximum OD value (ODmax) is ≥ 2.0 absorbance units. The LOD 1 is ≤ 0.2 ng/ml (at 1:10 dilution).
b) Positive control serum PEI 46 quantified at 2.2 ng/ml soy-specific IgE with a CV for inter-assay precision of less than 25 % (range 1.6 - 2.8 ng/ml).
c) The LOQ must be greater than both the LOD1 and LOD2.
d) The soy-specific serum IgE levels determined for the soy sample extracts were considered "positive" if the calculated IgE concentrations were larger than LOD 1 and LOD2, and if the %CV for

each triplicate was ≤ 25%. Sera not meeting these criteria were considered to be "negative" for the ELISA assay.

6.5   Data Evaluation and Statistical Analysis

Data evaluation was based on the IgE concentrations calculated for each extract. Values that failed to satisfy the ELISA acceptance criteria were treated as missing values for the purpose of the statistical analysis.

The proposed statistical model for the analysis was a randomized complete block design model with serum as the block and soybean variety as the treatment. The test for non-additivity was done using Tukey's one degree of freedom test for non-additivity (Snedecor and Cochran, 1980). The test was conducted using an SAS macro developed by Oliver Schabenberger (SAS Institute). The non-additivity test p-value < 0.0001 rejected the additivity assumption and, therefore, a randomized complete block design could not be used to analyze the data and consequently an alternate analysis was done.

The alternate statistical analysis consisted of calculating a tolerance interval. A tolerance interval is an interval that one can claim, with a specified degree of confidence, contains at least a specified proportion, p, of an entire sampled population for the parameter measured. Using the IgE binding values obtained for reference soybean extracts a 99% tolerance interval with 95% confidence was calculated. This interval is expected to contain with 95% confidence at least 99% of IgE binding values for the reference population. The test and control substances IgE binding values were then compared to the tolerance interval.

7.0   Control of Bias

Inclusion of a standard curve, positive and negative controls, and a control for inter-assay precision on each plate in addition to soybean extract served as a control of bias in this study.

8.0   Protocol Amendments

The protocol for the validated ELISA was added by Amendment 1 following the completion of the development work. The description of the statistical analysis used for data evaluation was added by Amendment 2 following the completion of method development. There was no negative impact on the study because of these changes.

9.0   Protocol Deviations

Throughout the experimental phase of the study, several protocol deviations occurred. The protocol stated that the fine powder of soybean test, control, and reference

substances was to be stored in a -80 ºC freezer until extraction. The contract facility stored the fine powder in a -70 ºC freezer. There was no impact of this deviation on the study data.

According to the protocol, the LOD 1 and LOD 2 should be determined for each plate. In several cases, the LOD 2 values were not calculated because reduced OD values were outside the standard curve range. However, all data calculated for tested soybean extracts with sera from allergic patients were above at least LOD1 and well within the standard curve. This deviation had no impact on the study data.

10.0 Quality Measures

The following quality measures were employed to ensure the integrity of the study: analytical methods were appropriate for the intended use in the study, validated method was utilized to produce study plan data, and highly trained personnel were involved in the production of the study plan data.

11.0 Results and Discussion

11.1 Sera from Soy Allergic Patients

A total of 26 sera from soy-allergic patients was collected (Table 2). All patients had positive allergic reactions during a DBPCFC with soy. The circulating level of total IgE antibody against soybean proteins was determined for each serum using capsulated hydrolic carrier polymer-fluoroenzyme immunoassay (CAP-FEIA). CAP-FEIA is a detection method which is generally used to assess food-specific IgE concentrations that are indicative of a patient being allergic to an allergenic food (Burks, 2000; Sampson, 2001). Sera from 18 patients had positive levels (>0.35 kU/l) of total IgE against soybean in the CAP-FEIA assay.

All 26 sera were tested for IgE antibody binding in the validated ELISA. Sera from 16 individuals yielded positive (i.e. above LOD) values, with the majority of soy protein extracts. Therefore, ELISA data for only 16 patients were used for the statistical analysis. A good correlation (15/16) was observed between CAP-FEIA values for IgE concentrations of >1 kU/l and positive ELISA tests for total soy-specific IgE against soybean proteins.

Although all sera were obtained from patients with a positive allergic reaction during DBPCFC, some of these patients did not have detectable levels of soy-specific IgE circulating in their serum. The inconsistency between clinical symptoms of soy allergy and the level of soy-specific IgE in serum has been observed in other studies (Perry et al., 2004).

11.2 ELISA Results for the Test and Reference Substances

The results of the ELISA assays are summarized in Tables 3 and 4. Sera from 16 soy-allergic patients yielded positive IgE values for the majority of the soy extracts. IgE values that failed one or more of the assay acceptance criteria were excluded from the statistical analysis.

None of the test, control, or reference substances showed IgE binding to sera from non-allergic patients (data not shown), therefore, this data was not submitted for statistical analysis.

11.3 Comparison of the Levels of IgE Binding Observed for the Test, Control, and Reference Substances

To compare levels of IgE binding, the ELISA values generated for the test, control, and reference substances were subjected to a statistical data evaluation as described in Appendix 2.

The IgE binding values obtained for the reference soybean extracts were used to calculate a 99% tolerance interval for each patient serum. The 99% tolerance interval represents the range of IgE binding for each patient serum to the extracts prepared from the commercial soybean varieties such that 99% of the IgE binding values are expected to fall within this range with a 95% confidence level. The IgE binding values obtained for protein extracts prepared from three SDA soybean varieties and a control variety were compared to the calculated tolerance intervals. All of the IgE values for SDA and control soybeans fall within the established tolerance intervals obtained for each serum (Figure 1 and Appendix 2) with three exceptions. The IgE binding values for serum # 6 to extract from the SDA soybean, MON 87769 and for serum #10 to extracts from SDA soybean MON 87769 and MON 87736 were below the assay's limit of detection (Table 3). None of the tested soybean varieties showed IgE binding to sera from non-allergic patients.

12.0 Conclusions

The results of this study demonstrate that the levels of endogenous soybean allergens in the SDA soybeans and non-transgenic control soybeans are comparable to the levels of endogenous soybean allergens in the soybean varieties that are currently on the market.

13.0 References

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Table 1. Test, Control, and Reference Substance Identification

Sample Identification	Soybean Variety	Regulatory Lot Number	Type
# 1	A211Q-1	REF-0412-15671-S	reference
# 2	A211Q-9	REF-0412-15679-S	reference
# 3	AG0101	REF-0507-16429-S	reference
# 4	AG3905	RDR-0409-15521-S	reference
# 5	AG4403	REF-0507-16428-S	reference
# 6	AG5605	REF-0507-16430-S	reference
# 7	M-SOY 7373	REF-0507-16433-S	reference
# 8	Beck	REF-0507-16425-S	reference
# 9	A2704	REF-0409-15504-S	reference
# 10	ST2788	REF-0409-15512-S	reference
# 11	A2869	REF-0409-15508-S	reference
# 12	A4922	REF-0507-16426-S	reference
# 13	Benning	REF-0504-16203-S	reference
# 14	M-SOY 8411	REF-0507-16434-S	reference
# 15	EXP2303REN	RDR-0503-16016-S	reference
# 16	EXP3103REN	RDR-0503-16017-S	reference
# 17	EXP2702REN	RDR-0503-16018-S	reference
# 18	EXP125	CON-0503-16019-S	reference
# 19	Prospect	CON-0503-16020-S	reference
# 20	Opal	REF-0507-16423-S	reference
# 21	AG1901	REF-0507-16442-S	reference
# 22	DKB20-52	REF-0507-16443-S	reference
# 23	DKB25-51	REF-0507-16445-S	reference
# 24	AG2403	REF-0507-16444-S	reference
# 30	MON 87714	GLP-0511-16694-S	test
# 31	MON 87769	GLP-0511-16696-S	test
# 32	MON 87736	GLP-0511-16697-S	test
# 33	A3525	REF-0409-15502-S	control

Table 2. Characteristics of Sera From Soybean-Allergic Patients

Serum #	CAP-FEIA (kU/l)[1]	ELISA Assay Result[2]	DBPCFC with Soy
#1	<0.35	negative	positive
#2	<0.35	negative	positive
#3	1.40	positive	positive
#4	1.03	positive	positive
#5	0.96	negative	positive
#6	2.10	positive	positive
#7	<0.35	negative	positive
#8	1.18	negative	positive
#9	<0.35	positive	positive
#10	1.97	positive	positive
#11	0.76	negative	positive
#12	<0.35	negative	positive
#13	<0.35	negative	positive
#14	6.42	positive	positive
#15	7.46	positive	positive
#16	<0.35	negative	positive
#17	6.08	positive	positive
#18	3.96	positive	positive
#19	5.43	positive	positive
#20	2.96	positive	positive
#21	<0.35	negative	positive
#22	32.60	positive	positive
#23	1.26	positive	positive
#24	1.15	positive	positive
#25	30.00	positive	positive
#26	25.50	positive	positive

[1]CAP-FEIA values were obtained for total soy-specific IgE
[2]Negative values for ELISA were defined as the values <LOD, positive values were defined as the values >LOD

Monsanto Company
Final Report
Product Characterization Center

Study Plan: 05-01-83-03
MSL: 20553
Page 22 of 42

Table 3. Levels of Soybean-Specific IgE Bound to Protein Extracts Prepared from Test, Control, and Reference Substances for Sera # 1-21

Serum	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20	#21	LOD 1	LOD 2
Extract									ng soy-specific IgE /ml serum													ng/ml at 1:10 dilution	
#1	<LOD	<LOD	2.395	0.701	<LOD	1.006	<LOD	<LOD	1.765	0.727	<LOD	<LOD	<LOD	3.615	3.428	<LOD	1.886	8.615	1.842	1.493	<LOD	0.057	0.040
#2	0.517	<LOD	2.678	0.560	<LOD	0.836	<LOD	<LOD	1.897	0.809	<LOD	<LOD	<LOD	4.067	3.946	<LOD	1.932	10.245	1.741	1.807	<LOD	0.051	0.047
#3	<LOD	<LOD	2.687	0.706	<LOD	0.945	<LOD	<LOD	2.031	0.696	<LOD	<LOD	<LOD	3.635	4.399	<LOD	1.828	8.515	1.602	1.714	<LOD	0.050	0.053
#4	<LOD	<LOD	2.732	0.579	<LOD	0.734	<LOD	<LOD	2.305	0.712	<LOD	<LOD	<LOD	3.584	4.481	<LOD	1.938	10.470	1.266	0.965	<LOD	0.035	0.054
#5	<LOD	<LOD	3.030	0.957	0.774	1.024	<LOD	<LOD	2.121	0.867	<LOD	<LOD	<LOD	3.546	3.891	<LOD	2.185	11.514	1.778	1.817	<LOD	0.057	0.057
#6	<LOD	<LOD	2.933	0.707	<LOD	0.759	<LOD	<LOD	2.184	0.841	<LOD	<LOD	<LOD	4.224	3.446	<LOD	1.746	12.474	1.378	1.494	<LOD	0.037	0.057
#7	<LOD	0.450	2.588	0.758	<LOD	0.718	<LOD	<LOD	2.532	0.837	<LOD	<LOD	<LOD	4.043	4.464	<LOD	1.976	10.369	1.333	1.688	<LOD	0.039	0.032
#8	<LOD	<LOD	2.878	0.703	<LOD	0.708	<LOD	<LOD	1.931	0.659	<LOD	<LOD	<LOD	4.445	3.067	<LOD	1.747	9.964	1.478	1.693	<LOD	0.048	0.029
#9	<LOD	<LOD	3.089	0.621	<LOD	0.976	<LOD	<LOD	2.248	0.562	<LOD	<LOD	<LOD	4.531	4.582	<LOD	1.972	11.206	1.591	1.294	<LOD	0.055	0.049
#10	<LOD	<LOD	3.401	0.741	<LOD	0.741	<LOD	<LOD	1.535	0.920	0.646	<LOD	<LOD	4.666	1.565	<LOD	1.568	10.343	2.015	1.935	<LOD	0.054	0.061
#11	<LOD	<LOD	3.310	1.116	<LOD	<LOD	<LOD	<LOD	2.371	<LOD	<LOD	<LOD	<LOD	5.631	4.588	<LOD	2.211	9.915	1.762	2.188	<LOD	0.092	0.102
#12	<LOD	<LOD	2.209	0.667	<LOD	0.963	<LOD	<LOD	1.547	<LOD	<LOD	<LOD	<LOD	5.034	3.429	<LOD	1.359	9.121	1.173	2.246	<LOD	0.058	0.055
#13	<LOD	<LOD	2.500	0.733	<LOD	0.894	<LOD	<LOD	1.838	0.694	<LOD	<LOD	<LOD	5.573	3.849	<LOD	1.935	10.141	1.663	1.582	<LOD	0.054	0.037
#14	<LOD	<LOD	2.667	0.591	<LOD	0.702	<LOD	<LOD	2.162	0.763	<LOD	<LOD	<LOD	4.622	4.362	<LOD	1.774	8.913	1.291	1.182	<LOD	0.034	0.037
#15	<LOD	<LOD	2.901	0.434	<LOD	0.544	<LOD	0.281	1.435	0.379	0.273	<LOD	<LOD	5.340	2.666	<LOD	1.188	7.420	1.033	1.063	<LOD	0.026	0.004
#16	<LOD	<LOD	2.442	0.572	<LOD	0.584	<LOD	<LOD	1.330	0.472	<LOD	<LOD	<LOD	2.977	2.581	<LOD	1.279	7.406	1.076	1.252	<LOD	0.046	0.007
#17	<LOD	<LOD	2.299	0.484	<LOD	0.635	<LOD	<LOD	1.691	0.588	<LOD	<LOD	<LOD	3.716	4.009	<LOD	1.561	9.793	1.265	1.443	<LOD	0.034	0.017
#18	<LOD	<LOD	3.213	0.728	<LOD	0.924	<LOD	<LOD	2.439	0.808	<LOD	<LOD	<LOD	4.347	4.683	<LOD	2.139	10.635	1.663	1.483	<LOD	0.059	0.043
#19	<LOD	<LOD	2.865	0.505	<LOD	0.534	<LOD	<LOD	2.255	0.660	<LOD	<LOD	<LOD	5.945	4.209	<LOD	2.010	8.975	1.674	1.353	<LOD	0.043	0.023
#20	<LOD	<LOD	2.813	0.530	0.458	0.801	<LOD	<LOD	1.977	0.567	<LOD	<LOD	<LOD	2.682	3.153	<LOD	1.673	7.853	1.406	1.105	<LOD	0.042	0.020
#21	<LOD	<LOD	3.164	0.829	<LOD	1.097	<LOD	<LOD	2.332	0.771	<LOD	<LOD	<LOD	5.032	5.503	<LOD	2.235	10.796	1.869	1.869	<LOD	0.071	0.076

Table 3. Continued from page 22.

Serum	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20	#21	LOD 1	LOD 2
Extract	ng soy-specific IgE /ml serum																					ng/ml at 1:10 dilution	
#22	<LOD	<LOD	3.557	0.935	<LOD	0.992	<LOD	<LOD	2.591	0.855	0.516	<LOD	<LOD	3.813	1.957	<LOD	2.220	11.892	1.882	1.712	<LOD	0.047	0.059
#23	<LOD	<LOD	3.052	0.501	<LOD	0.505	<LOD	<LOD	2.247	0.627	<LOD	<LOD	<LOD	4.126	4.778	<LOD	1.579	11.518	1.029	1.340	<LOD	0.048	0.014
#24	<LOD	<LOD	2.436	<LOD	<LOD	0.634	<LOD	<LOD	1.559	0.641	<LOD	<LOD	<LOD	3.279	3.903	<LOD	1.485	9.167	1.405	1.341	<LOD	0.051	0.050
#30	<LOD	<LOD	2.948	0.886	<LOD	1.030	<LOD	<LOD	2.313	0.838	<LOD	<LOD	<LOD	5.044	4.478	<LOD	2.029	9.137	1.734	1.991	<LOD	0.055	0.060
#31	<LOD	<LOD	3.631	0.946	<LOD	<LOD	<LOD	<LOD	2.389	<LOD	<LOD	<LOD	<LOD	5.667	5.145	<LOD	1.873	11.845	1.587	1.547	<LOD	0.091	0.035
#32	<LOD	<LOD	2.912	0.622	<LOD	0.910	<LOD	<LOD	1.814	<LOD	<LOD	<LOD	<LOD	5.322	4.097	<LOD	1.831	9.070	1.704	1.470	<LOD	0.060	0.019
#33	<LOD	<LOD	2.838	0.818	<LOD	1.090	<LOD	<LOD	2.447	0.511	<LOD	<LOD	<LOD	4.579	4.620	<LOD	2.252	10.665	2.051	1.807	<LOD	0.046	0.039

Table 4.   Levels of Soybean-Specific IgE Bound to Protein Extracts Prepared from Test, Control, and Reference Substances for Sera # 22-26

Serum	#22	#23	#24	#25	#26	LOD1	LOD2
Extract	ng soy-specific IgE /ml serum					ng/ml at 1:10 dilution	
#1	15.028	1.609	1.164	53.447	9.489	0.049	0.033
#2	14.534	1.868	1.099	73.118	12.502	0.050	0.015
#3	14.933	1.991	1.298	69.056	10.790	0.051	0.034
#4	14.085	1.672	1.303	70.419	13.028	0.047	0.037
#5	13.974	1.729	1.442	91.223	11.986	0.041	0.048
#6	13.796	1.397	1.265	155.572	17.841	0.038	0.018
#7	13.263	1.566	1.398	55.843	11.451	0.030	0.028
#8	14.925	1.797	1.238	109.62	11.737	0.029	0.013
#9	13.302	1.433	1.169	78.335	12.028	0.029	0.011
#10	14.869	1.813	1.239	56.878	12.136	0.030	0.042
#11	13.034	1.833	1.347	54.37	12.466	0.031	0.046
#12	13.486	1.916	1.327	57.287	14.978	0.030	0.041
#13	13.562	1.814	1.273	66.153	15.292	0.025	0.024
#14	13.816	1.837	1.348	63.563	11.993	0.034	0.027
#15	14.580	1.421	1.105	58.152	10.138	0.024	n/a [1]
#16	15.327	1.353	1.050	49.832	10.253	0.015	0.003
#17	14.331	1.955	1.255	79.83	12.907	0.020	0.016
#18	12.946	1.381	0.961	64.387	11.844	0.020	n/a
#19	17.664	1.611	1.276	52.421	11.763	0.036	0.029
#20	16.012	1.373	1.245	56.232	6.889	0.036	0.026
#21	16.223	1.775	1.372	67.211	12.005	0.035	0.052
#22	15.047	1.607	1.276	62.02	11.972	0.041	0.034
#23	13.728	2.097	1.336	216	13.190	0.041	0.024
#24	14.158	1.446	1.370	66.211	11.279	0.038	0.028
#30	13.074	1.610	1.000	51.252	11.762	0.031	0.032
#31	14.533	2.006	1.210	50.921	13.251	0.037	0.032
#32	12.645	1.882	1.142	72.167	12.680	0.037	0.012
#33	12.693	1.483	1.118	43.651	14.075	0.058	0.039

[1]n/a - not applicable (could not be calculated because reduced OD were below standard curve)

Monsanto Company
Final Report
Product Characterization Center

Study Plan: 05-01-83-03
MSL: 20553
Page 25 of 42
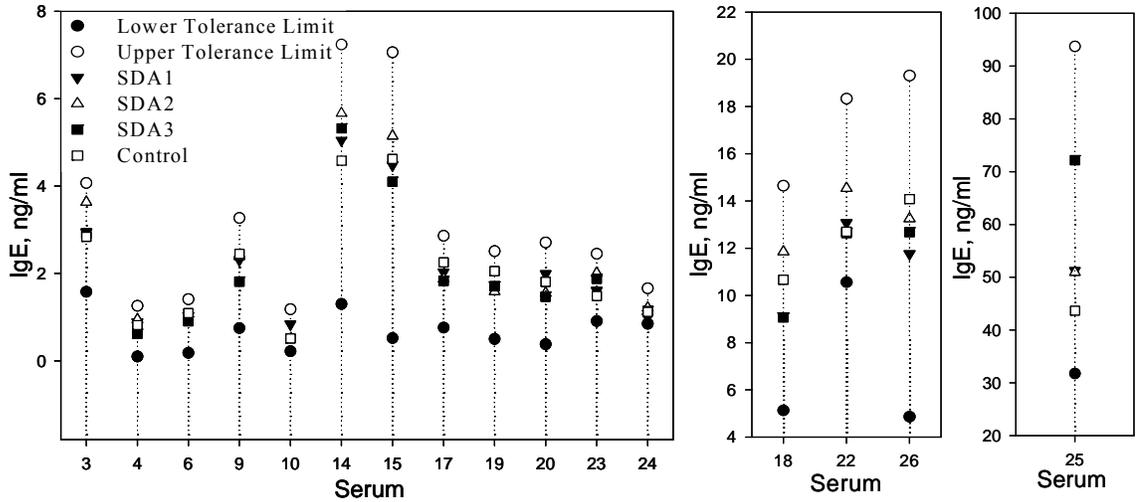
Figure 1. **Comparison of the Levels of IgE Bound to Protein Extracts Obtained for the Test (SDA-containing Soybeans) and Control Soybeans to the 99% Tolerance Interval with a 95% Confidence Level Calculated for 24 Commercial Soybean Varieties**

Sample Identification	Soybean Variety	Regulatory Lot Number
SDA1	MON 87714	GLP-0511-16694-S
SDA2	MON 87769	GLP-0511-16696-S
SDA3	MON 87736	GLP-0511-16697-S
Control	A3525	REF-0409-15502-S

Monsanto Company
Final Report
Product Characterization Center

Study Plan: 05-01-83-03
MSL: 20553
Page 26 of 42

Appendix 1.

Study Specific Work Procedure:
ELISA Protocol for Analysis of the Soybean-Specific Serum IgE Binding to Extracts from the Test, Control, and References Substances

This Study Specific Work Procedure describes the method used by the Paul-Ehrlich-Institute laboratory to extract proteins from soybean seed, test these protein extracts with serum from allergic and non-allergic patients using an ELISA method, and quantitate the level of IgE antibody bound to protein extracts. To ensure optimum extraction of the protein, performance of the assay, and reliability of the results, individual procedures will be preformed as outlined according to the parameters specified.

1. Reagents, buffers and consumables

Consumables and Reagents

- 96-well microtiter plate: Nunc Maxisorp F96 (order # 442404), lot # 083305
- IgE-standard: human serum from soybean-allergic individual PEI 163 (soybean-specific IgE according to Phadia CAP-FEIA 36 kU/L; PEI in-house serum collection) at 1:4 serial dilution; conversion into ng/ml IgE according to 2.4 ng/ml IgE equals 1 U/ml
- Positive human serum control PEI 46-4 (PEI in-house-serum collection)
- Study serum samples: serum of soy-allergic patients at 1:10 standard dilution in incubation buffer or as indicated
- Internal reference soybean extract (IRS): Yellow soybean "Hensel – GMO-free", W. Schoenenberger GmbH & Co. KG, Magstadt, Germany
- Non-allergic (NA) serum pool: mixture of equal volumes of 6 sera from non-atopic subjects (PEI serum bank: PEI 19, 208, 230, 231, 233, 245)
- Biotin conjugated anti-human IgE polyclonal antibody from goat (Kirkegaard & Perry Laboratories; KPL), via Medac, order No. 16-10-04, 0.5 mg; lot No. WJ 138). Reconstitution: with 1 ml double distilled water containing 0.1 % (v/v) sodium azide; aliquot each at 20 µl and store at –20 °C.
- NeutrAvidin-horseradish peroxidase conjugate (Pierce, order No. 31001). Reconstitution: with 1 ml double distilled water; aliquot each at 10 µl and store at –20 °C.

Solutions and Buffers

PBS buffer, 10 mM, pH 7.4,
(138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4)

PBST: PBS containing 0.05 % (v/v) Tween 20

Blocking solution: 2 % (w/v) BSA (Fraction IV) in PBS, pH 7.4

Incubation buffer: 2 % (w/v) BSA in PBS, 0.05 % (v/v) Tween20, pH 7.4

Wash buffer: PBS, 0.05 % (v/v) Tween20, pH 7.4

Coating buffer, 50 mM Carbonate, pH 9.6
(15 mM Na_2CO_3, 35 mM $NaHCO_3$)

Citrate buffer, pH 3.95
(210 mM citric acid monohydrate, 303 mM potassium hydroxide)

TMB-solution, 21 mM TMB
In Methanol

Substrate solution: 3 mM H_2O_2/ 1 mM TMB
(15 mL citrate buffer + 750 µL TMB-solution + 4.95 µL H_2O_2 (30 %))

Stop solution: 3 M sulfuric acid

2. Abbreviations and Definitions

CV	Coefficient of Variation
ELISA	Enzyme-Linked Immunosorbent Assay
HRP	Horse-Radish Peroxidase
IRS	Internal Reference Soybean extract
LOD	Limit Of Detection
LOQ	Limit Of Quantitation
NA serum pool	Non-Allergic serum pool
NSB	Non-Specific Binding
SD	Standard Deviation

3. Extraction of protein from test and reference substances and internal reference soybean

Each test, control, and reference substance will be ground to soybean flour in an analytical blender (Grindomix GM200, Retsch, Germany) and stored at -80 °C until extracted. From each ground test and reference substance, two independent aqueous extracts will be prepared with 1 x PBST as described in section 7 "Preparation of Soy Extracts". Both independent extracts will be pooled, centrifuged at 13,000 *g* and subsequently clarified by passage through a 0.22 µm cellulose acetate filter. The supernatant will be divided into 10 equal volumes of 750 µl and stored at -80 °C until used. Once thawed, extracts will be maintained on wet ice and analyzed within 6 hours. Total protein concentration will be determined using a commercially available ready-to-use Bradford reagent according to the manufacturer's instructions (Roti® Nanoquant, Carl Roth GmbH, Germany). An internal reference soybean extract will be quantified concurrently. The expected concentration of the internal reference soybean extract is 6.6 ± 1 mg/ml (≤ 15 % variance). For analysis by ELISA, test, control, and reference substances extracts will be diluted to 10 µg/ml in Coating buffer.

2. ELISA protocol

2.1 General considerations:
- One test, control, or reference substance extract will be analyzed for IgE-binding with serum samples per microtiter plate.
- Soybean-specific IgE will be quantified versus a soybean-specific IgE standard curve created by loading serial dilution of human serum PEI 163 into wells coated with internal reference soybean extract.
- All serum samples (standards and controls) will be tested in triplicate wells. All incubations will be performed at ambient temperature.
- Soybean extracts and all immunoreagents will be added at 100 µl/well.

Monsanto Company
Final Report
Product Characterization Center

Study Plan: 05-01-83-03
MSL: 20553
Page 29 of 42

- NA serum pool, positive serum control (PEI 46-4), and study serum samples will be diluted 1:10 in incubation buffer. Diluted study serum samples will be pre-incubated for 60 min before loading into plate.

2.2 Standard curve generation:

Standard curves will be generated with serial 4-fold dilutions of soy-specific IgE (soy allergic human serum PEI 163) in incubation buffer as follows: 21.6, 5.4, 1.35, 0.34, 0.080, and 0.021 ng/ml.

2.3 Plate coating:

For standard curve, NSB 1 reagent control, NA 1 negative serum control, and PEI 46-4 positive serum, control each well will be coated with 100 μl of IRS extract at a concentration of 10 μg/ml in Coating buffer.

For study serum samples, NSB 2, and NA 2 negative serum control, each well will be coated with 100 μl of the appropriate test, control, or reference substance extract at a concentration of 10 μg/ml in Coating buffer.

Coated plates will be incubated overnight and then washed 4 times with wash buffer at 300 μl per well.

2.4 Plate loading and development:

Following are the plate loading procedures conducted after plate coating, including blocking, load, incubation, and development steps:

Material	Procedure
Standards, positive and negative controls, study serum samples	- Add 100 μl/well of 2 % BSA in PBS - Incubate for 60-65 minutes - Wash the plate 4 times with 300 μl/well of wash buffer - Load 100 μl/well of standards, positive, negative controls, incubation buffer for non-specific reagent binding control, and study serum samples. - Incubate the plate for 2 hours - Wash 4 times with 300 μl/well of wash buffer

Biotinylated anti-IgE antibody	• Load 100 μl/well KPL goat anti-human IgE/Biotin (lot WJ138) diluted 1:4000 in incubation buffer • Incubate for 60-65 minutes • Wash 4 times with 300 μl/well of wash buffer
NeutrAvidin-HRP conjugate	• Load 100 μl/well of conjugate diluted 1:20,000 in incubation buffer • Incubate for 60-65 minutes • Wash 4 times with 300 μl/well of wash buffer
Plate development	• Add 100 μl/well substrate solution (TMB/Peroxide in citrate buffer) • Incubate for 10 minutes • Add 100 μl/well stop solution

3. Data generation

3.1 Data reduction.

Plates will be read bichromatically at 450 nm with a 630 nm reference wavelength. OD values recorded at 630 nm will be subtracted from OD values recorded at 450 nm for each well to produce a reduced OD values. The OD values will be reduced using Softmax Pro software (Molecular Devices), version 2.4.1. The raw data in the form of the completed data worksheets and the SoftmaxPro printouts will be retained. Mean values of reduced triplicate ODs from each sample will be calculated.

3.2 Soybean specific standard curve.

Reduced mean OD values for NSB control over IRS extract (designated as NSB1) will be subtracted from reduced mean OD values obtained for each standard concentration. The calculated OD values will be plotted as a semi-logarithmic curve versus concentration of the standards. The optimal sigmoidal standard curve will be derived with a 4-parameter logistic model using Softmax Pro software, version 2.4.1.

3.3 Calculations of the Limit of Detection for the standard curve (LOD1).

Reduced mean OD values for NSB1 will be subtracted from reduced mean OD values obtained for NA serum pool over IRS extract (designated as NA1). For NA1 the standard deviation (SD) of calculated mean OD values will be determined. The LOD1 will be calculated by interpolation versus the standard curve:

IgE, [ng/ml] at {[OD (NA 1) + 3 x SD (NA 1)] - OD (NSB 1)}

3.4 Quantification of soy-specific IgE in positive control PEI 46.

Reduced mean OD values for NSB1 will be subtracted from the reduced mean OD values for positive control PEI 46 and interpolated versus the standard curve.

3.5 Quantification of soy-specific IgE in study serum samples for each test, control, and reference substance extracts.

For each test, control, and reference substance extract, a specific LOD will be calculated (LOD2). Reduced mean OD values for non-specific NSB control over test or reference substance extract (designated as NSB2) will be subtracted from reduced mean OD values obtained for NA serum pool over test or reference substance extract (designated as NA2). For NA2, the SD of calculated mean OD values will be determined. The LOD2 will be calculated as follows:

IgE, [ng/ml] at $\{[OD (NA\ 2) + 3 \times SD (NA\ 2)] - OD (NSB\ 2)\}$

For each study serum, mean OD values will be reduced by the reduced OD values of NSB 2 and interpolated versus the standard curve.

4. ELISA acceptance criteria.

ELISA data will be considered valid if the following acceptance criteria are satisfied:

a) <u>Standard curve:</u>

Maximum OD value (ODmax) is ≥ 2.0 absorbance units. The LOD 1 is ≤ 0.2 ng/ml

b) <u>LOQ:</u>
The LOQ is defined as the lowest concentration of soy-specific IgE (ng/ml) that can be determined with a required % CV of $\leq 20\%$ of triplicate measurements. LOQ is derived from a precision profile of the standard curve where % CV of triplicate measurements of the standards plotted versus the logarithm of the concentration. The LOQ must be greater than both the LOD1 and LOD2. The LOD1, LOD2, and LOQ must be determined for each plate.

c) <u>Positive control serum PEI 46:</u>
Positive control serum PEI 46 is quantified at 2.2 ng/ml with a CV for interassay precision of less than 25 % (range 1.6 – 2.8 ng/ml)

The soybean-specific serum IgE level determined for the study serum samples will be considered positive if the following criteria are satisfied:

Monsanto Company
Final Report
Product Characterization Center

Study Plan: 05-01-83-03
MSL: 20553
Page 32 of 42

a) The calculated IgE concentrations are larger than the LOD 1 and LOD2;

b) The % CV for each triplicate is ≤ 25 %;

5. Plate Layout:

An example of the plate layout is as followed:

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Soy Reference Extract** **&** **IgE STD 1** 21.6 ng/ml			**Soy Reference Extract** **&** **PEI 46-4** positive control			**Test substance extract # x** **&** **Study serum sample # 6**			**Test substance extract # x** **&** **Study serum sample#14**		
B	**Soy Reference Extract** **&** **IgE STD 2** 5.4 ng/ml			**Test substance extract # x** **&** **NSB** reagent control			**Test substance extract # x** **&** **Study serum sample # 7**			**Test substance extract # x** **&** **Study serum sample#15**		
C	**Soy Reference Extract** **&** **IgE STD 3** 1.35 ng/ml			**Test substance extract # x** **&** **Non-Allergic serum**			**Test substance extract # x** **&** **Study serum sample # 8**			**Test substance extract # x** **&** **Study serum sample#16**		
D	**Soy Reference Extract** **&** **IgE STD 4** 0.34 ng/ml			**Test substance extract # x** **&** **Study serum sample # 1**			**Test substance extract # x** **&** **Study serum sample # 9**			**Test substance extract # x** **&** **Study serum sample#17**		
E	**Soy Reference Extract** **&** **IgE STD 5** 0.08 ng/ml			**Test substance extract # x** **Study serum sample # 2**			**Test substance extract # x** **&** **Study serum sample#10**			**Test substance extract # x** **&** **Study serum sample #18**		
F	**Soy Reference Extract** **&** **IgE STD 6** 0.02 ng/ml			**Test substance extract # x** **Study serum sample # 3**			**Test substance extract # x** **&** **Study serum sample#11**			**Test substance extract # x** **&** **Study serum sample #19**		
G	**Soy Reference Extract** **&** **NSB** reagent control			**Test substance extract # x** **&** **Study serum sample # 4**			**Test substance extract # x** **&** **Study serum sample#12**			**Test substance extract # x** **&** **Study serum sample #20**		
H	**Soy Reference Extract** **&** **Non-Allergic serum pool**			**Test substance extract # x** **&** **Study serum sample # 5**			**Test substance extract # x** **&** **Study serum sample#13**			**Test substance extract # x** **&** **Study serum sample #21**		

The suggested plate layout can be modified as needed. If more allergic serum is available, another plate will be utilized for the same test or reference substance extract.

Appendix 2.

Statistical Report:
Assessment of Human IgE Binding to SDA Soy, Control, and Reference Soy
Extracts
05-01-83-03

Purpose of the Statistical Analysis

Compare levels of IgE antibody from soy allergic patients for protein extracts prepared from the seeds of SDA soybeans, parental non-transgenic soybeans, and conventional soybeans grown in the United States.

Data

The data for the references, test substances, and the control substance, supplied in an Excel spreadsheet (IgE binding studies – Combined file for stats_10-26-06.xls) were directly read into SAS®, version 9.1, running under Windows XP Professional.

IgE values (ng/ml) were generated from sixteen different serums (individuals) over several different varieties. The varieties consisted of three test substances, one control, and twenty-four references. The varieties are listed in Table 1.

IgE values of zero were treated as missing. An outlier test on all the data was performed using studentized residuals. IgE values with an absolute studentized residual value of greater than six were deemed to be outliers and eliminated from the statistical analysis by designating them as missing values. Table 2 contains the four IgE values which were identified as outliers. All outliers were from serum 25 and this serum had substantially more variability than the other serums even after removal of the four outliers.

Statistical Analysis

The proposed statistical model for the analysis is a randomized complete block design model with serum as the block and variety as the treatment. Since this experiment was not designed as a randomized complete block design it is first necessary to check whether the data satisfy the randomized complete block design additivity assumption. The test for nonadditivity was done using Tukey's one degree of freedom test for nonadditivity

(Snedecor and Cochran, 1980). The test is conducted using a SAS macro developed by Oliver Schabenberger, SAS Institute (1997). The results from the SAS macro are in Table 3. The nonadditivity test p-value < 0.0001 rejects the additivity assumption and thus a randomized complete block design cannot be used to analyze the data.

Since the data cannot be analyzed using a randomized complete block design an alternate analysis was done. The analysis consists of calculating, for the references, a 99% tolerance interval with 95% confidence for each serum and then comparing the test and control substance IgE values to the tolerance interval.

Results

The results from the tolerance interval analysis are in Table 4. The column labeled Result indicates whether the test or control variety IgE value falls within (Yes) or outside (No) the tolerance interval. All the IgE test and control values fall within the tolerance interval.

References

SAS Software Release 9.1 (TS1M3). Copyright[©] 2002-2003 by SAS Institute Inc., Cary, NC.

SAS Macro NonAdd: © Oliver Schabenberger, January 1997.

Snedecor, G. W. and Cochran, W. G. (1980). Statistical Methods, Seventh Edition, pp. 283-287, Iowa State University Press, Ames, Iowa,

Table 1. List of Test Substances, Control Substance, and Reference Varieties

REF	A211Q-1
REF	A211Q-9
REF	AG0101
REF	AG3905
REF	AG4403
REF	AG5605
REF	M-SOY 7373
REF	Beck
REF	A2704
REF	ST2788
REF	A2869
REF	A4922
REF	Benning
REF	M-SOY 8411
REF	EXP2303REN
REF	EXP3103REN
REF	EXP2702REN
REF	EXP125
REF	Prospect
REF	Opal
REF	AG1901
REF	DKB20-52
REF	DKB25-51
REF	AG2403
SDA Test	MON 87714
SDA Test	MON 87769
SDA Test	MON 87736
SDA Control	A3525

Table 2. List of Four Outliers

Type	Variety	Serum	IgE	Residual
REF	AG5605	25	155.572	9.5553
REF	DKB25-51	25	216.000	20.5145
REF	AG4403	25	91.223	6.2858
REF	Beck	25	109.620	10.3826

Table 3. Results from the Nonadditivity Test in the Randomized Complete Block Model

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Block	15	56586.31969	3772.42131	1055.43	<.0001
Tx	27	215.18214	7.96971	2.23	0.0005
nonadd	1	749.98326	749.98326	209.83	<.0001

Monsanto Company
Final Report
Product Characterization Center

Study Plan: 05-01-83-03
MSL: 20553
Page 38 of 42

Table 4. 99% Reference Tolerance Intervals With 95% Confidence and Test Substance IgE Values

Variety	Serum	IgE ng/ml	n	Reference Mean IgE, ng/ml	Reference Minimum IgE, ng/ml	Reference Maximum IgE, ng/ml	Reference Standard Deviation IgE, ng/ml	Lower Tolerance Limit	Upper Tolerance Limit	Result
MON 87714	3	2.948	24	2.8266	2.209	3.557	0.35683	1.58	4.07	Yes
MON 87769	3	3.631	24	2.8266	2.209	3.557	0.35683	1.58	4.07	Yes
MON 87736	3	2.912	24	2.8266	2.209	3.557	0.35683	1.58	4.07	Yes
A3525	3	2.838	24	2.8266	2.209	3.557	0.35683	1.58	4.07	Yes
MON 87714	4	0.886	23	0.6808	0.434	1.116	0.16559	0.10	1.26	Yes
MON 87769	4	0.946	23	0.6808	0.434	1.116	0.16559	0.10	1.26	Yes
MON 87736	4	0.622	23	0.6808	0.434	1.116	0.16559	0.10	1.26	Yes
A3525	4	0.818	23	0.6808	0.434	1.116	0.16559	0.10	1.26	Yes
MON 87714	6	1.030	23	0.7937	0.505	1.097	0.17580	0.18	1.41	Yes
MON 87769	6	.	23	0.7937	0.505	1.097	0.17580	0.18	1.41	.
MON 87736	6	0.910	23	0.7937	0.505	1.097	0.17580	0.18	1.41	Yes
A3525	6	1.090	23	0.7937	0.505	1.097	0.17580	0.18	1.41	Yes
MON 87714	9	2.313	24	2.0135	1.330	2.591	0.36126	0.75	3.27	Yes
MON 87769	9	2.389	24	2.0135	1.330	2.591	0.36126	0.75	3.27	Yes
MON 87736	9	1.814	24	2.0135	1.330	2.591	0.36126	0.75	3.27	Yes

Table 4. Continued from page 38

Variety	Serum	IgE ng/ml	n	Reference Mean IgE, ng/ml	Reference Minimum IgE, ng/ml	Reference Maximum IgE, ng/ml	Reference Standard Deviation IgE, ng/ml	Lower Tolerance Limit	Upper Tolerance Limit	Result
A3525	9	2.447	24	2.0135	1.330	2.591	0.36126	0.75	3.27	Yes
MON 87714	10	0.838	22	0.7025	0.379	0.920	0.13605	0.22	1.18	Yes
MON 87769	10	.	22	0.7025	0.379	0.920	0.13605	0.22	1.18	.
MON 87736	10	.	22	0.7025	0.379	0.920	0.13605	0.22	1.18	.
A3525	10	0.511	22	0.7025	0.379	0.920	0.13605	0.22	1.18	Yes
MON 87714	14	5.044	24	4.2697	2.682	5.945	0.85171	1.30	7.24	Yes
MON 87769	14	5.667	24	4.2697	2.682	5.945	0.85171	1.30	7.24	Yes
MON 87736	14	5.322	24	4.2697	2.682	5.945	0.85171	1.30	7.24	Yes
A3525	14	4.579	24	4.2697	2.682	5.945	0.85171	1.30	7.24	Yes
MON 87714	15	4.478	24	3.7891	1.565	5.503	0.93762	0.52	7.06	Yes
MON 87769	15	5.145	24	3.7891	1.565	5.503	0.93762	0.52	7.06	Yes
MON 87736	15	4.097	24	3.7891	1.565	5.503	0.93762	0.52	7.06	Yes
A3525	15	4.620	24	3.7891	1.565	5.503	0.93762	0.52	7.06	Yes
MON 87714	17	2.029	24	1.8094	1.188	2.235	0.30109	0.76	2.86	Yes
MON 87769	17	1.873	24	1.8094	1.188	2.235	0.30109	0.76	2.86	Yes
MON 87736	17	1.831	24	1.8094	1.188	2.235	0.30109	0.76	2.86	Yes

Monsanto Company
Final Report
Product Characterization Center

Study Plan: 05-01-83-03
MSL: 20553
Page 40 of 42

Table 4. Continued from page 39

Variety	Serum	IgE ng/ml	n	Reference Mean IgE, ng/ml	Reference Minimum IgE, ng/ml	Reference Maximum IgE, ng/ml	Reference Standard Deviation IgE, ng/ml	Lower Tolerance Limit	Upper Tolerance Limit	Result
A3525	17	2.252	24	1.8094	1.188	2.235	0.30109	0.76	2.86	Yes
MON 87714	18	9.137	24	9.8858	7.406	12.474	1.36570	5.13	14.65	Yes
MON 87769	18	11.845	24	9.8858	7.406	12.474	1.36570	5.13	14.65	Yes
MON 87736	18	9.070	24	9.8858	7.406	12.474	1.36570	5.13	14.65	Yes
A3525	18	10.665	24	9.8858	7.406	12.474	1.36570	5.13	14.65	Yes
MON 87714	19	1.734	24	1.5090	1.029	2.015	0.28816	0.50	2.51	Yes
MON 87769	19	1.587	24	1.5090	1.029	2.015	0.28816	0.50	2.51	Yes
MON 87736	19	1.704	24	1.5090	1.029	2.015	0.28816	0.50	2.51	Yes
A3525	19	2.051	24	1.5090	1.029	2.015	0.28816	0.50	2.51	Yes
MON 87714	20	1.991	24	1.5441	0.965	2.246	0.33542	0.38	2.71	Yes
MON 87769	20	1.547	24	1.5441	0.965	2.246	0.33542	0.38	2.71	Yes
MON 87736	20	1.470	24	1.5441	0.965	2.246	0.33542	0.38	2.71	Yes
A3525	20	1.807	24	1.5441	0.965	2.246	0.33542	0.38	2.71	Yes
MON 87714	22	13.074	24	14.4426	12.946	17.664	1.11418	10.56	18.33	Yes
MON 87769	22	14.533	24	14.4426	12.946	17.664	1.11418	10.56	18.33	Yes
MON 87736	22	12.645	24	14.4426	12.946	17.664	1.11418	10.56	18.33	Yes
A3525	22	12.693	24	14.4426	12.946	17.664	1.11418	10.56	18.33	Yes

Table 4. Continued from page 40

Variety	Serum	IgE ng/ml	n	Reference Mean IgE, ng/ml	Reference Minimum IgE, ng/ml	Reference Maximum IgE, ng/ml	Reference Standard Deviation IgE, ng/ml	Lower Tolerance Limit	Upper Tolerance Limit	Result
MON 87714	23	1.610	24	1.6789	1.353	2.097	0.22144	0.91	2.45	Yes
MON 87769	23	2.006	24	1.6789	1.353	2.097	0.22144	0.91	2.45	Yes
MON 87736	23	1.882	24	1.6789	1.353	2.097	0.22144	0.91	2.45	Yes
A3525	23	1.483	24	1.6789	1.353	2.097	0.22144	0.91	2.45	Yes
MON 87714	24	1.000	24	1.2565	0.961	1.442	0.11576	0.85	1.66	Yes
MON 87769	24	1.210	24	1.2565	0.961	1.442	0.11576	0.85	1.66	Yes
MON 87736	24	1.142	24	1.2565	0.961	1.442	0.11576	0.85	1.66	Yes
A3525	24	1.118	24	1.2565	0.961	1.442	0.11576	0.85	1.66	Yes
MON 87714	25	51.252	20	62.7383	49.832	79.830	8.56558	31.75	93.73	Yes
MON 87769	25	50.921	20	62.7383	49.832	79.830	8.56558	31.75	93.73	Yes
MON 87736	25	72.167	20	62.7383	49.832	79.830	8.56558	31.75	93.73	Yes
A3525	25	43.651	20	62.7383	49.832	79.830	8.56558	31.75	93.73	Yes
MON 87714	26	11.762	24	12.0815	6.889	17.841	2.07291	4.86	19.31	Yes
MON 87769	26	13.251	24	12.0815	6.889	17.841	2.07291	4.86	19.31	Yes
MON 87736	26	12.680	24	12.0815	6.889	17.841	2.07291	4.86	19.31	Yes
A3525	26	14.075	24	12.0815	6.889	17.841	2.07291	4.86	19.31	Yes

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