Report Title

Quantitative ELISA Assessment of Human IgE Binding to MON 87705, Control, and Reference Soybean Using Sera from Soybean-Allergic Subjects

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This project does not meet the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160.

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Summary of Quality Control Review

This report was checked to ensure that it accurately reflects the raw data. The raw data were audited for compliance with the Monsanto Company Guidelines for Keeping Research Records (GRR 10/1/99), and where applicable, to Monsanto SOPs.

Date: 07/02/09

Quality Assurance Specialist
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Study Certification

This summary is an accurate and complete representation of the regulatory study activities

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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
IRS	Internal Reference Soybean extract
LOD	Limit of Detection
NA serum pool	Non-Allergic serum pool
NSB	Non-Specific Binding
OD	Optical Density
PEI	Paul-Ehrlich Institut
PBST	Phosphate Buffered Saline (containing Tween-20)
SD	Standard Deviation

[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

The purpose of this study was to quantitatively evaluate the soybean-specific IgE antibody in sera from clinically documented soybean allergic subjects. A quantitative evaluation of soybean-specific IgE provides an estimate of the endogenous allergens present in soybean seed. Protein extracts prepared from seeds of MON 87705, a conventional soybean control (variety A3525), and 17 commercial soybean varieties (references) were evaluated. The reference soybean varieties were used to establish the range for soybean-specific IgE binding. The reference varieties are commercially available and included high protein, high oil, and food-grade (tofu) soybean that are already on the market and are being used for human consumption.

Sera from 13 clinically documented, soybean-allergic subjects and five non-allergic subjects were used to assess IgE binding to each soybean extract. Only soybean-allergic subjects with a documented case history of soybean allergy with anaphylaxis and a positive Double-Blind Placebo Controlled Food Challenge (DBPCFC) were included as soybean positive subjects in this study.

Aqueous extracts were prepared from the ground soybean seeds of MON 87705, the conventional soybean control, and reference varieties. These extracts were then analyzed for soybean-specific IgE antibody binding by a validated enzyme-linked immunosorbent assay (ELISA). Each soybean extract was tested in triplicate. Soybean-specific IgE binding was quantified by interpolation against a soybean-specific IgE standard curve and was expressed as ng of IgE/ml of serum.

The IgE binding values obtained for the 17 reference soybean extracts were used to calculate a 99% tolerance interval for each subject's serum. The IgE binding values obtained for extracts prepared from MON 87705 and the conventional soybean control were compared to the tolerance interval derived for each serum. All of the IgE binding values for MON 87705 and the control were within the reference soybean tolerance limits for each subject's serum (Figure 1). None of the soybean varieties showed IgE binding to sera from non-allergic subjects.

The results of this assessment demonstrate that soybean-specific IgE binding to endogenous allergens in MON 87705 and the conventional soybean control are comparable with the IgE binding to commercially available soybean varieties currently on the market.

2.0 Introduction

Monsanto Company has developed soybean MON 87705 to generate nutritionally-improved soybean oil with decreased levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid) and increased levels of oleic acid (18:1). Specifically, MON 87705 uses gene suppression technology to decrease the RNA levels of *FATB* and *FAD2*.

MON 87705 also contains the 5-enolpyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium sp*. strain CP4 (*cp4 epsps*). Expression of the gene product, CP4 EPSPS protein, renders the soybean plant tolerant to glyphosate which is the active ingredient in the Roundup® family of agricultural herbicides.

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 did not cause an unintended change in the levels of endogenous allergenic proteins. This question can be addressed by comparing levels of soybean-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; Holzhauser et al., 2008).

Validated and calibrated ELISA assays were utilized in this study to determine the levels of endogenous soybean allergens in MON 87705, the conventional soybean control, (A3525), and in 17 commercial soybean varieties that are currently on the market.

3.0 Purpose

The purpose of this study was to evaluate the quantitative soybean-specific IgE antibody binding from soybean-allergic subjects to protein extracts prepared from the seeds of MON 87705 and conventional soybeans. This study was conducted on a contractual basis with the Paul Ehrlich Institut (PEI), Langen, Germany.

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

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Coeur, MO) to the Principal Investigator at Paul-Ehrlich-Institut where they were stored in a -20 ºC freezer.

4.1 Test Substance

The test substance was seed from MON 87705 soybean in an A3525 background (Orion ID# 10000885).

4.2 Control Substances

The control substance was seed from conventional soybean variety, A3525, which has a genetic background similar the test substance (Orion ID# 10000880).

4.3 Reference Substances

The reference substances were 17 commercially available soybean varieties (See Table 1 for identifiers).

4.4 ELISA Assay Internal Reference

The PEI internal reference substance (IRS) was conventional seed from yellow soybean "Hensel – GMO-free", W. Schoenenberger GmbH & Co. KG, Magstadt, Germany.

4.5 Characterization of Test, Control, and Reference Substances

The identity of the seed from MON 87705 test substance and the absence of the MON 87705 in the control substance were confirmed by event specific polymerase chain reaction (PCR). Copies of the "verification of identity" records for the seed from test and control substances are archived with this study.

Monsanto Chain of Custody (COC) records, denoting the assigned Monsanto Orion ID number served as the identification of the reference substances as conventional soybean varieties.

4.6 Sera

Sera for this experiment contained soybean-specific IgE antibody. The sera were collected from soybean-allergic subjects prior to this experiment by the principal investigator with the assistance of his clinical partners. The subjects had been diagnosed as soybean 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 subject is archived with the study file. A total of 20 sera from soybean-allergic subjects met the criteria for inclusion in the study.

Sera from 5 non-allergic individuals were also collected from either the clinical partners or obtained from the commercial supplier, PlasmaLab. Sera from soybean-allergic subjects were coded numerically as shown in Table 2. The level of total soybean-specific IgE was measured for screening purposes using capsulated hydrolic carrier polymer-fluoroenzyme immunoassay (CAP-FEIA) as shown in Table 2 (Phadia, Uppsala, Sweden).

5.0 Analytical Methods

5.1 Grinding of Soybean Seed

Seeds from the test, control, and reference substances were roughly ground at Monsanto and transported on dry ice to PEI. The roughly ground seed was stored in a -20 ºC freezer until it was re-ground to a fine powder in the PEI laboratories. The fine powder was stored in a -80 ºC freezer until extraction. After thawing, the fine powder was maintained on wet ice prior to extraction.

5.2 Preparation of Soybean Extracts

Aqueous protein extracts were prepared at PEI according to the following methodology. Finely ground, raw, full-fat soybean samples were extracted by shaking in 1× PBST (1 g tissue / 10mL PBST) at 4-8 ºC for 4-5 hours. Two independent extracts were prepared for each sample and pooled together, clarified by centrifugation at ~13, 000 X g and passed through a 0.22 µm cellulose acetate filter. The variability (%CV) in the total protein content between independent extractions using this method was demonstrated to be <10%. The clarified extracts were divided into 10 equal volumes of 750 µl and stored at -80 °C until used. Once thawed, the extracts were maintained on wet ice and used within 6 hours. The total protein concentrations in the clarified extracts were determined using a commercially available ready-to-use Bradford reagent according to the manufacturer's instructions. The stability of total protein in aqueous soybean extracts stored at -80°C has not been determined. However, test results at Monsanto support that ground soybean seed is stable when stored at -80 ºC, as indicated by a storage stability study where the concentration of immunoreactive CP4 protein remained constant after 335 days in storage at -80 ºC (Bhakta et al., 2001). Each pooled soybean extract, diluted with coating buffer to a total protein concentration of 10 µg/ml, was tested in triplicate wells and was added to a 96-well plate at 100 µl/well.

5.3 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 the amount of soybean-specific IgE in sera from soybean allergic and non-allergic subjects to extracts from test, control, and reference substances.

5.3.1 ELISA Plate Design

Each 96-well microtiter plate contained a standard curve, an internal reference soybean extract (Hensel), and human serum PEI 46 (PEI was the designation given to all sera collected at Paul-Ehrlich- Institut) containing soybean-specific IgE, which served as a positive control for inter-assay precision. Each plate also contained the appropriate test, control or reference soybean sample extracts. Controls utilized for data reduction included non-specific reagent binding (NSB) and IgE binding from the non-allergic (NA) serum pool. A mixture of equal volumes of five sera from non-allergic subjects were used to create the non-allergic serum pool (PEI designations; NA1, NA2, NA5, C, and E).

5.3.2 Standard Curve

Soybean-specific IgE binding was quantified by use of a soybean-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 contains a known amount of soybean-specific IgE into wells coated with internal reference soybean extract. Concentration of soybean-specific IgE in serum PEI 163 was 36 kU/l as measured by CAP-FEIA (Rice and Bannon, 2006). 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 soybean-specific IgE onto 96-well microtiter plates: 21.6, 5.4, 1.35, 0.34, 0.084, and 0.021 ng/ml.

5.3.3 Quantifying IgE Binding and Data Reduction

Plates were read bi-chromatically 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 Softmax Pro software (Molecular Devices ver. 5.2 Rev. C). 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 x 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 extract, a specific LOD was calculated (LOD2). 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 nonallergic 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 x 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.

5.3.4 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) was ≥ 1.5 absorbance units. The LOD1 was ≤ 0.2 ng/ml (at 1:10 dilution).

b) Positive control serum PEI 46 quantified at 3.31 ng/ml soybean-specific IgE with a CV for inter-assay precision of less than 25 % (range 2.48 – 4.14 ng/ml).

c) The minimum LOQ must be greater than LOD1 and LOD2.

d) The soybean-specific serum IgE levels determined for the soybean 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.

5.4 Statistical Analysis

Data evaluation was based on the IgE concentrations in each serum calculated for each extract (Appendix 2). 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 < 0.05 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 99% tolerance interval with 95% confidence for individual sera using the IgE binding values obtained from reference soybean extracts. The test and control substance IgE binding values were then compared to the tolerance interval (Figure 1).

6.0 Control of Bias

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

7.0 Protocol Amendments

The Study Specific ELISA Procedure, "ELISA for Measuring the Human IgE Binding Potential to Protein Extracts Prepared from Soybean Seed" reported in Appendix 1 is the same as used in REG-09-064, but was not signed separately prior to the start of REG-09-116. There was no negative impact on the study because of this change.

The description of work in sections 6.3.1, 6.3.2, and appendices 2 and 3 in the study protocol REG-09-116 referred to study activities supporting the western blot analysis of test and control substances. The western blot study activities were removed from study protocol REG-09-116 and were conducted as separate study activities. The 1D western blot analysis was conducted and reported under study REG-09-301. The 2D western blot analysis was conducted and reported under study REG-09-292. There was no negative impact on the study because of this change.

8.0 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 procedure was utilized to produce study plan data, the identities of the test and control substances were confirmed by event specific polymerase chain reaction assays, and highly trained personnel were involved in the production of the study plan data.

9.0 Results and Discussion

This study is an evaluation of the IgE binding potential of soybean-specific IgE antibody from soybean-allergic subjects to protein extracts prepared from the seeds of MON 87705 and conventional soybeans. The results of a quantitative assessment of IgE binding to soybean are reported herein.

9.1 Sera from Soybean Allergic Subjects

A total of 20 sera from soybean-allergic subjects were collected (Table 2). All subjects had clinically positive allergic reactions during a DBPCFC with soybean. The level of total IgE antibody against soybean proteins was determined for each serum using CAP-FEIA. CAP-FEIA is a detection method which is generally used to assess food-specific IgE concentrations that are indicative of a subject being allergic to an allergenic food (Burks, 2000; Sampson, 2001). Sera from 14 subjects had positive levels (>0.35 kU/l) of total IgE against soybean in the CAP-FEIA assay. All 20 sera were tested for IgE antibody binding in the validated ELISA.

Sera from 13 individuals yielded positive values (i.e. at or above LOQ) with all of the soybean extracts. A good correlation (12/13) was observed between CAP-FEIA values for IgE concentrations of >1 kU/l and consistently positive ELISA tests for total soybean-specific IgE. Although each of the 20 sera were obtained from subjects with a positive allergic reaction during DBPCFC (Table 2), some of these subjects did not have detectable levels of soybean-specific IgE circulating in their serum. The inconsistency between clinical symptoms of soybean allergy and the level of soybean-specific IgE in serum has been observed in other studies (Perry et al., 2004).

9.2 ELISA Results for Test, Control, and Reference Substances

The results of the ELISA assays are summarized in Table 3. Sera from 13 soybean-allergic subjects yielded positive IgE values for all of the soybean extracts and were included in the statistical analysis. Sera MS01, MS02, MS03, MS04, MS10, MS12, and MS15 had IgE binding values below the LOQ for at least three extracts (Table 4); IgE binding values below LOQ do meet study criteria for positive results. Therefore, IgE binding values from these sera were considered an incomplete data set and were excluded from the statistical analysis.

The IgE value for serum ME03 against a reference substance (soybean extract #3) was a statistical outlier (see Statistical Report, Appendix 2) and was considered a missing value and removed from the calculation of the tolerance interval.

Two sera, KB1 and MS05, produced IgE binding values that were below the LOQ (Table 3) for the control substance (soybean extract #18). Since they are not part of the tolerance interval calculation for the soybean references, these two values are included in Figure 1.

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

9.3 Comparison of IgE Binding for Test, Control, and Reference Soybean Extracts

To compare IgE binding for each of the 13 positive sera, 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 17 reference soybean extracts were used to calculate a 99% tolerance interval for each subject's serum. The 99% tolerance interval represents the range of IgE binding for each subject's serum to the reference soybean extracts. The tolerance interval describes the value range that includes 99% of the IgE binding values and that has a statistically predicted 95% confidence level. The IgE binding values obtained for extracts prepared from MON 87705 and the conventional soybean control were compared to the

tolerance interval derived for each serum. All of the IgE binding values obtained for MON 87705 and the control soybean are within the reference soybean tolerance limits for each subject's serum (Figure 1). None of the soybean varieties showed IgE binding to sera from non-allergic subjects.

10.0 Conclusions

The results of this study demonstrate that the levels of endogenous soybean allergens in the MON 87705 and the conventional soybean control, A3525, are comparable to the levels of endogenous soybean allergens in soybean varieties that are currently on the market.

11.0 References

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Table 1. Identification of Test, Control, and Reference Substances

Study Sample Identification	Soybean Variety	Regulatory Orion Identification	Type
1	A4922	10001425	Reference
2	A5427	10001395	Reference
3	Beck	10001424	Reference
4	Dwight	10001434	Reference
5	Hutcheson	10001432	Reference
6	M-SOY 8411	10001430	Reference
7	Pioneer 93B15	10001304	Reference
8	Stewart 3454	10001435	Reference
9	Stine ST2788	10001133	Reference
10	EXP125	10001433	Reference
11	Opal	10001431	Reference
12	A2553	10001295	Reference
13	A1900	10001299	Reference
14	A2442	10001297	Reference
15	A2824	10001294	Reference
16	AJB2501KOC	10001503	Reference
17	A241QT-211	10001504	Reference
18	A3525	10000880	Control
19	MON 87705	10000885	Test

Table 2. Characteristics of Sera from Soybean-Allergic Subjects

Serum ID	CAP-FEIA (kU/l)[1]	IgE ELISA Result	DBPCFC with Soybean
KB 1	0.68	Positive	Positive
KB 2	2.64	Positive	Positive
ME 1	25.0	Positive	Positive
ME 2	<100	Positive	Positive
ME 3	38.7	Positive	Positive
MS 01	0.09	Negative[2]	Positive
MS 02	0.17	Negative[2]	Positive
MS 03	0.23	Negative[2]	Positive
MS 04	1.05	Negative[2]	Positive
MS 05	1.73	Positive	Positive
MS 06	4.62	Positive	Positive
MS 07	4.70	Positive	Positive
MS 08	7.12	Positive	Positive
MS 09	2.76	Positive	Positive
MS 10	0.28	Negative[2]	Positive
MS 11	12.7	Positive	Positive
MS 12	0.06	Negative[2]	Positive
MS 13	2.10	Positive	Positive
MS 14	1.87	Positive	Positive
MS 15	0.02	Negative[2]	Positive

[1]CAP-FEIA values were obtained for total soybean-specific IgE.
[2]These sera had 3 or more IgE binding values against the 17 conventional soybean references below the ELISA limit of quantitation (LOQ)[3].
[3]LOQ is greater than LOD1 and LOD2.

Table 3. Soybean-Specific IgE Bound to Protein Extracts Prepared from Test, Control, and Reference Substances for Soybean Allergic Sera.

	Serum									
Extract	KB 1	KB 2	ME 1	ME 2	ME 3	MS05	MS06	LOQ	LOD1	LOD2
								ng/ml at 1:10 dilution		
1	0.677	3.686	57.419	200.754	304.025	0.720	7.364	0.035	0.034	0.028
2	0.675	3.272	60.357	205.103	332.570	0.551	7.372	0.042	0.041	0.038
3	0.552	2.589	58.930	154.827	158.722	0.477	5.999	0.028	0.027	0.023
4	0.684	1.720	60.012	167.993	186.808	0.696	6.276	0.034	0.033	0.030
5	0.610	2.933	60.000	166.297	198.268	0.626	6.838	0.035	0.034	0.022
6	0.820	3.666	71.013	194.110	221.817	0.806	6.473	0.068	0.034	0.067
7	0.909	4.172	72.189	196.566	227.815	0.849	6.030	0.042	0.038	0.041
8	0.766	3.369	66.549	187.269	234.202	0.665	6.244	0.032	0.031	0.025
9	0.687	1.508	71.386	156.588	234.571	0.677	6.704	0.046	0.045	0.030
10	0.839	3.486	90.108	231.531	353.648	0.752	7.519	0.049	0.048	0.044
11	0.862	1.828	73.225	166.311	257.072	0.840	6.643	0.040	0.039	0.034
12	0.840	3.123	62.901	217.216	264.275	0.905	5.658	0.043	0.042	0.041
13	1.044	4.009	74.894	176.490	352.677	0.914	6.865	0.051	0.044	0.050
14	0.693	4.326	62.627	199.664	279.766	0.791	6.074	0.050	0.049	0.040
15	0.829	3.580	66.639	217.984	321.875	0.790	5.809	0.053	0.052	0.040
16	0.796	4.180	74.802	205.931	272.795	0.826	6.865	0.045	0.044	0.035
17	0.631	3.311	75.616	241.355	264.880	0.627	6.027	0.061	0.060	0.032
18	0.850[2]	3.055	73.130	143.288	303.569	0.864[2]	6.284	0.096	0.042	0.095
19	0.906	3.591	67.748	240.793	298.539	0.977	6.871	0.045	0.044	0.038

[2] Values are shown here even though they are below the LOQ at a 1:10 dilution because they are control substance.

Table 3 Continued. Soybean-Specific IgE Bound to Protein Extracts Prepared from Test, Control, and Reference Substances for Soybean Allergic Sera.

	Serum								
Extract	**MS07**	**MS08**	**MS09**	**MS11**	**MS13**	**MS14**	**LOQ**	**LOD1**	**LOD2**
	ng/ml at 1:10 dilution								
1	4.440	6.594	12.515	12.774	1.628	1.211	0.035	0.034	0.028
2	2.133	7.019	15.624	17.410	1.158	1.124	0.042	0.041	0.038
3	3.292	6.336	11.983	11.589	1.362	1.008	0.028	0.027	0.023
4	5.673	6.530	13.982	10.261	1.074	1.409	0.034	0.033	0.030
5	3.953	6.321	13.037	13.260	1.425	1.041	0.035	0.034	0.022
6	6.279	7.432	14.021	14.077	1.715	1.335	0.068	0.034	0.067
7	5.488	7.426	13.560	11.301	1.860	1.552	0.042	0.038	0.041
8	6.032	7.058	13.985	11.825	1.547	1.255	0.032	0.031	0.025
9	2.345	7.097	18.217	9.757	1.624	1.184	0.046	0.045	0.030
10	5.206	7.253	14.293	12.427	1.683	1.315	0.049	0.048	0.044
11	3.682	7.510	14.286	8.547	1.722	1.374	0.040	0.039	0.034
12	3.566	6.881	14.770	9.666	1.734	1.314	0.043	0.042	0.041
13	5.002	7.977	16.996	11.662	1.990	1.573	0.051	0.044	0.050
14	4.965	7.150	14.481	12.643	1.688	1.094	0.050	0.049	0.040
15	4.987	6.978	15.204	11.328	1.687	1.215	0.053	0.052	0.040
16	5.075	7.134	13.996	13.113	1.697	1.318	0.045	0.044	0.035
17	4.407	6.273	14.639	10.668	1.479	1.069	0.061	0.060	0.032
18	3.100	7.486	14.896	9.795	1.777	1.363	0.096	0.042	0.095
19	5.214	7.374	14.878	11.352	1.911	1.399	0.045	0.044	0.038

Table 4. Soybean-Specific IgE Bound to Protein Extracts Prepared from Test, Control, and Reference Substances for Soybean Allergic Sera that were Considered Negative[1]

	Serum									
Extract	**MS01**	**MS02**	**MS03**	**MS04**	**MS10**	**MS12**	**MS15**	**LOQ**	**LOD1**	**LOD2**
	ng/ml at 1:10 dilution							ng/ml at 1:10 dilution		
1	0.027	0.013	0.033	0.042	0.026	0.011	0.014	0.035	0.034	0.028
2	0.032	0.023	0.038	0.043	0.038	0.015	0.028	0.042	0.041	0.038
3	0.021	0.011	0.024	0.032	0.023	0.004	0.010	0.028	0.027	0.023
4	0.027	0.011	0.028	0.047	0.039	0.010	0.013	0.034	0.033	0.030
5	0.020	0.007	0.025	0.035	0.026	0.000	0.008	0.035	0.034	0.022
6	0.032	0.014	0.036	0.054	0.037	0.006	0.014	0.068	0.034	0.067
7	0.033	0.014	0.042	0.066	0.040	0.007	0.020	0.042	0.038	0.041
8	0.025	0.008	0.033	0.046	0.033	n/a	0.014	0.032	0.031	0.025
9	0.030	0.016	0.037	0.043	0.040	0.008	0.012	0.046	0.045	0.030
10	0.033	0.021	0.042	0.055	0.036	0.013	0.019	0.049	0.048	0.044
11	0.028	0.022	0.039	0.056	0.037	0.012	0.016	0.040	0.039	0.034
12	0.036	0.019	0.043	0.065	0.049	0.011	0.017	0.043	0.042	0.041
13	0.047	0.028	0.050	0.071	0.059	0.017	0.030	0.051	0.044	0.050
14	0.028	0.015	0.037	0.051	0.039	0.005	0.017	0.050	0.049	0.040
15	0.037	0.020	0.043	0.064	0.047	0.012	0.018	0.053	0.052	0.040
16	0.027	0.015	0.038	0.060	0.045	0.007	0.017	0.045	0.044	0.035
17	0.015	0.001	0.021	0.040	0.031	n/a	0.009	0.061	0.060	0.032
18	0.030	0.013	0.045	0.066	0.044	0.003	0.013	0.096	0.042	0.095
19	0.037	0.018	0.046	0.071	0.057	0.008	0.015	0.045	0.044	0.038

n/a – Value not reportable due to reduced OD value < 0.00.
[1] Below LOQ for 3 or more reference soybean extracts

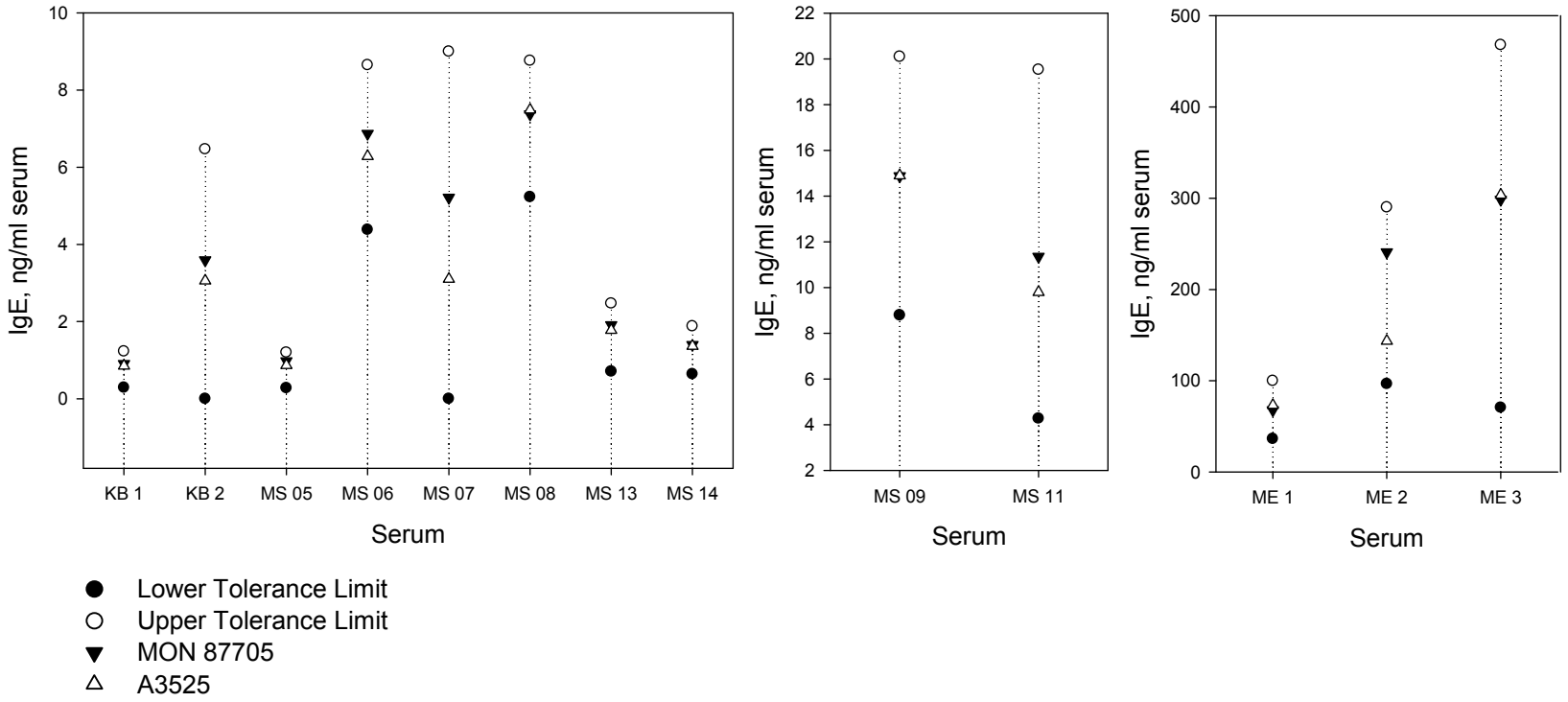


Figure 1. Serum IgE Binding Values for MON 87705, Conventional Control (A3525), and the Tolerance Limits for 17 Conventional References.

The lower and upper tolerance limits for 99% tolerance intervals with 95% confidence for each serum are the result of a tolerance interval analysis for 17 commercial soybean varieties. Lower limits of the tolerance intervals that were calculated as less than zero were reported as zero in the analysis. Data are presented in three graphs due to the difference in IgE concentration range between sera.

Appendix 1. ELISA Study Specific Procedure

ELISA for Measuring the Human IgE Binding Potential to Protein Extracts Prepared From Soybean Seed

1.0 Purpose

This Study Specific Work Procedure (SSWP) describes the ELISA method that will be used to assess the IgE binding potential of IgE antibody from soybean allergic subjects' sera to protein extracts prepared from the seeds of MON 87705 soybeans, parental non-transgenic soybeans and conventional reference soybeans grown in the United States. This SSWP will support a regulatory study, REG-09-064.

2.0 Soybean Sample Preparation

2.1 Grinding of Soybean Seed

At Paul-Ehrlich-Institut roughly ground seeds will be stored in a -20 ºC freezer until study initiation. Upon study initiation, the seeds will be re-ground to a fine powder. Fine powder of test, control, and reference substances will be labeled with the plan number, lot number, preparation date, and preparers' initials and stored in a -80 ºC freezer until extraction. After thawing, the finely ground seed must be maintained on wet ice prior to extraction.

2.2 Preparation of Soybean Extracts

Aqueous extracts will be prepared at Paul-Ehrlich-Institut according to the following methodology. Finely ground, raw, full-fat soybeans will be extracted with shaking in 1× PBST (1 g tissue / 10mL PBST) at 4-8 ºC for 4-5 hours. Extracts will be clarified by centrifugation at ~13,000 × g followed by passage through a 0.22 µm cellulose acetate filter. Two extracts will be prepared separately for each ground seed sample and pooled. The clarified extracts will be divided into several equal volume aliquots and stored at -80 °C until used. Once thawed, the extracts will be maintained on wet ice and used within 6 hours. The protein concentrations of the clarified extracts will be determined using a commercially available ready-to-use Bradford reagent according to the manufacturer's instructions (Roti® Nanoquant, Carl Roth GmbH, Germany). Each aliquot will only be thawed and analyzed once. If repeated analysis is required, another identical aliquot will be thawed and analyzed.

3.0 ELISA Analytical Methods

The Paul-Ehrlich-Institut laboratory will perform the human IgE immunoassays to be used in this study. All sera from soybean allergic and non-allergic subjects will be tested for IgE binding to test, control, and reference substances using the ELISA protocol developed by Dr. Stefan Vieths' laboratory (Paul-Ehrlich-Institut, Langen, Germany).

3.1 General Considerations

3.1.1 One test, control, or reference substance extract will be analyzed for IgE-binding with serum samples per microtiter plate. Standard curve will be run on each plate.

3.1.2 For standard curve and appropriate controls, wells will be coated with internal reference soybean extract (IRS) (Yellow soybean "Hensel – GMO-free", W. Schoenenberger GmbH & Co. KG, Magstadt, Germany).

3.1.3 Soybean-specific IgE will be quantified versus a soybean-specific IgE standard curve created by loading serial dilution of human serum PEI 163 containing 36 kU/l of soybean-specific IgE measured by CAP-FEIA.

3.1.4 All serum samples (standards and controls) will be tested in triplicate wells. All incubations will be performed at ambient temperature.

3.1.5 Soybean extracts and all immunoreagents will be added at 100 µl/well.

3.1.6 Following controls will be used with each standard curve: control for reagents Non-Specific Binding (NSB 1), control for non-specific binding to non-allergic serum pool (NA 1), and PEI 46-4 positive serum which will serve as a positive control for ELISA performance.

3.1.7 Following controls will be used with each test, control, or reference extract: control for reagents Non-Specific Binding (NSB 2), control for non-specific binding to non-allergic serum pool (NA 2).

3.2 Generation of a Standard Curve

Soybean-specific IgE binding will be quantified by use of a soybean-specific IgE standard curve and expressed as ng/ml of serum. The standard curve will be created by loading serial dilutions of human serum PEI 163 that contained a known amount of soybean-specific IgE into wells coated with internal reference soybean extract. Conversion of IgE concentration expressed as U/ml into ng/ml

will be done according to the following conversion ratio: 2.4 ng/ml IgE = 1 U/ml. Standard curves will be generated with serial 4-fold dilutions of human serum PEI 163 in an incubation buffer and then loading 6 dilutions of soybean-specific IgE to create a six-point standard curve.

3.3 Plate Coating

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

For each study serum sample, 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 µg per well.

3.4 Plate Loading and Development

See Table 1 for plate loading procedures conducted after plate coating, including blocking, load, incubation, and development steps.

3.5 Data Reduction

Plates will be read bi-chromatically 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 v5.2revC. 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.

For standard curve, reduced mean OD values for NSB 1 control 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 v5.2revC.

3.6 Calculations of the Limit of Detection for the Standard Curve (LOD 1)

To determined standard curve LOD (LOD 1), mean OD values for NSB1 will be subtracted from mean OD values obtained for NA 1. For NA1 the standard deviation (SD) will be determined. LOD 1 will be calculated using the following equation:

- LOD1 = [OD (NA 1) + 3 x SD (NA 1)] - OD (NSB 1)

The LOD1 will be converted into ng/ml of IgE by interpolation versus the standard curve.

3.7 Quantification of Soybean-Specific IgE in Positive Control PEI 46

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

3.8 Quantification of Soybean-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 (LOD 2). Mean OD values for NSB2 will be subtracted from mean OD values obtained for NA 2. For NA2, the SD will be determined. The LOD 2 will be calculated as follows:

- LOD 2 = [OD (NA 2) + 3 x SD (NA 2)] - OD (NSB 2)

For each study serum, mean OD values will be reduced by the OD values of NSB 2 and interpolated versus the standard curve to be expressed in ng/ml of IgE.

3.9 ELISA Acceptance Criteria.

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

- Standard curve: Maximum OD value (ODmax) is $\geq$ 1.5 absorbance units. The LOD 1 is $\leq$ 0.2 ng/ml

- Limit of Quantitation (LOQ). The LOQ is defined as the lowest concentration of soybean-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.

- Positive control serum PEI 46: Positive control serum PEI 46 is quantified at 3.3 ng/ml with a CV for inter-assay precision of less than 25 % (range 2.48 – 4.14 ng/ml).

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

  The calculated IgE concentrations are larger than the LOD 1 and LOD2;
  The % CV for each triplicate is ≤ 25 %.

Table 1. ELISA Plate Loading and Development	
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
Horseradish peroxidase-labeled anti-IgE antibody	• Load 100 µl/well SBA mouse anti-human IgE (ε-chain specific, lot J681-RB83F) diluted 1:1000 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 10-11 minutes • Add 100 µl/well stop solution

Appendix 2. Statistical Report

Statistical Report For:

Assessment of Human IgE Binding to MON 87705, Control, and Reference Soybean Extracts

Study Number: REG-09-116

Purpose of the Statistical Analysis

The purpose of this analysis is to compare the amount of IgE antibody in sera from soybean allergic subjects that is specific for protein extracts prepared from soybean seeds of MON 87705, a parental control, and 17 conventional references.

Data

The data for the references, MON 87705 test substance (Orion ID 10000885), and the control substance (Orion ID 10000880), supplied in an Excel spreadsheet (REG_09_064_116_121_data_stats040909.xlsx), were directly read into SAS®, version 9.2, running under Windows XP Professional.

IgE values (ng/ml) were generated from thirteen different sera (individuals) over several different soybean varieties. The varieties consisted of one test substance, one control, and seventeen references. The test, control, and reference substances are listed in Table 1.

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 IgE value which was identified as an outlier. The outlier was from serum ME 3 and this serum had substantially more variability than the other sera even after removal of the outlier.

Statistical Analysis

The proposed statistical model for the analysis is a randomized complete block design model with serum as the block and substance 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. If a lower limit value was less than zero the lower limit was set to zero.

Results

The results from the tolerance interval analysis are in Table 4. The column labeled Result indicates whether the test or control substance 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 9.2 (TS1M0). Copyright© 2002-2008 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, Control, and Reference Substances

Type	Substance	Orion ID	Secondary ID
REF	A4922	10001425	1
REF	A5427	10001395	2
REF	Beck	10001424	3
REF	Dwight	10001434	4
REF	Hutcheson	10001432	5
REF	M-SOY 8411	10001430	6
REF	Pioneer 93B15	10001304	7
REF	Stewart 3454	10001435	8
REF	Stine ST2788	10001133	9
REF	EXP125	10001433	10
REF	Opal	10001431	11
REF	A2553	10001295	12
REF	A1900	10001299	13
REF	A2442	10001297	14
REF	A2824	10001294	15
REF	AJB2501KOC	10001503	16
REF	A241QT-211	10001504	17
Omni Control	A3525	10000880	18
Omni Test	MON 87705	10000885	19

Table 2: List of Outliers

Orion ID	Secondary ID	Serum	Type	IgE ng/ml	Studentized Residual
10001424	3	ME 3	REF	158.722	-6.52045

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	12	1587000.284	132250.024	1208.30	<.0001
Tx	18	8651.232	480.624	4.39	<.0001
nonadd	1	29062.853	29062.853	265.53	<.0001

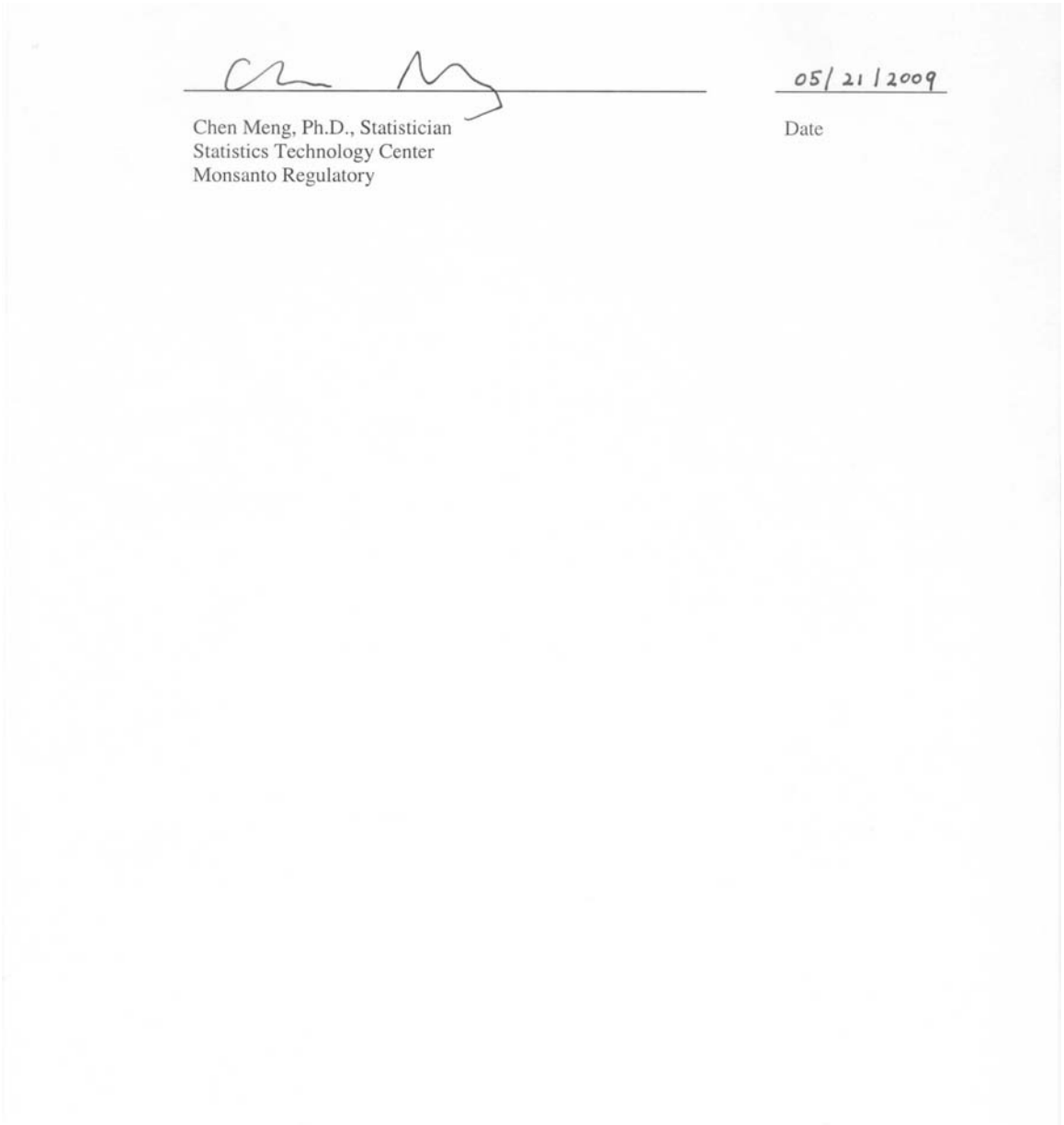
Table 4: 99% Reference Tolerance Intervals With 95% Confidence and Test (Orion ID 10000885) and Control (Orion ID 10000880) Substance IgE Values. N=Number of References.

Serum	Orion ID	Secondary ID	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
KB 1*	10000885	19	0.906	17	0.760	0.552	1.044	0.1244	0.29	1.23	Yes
KB 2	10000880	18	3.055	17	3.221	1.508	4.326	0.8636	0.00	6.47	Yes
	10000885	19	3.591	17	3.221	1.508	4.326	0.8636	0.00	6.47	Yes
ME 1	10000880	18	73.130	17	68.157	57.419	90.108	8.4588	36.36	99.95	Yes
	10000885	19	67.748	17	68.157	57.419	90.108	8.4588	36.36	99.95	Yes
ME 2	10000880	18	143.288	17	193.293	154.827	241.355	25.7431	96.53	290.06	Yes
	10000885	19	240.793	17	193.293	154.827	241.355	25.7431	96.53	290.06	Yes
ME 3	10000880	18	303.569	16	269.192	186.808	353.648	52.0380	70.51	467.87	Yes
	10000885	19	298.539	16	269.192	186.808	353.648	52.0380	70.51	467.87	Yes
MS 05*	10000885	19	0.977	17	0.736	0.477	0.914	0.1221	0.28	1.20	Yes
MS 06	10000880	18	6.284	17	6.515	5.658	7.519	0.5681	4.38	8.65	Yes
	10000885	19	6.871	17	6.515	5.658	7.519	0.5681	4.38	8.65	Yes
MS 07	10000880	18	3.100	17	4.501	2.133	6.279	1.1978	0.00	9.00	Yes
	10000885	19	5.214	17	4.501	2.133	6.279	1.1978	0.00	9.00	Yes
MS 08	10000880	18	7.486	17	6.998	6.273	7.977	0.4698	5.23	8.76	Yes

Serum	Orion ID	Secondary ID	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
	10000885	19	7.374	17	6.998	6.273	7.977	0.4698	5.23	8.76	Yes
MS 09	10000880	18	14.896	17	14.446	11.983	18.217	1.5053	8.79	20.10	Yes
	10000885	19	14.878	17	14.446	11.983	18.217	1.5053	8.79	20.10	Yes
MS 11	10000880	18	9.795	17	11.900	8.547	17.410	2.0293	4.27	19.53	Yes
	10000885	19	11.352	17	11.900	8.547	17.410	2.0293	4.27	19.53	Yes
MS 13	10000880	18	1.777	17	1.593	1.074	1.990	0.2338	0.71	2.47	Yes
	10000885	19	1.911	17	1.593	1.074	1.990	0.2338	0.71	2.47	Yes
MS 14	10000880	18	1.363	17	1.258	1.008	1.573	0.1654	0.64	1.88	Yes
	10000885	19	1.399	17	1.258	1.008	1.573	0.1654	0.64	1.88	Yes

*: The control substance (Orion ID 10000880) IgE value for the serum fell below the limit of quantitation and was not reported.

Statistical Report Submitted By:



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