

**Study Title**

**Additional Bioinformatic Evaluation of DNA Sequences Flanking the 5' Junction of  
the Inserted DNA in MON 87705: Assessment of Putative Polypeptides**

**Authors**

**Andre Silvanovich  
Haidi Tu**

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**Sponsor and Performing Laboratory**

**Monsanto Company  
Regulatory Product Characterization Center  
800 North Lindbergh Blvd.  
St. Louis, MO 63167**

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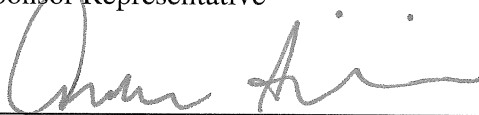
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\_\_\_\_\_  
Submitter

Date: \_\_\_\_\_

  
\_\_\_\_\_  
Daniel J. Jenkins  
Sponsor Representative

Date: 10/20/09

  
\_\_\_\_\_  
Andre Silvanovich  
Author

Date: Oct 20, 2009

### Quality Assurance Statement

Study Title: Additional Bioinformatics Evaluation of DNA Sequences  
Flanking the 5' Junction of the Inserted DNA in MON 87705:  
Assessment of Putative Polypeptides

Study Number: REG-09-471

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

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

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
10/15/2009	Draft Report and Data Audit	10/19/2009	10/19/2009



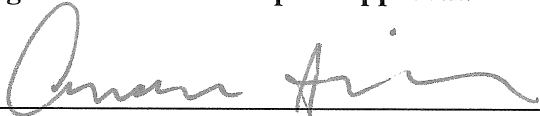
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Quality Assurance Unit  
Monsanto Regulatory, Monsanto Company

Date: 10/20/2009

### Study Certification


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

#### Signatures of Final Report Approval:



Andre Silvanovich, Ph. D.  
Author

Date: Oct 20, 2009



Gary Bannon, Ph.D.  
Lead, Regulatory Product Characterization Center

Date: Oct. 20, 2009

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**Study Information**

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**Facility:** Monsanto Company  
Product Characterization Center  
800 North Lindbergh Blvd.  
St. Louis, MO 63167

**Regulatory Product  
Characterization  
Center Lead:** Gary A. Bannon, Ph.D.

**Sponsor Representative:** Daniel J. Jenkins

**Authors:** Andre Silvanovich, Ph.D.  
Haidi Tu

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## Abbreviations and Definitions

aa	Amino acid
AD_2009	Allergen, gliadin, and glutenin protein sequence database
BLOCKS	A database of amino acid motifs found in protein families
BLOSUM	BLOcks SUBstitution Matrix, used to score similarities between pairs of distantly related protein or nucleotide sequences
<i>E</i> -Score	Expectation score
FASTA	Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
GenBank	A public genetic database maintained by the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA
GI	Gene sequence identification number
NCBI	National Center of Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA
PRT_2009	A protein sequence database derived from GenBank release 169
TOX_2009	Toxin protein sequence database

## 1.0 Summary

Monsanto Company has developed biotechnology-derived soybean, MON 87705, to generate nutritionally-improved soybean oil with decreased levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid), increased levels of oleic acid (18:1) and an associated decrease in linolenic acid (18:2). Specifically, MON 87705 uses gene suppression technology to decrease the levels of two key oil biosynthetic enzymes, FATB1-A and FAD2-1A. Suppression of the FATB1-A enzyme results in a decrease in the levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid), while suppression of the FAD2-1A enzyme results in an increase of oleic acid (18:1).

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) renders the plant tolerant to glyphosate, which is the active ingredient in the Roundup<sup>®</sup> family of agricultural herbicides. Glyphosate binds to the endogenous plant EPSPS enzyme and blocks the biosynthesis of shikimate-3-phosphate, thereby depriving plants of aromatic amino acids (Haslam, 1993; Steinrücken et al., 1984). The CP4 EPSPS protein is structurally similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate relative to endogenous plant EPSPS (Padgett et al., 1996). Introduction of the *cp4 epsps* gene into MON 87705 allows for the production of aromatic amino acids and other metabolites even in the presence of glyphosate (Padgett et al., 1996).

As part of a comprehensive safety assessment, bioinformatic analyses were performed to assess the potential for allergenicity, toxicity, or biological activity of putative polypeptides encoded by the 5' and 3' inserted DNA-soybean genomic DNA junctions (Tu and Silvanovich, 2009). In this report, additional sequence located at a junction that was created as part of a deletion and likely sequence duplication is evaluated. This additional junction is located 2374 nucleotides upstream of the 5' genomic DNA-insert DNA previously analyzed by Tu and Silvanovich (2009). Putative polypeptides from each reading frame, eight amino acids or greater in length were compared to allergen (AD\_2009), toxin (TOX\_2009), and public domain protein (PRT\_2009) database sequences using bioinformatic tools.

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and any protein sequences in the AD\_2009, TOX\_2009, and PRT\_2009 databases. Structural similarities shared between each putative polypeptide with each sequence in the database were examined. The extent of structural relatedness

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was evaluated by detailed visual inspection of the alignment, the calculated percent identity, and the *E*-score. In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope.

No biologically relevant structural similarity to any sequence in the AD\_2009, TOX\_2009 or PRT\_2009 databases were observed for any of the putative polypeptides. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the AD\_2009 database. These data demonstrate the lack of both structurally and immunologically relevant similarity to known allergens for all of the putative polypeptides analyzed. These data also demonstrate the lack of structurally relevant correlates to toxins or other biologically active proteins for the putative polypeptides analyzed.

This bioinformatics analysis is theoretical. No empirical evidence exists to suggest that transcription of DNA sequence at the additional junction described above for MON 87705 occurs. Rather, the results of these bioinformatic analyses indicate that in the highly unlikely occurrence that any of the junction sequences were to be transcribed and that a transcript were to be translated, the translation product would not share a sufficient degree of sequence similarity or identity to indicate that it would be potentially allergenic, toxic, or have other safety implications.

## 2.0 Introduction

Monsanto Company has developed biotechnology-derived soybean, MON 87705, to generate nutritionally-improved soybean oil with decreased levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid), increased levels of oleic acid (18:1) and an associated decrease in linolenic acid (18:2). Specifically, MON 87705 uses gene suppression technology to decrease the levels of two key oil biosynthetic enzymes, FATB1-A and FAD2-1A. Suppression of the FATB1-A enzyme results in a decrease in the levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid), while suppression of the FAD2-1A enzyme results in an increase of oleic acid (18:1).

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) renders the plant tolerant to glyphosate, which is the active ingredient in the Roundup family of agricultural herbicides. Glyphosate binds to the endogenous plant EPSPS enzyme and blocks the biosynthesis of shikimate-3-phosphate, thereby depriving

plants of aromatic amino acids (Haslam, 1993; Steinrücken et al., 1984). The CP4 EPSPS protein is structurally similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate relative to endogenous plant EPSPS (Padgett et al., 1996). Introduction of the *cp4 epsps* gene into MON 87705 allows for the production of aromatic amino acids and other metabolites even in the presence of glyphosate (Padgett et al., 1996).

As part of a comprehensive safety assessment, bioinformatic analyses were performed to assess the potential for allergenicity, toxicity, or biological activity of putative polypeptides encoded by the additional DNA junction contained in MON 87705. Sequences spanning a junction that was created as part of a deletion and likely sequence duplication were evaluated. This junction is located located 2374 nucleotides upstream of the T-DNA insertion site. Sequence spanning this junction was translated from stop codon to stop codon in all six reading frames. Putative polypeptides from each reading frame, eight amino acids or greater in length were compared to allergen (AD\_2009), toxin (TOX\_2009), and public domain protein (PRT\_2009) database sequences using bioinformatic tools.

Exposure to allergens in foods may cause sudden, medically significant reactions in susceptible individuals. Gliadins and glutenins are suspected to cause celiac disease, a non-IgE mediated disorder (gluten-sensitive enteropathy), and are also considered important immunologically active proteins. Screening the amino acid sequences of proteins introduced into plants by modern biotechnology for similarity to sequences of known allergens, gliadins, and glutenins is one of many assessments performed to support product safety. Similarly, the amino acid sequences of introduced proteins are also screened against known toxins as well as all known proteins in publicly available genetic databases.

The FASTA algorithm can be used to evaluate the extent of sequence alignment between a query protein sequence and a database sequence. In principle, if two proteins share sufficient linear sequence similarity and identity, they will likely share three-dimensional structure and, therefore, functional homology. By definition, homologous proteins share secondary structure and common three-dimensional folds (Pearson, 2000). Because the degree of relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 25% amino acid identity in a 200 amino acid overlap (Pearson, 2000), this is not generally sufficient to indicate IgE mediated cross-reactivity (Aalberse et al., 2001). Indeed, allergenic cross-reactivity caused by proteins is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is

currently applied by which related protein sequences are identified as potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al, 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

A second bioinformatics tool, an eight amino acid sliding window search, is used to specifically identify short linear polypeptide matches to known or suspected allergens. It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may still contain smaller immunologically significant epitopes. A query sequence may be considered allergenic if it has an exact sequence identity of at least eight contiguous amino acids with a potential allergen epitope (Metcalf et al., 1996; Hileman et al., 2002; Goodman et al., 2002). However, most allergen epitopes have not been confirmed and the amino acid length for those that have been identified can vary widely, thus the relevance of an exact match of eight amino acids may have limited immunological relevance (Thomas et al., 2005). The eight amino acid bioinformatic strategy is currently an *in silico* search that can produce matches containing significant uncertainty depending on the length of the query sequence (Silvanovich et al., 2006).

This report describes the bioinformatics assessment of putative polypeptides encoded at the additional junction of MON 87705. Inspection of the bioinformatic analysis data can be used to indicate whether the putative polypeptides have biologically relevant sequence similarity to known allergens, toxins, or other biologically active proteins.

### **3.0 Purpose**

The purpose of this study was to evaluate the amino acid sequences of putative polypeptides obtained from all reading frames that span a junction that was created as part of a deletion and likely sequence duplication. This junction is located 2374 nucleotides upstream of the MON 87705 T-DNA insertion site. Structural relatedness between the putative polypeptides and known allergens, toxins, and biologically active proteins was assessed using the FASTA sequence alignment tool. Using each putative polypeptide as a query sequence, FASTA searches were performed with the allergen (AD\_2009), toxin (TOX\_2009), and public domain (PRT\_2009) sequence databases. Immunologically relevant correlates were assessed using the pair wise comparison algorithm using the putative polypeptide as a query sequence to search against the AD\_2009 database.

## **4.0 Methods**

### **4.1 Sequence Database Preparation:**

The allergen, gliadin, and glutenin sequence database (AD\_2009) was obtained from FARRP (2009)<sup>1</sup> and was used as provided. The AD\_2009 database contains 1,386 sequences. A complete description of the AD\_2009 database can be found in Silvanovich (2009).

GenBank protein database, release 169.0 (December 16, 2008), was downloaded from NCBI and formatted for use in these bioinformatic analyses. It is referred to herein as the PRT\_2009 database and contains 14,717,352 sequences. A complete description of the PRT\_2009 database can be found in Silvanovich (2009).

The toxin database is a subset of sequences derived from the PRT\_2009 database that was selected using a keyword search and filtered to remove likely non-toxin proteins. It is referred to herein as the TOX\_2009 database and contains 7,651 sequences. A complete description of the TOX\_2009 database can be found in Silvanovich (2009).

### **4.2 Translation of Putative Polypeptides:**

DNA sequence spanning the genomic DNA-likely duplicated DNA junction 2374 nucleotides upstream of the MON 87705 insertion site (Skipwith et al., 2009) was imported with DNASTar, version 7.2.1 (1), 410 for translation with SeqBuilder 7.2.1 (1). All six reading frames originating or terminating within the duplicated DNA and spanning the above described junction were translated using the standard genetic code from stop codon to stop codon. Each putative polypeptide greater than eight amino acids in length was designated as 5 (representing the 5' of the T-DNA insert), separated with an underscore from a numerical value 1 to 6 representing the respective reading frame and appended with the letter "a" to represent additional (see Figure 1 for reading frame assignment). Reading frame assignment is shown in Figure 1 and translated sequence can be found in Table 1 and Appendices 1-6.

### **4.3 Sequence Database Searches:**

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<sup>1</sup> located at <http://www.allergenonline.com>

FASTA analyses using the AD\_2009, TOX\_2009 and PRT\_2009 databases were performed on a desktop computer loaded with a SUSE LINUX version 10.1 operating system and FASTA version 3.4t26 July 7, 2006. The structural similarity of the translated protein sequences to sequences in each database (AD\_2009, TOX\_2009, and PRT\_2009) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

FASTA comparisons are initiated by aligning the first match of a specific wordsize. The alignment is then extended based on the chosen scoring matrix. Default FASTA comparison parameters for wordsize (*k-tuple*), gap creation penalty and gap extension penalty were used. The expectation threshold (*E*-score) limit was set to one. The *E*-score (expectation score) is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences will need to have an *E*-score of less than  $1e-5$  ( $1 \times 10^{-5}$ ) or smaller to be considered to have significant homology. FASTA comparisons were performed using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). Multiple alignments are made between the query sequence and each sequence in the database with a score calculated for each alignment. Only the top scoring alignment is extensively analyzed for each database sequence. The BLOSUM matrix series (Henikoff and Henikoff, 1992) was derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. Sequences from each block were clustered based on the percent of identical residues in the alignments (Henikoff and Henikoff, 1996). The BLOSUM50 matrix will identify blocks of conserved residues that are at least 50% identical. BLOSUM50 works well for identifying sequence similarities that include gaps, and thus recognizes distant evolutionary relationships (Pearson, 2000).

If two proteins share sufficient linear sequence similarity and identity, they will also share three-dimensional structure and, therefore, functional homology. By definition, homologous proteins share secondary structure and common three-dimensional folds (Pearson, 2000). Because the degree of relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 25% amino acid identity in a 200 amino acid overlap (Pearson, 2000), this is not generally sufficient to indicate IgE mediated cross-reactivity (Aalberse et al., 2001). Indeed, allergenic cross-reactivity caused by proteins is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is currently applied by which related protein sequences are identified as

potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al., 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

In addition to the FASTA comparisons of each putative polypeptide to known allergens (to assess overall structural similarity), an eight amino acid sliding window search was performed. An algorithm was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (AD\_2009). This program compares the query sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. While there have been recommendations for using a shorter scanning window (Gendel, 1998; Kleter and Peijnenburg, 2002), only a few studies have actually investigated the ability of six, seven, or eight amino acid search windows to identify allergens (Hileman et al., 2002; Goodman et al., 2002; Stadler and Stadler, 2003). In these studies, randomly or specifically selected protein sequences were used as query sequences in FASTA and six, seven, and eight amino acid window searches against allergen databases. The results demonstrated that searches with six and seven amino acid windows led to high rates of false positive matches between non-allergenic query sequences and allergen database sequences. Additionally, searches with a six or seven amino acid window identified apparently random matches between totally unrelated proteins, such that the matched proteins were not likely to share any structural or sequence similarities that could act as cross-reactive epitopes. These studies concluded that six or seven amino acid sliding-window searches yielded such a high rate of false positive hits that they were of no predictive value. Furthermore, Silvanovich et al. (2006) recently demonstrated the lack of value of six or seven amino acid sliding-window searches in a comprehensive analysis of short peptide match frequencies by analyzing the match frequencies of peptides derived from ~1.95 million published protein sequences. In order to provide the best predictive capability to identify potentially cross-reactive proteins, a window of eight contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalf et al., 1996).

#### **4.4 Significance of the Alignment:**

An *E*-score of  $1 \times 10^{-5}$  was set as an initial high cut-off value for alignment significance. Although all alignments were inspected visually, any aligned



sequence that yielded an *E*-score less than or equal to  $1 \times 10^{-5}$  was analyzed further to determine if such an alignment represented significant sequence homology.

## 5.0 Results and Discussion

Bioinformatics analyses were performed on putative polypeptides deduced from DNA sequence spanning a junction that was created as part of a deletion and likely sequence duplication to assess the potential for similarity towards known allergens, toxins, or other biologically active proteins. This junction is located 2374 nucleotides upstream of the T-DNA insertion site. DNA sequence spanning the additional junction in MON 87705 was translated from stop codon to stop codon in all possible reading frames and six sequences greater than eight amino acids in length (see Figure 2 and Table 1) were evaluated. Supporting dataset output files for each putative polypeptide are contained in Appendices 1-6.

### 5.1 *Assessment of Potential Allergenicity:*

The results of the allergenicity assessment are shown in Table 2. Potential allergenicity of the six putative polypeptides was assessed using the FASTA and eight amino acid sliding window search algorithms. Using the FASTA algorithm to search the AD\_2009 database, no alignments with any of the six query sequences generated an *E*-score of less than or equal to  $1 \times 10^{-5}$ . Likewise, no alignment met or exceeded the Codex Alimentarius (2003) FASTA alignment threshold for potential allergenicity of 35% identity over 80 amino acids. Finally, no alignments of eight or more consecutive identical amino acids were found between any query sequence and the AD\_2009 database. As a result, these six putative polypeptides are unlikely to contain any cross-reactive IgE binding epitopes with known allergens.

### 5.2 *Assessment of Potential Toxicity:*

The results of the toxicity assessment are shown in Table 3. Potential toxicity of the six putative polypeptides was assessed using the FASTA algorithm. Using the FASTA algorithm to search the TOX\_2009 database, no alignments with any of the six query sequences generated an *E*-score of less than or equal to  $1 \times 10^{-5}$ .

### **5.3** *Assessment of Potential Adverse Biological Activity:*

The results of the potential adverse biological activity assessment are shown in Table 4. Potential adverse biological activity of the six putative polypeptides was assessed using the FASTA algorithm. Among all FASTA alignments between the six query sequences and the PRT\_2009 database, none showed potentially significant alignments.

## **6.0 Conclusions**

Analyses of putative polypeptides encoded by DNA spanning the additional junction in MON 87705 was performed using bioinformatic tools. Results of the FASTA sequence alignments demonstrated a lack of structurally relevant similarity between any known allergenic, toxic or biologically active proteins and the six putative polypeptides. Moreover, results from eight amino acid sliding window search demonstrated the lack of potential immunologically relevant sequence matches between any of the putative polypeptides and the AD\_2009 database. The results of these bioinformatic analyses demonstrate that even in the highly unlikely occurrence that any of the junction polypeptides were translated; they would not share a sufficient degree of sequence similarity with other proteins to indicate that they would be potentially allergenic, toxic, or have other safety implications.

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## **[CBI Cross Reference Number 1]**

Deleted Figures 1 and 2, and Table 1

Deleted pages 21 – 23 are found in the Confidential Attachment, pages 4 – 6.

**Table 2.** Summary of alignments for the FASTA searches of the AD\_2009 database using putative polypeptide sequences encoded by the genomic DNA-likely duplicated DNA junction in MON 87705.

Appendix	Query	Sliding 8-mer	FASTA					
			Hits	gi-number	Description	Percent Identity	aa overlap	E-score
1	5_1a	No	2	5059162	AF144060_1 alpha-amylase [Der (496 aa)	26.087	69	0.54
2	5_2a	No	-	-	-	-	-	-
3	5_3a	No	-	-	-	-	-	-
4	5_4a	No	-	-	-	-	-	-
5	5_5a	No	-	-	-	-	-	-
6	5_6a	No	1	729979	MAG_DERFA Allergen Mag (341 aa)	32.258	31	0.29

**Table 3.** Summary of alignments for the FASTA searches of the TOX\_2009 database using putative polypeptide sequences encoded by the genomic DNA-likely duplicated DNA junction in MON 87705.

Appendix	Query	FASTA					
		Hits	gi-number	Description	Percent Identity	aa overlap	E-score
1	5_1a	-	-	-	-	-	-
2	5_2a	-	-	-	-	-	-
3	5_3a	-	-	-	-	-	-
4	5_4a	-	-	-	-	-	-
5	5_5a	-	-	-	-	-	-
6	5_6a	-	-	-	-	-	-

**Table 4.** Summary of alignments for the FASTA searches of the PRT\_2009 database using putative polypeptide sequences encoded by the genomic DNA-likely duplicated DNA junction junction in MON 87705.

Appendix	Query	FASTA					
		Hits	gi-number	Description	Percent Identity	aa overlap	E-score
1	5_1a	4	124360394	Peptidase aspartic, active (435 aa)	45.455%	33	0.051
2	5_2a	-	-	-	-	-	-
3	5_3a	-	-	-	-	-	-
4	5_4a	-	-	-	-	-	-
5	5_5a	-	-	-	-	-	-
6	5_6a	-	-	-	-	-	-

## **[CBI Cross Reference Number 2]**

Deleted Appendices 1-6

Deleted pages 26 – 43 are found in the Confidential Attachment, pages 7 – 24.