



Herculex® I Product Overview

Herculex® I insect protection for corn, co-developed by Dow AgroSciences and Pioneer Hi-Bred is a superior example of the use of a natural Bt protein. The Bt protein (Cry1F) produced in corn with the Herculex I trait offers the broadest spectrum of above-the-ground insect pest control on the market.

These pests include European corn borer, southwestern corn borer, black cutworm, western bean cutworm, lesser corn stalk borer, southern corn stalk borer, sugarcane borer, corn earworm and fall armyworm.

Insect infestation of crops severely impacts the health of the plants, reducing yield (measured in bu/acres) and grain quality. Major pests for corn are European corn borer, western bean cutworm, black cutworm and corn rootworm. The European corn borer alone destroys an estimated \$1 to \$2 billion worth of corn each year.

Insects cause many other forms of damage when they feed on parts of the corn plant, including breakage of the plant, eardrop and kernel damage. Damaged kernels can lead to growth of fungi, which may produce biologically active compounds called mycotoxins (e.g. aflatoxin, fumonisin). Dependent upon their accumulation in kernels, mycotoxins pose a potential threat to human and animal health if consumed.

Corn with the Herculex I trait provides many benefits for farmers, including improving yield and reducing the need for spray pesticides.

In field tests, corn hybrids with Herculex I insect protection averaged 8.3-13.9 bu/acres more than the same hybrids without protection. This means an increased yield of 5 to 9 percent for farmers.

With Herculex I insect protection, farmers can use fewer spray pesticides. For years, farmers have used sprays to fight insects. Use of these sprays can be time consuming, costly and not always effective. The pesticide application for corn borers, for example, has to be done during the short period between the time the caterpillars hatch and when they bore into the stalks where the pesticide doesn't reach them.

It has been long known that a common soil bacterium called *Bacillus thuringiensis* (often referred to as Bt) produces proteins (often referred to as Bt proteins) that kill specific insect larvae. Many of these proteins are found in crystals formed by the bacteria during one stage of its lifecycle. These are referred to as "Cry" proteins. The insecticidal activities of many Bt and Cry proteins have been studied in great detail and the majority are very specific, affecting only certain insects. Studies of many Bt bacterial strains, as well as their Bt proteins, have shown them to be harmless to other animals and humans. In fact, numerous spray formulations containing either Bt bacteria or Bt proteins have been used for more than 40 years for crop protection, including organic farming operations, and have been approved as an insecticide by the EPA.

Researchers have isolated the genes responsible for making the Bt proteins and transferred them into corn plants. Compared to conventional chemicals, these plants with the Bt protein provide insect protection much more effectively.

In addition to insect protection, corn with the Herculex I trait also provides protection against the herbicide glufosinate (Liberty®) by the use of a bacterial protein, generally referred to as PAT. Following U.S. federal agency guidelines, Herculex I went through extensive testing and regulatory evaluation for food, feed and environmental safety prior to commercialization.

Herculex® I Safety Assessment Summary

Throughout the development of products derived through biotechnology, DuPont strives to maintain the highest standards to ensure product quality and product safety for farmers and consumers. On the basis of rigorous testing, regulatory agencies concluded that corn with Herculex* I insect protection is as safe as non-biotech corn. The Herculex I trait has received full food, feed and environmental approval by the United States and Canada. It has been approved for import by respective regulatory bodies in the European Union, Japan, Australia, New Zealand, South Korea, South Africa, and Philippines. Work to ensure our products will have appropriate regulatory approvals with other countries is continuing.

Before a biotech product can be introduced to the market, approval from appropriate governmental agencies is required. Following criteria established by the agencies, DuPont conducts extensive, validated tests for its biotech products. For the approval of corn with the Herculex I trait, extensive safety data was provided by Pioneer Hi-Bred International, Inc., and Dow AgroSciences LLC to the U.S. Department of Agriculture (USDA), Environmental Protection Agency (EPA), Food and Drug Administration (FDA) and the regulatory systems of other countries. Data on the genes and proteins used to develop the trait, crop characteristics, food and feed composition and environmental impacts were compiled over several years and from multiple environments in the United States and abroad.

The following tests were conducted on corn with the Herculex I trait:

Molecular Analysis

The plant genetic material (DNA) in corn with the Herculex I trait was analyzed to determine the functional integrity of the introduced genes that confer insect and herbicide resistance (*cry1F* and *pat* genes). The molecular analysis proved functional integrity by showing that the DNA segment used was properly integrated into the plant DNA.

Trait Stability

Trait stability and agronomic performance were monitored over multiple generations and environments. Plants of every generation were tolerant to the herbicide and resistant to infestation of target insects (European and southwestern corn borer, black and western bean cutworm, lesser corn stalk borer, southern corn stalk borer, sugarcane borer, corn earworm and fall armyworm). Continued stability is demonstrated through product performance.

Trait Specificity

Field trials and insect feeding studies demonstrated specificity of Cry1F protein to inhibit growth of the target insects. No adverse effects were observed when non-target animals (e.g. birds, fish and earthworms) and beneficial insects (e.g. ladybugs, honeybees and butterflies) were fed either purified Cry1F protein or appropriate plant material containing Cry1F (e.g. pollen).

Toxicity and Allergenicity

Both proteins used in corn with the Herculex I trait, Cry1F and PAT, are present in soil bacteria and are not considered as pathogens for humans or animals. Bt proteins similar to Cry1F have been used safely for many years as natural pesticides in commercial agriculture, including organic farming. PAT is used in several biotech crops and is not associated with toxicity or allergenicity.

Several studies demonstrated the safety of Cry1F and PAT proteins for human and animal consumption. Healthy mice demonstrated lack of acute toxicity after ingesting a dose of Cry1F or PAT protein many

thousand times the estimated dietary intake of humans. In addition, a thirteen-week (90-day) oral toxicity feeding study in rats was carried out with grain from corn with the Herculex I trait and non-biotech corn grain. No significant diet-related differences were observed in the development or well-being of the rats.

Both proteins were checked for characteristics typical of known allergenic proteins. There was no indication of allergenic potential.

Substantial Equivalent

The FDA reviews information showing that a biotech crop is equivalent to a non-biotech counterpart that has a history of safe food use. This is known as "substantial equivalence."

Compositional analysis and nutritional assessment of corn with the Herculex I trait were compared with non-biotech counterparts. Nutrients, like fat, protein, fiber, minerals, and vitamins, were measured. Long-term feeding studies also were conducted. There was no significant difference between corn with the Herculex I trait and the non-biotech corn. This finding means non-biotech corn and corn with the Herculex I trait are substantially equivalent. Another test for substantial equivalence involved long-term feeding studies with chickens and corn with the Herculex I trait. The studies indicated no difference in growth and health of the animals compared to those fed with non-biotech grain.

Herculex® I Detailed Safety Assessment

1. Molecular and Protein Analysis

1.1 Genes Used and Their Molecular Analysis in Corn with the Herculex® I Trait

A purified DNA segment with the two genes -- *cry1F* (insect-protection) and *pat* (glufosinate herbicide tolerance) -- was used for biolistic transformation.

The source of the *cry1F* gene is the common soil bacterium *Bacillus thuringiensis* (Bt) ssp. *aizawai*. Bt organisms, or purified Bt proteins, have been used safely in spray form as a crop protective agent for at least 40 years (Schnepf et al., 1998; U.S. EPA, 1996). The ssp. *aizawai* is used commercially to control wax moth larvae and various caterpillars (Cornell University, 1996). In its natural form found in the bacterium, Cry1F protein is produced as a protoxin of 1174 amino acids (Chambers et al., 1991). After ingestion by the insect larvae, the protein is proteolytically cleaved to its active form like many other Cry proteins studied (Gill et al., 1992; English and Slatin, 1992; Knowles, 1994; Dean et al., 1996). Studies of several activated Cry protein toxins similar to Cry1F have demonstrated that they interact specifically with insect midgut cells, causing pore formation and disruption of the osmotic balance of these cells, ultimately leading to the death of the larvae (Lambert et al. 1996; Van Rie et al. 1990; Van Rie et al. 1989; Wolfersberger et al. 1986; Hofmann et al., 1988a and b). The Cry1F Bt protein is highly selective for lepidopteran species and prevents growth of the larvae of European corn borer, southwestern corn borer, black cutworm, western bean cutworm, lesser corn stalk borer, southern corn stalk borer, sugarcane borer, corn earworm and fall armyworm.

The source of the *pat* gene is the ubiquitous soil fungus *Streptomyces viridochromogenes*. The gene encodes for a phosphinothricin N-acetyltransferase of 183 amino acids that converts L-PPT (L-phosphinothricin), the active ingredient in glufosinate-ammonium herbicide, to its inactive form N-acetyl-L-PPT (De Block et al., 1988; Wehrmann et al., 1996; OECD, 1999). L-PPT, binds to, and inactivates, the enzyme glutamine synthetase in plants, preventing the detoxification of excess ammonia, which ultimately results in plant death. Plants

expressing the PAT enzyme are therefore tolerant to the herbicide, enabling treatment of surrounding weeds without harm to the crop. The herbicide tolerance trait was also used as a selectable marker to facilitate generation of transgenic plants.

To confer insect resistance, a less than full length form of the *cry1F* gene, encoding only the active toxin moiety of 605 amino acids, was inserted into tissue.

The codon usage of both genes, *cry1F* and *pat*, was altered at certain locations. The amino acid sequence was unchanged with regards to the naturally found protein, with the exception of the next to the last amino acid of Cry1F. This amino acid change was made to facilitate production of large quantities of a microbially produced Cry1F needed for toxicology studies. Expression of the introduced genes is through constitutive promoters -- one derived from corn (*ubiZM1*; Christensen et al., 1992) and the other from the cauliflower mosaic virus (35S; Odell et al., 1985). In order to stop gene transcription, terminator regions from *Agrobacterium tumefaciens* (ORF25PolyA; Fraley et al., 1983) and from cauliflower mosaic virus (35S; Pietrzak et al., 1986) were used.

Polymerase chain reaction (PCR) and sequence data analysis identified that the transformation resulted in the insertion of one, almost full-length copy of the DNA segment (6186 bp from the 6235 bp of the DNA segment used for transformation) into the plant nuclear genome. In addition, the plant insert contains the following non-functional elements:

- one fragment (335 bp) of the *cry1F* gene, with no *ubiZM1* promoter sequence, and one fragment (15 bp) of the *cry1F* gene, both located at the 5' end of the almost full-length insert;
- two fragments (201 bp and 138 bp long, respectively) of the *pat* gene, without regulatory sequences associated, located at the 5' border and, one fragment (188 bp) of the *pat* gene, located at the 3' border;
- one fragment (118 bp) of the polylinker region and *ubiZM1* promoter sequence located at the 5' border;
- one fragment (550 bp) of the ORF25PolyA terminator sequence in inverted position located immediately at the 3' end of the almost full-length insert.

Fig. 1: DNA segment used to produce Corn with the Herculex I trait (6235 bp)

1.2 Stability of Inserted DNA Segment and Agronomic Performance

No product is commercialized without rigorous performance review during development. Agronomic performance, particularly stalk and root strength, continues to be monitored after commercialization.

The presence of the transferred genes in corn with the Herculex I trait was investigated over multiple generations to ascertain genetic stability. The results from several rounds of backcrossing and self-crossing demonstrate that the *cry1F* and *pat* genes are stable in this line. Observations of the phenotype indicated that the transgenes are inherited as dominant genes according to Mendelian segregation patterns. All of the plants determined to be tolerant to glufosinate-ammonium also were found to be resistant to European corn borer infestation.

General agronomic performance for a broad range of traits was evaluated in more than 5,000 field trials in the United States, Europe, Canada and South America. Several characteristics were measured, including plant height, tassel size, leaf color and ear shape. The results indicated no difference between corn with the Herculex I trait and its non-biotech counterpart, except for the introduced traits.

1.3 Cry1F and PAT Protein Expression

Field studies representative of the conditions and growth stages corresponding to commercial corn production were undertaken during the 1998-99 growing season in Chile; 1999 in France, Italy and the United States; and 2000 in France, Italy and Bulgaria.

The expression levels of Cry1F and PAT proteins in leaf, pollen, silk, stalk, whole plant, grain and senescent whole plant tissues from corn with the Herculex I trait and a non-biotech corn with comparable background genetics were measured using ELISA (Enzyme Linked Immunosorbent Assay) specifically developed for each protein. Western immunoblot analysis was used to confirm the integrity of the newly expressed proteins.

Cry1F was detectable in all plant tissues with average levels of 1064 ng/mg total protein in whole plant, 550 ng/mg in stalk, 111 ng/mg in leaf, 90 ng/mg in grain, 50 ng/mg in silk and 136 ng/mg in pollen.

Expression of the PAT protein was found in leaf tissue samples at detectable levels up to approximately 54 ng/mg total protein. These levels are sufficient to confer tolerance to glufosinate-ammonium herbicide at the level of the whole plant. In all other tissues, the levels of PAT protein were below the limit of detection (20 ng/mg).

As expected, expression of the Cry1F and PAT proteins was not detected in any samples from the control plants.

2. Food and Feed Safety Analysis

Following federal agency guidelines, corn with the Herculex I trait was rigorously tested to determine if there were any toxicological, allergenicity and nutritional issues. In all cases, corn with the Herculex I trait proved to be comparable to non-biotech corn.

To obtain quantities of the Cry1F and PAT protein sufficient for biochemical and toxicological tests, the proteins were produced microbially in the laboratory. A range of analyses was subsequently undertaken to establish that the microbially produced proteins were equivalent to the proteins produced by the transformed corn plants. Several characteristics were analyzed, such as molecular weight, immunoreactivity, post-translational modification, N-terminal amino acid sequence and bioactivity on susceptible insect larvae.

2.1 Lack of Toxicity Potential

The potential toxicity of a novel protein is evaluated by comparing the novel protein's amino acid sequence to that of known toxic proteins and by conducting acute oral toxicity studies with mammals. Amino acid sequence comparisons showed no similarity between Cry1F and known toxic proteins available in public databases.

When proteins are toxic, they are known to act via acute mechanisms and at very low levels (Sjoblad et al., 1992).

Mice were fed a diet containing a single dose of the Cry1F protein that was equivalent to 576 mg/kg body weight. A dose equivalent to 5000 mg/kg was used for the PAT toxicity study. These doses are equivalent to many thousand times the estimated dietary intake for human consumption (corn with the Herculex I trait seeds contain 0.0017 to 0.0034 mg of Cry1F/gram of corn kernel tissue). Observations for mortality and clinical or behavioral signs of toxicity as well as individual body weights were carried out over a period of 14 days.

In addition, a thirteen-week (90-day) oral toxicity feeding study in rats was carried out with grain from corn with the Herculex I trait. The animals were fed diets containing 33% or 11% grain from corn with the Herculex I trait, non-biotech maize with comparable genetic background or other commercial non-biotech maize. No biologically

significant, diet-related differences were observed in mean body weight, body weight gain or food efficiency of the mice and rats fed any of the different diets. At the conclusion of the studies, the animals were examined for gross necropsy. No toxicologically significant diet-related differences were observed.

2.2 Lack of Allergenicity Potential

The two new proteins in corn with the Herculex I trait were evaluated according to criteria established by a panel of food allergy experts with the Allergy and Immunology Institute of the International Life Science Institute (ILSI), in collaboration with the International Food Biotechnology Council (IFBC). The panel developed a decision-tree approach for food allergy assessment that has been accepted by U.S. regulatory agencies, and applied to every protein introduced into products developed through biotechnology. The assessment focuses on potential allergenic history of the source of the novel protein, significant amino acid similarity between the novel protein and that of known allergens, and the structural properties of the novel proteins. Applying such criteria systematically provides reasonable evidence about the potential of the newly introduced proteins to act as an allergen.

The percentage of proteins that are allergenic is very small. Only approximately 200 of the hundreds of thousands of proteins humans consume in food are food allergens (Day, 1992). Hefle et al. (1996) surveyed the literature and identified approximately 180 foods reported to be allergenic. Of those, eight food groups (peanuts, soybeans, crustacea, fish, cow's milk, eggs, tree nuts and wheat) account for 90% of all reported food allergies worldwide (Metcalf, et al., 1996).

Cry1F and *pat* genes were derived from common soil bacteria. Cry1F-like proteins (Cry proteins) and PAT, as novel protein in plants, have a history of safe use in agricultural crop commodities and are not associated with allergenicity.

The Wisconsin Genetics Computer Group (GCG) sequence analysis computer program was used to search standard public domain DNA and protein sequence databases for significant similarity of Cry1F and PAT with known allergens. The results of this comprehensive search demonstrated that neither the CRY1 nor PAT proteins share significant amino acid sequence similarity with known allergen proteins. Significant similarity is defined as sequence identity of 8 or more contiguous amino acids (Metcalf et al., 1996).

Another allergenicity test focused on the digestibility of proteins in corn with the Herculex I trait. To elicit an allergic response after reaching and passing through the intestinal mucosa, most food allergens tend to be stable in the peptic and acidic conditions of the digestive system (Astwood et al., 1996; Metcalf et al., 1996; Kimber et al., 1999).

The *in vitro* digestibility of bacterially derived Cry1F protein was determined in laboratory experiments. In the experiments, the protein was exposed to simulated mammalian gastric fluid, with variations in either the pepsin concentration or the incubation time. The samples were analyzed by SDS-PAGE and visualized following Coomassie blue staining.

The experiments showed that Cry1F was completely broken down into amino acids and small peptides within 5 minutes at molar ratios approximating 1:100 (Cry1F:pepsin). In separate experiments, the same molar ratio of 1:100 effected nearly complete proteolysis of the Cry 1F within 1 minute.

In a separate study, PAT protein was undetectable within 5 seconds of incubation with 0.3% (weight /volume) pepsin in the simulated gastric system.

As some protein allergens are glycosylates, studies were carried out to determine whether Cry1F is a glycoprotein. The potential glycosylation of Cry1F protein *in planta* and from a microbial source was examined by a sensitive immuno-blot technique used for glycoprotein detection. The studies provided no evidence that Cry1F is a glycoprotein.

2.3 Compositional Analysis

A key component in the regulatory approval process is the demonstration that a crop derived through biotechnology is "substantially equivalent" to a non-biotech crop. The key constituents in corn grain have been evaluated in order to compare them to corresponding data from corn with the Herculex I trait expressing Cry1F and PAT proteins, as well as a non-transgenic counterpart and published literature values obtained for non-transgenic varieties of corn. As a reference tool, the Organization for Economic Co-operation and Development (OECD) has produced a consensus document on compositional considerations for new varieties of maize, which looks at nutrients and plant metabolites (OECD, 2002).

This evaluation includes a study of the major constituents that are characteristic of whole corn grain, taking into account the natural variation in composition that is known to occur due to genetic variability and geographical or environmental factors.

Two major studies were conducted at different geographical areas to determine the compositional profile of key corn tissues collected from Corn with the Herculex I trait and non-transgenic control lines with comparable genetic background. The studies were conducted at four trial sites located in the major corn growing regions of Chile (1989/99) and in field sites in France and Italy (1999). In previous studies, Pioneer Hi-Bred International, Inc., demonstrated that corn plants grown in Chile are equivalent to those grown in the United States.

A nutrient analysis focused on a number of components from corn forage and grain, including fat, protein, fiber content, moisture and ash. In addition, grain was measured for fatty acid content, amino acid profile, mineral content, vitamin content and two potential antinutrients. The statistical analysis included means for the test line (corn with the Herculex I trait) and a control line, as well as a comparison of means. Statistically significant differences ($p < 0.05$) between the test line and the control line were considered. A comparison of the means for corn with the Herculex I trait with the range for commercial maize hybrids published in the literature also were taken into consideration.

The following nutrient analysis information is based on data collected from 1989/99 Chile field trials.

- **Proximate Analysis of Corn Forage and Corn Grain**

An analysis of forage and grain of Herculex I showed that its nutritional composition is comparable to non-biotech hybrids. The analysis focused on the protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash and moisture levels of forage and of grain from corn with the Herculex I trait and the control hybrid (See Table 1 and Table 2 below). The moisture was analyzed only for calculation purposes, and these values are not presented since the forage was dried before shipping.

Table 1

Proximate Analysis of Corn Forage				
Variable*	Herculex® I Trait (Mean)	Control (Mean)	P-Value	Literature Range
Fat	2.44	2.42	0.653	0.7 - 6.7**

Protein	7.83	7.85	0.932	3.5 - 15.9**
ADF	26.97	27.96	0.039	30^
NDF	47.96	48.28	0.698	51^
Ash	5.66	5.72	0.724	1.3 - 10.5**

* - Percentage on a dry weight basis

** - Watson, 1982

^ - Watson, 1982 reports an average value for ADF of 30% and NDF of 51%

Table 2

Proximate Analysis of Corn Grain				
Variable*	Herculex® I Trait (Mean)	Control (Mean)	P-Value	Literature Range
Fat	3.83	3.94	0.046	3.1 - 5.7**
Protein	11.20	11.32	0.611	6.0 - 12**
ADF	3.55	3.68	0.250	3.0 - 4.3^
NDF	10.47	10.08	0.315	8.3 - 11.9^
Ash	1.51	1.50	0.335	1.1 - 3.9^

* - Percentage on a dry weight basis

** - Watson, 1987

^ - Watson, 1982

The levels of protein, fat, and ash in corn with the Herculex I trait were all within the reported literature ranges for maize forage. The published literature report means for forage ADF and NDF are 30% and 51%, respectively; however, no range of values is available. The forage ADF and NDF levels in corn with the Herculex I trait of 26.97% and 47.96%, respectively, are similar to the means reported in the literature. Levels of protein, fat, ADF, NDF and ash measured in grain from Herculex I and the control hybrid were all in the reported literature range.

- **Protein Composition**

The level of 18 amino acids in the grain from corn with the Herculex I trait and the non-transgenic control was compared. The results are presented in Table 3. With the exception of threonine and glutamic acid, levels of all other essential amino acids were within the range published in the literature or determined by the analysis of 22 commercial Pioneer® brand hybrids. Levels for threonine and glutamic acid in corn with the Herculex I trait and the control hybrid were slightly above the published range. However, there was no statistically significant difference in threonine and glutamic acid levels between the test and control lines, indicating that the slightly elevated level in corn with the Herculex I trait was not due to the presence of the transgenes.

Table 3

Protein Composition of Corn Grain	
-----------------------------------	--

Variable*	Herculex® I Trait (Mean)	Control (Mean)	P-Value	Literature Range
Glycine	0.39	0.40	0.150	0.26 - 0.47** 0.24 - 0.41^
Threonine	0.40	0.41	0.302	0.29 - 0.39** 0.21 - 0.37^
Valine	0.51	0.52	0.902	0.21 - 0.52** 0.25 - 0.67^
Isoleucine	0.40	0.40	0.952	0.26 - 0.40** 0.19 - 0.39^
Leucine	1.42	1.43	0.880	0.78 - 1.52** 0.43 - 1.35^
Phenylalanine	0.56	0.57	0.479	0.29 - 0.57** 0.04 - 0.54^
Histidine	0.29	0.30	0.822	0.20 - 0.28** 0.21 - 0.32^
Lysine	0.32	0.32	0.522	0.20 - 0.38** 0.19 - 0.36^
Arginine	0.44	0.45	0.672	0.29 - 0.59** 0.28 - 0.55^
Cysteine	0.21	0.23	<0.0001</td>	0.12 - 0.16** 0.13 - 0.27^
Methionine	0.19	0.20	0.020	0.10 - 0.21** 0.12 - 0.26^
Tryptophan	0.08	0.08	0.065	0.05 - 0.12** 0.05 - 0.10^
Serine	0.54	0.55	0.390	0.42 - 0.55** 0.25 - 0.46^
Alanine	0.84	0.85	0.727	0.64 - 0.99** 0.37 - 0.81^
Glutamic Acid	2.14	2.18	0.472	1.24 - 1.96** 0.89 - 2.02^
Proline	1.01	1.03	0.679	0.66 - 1.03** 0.43 - 1.01^
Aspartic Acid	0.77	0.81	0.102	0.58 - 0.72** 0.37 - 0.80^
Tyrosine	0.20	0.20	0.954	0.29 - 0.47** 0.17 - 0.31^^

* - Percentage on a dry weight basis

** - Watson, 1982

^ - Data from analysis of 22 commercial Pioneer® Brand Hybrids

^^ - Iowa Gold Catalog, 1994

- **Oil Composition**

Analyses of five major fatty acids in corn grain was conducted: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) (Table 4). There was a small statistically significant difference between corn with the Herculex I trait and the non-transgenic control in all of

these measurements, with the exception of palmitic acid. However, the values for these fatty acids in corn with the Herculex I trait and the control were within the literature-reported range for maize.

Table 4

Fatty Acid Analysis of Corn Grain				
Variable*	Herculex® I Trait (Mean)	Control (Mean)	P-Value	Literature Range**
Palmitic	11.07	10.92	0.091	7 - 19
Stearic	2.28	2.44	0.007	1 - 3
Oleic	30.61	32.53	0.002	20 - 46
Linoleic	53.10	51.16	0.002	35 - 70
Linolenic	1.29	1.21	0.0001	0.8 - 2

* - Value of fatty acid is % of total lipid

** - Watson, 1982

- Minerals**

The levels of nine minerals -- calcium, phosphorus, copper, iron, magnesium, manganese, potassium, sodium and zinc -- were analyzed in grain from corn with the Herculex I trait and the non-transgenic control line (Table 5). The levels of sodium were below the limit of quantitation in all samples. There was a small statistically significant difference between corn with the Herculex I trait and the non-transgenic control in the levels of manganese and potassium. However, these two minerals, as well as the others, were in the range of values found in the literature.

Table 5

Mineral Analysis of Corn Grain				
Variable*	Herculex® I Trait (Mean)	Control (Mean)	P-Value	Literature Range
Calcium %	0.0036	0.0031	0.620	0.01 - 0.10** 0.002 - 0.011^
Phosphorus %	0.33	0.32	0.161	0.26 - 0.75** 0.21 - 0.37^
Copper ppm	2.03	2.11	0.845	0.9 - 10**
Iron %	0.0025	0.0025	0.549	0.0001 - 0.01**
Magnesium %	0.12	0.13	0.524	0.09 - 1.0**
Manganese %	0.0005	0.0006	0.0003	0.00007 - 0.0054**
Potassium %	0.40	0.36	0.023	0.32 - 0.72**
Zinc %	0.002	0.002	0.141	0.0012 - 0.0030**

* - Percentage on a dry weight basis or parts per million (ppm) on a dry weight basis

** - Watson, 1982

^ - Data from analysis of 22 commercial Pioneer® Brand Hybrids

- Vitamins**

Grain from corn with the Herculex I trait and the control corn was analyzed for content of four vitamins: B1, B2, total tocopherols (Vitamin E) and folic acid (Table 6). The results show small, statistically

significant variations between the transgenic and non-transgenic corn in terms of the total tocopherols (slightly higher in corn with the Herculex I trait) and in vitamin B1 levels (slightly lower in corn with the Herculex I trait). However, the values obtained for all vitamin analyses were within the respective range previously reported in the literature. No typical range in the literature is available for folic acid in grain, although an average value of 0.3 ppm is reported. Levels of folic acid in corn with the Herculex I trait and the control corn were not significantly different.

Table 6

Vitamin Analysis of Corn Grain				
Variable*	Herculex® I Trait (Mean)	Control (Mean)	P-Value	Literature Range
Thiamine Hydrochloride (B1) ppm	3.64	4.06	0.002	3.0 - 8.6**
Riboflavin (B2) ppm	1.67	1.66	0.827	0.25 - 5.6**
Folic Acid ppm	0.151	0.144	0.676	0.3^
Total Tocopherols ppm	48.2	41.6	0.0005	42 - 87^

* - Parts per million (ppm) on a dry weight basis

** - Watson, 1982

^ - Watson, 1987 reports an average value for folic acid in grain as 0.3 mg/kg

- **Antinutrients**

Two potential anti-nutrients in maize -- phytic acid and trypsin inhibitor -- were analyzed in grain from corn with the Herculex I trait and the control line (Table 7). Phytic acid levels in corn with the Herculex I trait were found to be within the published literature range for maize grain. Maize contains only low levels of the antinutrient trypsin inhibitor (Watson, 1987; Del Valle, 1983). Trypsin inhibitor levels in both corn with the Herculex I trait and the control line were below the limit of quantitation of the enzyme assay that was used in this analysis. This confirmed that no unusually high levels of trypsin inhibitor are present in corn with the Herculex I trait.

Table 7

Anti-Nutrient Analysis of Corn Grain				
Variable*	Herculex® I Trait (Mean)	Control (Mean)	P-Value	Literature Range**
Phytic acid %	0.99	0.96	0.146	0.7 - 1.0
Trypsin inhibitor TIU/g	<LOQ^	<LOQ	--	NA

* - Percentage (%) or mg/100 g on a dry weight basis. Trypsin inhibitor is expressed in units of trypsin inhibitor enzyme activity per gram on a dry basis

** - Watson, 1982

^ - Below level of quantitation for trypsin inhibitor of 2,000 TIU/g

NA - Ranges are not available in the published literature

- **Animal Feeding Study**

In assessing the safety of a food and feed derived through biotechnology it is important to establish that the food and feed is nutritionally adequate and will support typical growth and well being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, coupled with an extensive compositional analysis of the food. Carefully designed feeding studies in animals may provide further reassurance that the food is nutritionally adequate.

Rapidly growing broiler chickens are sensitive to changes in nutrient quality in diets, and, therefore, serve as a useful model species to evaluate the wholesomeness of food and feed.

A total number of 245 healthy male chickens (Cobb x Cobb) were randomly assigned to the various treatment groups. All diets were formulated to meet nutritional recommendations (National Research Council, 1994), and consisted of a commercial corn/soy ration. Corn with the Herculex I trait, non-transgenic corn with comparable genetic background and commercially available corn were used. On days 0-20, the chickens were fed a starter diet containing 54.21% corn. On days 21- 42, the chickens were fed a grower/finisher diet containing 57.03% corn. These rations applied to all treatments. Both the solid diets and water were provided to the chickens *ad libitum*, and no medication was administered during the course of the study.

The results of the broiler feeding study show there were no differences in parameters tested between birds fed a diet containing corn with the Herculex I trait and the other corn as control (Table 8). These results support the findings of the compositional analyses and indicate that corn with the Herculex I trait is equivalent to non-transgenic corn in the ability to provide adequate nutrition to rapidly growing broiler chickens.

Table 8

Performance of Chickens in Corn Feeding Study						
Variable	Corn Grain #1	Corn Grain #2	Corn Grain #3	Corn Grain #4	Corn Grain Hybrid 7250	Corn Grain Herculex® Trait
Mortality %	5.71	5.71	2.86	5.71	2.86	5.71
Body Weight (kg) day 42	0.044	0.043	0.043	0.043	0.044	0.043
Body Weight (kg) day 42	1.730	1.739	1.738	1.738	1.739	1.757
Daily Gain (g/bird/day)	0.040	0.040	0.040	0.040	0.040	0.040
Feed Conversion (body wt. corrected)	1.797	1.806	1.808	1.804	1.802	1.775

Control Grain Hybrid 7250 - Non-transgenic corn with comparable genetic background to Herculex® I

Control Grain #1 to #4 - Batches of commercial corn

3. Environmental Impact Analysis

Following federal agency guidelines, corn with the Herculex I trait was extensively tested to evaluate its environmental safety. All tests indicated that corn with the Herculex I trait had no adverse effects on soil organisms, animals and insects.

3.1 Soil Metabolism

Some of the Cry1F protein exposed to the soil or soil organisms in the field will be from post-harvest crop debris in the field. A bioassay based on the insecticidal activity of Cry1F against tobacco budworm was used to determine the biodegradation in the soil and a DT50 (time to 50% reduction of activity) of 3.13 days was obtained. This value is comparable with the metabolism of other Bt proteins published in the literature (Pruett et al., 1980; West, 1984; West et al., 1984; Palm et al., 1994; Sims and Holden, 1996). The maximum estimated

amount of Cry1F in the field after harvest is 0.023 mg/kg dry soil, which is well below levels used in non-target toxicity studies. (The value of 0.023 mg/kg dry soil is based on the assumption that 25,000 corn plants/acre are left in the field.)

3.2 Non-target Toxicity Studies

Feeding studies with either purified Cry1F protein mixed into prescribed diet material or with plant tissue derived from corn with the Herculex I trait, expressing Cry1F, were performed on EPA-recommended representative animals. A selection of those animals tested included: avian (bobwhite quail), aquatic (waterflea), soil (earthworm) and terrestrial (honeybee, green lacewing, parasitic wasp, ladybird beetle, monarch butterfly). No adverse effects on the well-being of the animals was detected in the feeding studies.

For example, adult ladybird beetles fed a concentration of Cry1F protein at 15x the expected rate found in corn pollen (pollen expressing 32 ng/mg pollen) resulted in no mortality or signs of toxicity over a 29-day period. Earthworms were kept in artificial soil supplemented with 2.26 mg protein/kg dry soil, which is about 100 times more than the maximal expected concentration of Cry1F in the field after harvest. No apparent effects on mortality or body weight of the earthworms were observed.

3.3 Non-target Field Study

A field study was conducted to determine whether corn with the Herculex I trait had any significant negative impact on natural non-target insect populations. Sticky traps, which attract and capture flying insects, were set out weekly in corn with the Herculex I trait plots for six weeks. In addition, plants in the center of the plot were visually evaluated for the presence of beneficial insects. The results were compared with non-biotech corn and indicated no adverse effect of corn with the Herculex I trait plants on the number of beneficial insects like ladybird beetles, predacious carabid beetles, lacewings, predatory wasps, parasitic wasps, damselflies, dragonflies and spiders.

3.4 Endangered Species Risk Assessment

Because of the selectivity of Cry1F protein for lepidopteran species, endangered species concerns primarily focus on the order Lepidoptera. The distribution of most endangered lepidopteran species listed by the U.S. Fish and Wildlife Service does not overlap with corn production areas. The exception is the Karner blue butterfly, which lives in areas where corn is grown. After reviewing the preferred habitats of the Karner blue butterfly, the EPA concluded that there would be no exposure to significant levels of Cry1F protein. Also, corn is not considered to be a host plant for endangered Lipidoptera species.

4. Post Marketing Stewardship

DuPont is committed to rigorous stewardship of products derived through biotechnology through the development process and after a product has become commercialized.

4.1 Availability of Diagnostics

Validated methods to detect the *Cry1F* gene and Cry1F protein in transgenic plant material were developed by independent companies. Two methods based on the use of antibodies against the Cry1F protein, a lateral flow test kit (dip stick) and an ELISA assay were made commercially available even before planting of the first Herculex I crop (2003 season). Qualitative and quantitative PCR-based detection kits are commercially available.

4.2 Insect Resistant Management (IRM)

Companies registering biotech corn products expressing a Bt trait for corn borer protection, such as corn with the Herculex I trait, are required by the EPA to develop and implement an insect resistant management (IRM) plan. The purpose of an IRM plan is to prevent or delay the development of insect resistance to Bt proteins. This

can be achieved by planting a refuge of corn that does not contain a gene that produces a protein for controlling corn borers. The refuge provides a habitat where corn borers will not be exposed to plants producing the Bt corn borer protein. This population, which contains a large number of Bt susceptible corn borer moths, will mate with potentially resistant moths that emerge from a corn borer protected field. The mating of susceptible and resistant corn borer moths results in offspring that are susceptible to the Bt corn borer protein. These offspring will die as larvae if they feed on tissue of a Bt corn borer protected plant. This use of refuges is one strategy to slow the evolution of resistance to Bt proteins and preserve the efficacy of Bt corn borer technology and Bt sprays

Additional research is being conducted to further define practical and effective long-term management strategies.

As a condition of registration, Pioneer Hi-Bred International, Inc., requires that all growers purchasing hybrids with the Bt corn borer trait sign a *Pioneer Hi-Bred Technology Agreement*. By signing the agreement, growers agree to plant at least 20% of their corn acres in corn refuge (50% in certain corn/cotton growing counties). In addition, the Bt corn borer protected corn must be within ½ mile (preferably ¼ mile) of this corn refuge. Bt spray-able insecticides must not be applied to refuge corn, but other insecticides may be applied if the economic threshold has been reached for specific target insects.

4.3 Reporting of Adverse Effects

Any information obtained by Pioneer Hi-Bred International, Inc., regarding unreasonable adverse effects of biotech crops containing plant-incorporated pesticides on human health or the environment will be reported, as required, to the EPA. EPA has established regulations interpreting what types of information would be considered "unreasonable."

Herculex Insect Protection technology by Dow AgroSciences and Pioneer Hi-Bred. ®Herculex is a registered trademark of Dow AgroSciences LLC.