11) Kolwyck, D., B-P. Tonnu, Y. A. Dudin, T. Ploesser and K. Gustafson (2001). Validated method for extration and direct ELISA analysis of Cry3Bb1 in corn grain. An unpublished method developed by Monsanto Company.

TITLE

Validated Method for Extraction and Direct ELISA Analysis
of Cry3Bb1 in Corn Grain

DATA REQUIREMENT

Petition for an Exemption from the Requirement of a Tolerance for Cry1Ab, Cry1Ac, Cry2Ab, Cry3A and Cry3Bb in All Food Commodities
EPA Tolerance Petition Number: 7F4888

AUTHORS

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DATE

April 7, 2001

PERFORMING LABORATORY

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DOCUMENT REFERENCE No.

99-640E-1

Statement of No Data Confidentiality Claim

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d)(1)(A), (B) or (C).

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Company: Monsanto Company

Company Agent: _____

Dennis P. Ward, Ph.D.
Regulatory Affairs Manager

Date: April 6, 2001

Statement of Compliance

The development of this analytical method and subsequent validation was not conducted under the Good Laboratory Practice Standards, 40 CFR Part 160, as set down in the Federal Register, 54:34052-74 of August 17, 1989. The method development and validation are not considered studies and therefore do not fall within the scope of the Good Laboratory Practice Standards. The validation summary describes the experiments that were performed to determine the precision, accuracy, sensitivity and specificity of the analytical method. However, a quality control review was conducted to ensure the quality and integrity of the data generated and the documentation of the results of the validation experiments.

Sponsor/Submitter

7 April 2001
Date

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Validated Method for Extraction and Direct ELISA Analysis of Cry3Bb1 in Corn Grain

Overview

Purpose & Scope

This analytical method describes the extraction and quantitation by direct sandwich ELISA of Cry3Bb1 protein in corn grain. This method is not intended to be used as an enforcement method. To ensure optimum extraction of the tissue, performance of the assay, and reliability of the results, perform the procedures as outlined according to the parameters specified.

The method is presented in four portions: sample preparation, extraction of Cry3Bb1 protein from corn grain, preparation and storage of extracts and ELISA.

Important Safety Precautions

Some important precautions include:

- Follow all appropriate personal protective equipment (PPE) procedures. Safety glasses are required at **all times** in the laboratory. Laboratory coat and gloves are recommended.
- Wear thermal gloves to avoid skin contact with dry ice, which may cause **burns**.
- Phosphoric acid can **irritate skin** and permanently **damage clothing**. Rinse with copious amounts of water if the acid comes in contact with skin or clothing.
- The 3,3′,5,5′-tetramethylbenzidine (TMB) substrate is a suspected **carcinogen** and is a skin and eye **irritant**. Use appropriate PPE (*e.g.*, protective gloves, safety glasses) when handling.
- Consult each manufacturer's MSDS for further information.

Abbreviations

The following abbreviations are used in this analytical method:

Abbreviation	Definition
Ab	antibody
ACS	American Chemical Society
BSA	bovine serum albumin
DF	Dilution Factor
fwt	fresh weight
HRP	horse radish peroxidase
LOD	limit of detection
LOQ	limit of quantitation
mQ	milli-Q
MSDS	Material Safety Data Sheet
OD	optical density
PBST	phosphate buffered saline + 0.05% Tween-20
% CV	percent coefficient of variation (SD/mean x 100)
PPE	personal protective equipment

Abbreviations (Continued)

The following abbreviations are used in this analytical method (continued):

Abbreviation	Definition
QC	quality control
QS	quantity sufficient to make the total volume
R^2	coefficient of determination for line regression
RT	room temperature
SD	standard deviation
T:B	tissue weight to buffer volume ratio
TBA	Tris-borate + 0.2% L-ascorbic acid
TMB	3,3′,5,5′-tetramethylbenzidine
Tween-20	polyoxyethylene-sorbitan monolaurate

Sample Preparation

Order of Preparation

To avoid contamination, samples must be processed in the following order:

- conventional
- transgenic

General Procedure

Following is the procedure for grain sample preparation. Process at least 15-20 grams of each sample.

Task	Description
1	To ensure the samples are properly identified, compare the sample material with the container label.
2	Homogenize the desired amount of grain sample, with dry ice, using a vertical cutter mixer (Waring blender).
3	Collect a processed sample sufficient for analysis and place into a plastic bag. Label the bag with the unique identification of the sample. Place the remaining homogenized sample (bulk) back into the original bag.
4	Corn grain samples processed with dry ice should remain outside the storage freezer until the dry ice dissipates for safety reasons (the sample remains frozen).
5	Store the processed sample in a -80°C freezer until the processed sample is required for extraction.
6	Rinse equipment with hot water. Clean with a suitable detergent (*e.g.*, Labtone). Rinse with hot water followed by acetone. Air dry or wipe dry using a clean disposable towel (*e.g.*, Accudry). Ensure equipment is clean prior to preparing each sample.

Extraction of Cry3Bb1 Protein from Corn Grain

Overview

Following are the procedures to extract Cry3Bb1 protein from corn grain.

Preparing the Grain

Following are the steps to prepare the corn grain prior to extraction:

Step	Action
1	Choose a vessel appropriate for the volume of buffer needed for the extraction (*e.g.* 15 mL Sarstead centrifuge tube).
2	Obtain frozen, processed grain and place on dry ice until weighing.
3	Weigh enough grain for the desired volume of extract. **Note:** Use a pre-chilled spatula to remove frozen tissue and weigh quickly to prevent thawing of the tissue. **Record the Weight and Buffer Volume** added to each sample.

Tissue Type	T:B Ratio	Extraction Buffer	Recommended Grain Amount per sample
grain	1:100	PBST	0.1 g ± 0.05 g

Step	Action
4	Place the extraction vessel on dry ice or in a -80°C freezer until performing the tissue extractions.

Initially Inspecting & Cleaning the Blade

Following are the steps to inspect and clean the blade on the Brinkman Polytron® (PT-3000) homogenizer prior to extracting any samples:

Step	Action
1	Examine the homogenizer blade to ensure it rotates freely.

IF the blade...	THEN...
rotates freely and no obstructions are observed	continue with step 2.
does not rotate freely	select another generator and repeat inspection.

Step	Action
2	Immerse the blade in ethanol above the level that will contact the extraction mixture and run the generator for 15-25 seconds at maximum speed.
3	Rinse the generator with mQ H_2O using a squirt bottle to remove ethanol.
4	Immerse the blade in mQ H_2O above the level that will contact the extraction mixture and run the generator for 15-25 seconds at maximum speed.

Extracting the Grain

Following are the steps to extract the grain. **Note:** Extract all non-transgenic samples prior to transgenic samples to minimize the possibility of cross-contamination.

Step	Action
1	Place the extraction vessel on wet ice and add the appropriate volume of extraction buffer (determined by T:B ratio in step 3 of **"Preparing the Grain"**).
2	Homogenize the sample for 60 seconds (or twice for 30 seconds) by immersing the tip of the blade ≥½" into the extraction mixture and homogenize at 17,500 rpm. -**Note:** If performing twice, allow the extraction mixture to cool 10-15 seconds between homogenizations.
3	Place the extraction vessel on wet ice. **Note:** After 4 hours on wet ice, the extracts must not be used and should be discarded.

Preparation and Storage of the Extracts

Cleaning the Blade

Following are the steps to clean the blade between grain extractions and prior to storage. **Note:** It is important to wash the blade in ethanol prior to storage to prevent microbial growth and rusting.

Step	Action
1	Rinse the blade with mQ H_2O using a squirt bottle to remove any remaining extract material.
2	Immerse the blade in mQ H_2O above the level that will contact the extraction mixture and run the generator for 15-25 seconds at maximum speed.
3	Check for debris caught in the blade. If necessary, remove the blade, dislodge any particles, and repeat steps 1 and 2.
4	Use the table below to determine the next step.

IF cleaning between extractions of...	THEN...
replicate tissue	extract the next tissue sample.
non-replicate tissue or prior to storage	continue with step 5.

Step	Action
5	Immerse the blade in ethanol above the level that will contact the extraction mixture and run the generator for 15-25 seconds at maximum speed.
6	Use the table below to determine the next step.

IF cleaning...	THEN...
between extractions of non-replicate tissue	repeat steps 1-3 before extracting the next tissue sample.
prior to storage	the homogenizer is ready for storage.

Preparing & Storing the Extracts

Following are the steps to prepare and store the extracts for analyses:

Step	Action
1	Remove particulate matter from the extract by centrifuging the extract to a pellet using the recommended parameters: 10 minutes, , approximately 9,700g and at 4-8°C. Carefully withdraw the supernatant. **Note:** If a pellet does not form, this step may be repeated 2X.
2	Aliquot the samples into appropriately labeled tubes. **Note:** Generate ≥1.5 times the number of aliquots required for analyses (usually need at least 0.35 mL per ELISA).
3	Store the aliquots in a -80°C freezer until analyses.

ELISA Analysis of Cry3Bb1 Protein in Corn Grain

The assay format is a one-step, two-site sandwich ELISA. Protein-A purified polyclonal rabbit anti-Cry3Bb1 is prepared in carbonate/bicarbonate buffer and dispensed onto 96-well microtiter plates. The plates are coated overnight at ~4°C and are washed prior to use. The ELISA is performed in triplicates. The incubation step consists of a simultaneous addition of standards, quality control samples and unknowns with Protein-A purified rabbit anti-Cry3Bb1 conjugated to horseradish peroxidase. The plates are covered with adhesive sealer and incubated for approximately 1 hour at 37°C. Plates are then washed and developed with TMB for about 10 minutes at room temperature. The enzymatic reaction is terminated by the addition of phosphoric acid. Absorbance readings are determined immediately following the termination of the reaction. The concentration of the Cry3Bb1 protein levels is determined by reference to a 7-point standard curve ranging from 1 to 64 ng/mL. Absorbance readings and protein concentrations are fitted with a 4-parameter regression model. It is recommended to test each event for optimal dilution(s) such that samples fall within the range of the quantitation standards to eliminate any prozone effects or hook effects associated with one-step assays. The validation summary is provided in the attachments.

The following ELISA method is presented in worksheet format for the convenience of the analyst.

Plate _____ of _____

Study No.: _____ Initials: _____ Date: _____

1. Plate Coating

Plates expire at 15 days post-coating day when stored sealed at 4-8°C.

Lot: _____ Exp. date: _____

2. Standards Preparation

Stock Cry3Bb1 standard:

Concentration: _____ mg/mL.

- Dilute stock standard to 0.128 mg/mL in PBST/0.1% BSA (*e.g.*, 11.8 µL of 0.271 mg/mL stock standard in 13.2 µL of buffer). Further dilute standard at 1:100 to 1.28 µg/mL in appropriate sample buffer (*e.g.*, 10 µL of 0.128 mg/mL standard in 990 µL of buffer).

Sample and Standard buffer diluent: PBST/0.1% BSA:

Lot: _____ Exp. date: _____

_____ µL of stock standard in _____ µL of PBST/0.1% BSA to make 0.128 mg/mL.

_____ µL of 0.128 mg/mL standard in _____ µL of sample buffer to make 1.28 µg/mL.

- Generate the quantitation standards according to the following table.

Std. No.	Quantitation Standards Scheme (mL)	Sample Buffer (mL)	Cry3Bb1 Concentration (ng/mL)
1	0.05 mL of 1.28 µg/mL working standard	0.950	64
2	0.500 mL of standard 1	0.500	32
3	0.500 mL of standard 2	0.500	16
4	0.500 mL of standard 3	0.500	8
5	0.500 mL of standard 4	0.500	4
6	0.500 mL of standard 5	0.500	2
7	0.500 mL of standard 6	0.500	1

3. Conjugate Preparation

HRP-rabbit anti-Cry3Bb1 detection antibody:

Conjugate used

Lot: _____ Exp. date: _____

- Dilute conjugate further at appropriate dilution and record the scheme:

_____ µL of above conjugate in _____ mL of StabilZyme HRP conjugate stabilizer.

StabilZyme: Lot: _____ Exp. date: _____

4. Plate Loading.

- Wash plate 3 times quickly with wash buffer (PBST)
- Dilute samples in sample buffer as described below.

Note: Samples, Standards and QCs should be prepared on wet ice. It is recommended to test each event for optimal dilution(s) such that samples fall within the range of the quantitation standards. The first time samples are tested as serial dilutions at 1:5, 1:10, 1:30, 1:50, 1:100 to eliminate any prozone effects or hook effects associated with one-step assays. If transgenic samples do not fall within the range of quantitation, repeat using appropriate dilutions.

Tissue Type	Sample Buffer (for Standard & Sample Analyses)	Suggested Pre-dilution	Maximum Volume of Undiluted Extract
corn grain	PBST/0.1% BSA	1:30	50 µL/well

- Record unique identifier, dilution factor of each sample, and dilution of quality control samples on plate template as appropriate.

Positive QC: Lot: _____ Exp. date: _____

Negative QC: Lot: _____ Exp. date: _____

- Load 50 µL/well of buffer blank (PBST+0.1% BSA), standards, samples, and QCs in triplicate. Add 50 µL of appropriately diluted conjugate to all appropriate wells. Gently tap the plate to mix the reagents in the well. **Note:** Refer to plate template for loading locations.
- Cover plate with sealer and incubate in 37°C incubator for 65 ± 5 minutes.

Incubation time: _____ minutes.

5. TMB Substrate Preparation (use only Kirkegaard & Perry Cat. No. 50-76-03)

- Ensure TMB substrate components TMB and H_2O_2 are at RT before using.
- Mix the two components together at 1:1 ratio (prepare ≥10 mL/plate).

_____ mL of **TMB:** Lot: _____ Exp. date: _____

_____ mL of **H_2O_2:** Lot: _____ Exp. date: _____

6. Development

- Wash plate 3 times quickly with PBST wash buffer.
- Add 100 µL of TMB substrate solution to all appropriate wells.
- Develop plate for 10 minutes (±60 seconds)
- Add 100 µL of stop solution/well to stop development. **Note:** Mix if necessary. Termination of the reaction is evidenced by an even yellow color in all wells.

Stop solution (6M Phosphoric Acid): Lot: _____ Exp. date: _____

Development time: _____ minutes.

7. Data Generation

- Wipe smudges from bottom of plate and read at 450 nm with 650 or 655 nm reference wavelength.

Software used for data reduction: SOFTmax PRO, version 2.4.1 (4-parameter curve fit)

Filename for data reduction: _____

Comments: _____

_____

Materials

Equipment

The following equipment is used in this procedure (**equivalents may be substituted**):

Equipment	Number/Specification
Analytical balance	Mettler AE250
Borosilicate glass culture tube (12 *x* 75 mm)	Kimax Cat. No. 60825-550
Centrifuge (refrigerated super-speed)	Sorvall RC-5B
Homogenizer	Brinkmann Polytron PT3000
Incubator	VWR 1535
Microcentrifuge tubes	VWR Scientific Cat. No. 20170-369
Centrifuge tubes (13 mL)	Sarstedt 60.540
Microtiter plate (96-well polystyrene)	Nunc Maxisorp Cat. No. 472230
Microtiter plate reader	Molecular Devices SPECTRAmax Plus
Microtiter plate sealer	Dynatech Cat. No. 001-010-5701
Microtiter plate washer	SLT 96PW
Reservoir trays (multi-channel pipet)	Costar Cat. No. 4870
Commercial Blender	Waring, Commercial

Reagents

The following reagents are used in this procedure (**equivalents may be substituted**):

Reagent	Number/Specification
Ethanol	Aaper 200 proof
BSA	Sigma Cat. No. A7888
Concentrated HCl	Fisher Cat. No. A144-500
Concentrated H_3PO_4 (approximately 85%)	Mallinckrodt Cat. No. 2796-1
KCl	Sigma Cat. No. P4504
L-ascorbic acid	Sigma Cat. No. A-7631
$MgCl_2$	Mallinckrodt Cat. No. 5958t
mQ H_2O	-
NaCl (ACS crystals)	Fisher Cat. No. 271-500
• NaOH • 2.5 N NaOH	• Sigma Cat. No. S8045 • Fisher Cat. No. SS414-1
10X PBS	Boehringer Mannheim Cat. No. 16666789
Potassium phosphate monobasic, KH_2PO_4, (ACS crystals)	Mallinckrodt Cat. No. 7100
Sodium bicarbonate (Na_2HCO_3)	Fisher Cat. No. S233-500
Sodium carbonate (Na_2CO_3)	Fisher Cat. No. S263-500
Sodium phosphate dibasic, heptahydrate Na_2HPO_4 *x* 7 H_2O, (ACS crystals)	Fisher Cat. No. S373-500
Na_2CO_3/Na_2HCO_3 buffer capsules	Sigma Cat. No. C3041
Sodium tetraborate ($Na_2B_4O_7$ *x* 10 H_2O)	Fisher Cat. No. BP175-500
StabilZyme HRP conjugate stabilizer	Surmodics Product No. SZ02-1000
TMB substrate	Kirkegaard & Perry Cat. No. 50-76-03 (**no substitutions**)
Tris base (Trizma)	Sigma Cat. No. T-1503
Tween-20	Sigma Cat. No. P-1379

Assay Reagents

Overview

The following describes the appropriate dilutions, stability, and storage of current assay reagents used in this procedure. All new reagent lots must be evaluated/ bridged against previous lot for performance and appropriate dilution.

Reference Standard

Cry3Bb1 Purified Protein Standard:

- Standard is stored in 50 mM Na_2HCO_3/0.05% Tween 20 Buffer
- Store in -80°C freezer. Do not subject standard to multiple freeze/thaw cycles.
- Store diluted or thawed standard in 4°C refrigerator for up to 7 days.

Coating Antibody

Polyclonal Rabbit Anti-Cry3Bb1 Purified Antibody:

- Need ≥10 mL/plate.
- Store in -20°C (or below) freezer.
- Store thawed Ab Stock in 4°C refrigerator for up to 1 year.

Conjugate

HRP-Rabbit Anti-Cry3Bb1 Detection Antibody:

- Dilute in StabilZyme before use.
- Store in 4°C refrigerator for up to 1 year.
- Store in 4°C refrigerator for up to 3 months if the conjugate is made as a working dilution aliquot in StabilZyme.

Positive QC

Cry3Bb1 Positive QC:

- Store in -80°C freezer.
- Do not subject reagent to multiple freeze/thaw cycles.

Buffers and Solutions

Overview

The following describes the preparation and storage of the buffers and solutions used in this procedure. **Note:** Volumes may be scaled as needed. Equivalent reagents may be substituted, however, verification of equivalence may be required. To filter solutions, use a 0.45 or 0.22 µm filter and a sterile receptacle.

10X PBST

10X PBST - For 1 liter:

- 80 g NaCl (1.38 M)
- 21.68 g $Na_2HPO_4 \times 7\ H_2O$ (0.081 M)
- 2 g KH_2PO_4 (0.015 M)
- 2 g KCl (0.027 M)
- 5 mL Tween-20™ (0.5% v/v)
- QS to 1 liter with mQ H_2O, pH 7.4 (no pH adjustment should be needed)

Store at RT for up to 1 year if filtered or for up to 6 months if not filtered.

Note: If using an equivalent reagent (10X PBS from Boehringer Mannheim), follow storage and expiration according to the manufacturer's instructions.

Ab Coating Buffer

Ab Coating Buffer - For 1 liter:

- 1.59 g Na_2CO_3 (0.015 M)
- 2.93 g $NaHCO_3$ (0.035 M)
- QS to 1 liter with mQ H_2O, pH 9.6 (no pH adjustment should be needed)

Alternatively, dissolve one capsule of $Na_2CO_3/NaHCO_3$ buffer per 100 mL of mQ H_2O, pH 9.6, at 25°C as stated by manufacturer.

Store at RT for up to 6 months.

Ab Coated Plates

Anti-Cry3Bb1 Ab Coated Plates - For 1 plate:

- Dilute anti-Cry3Bb1 Ab in Ab coating buffer. Below is an example calculation for Ab dilution. NOTE: This depends on the Lot # used for assay reagent preparation.
- Load 100 µL of diluted coated Ab/well.
- Cover plate with sealer or lid and incubate in 4°C refrigerator ≥8 hours or 37°C incubator for 1 hour followed by 1 hour at RT.

Store in 4°C refrigerator for up to 14 days.

__A__ µg of Ab/well / 100 µL of Ab/well = __B__ µg/µL of Ab solution.
__B__ µg/µL of Ab solution x 10 mL of Ab solution x 1000 µL/mL = __C__ µg of Ab needed.
__C__ µg of Ab needed / __B__ µg/µL of Ab = __D__ µL of Ab.

Extraction Buffer (Grain)	**PBST** - For 1 liter: • 100 mL 10X PBST • 900 mL mQ H_2O Store at RT for up to 1 year if filtered or for up to 6 months if not filtered.
Sample Buffer (Grain)	**PBST/0.1% BSA** - For 1 liter: • 100 mL 10X PBST • 900 mL mQ water • 1 g BSA (0.1% w/v) **Note:** PBST may be used instead of 10X PBST and water. Store in 4°C refrigerator for up to 3 months if filtered or for up to 4 weeks if not filtered.
TMB Substrate Solution	**TMB Substrate/H_2O_2** - Use only Kirekegaard solution . **Note:** The TMB substrate is unstable in light. Avoid prolonged exposure of the mixture to light. Use within 10-15 minutes of mixing the two substrate components.
Stop Solution	**6 M H_3PO_4** - For 500 mL: • 296.5 mL mQ H_2O • 203.5 mL concentrated H_3PO_4 Store at RT for up to 1 year. **Caution:** Exothermic reaction. Add acid slowly to water and prepare in the fume hood.
Wash Buffer	**PBST** - For 1 liter: • 100 mL 10X PBST • 900 mL mQ H_2O Store at RT for up to 1 year if filtered or for up to 6 months if not filtered.

Assay Evaluation and Performance

Assay Acceptance Criteria

The following criteria are used to evaluate the performance of the assay.
Note: An assay that does not meet these criteria cannot be considered reliable and all samples must be repeated.

Criteria	Value/Range
Mean absorbance of the buffer blank: (L_1-L_2) +3 SD	• **PBST/0.1% BSA:** ≤0.094
Mean absorbance of the highest standard (with blank subtracted): (L_1-L_2) +3 SD	• **PBST/0.1% BSA:** ≥0.950
% error of back-calculated concentration of the standards	• **1 ng/mL standard:** ≤20% • **2-64 ng/mL standards:** ≤15%
R^2 of the standard curve	≥0.98
Positive QC (±3 SD)	Lot specific criteria
Negative QC (+3 SD)	Lot specific criteria

Sample Acceptance Criteria

The following criteria are used to evaluate the results of the samples.
Note: A sample that does not meet the appropriate criterion cannot be considered reliable and must be repeated. If the criterion is not met after running the sample twice, report the mean of all analyses.

Criteria	Value
% CV of quantifiable triplicate sample wells (based on concentration, value given as absorbance with blank subtracted):	
• In the range of standards ≥1 and <2 ng/mL	• ≤20%
• In the range of standards ≥2 and ≤64 ng/mL	• ≤15%

Performance Parameters

Following are the performance parameters of the ELISA:

Parameter	Value/Range		
LOD (negative extract tissue +3 SD)	**Tissue**	**ng/mL**	**µg/g fwt**
Note: Non-transgenic or negative extracts were used neat (*i.e.*, no dilution and loaded at 50 µL/well).	corn grain	0.96	0.096
Range of quantitation	1-64 ng/mL		
Method for curve fit (SOFTmax PRO software version 2.4.1)	4-parameter		

fwt Expression Level

Following is the calculation used to determine the µg/g fwt expression level prior to correction for method bias. After obtaining this value, use the appropriate bias correction factor (stated above) to determine the actual expression level in the grain:

µg/g fwt = (ng/mL of extract from SOFTmax PRO)(DF*)(T:B ratio) / 1000

***DF = Insert the dilution factor used before samples were loaded on the plate**

Correction for Method Bias

Correct all expression data generated for the sample for method bias by dividing the µg/g fwt value by the appropriate bias correction factor. **Note:** The bias correction factor is determined during ELISA validation.

Grain Type	Spike & Recovery	Extraction Efficiency	Bias Correction Factor (Spike & Recovery)(Extraction Efficiency)
corn grain	87.7%	70.9%	0.62

Cry3Bb1 ELISA Validation Summary

I. Accuracy

Tissue Type	Tissue to Buffer Ratio	Extraction Variability[1] (CV%)	Extraction Efficiency[2] (%)	Spike and Recovery[3] (%)	Method Bias Correction Factor[4]
Corn grain	1:100	21.1	87.7	70.9	0.62

II. Precision

Standard Curve Precision Range[5]:	4.4 – 14.6% CV
Intra-Assay Variability[5]:	6.1% CV
Inter-Assay Variability[5]:	8.4% CV

III. Range of Quantitation

1.0 – 64 ng/mL

IV. Sensitivity

Tissue Type	Dilution or Max Sample Volume (µL/well)	LOD (ng/mL)[6]	LOD (µg/g fwt)[7]
Corn grain	Neat / (50)	0.96	0.096

1. Extraction variability was assessed by determining the %CV (standard deviation, SD divided by the average and multiplied by 100%) of the Cry3Bb1 protein levels based on fourteen extractions of the same sample of corn grain.
2. Extraction efficiency for each tissue type was determined by extracting three replicates and re-extracting the same tissue several successive times.
3. Spike and recovery: grain from control corn plants were extracted with assay buffer spiked with known quantities of Cry3Bb1 protein at three concentrations spanning the range of quantitation. Each spike was performed in three replicates.
4. Method Bias Correction Factor: (defined as two components of accuracy) More specifically, mean recovery of the standard protein from spiked control tissues (see note 3) and extraction efficiency for transgenic tissues (see note 2) comprised the method bias which was calculated from the combined measurement of these two parameters. The following formula was applied to all the Cry3Bb1 protein measurements after conversion to µg/g fwt values: Total Cry3Bb1 protein (µg/g fwt) = µg/g fwt / [(% extraction efficiency)/100 × (% spike and recovery)/100]. All of the protein data was corrected by the grain specific factor, unless value was below LOD.
5. The intra-assay precision was assessed by determining the %CV of the protein level in a particular sample within a single assay in triplicate wells. The inter-assay precision was assessed by measuring the concentration of a positive sample in 10 ELISAs. Intra- and inter-assay variability were calculated using a one way analysis of variance (ANOVA).
6. The limit of detection (LOD) was calculated using the non-transgenic corn grain sample extracts. The LOD was calculated at the maximum sample loading volume of 50 µL/well as follows: LOD = mean (ng/mL) + 3 standard deviations.
7. LOD (µg/g fwt): the ng/mL values were converted to µg/g fwt by multiplying the LOD (ng/mL; note 6) by the tissue weight to buffer volume ratio (g/mL) and dividing by a conversion factor of 1000 ng/µg.

Extraction Worksheet for Processed Corn Grain

Experiment #: _____ **Initials:** _____ **Date:** _____

Crop:	Buffer Name:	
Tissue Type:	Buffer Lot:	Exp. Date:
Generator/Homogenizer:	Serial #:	Location:
Centrifuge:	Serial #:	Location:

Explain if other equipment is used for extraction_____

Sample #	Line ID	Tissue Wt *Record Actual Weight*	Tissue to Buffer Ratio	Actual Buffer Volume (µL)	No. of Vials	Comments

(*Check off as actual buffer volume is added. Check off each extract in the last column as received.)

Comments: _____

	Initials:	Date:	Total # Vials	Storage Conditions
Extracts Prepared by:				