ENVIROLOGIX™

QuantiPlate™ Kit for Cry9C

Catalog Number AP 008

Highlights:

- **High Sensitivity Protocol**
  has a range of 0.01% to 0.125% StarLink corn (by weight) and takes 3 hours to run
- **Rapid Protocol**
  has a range of 0.04% to 0.25% StarLink Corn, and takes only 1.75 hours to run.

Contents of Kit:

- *12 strips of 8 antibody-coated wells each, in plate frame*
- *Cry9C Negative Control*
- *0.01% StarLink (0.2 ppb Cry9C) Calibrator*
- *0.04% StarLink (0.8 ppb Cry9C) Calibrator*
- *0.125% StarLink (2.5 ppb Cry9C) Calibrator*
- *0.25% StarLink (5 ppb Cry9C) Calibrator*
- *Cry9C-Enzyme Conjugate*
- *20X Grain Extraction Concentrate*
- *1 packet of Buffer Salts*
- *Substrate*
- *Stop Solution*

Intended Use

The EnviroLogix QuantiPlate Kit for Cry9C is designed for the quantitative or qualitative laboratory detection of Cry9C endotoxin in bulk corn grain or seed. The most prevalent Cry9C varieties in corn are StarLink™ and StarLink licensees. For ease of reference only, corn modified with Cry9C is referred to throughout this Product Insert as StarLink. Two assay protocols are presented: High Sensitivity and Rapid Protocols.

For instructions on use of this kit with corn leaves, single corn seeds, corn flour, cornmeal, and corn grits, please see the Appendices, Pages 11-13.

How the Test Works

This QuantiPlate Kit is a "sandwich" Enzyme-Linked ImmunoSorbent Assay (ELISA). In the test, corn product sample extracts are added to test wells coated with antibodies raised against Cry9C toxin. Any residues present in the sample extract bind to the antibodies, and are then detected by addition of enzyme (horseradish peroxidase)-labeled Cry9C antibody.

After a simple wash step, the results of the assay are visualized with a color development step; color development is proportional to Cry9C concentration in the sample extract.

Lighter color = Lower concentration
Darker color = Higher concentration

Limit of Detection

The Limit of Detection (LOD) of the QuantiPlate Kit for Cry9C, High Sensitivity protocol, is 0.070 parts per billion (ppb) Cry9C in corn product extract. The LOD was determined by interpolation at 0.079 OD (optical density absorbance) units from a Cry9C standard curve. 0.079 OD units was determined to be 3 standard deviations from the mean of a population of negative corn product samples in the High Sensitivity assay.

The LOD of the EnviroLogix QuantiPlate Kit for Cry9C, Rapid protocol, is 0.250 ppb Cry9C in corn product extract. The LOD was determined by interpolation at 0.083 OD units from a Cry9C standard curve. 0.083 OD units was determined to be 3 standard deviations from the mean of a population of negative corn product samples in the Rapid assay.

Limit of Quantification

The Limit of Quantification (LOQ) of the QuantiPlate Kit for Cry9C was validated at 1.5 ppb in the High Sensitivity assay, and at 6 ppb in the Rapid protocol. The LOQ was determined by fortifying a population of ground corn, cornmeal, corn flour and corn grits samples at the above concentrations of Cry9C protein. In the High Sensitivity protocol, the mean recovery was 101% with a coefficient of variation [CV, (standard deviation/mean) x 100] of 19%. In the Rapid assay, the mean recovery was 110%, with a CV of 15%.

Precision

	Recov. (%CV)	OD (%CV)	Recov. (%CV)	OD (%CV)
	Intra-Assay		**Inter-Assay**	
	High-Sensitivity protocol			
Ctl #1	8.1%	5.6%	23.8%	n/a
Ctl. #2	3.7%	3.4%	20.1%	n/a
	Rapid protocol			
Ctl. #1	2.4%	2.5%	15.2%	n/a
Ctl. #2	1.7%	1.7%	14.2%	n/a

Prepare wash buffer and grain extraction solutions

Precision

Cry9C-fortified control solutions were repetitively analyzed both within a single assay (Intra-Assay), and in different assays on different days (Inter-Assay). The data is expressed as % CV for both the recovered concentration (Recov.) and for absorbance (OD).

Fortification and Recovery

For the High Sensitivity protocol, six ground corn, two cornmeal, two corn flour, and two corn grits samples were fortified with Cry9C to a concentration of 6 ppb. The average recovery was 116%, with a CV of 16%.

For the Rapid protocol, six ground corn, two cornmeal, two corn flour, and two corn grits samples were fortified with Cry9C to a concentration of 15 ppb. The average recovery was 113%, with a CV of 9%.

Materials Not Provided

- distilled or deionized water for preparing Wash Buffer and diluting 20X Grain Extraction Concentrate
- glass bottles or flasks with 1.2 liter capacity for preparation of 1X Grain Extraction Solution and 1 liter capacity for preparation of Wash Buffer; plus graduated cylinders for measuring the components of these solutions
- test or centrifuge tubes for extraction of grain and dilution of sample extracts (optional)
- centrifuge capable of 5000 x g, or low protein-binding hydrophilic syringe filters, 0.45 µm (such as Pall Gelman Sciences – Product No. 4184) (optional)
- disposable tip, adjustable air-displacement pipettes which will measure 100 and 1000 microliters (µL), and 5 mL
- marking pen (indelible)
- tape or Parafilm®
- timer
- microtiter plate reader or strip reader
- wash bottle, or microtiter plate or strip washer
- multi-channel pipette that will measure 100 µL (optional)
- racked dilution tubes for loading samples into the plate with a multi-channel pipette (optional)
- orbital plate shaker (optional)

Preparation of Solutions

Wash Buffer:

Add the contents of the packet of Buffer Salts (phosphate buffered saline, pH 7.4 - Tween 20) to 1 liter of distilled or deionized water, and stir to dissolve. Store refrigerated when not in use; warm to room temperature prior to assay.

1X Grain Extraction Solution:

To prepare 1X working Grain Extraction Solution, either:

- Mix 5 mL of Grain Extraction Concentrate (20X) plus 95 mL distilled or deionized water for every 100 mL required, or
- Add the entire contents of the bottle of Grain Extraction Concentrate (60 mL) supplied in the kit to 1140 mL of distilled or deionized water in a suitable container.

Extract Sample

Centrifuge to clarify sample extract

Mix thoroughly to dissolve. May be stored at room temperature, but use within 30 days of preparation.

Sample Preparation

Sample Extraction:
Testing of bulk corn grain for Cry9C endotoxin is an indicator of the presence or absence of StarLink GM-modified corn in a given sample. A negative test with this kit is not an indicator of the absence of other genetic modifications.

This protocol calls for a small sample to be analyzed. It is essential that this sample be well mixed and representative of the larger bulk. Note that sampling to detect 0.01% is the equivalent of detecting one kernel of Cry9C corn in a sample of 10,000 kernels.

NOTE: Thorough mixing of the bulk grain sample and determination of an appropriate sampling plan are critical to the results of this testing, and are the responsibility of the user of this test kit. The USDA/GIPSA has prepared several guidance documents to address the issues involved in obtaining representative grain samples from static lots (such as trucks, barges, railcars) and for taking samples from grain streams. Sampling plans should be chosen that best meet the needs of both the buyer and seller in terms of acceptable risks. Increasing the number of kernels in the sample and taking multiple samples will increase the likelihood of obtaining representative samples, and maximize the probability of detecting contamination in the grain lot. For further information on USDA/GIPSA guidelines for obtaining representative samples and assessing detection probabilities for biotech grain, see the websites listed in the margin on page 4.

It is the responsibility of the user to ensure proper sampling and thorough mixing prior to analysis.

Once representative samples have been obtained from the truck or container, they can be reduced in size using a splitter and uniformly ground and mixed.

1. Weigh 1 gram of ground corn into a 10 mL capacity vial or tube.

2. Add 5 mL of 1X Grain Extraction Solution to each tube. Cap and shake vigorously by hand for 20-30 seconds. Let stand at room temperature to extract for at least 60 minutes. Optimal extraction requires 3 hours to overnight soaking.

3. The extracted samples may be quickly clarified by: a) centrifuging the extract at 5000 x g for 5 minutes, or, b) filtering the material through a low protein binding hydrophilic syringe filter, 0.45 µm (such as Pall Gelman Sciences - Product No. 4184). Alternatively, the extract may be allowed to settle undisturbed for a full hour of extraction (after the initial shaking) instead of clarifying mechanically. Pipette the liquid off of the settled sample without disturbing the particulate matter.

Sample Dilution:
Concentrations of Cry9C endotoxin in StarLink corn can range from 8 to 24 micrograms per gram (ppm), and average 10 to 12 ppm. If screening bulk grain for the presence or absence of Cry9C and the maximum sensitivity is desired, use undiluted extracts in the High-Sensitivity Protocol described below. If sample extracts produce more color than the highest calibrator, to

USDA Websites

http://archive.gipsa.usda.gov/reference-library/handbooks/grain-insp/grbook1/bk1.pdf
USDA Grain Inspection Handbook, Book 1, Grain Sampling. This document provides a comprehensive overview of recommended sampling guidelines for static lots and grain streams. It reviews the various types of equipment and strategies that can be used to obtain a representative grain sample from different types of containers.

http://archive.gipsa.usda.gov/biotech/sample2.htm
Guidance document entitled Sampling for the Detection of Biotech Grains, which provides important statistical sampling considerations when testing for the presence of biotech grains. It covers the basis for making probability determinations in accepting lots based upon different assumptions with respect to sample size, number of samples, sample preparation, etc.

http://archive.gipsa.usda.gov/biotech/sample1.htm
Practical Application of Sampling for the Detection of Biotech Grains. This one-page application guide provides a table that gives sample sizes for selected lot concentrations and probability of rejecting the specified concentrations. It also provides a formula for making the calculation for other combinations.

http://archive.gipsa.usda.gov/biotech/samplingplan1.xls
This website provides a simple to use Sample Planner (29k Excel Spreadsheet). The planner allows you to enter different assumptions in terms of sample size, number of samples, acceptable quality level and to determine the probability of accepting lots with given concentration levels. It also plots the probabilities in graph form for easy interpretation. Specific data can be saved for documentation and future analyses.

quantitatively determine the Cry9C endotoxin in that sample, dilute the sample extract in 1X Grain Extraction Solution and run the Rapid Protocol. Multiple dilutions may be required to get the sample extract within the range of calibration. Pure StarLink corn requires at least a 1:1000 additional dilution in order for the extract to be quantitated in the Rapid Protocol. Very strong positive samples may give erroneously low results if they are not diluted sufficiently.

How to Run the Assay

- Read all of these instructions before running the kit.
- Allow all reagents to reach room temperature before beginning (at least 30 minutes with un-boxed strips and reagents at room temperature - do not remove strips from bag with desiccant until they have warmed up).
- Organize all Calibrators and clarified sample extracts, and pipettes so that step 1 can be performed in 15 minutes or less.
- If more than four strips are to be run at one time, the 15 minutes is likely to be exceeded, and the use of a multi-channel pipette is recommended (see "Note" below).
- If four or fewer strips are to be run, use a disposable-tip air-displacement pipette and a clean pipette tip to add each Calibrator and sample extract to the wells. Conjugate, Substrate, and Stop Solution may be added in the same manner; alternatively, use a repeating pipette with a disposable tip on the end of the Combitip for these three reagents.
- If fewer than all twelve strips are used, reseal the unneeded strips and the desiccant in the foil bag provided, and refrigerate.
- Use the well identification markings on the plate frame to guide you when adding the samples and reagents. In a qualitative assay, the Negative Control (NC), the lowest calibrator and 46 sample extracts (S) may be run in duplicate wells on one plate. (See the Qualitative Assay Example Plate Layout - Figure 1A). For a quantitative assay the Negative Control (NC) and three Calibrators (C1-C3), along with 44 sample extracts (S) may be run in duplicate wells on one plate. (See the Quantitative Assay Example Plate Layout - Figure 1B).

Choose the assay protocol that best fits your needs for detection limits and your time constraints.

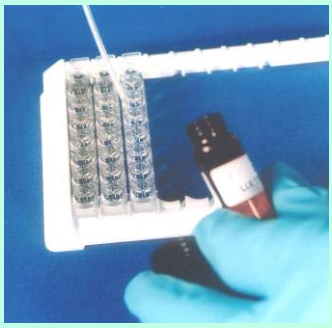
The **High Sensitivity Protocol** is for screening of bulk grain, with the ability to detect 0.01% to 0.125% StarLink corn (by weight) and requires 3 hours of total assay incubation time.

The **Rapid Protocol** is slightly less sensitive (able to detect 0.04% to 0.25% StarLink corn) but only requires one hour and 45 minutes of total assay incubation time.

NOTE: Estimates of sensitivity and calibrator equivalents in per cent contamination are based upon the assumption that the average StarLink corn expresses 10 ppm of Cry9C endotoxin. Since StarLink corn can express anywhere from 8 to 24 ppm endotoxin, per cent contamination estimates can be expected to vary by 2-fold or more.

Remove unneeded strips

Select Calibrators and Control



Add calibrators and sample extracts

HIGH SENSITIVITY PROTOCOL

Procedure

1. For this protocol, use the Negative Control, 0.01% StarLink (0.2 ppb Cry9C), 0.04% StarLink (0.8 ppb Cry9C), and 0.125% StarLink (2.5 ppb Cry9C) Calibrators. Do not use the 0.25% StarLink (5 ppb Cry9C) Calibrator.

   Add **100 µL** of **Negative Control, 100 µL** of each **Calibrator**, and **100 µL** of each clarified **sample extract** to their respective wells, as shown in the Example Plate Layouts (Figures 1A and 1B). Follow this same order of addition for all reagents.

NOTE: In order to minimize setup time it is recommended that a multi-channel pipette be used in steps 1, 5, 9 and 11 when more than 4 strips are used.

2. Thoroughly mix the contents of the wells by moving the strip holder in a rapid circular motion on the bench top for a full 20-30 seconds. Be careful not to spill the contents!

3. Cover the wells with tape or Parafilm to prevent evaporation and **incubate** at **ambient temperature for 30 minutes**. If an orbital plate shaker is available shake plate at 200 rpm.

4. After incubation, carefully remove the covering and vigorously shake the contents of the wells into a sink or other suitable container. Flood the wells completely with Wash Buffer, then shake to empty. Repeat this wash step three times. Alternatively, perform these four washes (300 µL/well) with a microtiter plate or strip washer. Slap the plate on a paper towel to remove as much water as possible.

5. Add **100 µL** of **Cry9C-enzyme Conjugate** to each well.

6. Thoroughly mix the contents of the wells as described in step 2. Be careful not to spill the contents!

7. Cover the wells with new tape or Parafilm to prevent evaporation and **incubate** at **ambient temperature for 2 hours**. If an orbital plate shaker is available shake plate at 200 rpm.

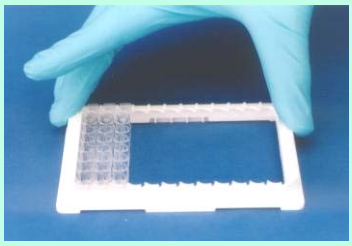
8. After incubation, carefully remove the covering and vigorously shake the contents of the wells into a sink or other suitable container. Flood the wells completely with Wash Buffer, then shake to empty. Repeat this wash step three times. Alternatively, perform these four washes (300 µL/ well) with a microtiter plate or strip washer. Slap the plate on a paper towel to remove as much water as possible.

9. Add **100 µL** of **Substrate** to each well.

10. Thoroughly mix the contents of the wells, as in step 2. Cover the wells with new tape or Parafilm and **incubate** for **30 minutes at ambient temperature**. Use orbital shaker if available.

Caution: Stop Solution is 1.0N Hydrochloric acid. Handle carefully.

11. Add **100 µL** of **Stop Solution** to each well and mix thoroughly. This will turn the well contents yellow.



Mix plate

Incubate

Bottle Wash method

Strip Plate Wash option

RAPID PROTOCOL

Procedure

1. For this protocol, use the Negative Control, 0.04% StarLink (0.8 ppb Cry9C), 0.125% StarLink (2.5 ppb Cry9C), and 0.25% StarLink (5 ppb Cry9C) Calibrators. Do not use the 0.01% StarLink (0.2 ppb Cry9C) Calibrator.

   Add **100 µL** of **Negative Control, 100 µL** of each **Calibrator**, and **100 µL** of each clarified **sample extract** to their respective wells, as shown in the Example Plate Layouts (Figures 1A and 1B). Follow this same order of addition for all reagents.

NOTE: To minimize setup time, it is recommended that a multi-channel pipette be used in steps 1, 4, 8 and 10 when more than 4 strips are used.

2. Thoroughly mix the contents of the wells by moving the strip holder in a rapid circular motion on the bench top for a full 20-30 seconds. Be careful not to spill the contents!

3. Cover the wells with tape or Parafilm to prevent evaporation and **incubate at ambient temperature for 15 minutes**. If an orbital plate shaker is available shake plate at 200 rpm.

4. Add **100 µL** of **Cry9C-enzyme Conjugate** to each well. Do not empty the well contents or wash the strips at this time.

5. Thoroughly mix the contents of the wells as described in step 2. Be careful not to spill the contents!

6. Cover the wells with new tape or Parafilm to prevent evaporation and **incubate at ambient temperature for 1 hour**. If an orbital plate shaker is available shake plate at 200 rpm.

7. After incubation, carefully remove the covering and vigorously shake the contents of the wells into a sink or other suitable container. Flood the wells completely with Wash Buffer, then shake to empty. Repeat this wash step three times. Alternatively, perform these four washes (300 µL/well) with a microtiter plate or strip washer. Slap the plate on a paper towel to remove as much water as possible.

8. Add **100 µL** of **Substrate** to each well.

9. Thoroughly mix the contents of the wells, as in step 2. Cover the wells with new tape or Parafilm and incubate for 30 minutes at ambient temperature. Use orbital shaker if available.

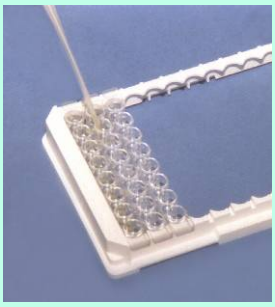
Caution: Stop Solution is 1.0N Hydrochloric acid. Handle carefully.

10. Add **100 µL** of **Stop Solution** to each well and mix thoroughly. This will turn the well contents yellow.

How to Interpret the Results

Spectrophotometric Measurement

1. Set the wavelength of the microtiter plate reader to 450 nanometers (nm). (If it has dual wavelength capability, use 600, 630 or 650 nm as the reference wavelength.)



Complete protocol and add Stop Solution

Read plates in a Plate Reader within 30 minutes of the addition of Stop Solution.

2. Set the plate reader to blank on the Negative Control wells (this should automatically subtract the mean optical density (OD) of the Negative Control wells from each calibrator and sample OD). If the reader cannot do this, it must be done manually.

3. For a quantitative Cry9C assay, a **linear or quadratic** curve fit for the standard curve should be used if the microtiter plate reader you are using has data reduction capabilities. If not, calculate the results manually as described in the "How to Calculate the Quantitative Cry9C Results" section. Be sure to use the appropriate calibrator labels for the Protocol that you ran.

General Test Criteria

- The mean OD of the BLANK wells should not exceed 0.2.
- The coefficient of variance (%CV) between the duplicate Calibrator and sample wells should not exceed 15%.

$$\%CV = \frac{\text{std. deviation of OD's}}{\text{mean OD}} \times 100$$

If the results of an assay fail to meet these criteria, consult EnviroLogix' Technical Service for suggestions on improving the test when you repeat the assay.

How to Interpret the Semi-Quantitative Results

Compare the OD's of the sample extracts to those of the Calibrators to obtain an estimate of the %StarLink or ppb Cry9C endotoxin in your sample extract. Samples with OD's greater than that of the lowest calibrator are considered positive. Those with OD's lower than that of the lowest calibrator either contain no, or less than 0.01% StarLink (1 ppb Cry9C) in the High Sensitivity Protocol, or less than 0.04% StarLink (0.8 ppb Cry9C) in the Rapid Protocol.

How to Calculate the Quantitative Cry9C Results

1. After reading the wells, average the OD of each set of calibrators and samples, and subtract the average OD of the Negative Control wells from all (if your reader has not automatically done so).
2. Graph the mean OD of each Calibrator against its % StarLink content (or Cry9C concentration) on a linear scale (see Figures 3a & 3b). Be sure to label the calibrator levels appropriately for the protocol you ran.
3. Determine the %StarLink content (or Cry9C concentration) of each sample by finding its OD value and the corresponding concentration level on the graph. Multiply the ppb Cry9C result by 5 for the dilution factor incurred during extraction; the %StarLink labels have this dilution taken into account, so do not multiply these by 5. Then multiply ppb Cry9C or %StarLink by any additional dilutions you may have made.
4. Interpolation of sample concentration is only possible if the OD of the sample falls within the range of OD's of the Calibrators.

If the OD of a sample is lower than that of the Low Calibrator (0.2 ppb in the High Sensitivity Protocol, 0.8 ppb in the Rapid Protocol), the sample must be reported as less than:

High Sensitivity Protocol:
0.01%StarLink corn, or
0.2 ppb x 5 (dilution factor during extraction) = 1 ppb Cry9C.

Rapid Protocol:
0.04% StarLink corn, or,
0.8 ppb x 5 (dilution factor during extraction) = 4 ppb Cry9C.

If any additional dilutions were performed, multiply by these factors as well.

If the OD of a sample is higher than that of the High Calibrator (2.5 ppb in the High Sensitivity Protocol, 5 ppb in the Rapid Protocol), the sample must be reported as greater than:

High Sensitivity:
0.125% StarLink corn, or,
2.5 ppb x 5 (dilution factor during extraction) = 12.5 ppb Cry9C.

Rapid Protocol:
0.25% StarLink corn, or
5 ppb x 5 (dilution factor during extraction) = 25 ppb Cry9C.

If any additional dilutions were performed, multiply by these factors as well.

If a concentration must be determined for these high level samples, dilute the sample extract 10 to 1000-fold more than executed in the original assay, in 1X Grain Extraction Solution. Run this dilution in a repeat of the Rapid Protocol. If the result now falls within the range of the OD's of the Calibrators, you must then be sure to use this new dilution factor of sample extract in the calculations described above.

Figure 1a. Example of a typical Qualitative assay setup.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	NC	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
B	C1	C1	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
C	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
D	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
E	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
F	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44
G	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37	S45	S45
H	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38	S46	S46

Figure 1b. Example of a typical Quantitative assay setup.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	NC	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
B	C1	C1	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
C	C2	C2	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
D	C3	C3	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
E	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
F	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
G	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
H	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44

Actual values may vary; this data is for demonstration purposes only.

Figure 3a.
Illustrative standard curve – High Sensitivity Protocol

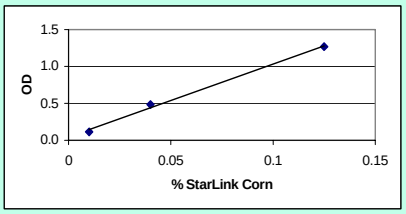


Figure 3b.
Illustrative standard curve – Rapid Protocol

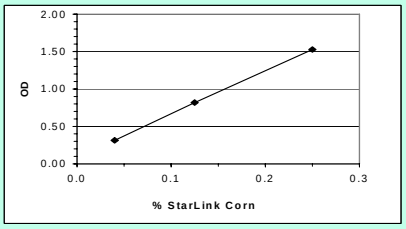


Figure 2a. Illustrative quantitative calculations – High Sensitivity Protocol

Well contents	OD	Avg OD ± sd	% CV	Cry9C Conc. (ppb)
Negative Control	0.093 0.101	0.097 ± 0.006	5.8	NA
0.01%StarLink (0.2 ppb Cry9C) Cal.	0.110* 0.120	0.115 ± 0.007	6.1	NA
0.04% StarLink (0.8ppb Cry9C) Cal.	0.471* 0.496	0.484 ± 0.018	3.7	NA
0.125% StarLink (2.5 ppb Cry9C) Cal.	1.314* 1.219	1.267 ± 0.067	5.3	NA
Sample**	0.604* 0.595	0.599 ± 0.006	1.1	0.056% StarLink 1.12 ppb Cry9C

* Figures are after subtraction of Negative Control values.

**Concentration from curve = 1.12 ppb Cry9C, multiplied by 1:5 dilution during extraction = 5.6 ppb Cry9C.

Figure 2b. Illustrative quantitative calculations – Rapid Protocol

Well contents	OD	Average OD ± sd	% CV	Cry9C Conc. (ppb)
NegativeControl	0.061 0.061	0.061 ± 0.000	0	NA
0.04%StarLink (0.8ppb Cry9C) Cal.	0.296* 0.331	0.314 ± 0.025	7.9	NA
0.125% StarLink (2.5 ppb Cry9C) Cal.	0.778* 0.860	0.819 ± 0.058	7.1	NA
0.25% StarLink (5 ppb Cry9C) Cal.	1.518* 1.535	1.527 ± 0.012	0.8	NA
Sample**	0.822 * 0.803	0.813 ± 0.013	1.7	0.13% StarLink 2.51 ppb Cry9C

* Figures are after subtraction of Negative Control values.

**Concentration from curve = 2.51 ppb Cry9C, multiplied by 1:5 dilution during extraction = 12.5 ppb Cry9C.

Precautions and Notes

- Store all Kit components at 4°C to 8°C (39°F to 46°F) when not in use.
- Do not expose Kit components to temperatures greater than 37°C (99°F) or less than 2°C (36°F).
- Allow all reagents to reach ambient temperature (18°C to 27°C or 64°F to 81°F) before use.
- Do not use kit components after the expiration date.
- Do not use reagents or test well strips from one Kit with reagents or test well strips from a different Kit.
- **Do not expose Substrate to sunlight** during pipetting or while incubating in the test wells.
- Do not dilute or adulterate test reagents or use samples not called for in the test procedure. Observe any applicable regulations when disposing of samples and kit reagents.
- Cry9C endotoxin is a protein that can be degraded by heat and sunlight. Samples that cannot be extracted immediately may be stored frozen for up to 1 week prior to analysis.
- As with all tests, it is recommended that results be confirmed by an alternate method when necessary.

For Technical Support Contact Us At:

EnviroLogix
500 Riverside Industrial Parkway
Portland, ME 04103-1486
USA

Tel: (207) 797-0300
Toll Free: 866-408-4597
Fax: (207) 797-7533

e-mail:
info@envirologix.com

website:
www.envirologix.com

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APPENDIX A – APPLICATION GUIDE

Testing for Cry9C in corn seeds and in corn leaf tissue

Background:

The EnviroLogix Cry9C Plate Kit is designed for the detection of this Bt endotoxin in bulk corn grain. This Application Guide describes the sample extraction and preparation steps required to use this kit to detect Cry9C endotoxin (found in StarLink™ and other Cry9C genetically modified corn varieties) in individual seeds and in corn leaf tissue. Read the entire kit Product Insert before using this Application Guide.

Corn Leaf Tissue Protocol:

This application describes qualitative and quantitative procedures for testing corn leaf samples for Cry9C endotoxin. A negative test with this kit is not an indicator of the absence of other genetic modifications.

A bottle of 5X Extraction/Dilution Buffer is required for this application and is available upon request from EnviroLogix. To prepare 1X Extraction/Dilution Buffer, add the entire contents of the bottle (35 mL) to 140 mL of distilled or deionized water in a suitable container. Mix thoroughly to dissolve. Store refrigerated when not in use; warm to room temperature prior to assay. (Note: this same buffer is also supplied in both the EnviroLogix Cry1Ab/Cry1Ac and Cry2A Plate Kits. Leaf samples may be extracted with 1X Buffer from any of the kits, and the same extract can be assayed in each of the three plate kits.)

Sample Extraction:

1. Take 2 leaf punch samples by snapping the tube cap of the Disposable Sample Extractor (Cat# ACC 002) down on the leaf. (Note: To quantitate Cry9C endotoxin in corn leaf, the weight of each sample must be recorded.) Insert the pestle into the tube and grind the tissue by rotating the pestle against the sides of the tube with twisting motions. Continue this process for 20-30 seconds, or until the leaf tissue is well ground. Use a new extraction device for each sample to avoid sample-to-sample cross-contamination.
2. Add 0.5 mL of 1X Extraction/Dilution Buffer to each tube. Repeat the grinding step to mix tissue with Extraction/Dilution Buffer. Allow the solids to settle in each tube for a few minutes.

Sample Extract Dilution:

3. Concentrations of Cry9C endotoxin will vary from plant to plant, but will be in the microgram per gram tissue range. To screen plants for presence or absence of Cry9C modification, dilute leaf extracts 1:11 in 1X Extraction/Dilution Buffer (50 µL extract added to 500 µL Buffer). To quantitate positive samples, a 1:2200 dilution is recommended (add 10 µL of the 1:11 dilution to 2 mL of 1X Extraction/Dilution Buffer). Failure to sufficiently dilute positive extracts can result in erroneously low Cry9C concentrations.

Assay Protocol and Interpretation:

4. Pipet 100 µL of diluted sample extract into the ELISA microplate as directed in the Product Insert under “How to Run the Assay”, in the “Rapid Protocol” section. Continue with the Rapid Protocol instructions as stated in the Product Insert.
5. To interpret a qualitative screening assay, any sample extracts that have been diluted 1:11 and produce lower OD results than that of the lowest calibrator in the assay (1 ppb Cry9C) are presumed to be negative for Cry9C modification. Cry9C-modified corn leaf should result in OD’s substantially greater than that of the 1 ppb Calibrator.
6. For quantification of Cry9C endotoxin, follow the Product Insert Instructions for graphing Cry9C Calibrator (use ppb Cry9C levels) vs. OD, and interpolating each sample’s Cry9C concentration from the curve. Multiply the ppb Cry9C results from the curve by the dilution incurred during extraction (0.5 mL buffer divided by x grams of leaf), and also by the 1:2200 dilution of sample extract that was made. For example, if a 0.020 gram leaf sample was assayed as described above and resulted in a concentration of 2.8 ppb Cry9C from the standard curve, multiply 2.8 ppb x 25 (dilution during extraction) x 2200 = 154,000 ppb, or 154 ppm Cry9C.

Individual Corn Seed Protocol:

The ability to test individual seeds is a valuable quality control tool for producers of Cry9C endotoxin-containing corn (such as StarLink™, CBH-351). This application describes qualitative and quantitative procedures for testing corn seeds for Cry9C endotoxin. A negative test with this kit is not an indicator of the absence of other genetic modifications.

To prepare 1X working Grain Extraction Solution, either: (a) Mix 5 mL of Grain Extraction Concentrate (20X) plus 95 mL distilled or deionized water for every 100 mL required, or (b) add the entire contents of the bottle of Grain Extraction Concentrate (60 mL) supplied in the kit to 1140 mL of distilled or deionized water in a suitable container. Mix thoroughly to dissolve. May be stored at room temperature, but use within 30 days of preparation.

1. Crush a single seed (place in small plastic bag and crush with hammer or pliers; take precautions to avoid sample-to-sample cross-contamination) and transfer to a small tube. To use the kit quantitatively, each sample must be weighed.
2. Add 2 mL of 1X Grain Extraction Solution to each tube. Cap and shake vigorously by hand for 20-30 seconds. Note: Optimal extraction of the endotoxin requires soaking the sample in the extraction buffer for at least 4 hours, preferably overnight. If accurate quantitation of endotoxin present in the seed is required, this soaking step must be incorporated. If a simple presence/absence result is sufficient, the extra extraction time is not necessary; proceed directly to step 3.
3. The extracted samples must be clarified by one of two methods: (a) centrifuge the tubes at 5000 x g for 5 minutes, or, (b) filter the material through a low protein binding hydrophilic syringe filter, 0.45 µm (such as Pall Gelman Sciences - Product No. 4184).
4. To screen for presence or absence of Cry9C in seeds, pipet 100 µL of clarified extract into the wells of the ELISA plate as directed under "How to Run the Assay" in the Rapid Protocol section of the Product Insert. Continue with the Rapid Protocol instructions as stated in the Product Insert.

   To interpret the qualitative results, sample wells with more color than that of the lowest calibrator indicate that the seed contains Cry9C endotoxin; sample wells with less color than that of the lowest calibrator indicate that the seed is negative or contains less Cry9C endotoxin than is commonly seen in GM-seed.
5. To quantitate Cry9C endotoxin in individual seeds, any seed that gave a positive result in the above screen must be re-run in the Rapid Protocol after diluting the clarified sample extract 1:1000 in 1X Grain Extraction Solution. Failure to perform this dilution can result in erroneously low results for the high-expressing StarLink seeds.

   Follow the Product Insert instructions for graphing Cry9C Calibrator (use ppb Cry9C levels) vs. OD, and interpolating each sample's Cry9C concentration from the curve. Multiply the ppb Cry9C results from the curve by the dilution incurred during extraction (2 mL buffer divided by x grams of seed), and also by the 1:1000 dilution of sample extract that was made. For example, if a 0.30 gram seed sample was assayed as described above and resulted in a concentration of 1.5 ppb Cry9C from the standard curve, multiply 1.5 ppb x 6.667 (dilution during extraction) x 1000 = 10,000 ppb, or 10 ppm Cry9C.

APPENDIX B – APPLICATION GUIDE

Testing for Cry9C in corn meal, flour and grits

Background:

1. The EnviroLogix QuantiPlate Kit for Cry9C is designed for the detection of this Bt endotoxin in bulk corn grain. This Application Guide describes the sample extraction and preparation steps required to use this kit to detect Cry9C endotoxin (found in StarLink and other Cry9C modified corn varieties) in processed corn products (cornmeal, corn flour, corn grits). Read the entire Product Insert before using this Application Guide.

Note: A negative test with this kit is not an indicator of the absence of other genetic modifications.

Sample Preparation and Extraction:

2. This protocol calls for a small sample to be analyzed. It is essential that this sample be well mixed and representative of the larger bulk that it is drawn from. Thorough mixing and determination of an appropriate sampling plan are critical to the results of this testing, and are the responsibility of the user of this test kit.
3. Cornmeal and corn flour samples are ready to extract without further treatment. Corn grits should be ground to the consistency of cornmeal, using the blender and jars described in the product insert.
4. Weigh 5 grams of cornmeal, flour or ground grits into a 50 mL capacity tube or vial. Note: larger sample sizes may be used; just be sure to maintain the ratio of extraction solution to weight of sample described in the next paragraph. If you require additional 20X Grain Extraction Concentrate, please contact EnviroLogix for Cat# KR 004.
5. To cornmeal and ground corn grits, add **7.5 mL/gram** of **1X Grain Extraction Solution** (37.5 mL to 5 grams). Add **10 mL/gram** of **1X Grain Extraction Solution** to corn flour samples (50 mL to 5 grams). Mix vigorously and allow to extract for 1 hour. Mix again at the end of the 1 hour.
6. An aliquot of the extracted sample must be clarified by centrifugation at 5000 x g for 5 minutes.

Sample Extract Dilution:

7. Concentrations of Cry9C endotoxin in StarLink corn can vary greatly. To screen products for presence or absence of Cry9C modification, use undiluted clarified extracts in the High Sensitivity Protocol for maximum sensitivity. If sample extracts produce more color than the highest calibrator, to quantitatively determine the Cry9C endotoxin in that sample, dilute the sample extract in 1X Grain Extraction Solution and run it in the Rapid Protocol. Multiple dilutions may be required to get the sample extract within the range of calibration. Failure to sufficiently dilute positive extracts can result in erroneously low Cry9C concentrations.

Assay Protocol and Interpretation:

8. Pipet 100 µL of clarified sample extract into the ELISA microplate as directed in the Product Insert under "How to Run the Assay", in the "High Sensitivity Protocol" section of the Product Insert. Continue with the High Sensitivity Protocol instructions as stated in the Product Insert.
9. To interpret a qualitative screening assay, any sample extracts that produce lower OD results than that of the lowest calibrator in the assay (0.01% StarLink/0.2 ppb Cry9C) are presumed to be negative for Cry9C modification. Cry9C-modified corn products containing 0.01% StarLink or more should result in OD's greater than that of the lowest Calibrator.
10. For quantification of Cry9C endotoxin, follow the Product Insert instructions for graphing Cry9C Calibrator (%StarLink or ppb Cry9C levels) vs. OD, and interpolating each sample's StarLink/Cry9C concentration from the curve. Multiply the ppb Cry9C results from the curve by the dilution incurred during extraction (7.5 for meal and grits, 10 for flour). If using the %StarLink calibrator values, do not multiply by this extraction buffer to sample weight ratio. If any post-extraction dilutions were made, multiply both the ppb Cry9C and the %StarLink by this factor.