

Highlights:

- Will detect 0.1% (1 seed in 1000) of YieldGard Rootworm corn
- Results in one hour

Contents of Kit:

- 10 antibody-coated 96-well plates
- 1 vial of Positive Control ground corn
- 1 bottle of Cry3Bb1 Enzyme Conjugate
- 1 bottle of Substrate

Precision

	OD (%CV)	Pos. Ctl. Ratio (%CV)
Inter-Assay n=30		
0.05%	15.5%	20.6%
0.20%	13.6%	22.6%

Catalog Number AP 015 NW V10

Intended Use

The EnviroLogix QualiPlate Kit for Cry3Bb1 is designed for the qualitative laboratory detection of Cry3Bb protein in single leaf, single seed, or bulk grain samples. This test will detect Cry3Bb1 protein found in 0.1% YieldGard Rootworm corn (1 seed in 1000) and requires 1 hour to run.

How the Test Works

This QualiPlate Kit is a “sandwich” Enzyme-Linked ImmunoSorbent Assay (ELISA).

In the test, **corn** sample extracts are added to test wells coated with antibodies raised against Cry3Bb1 protein. Any Cry3Bb1 protein present in the sample extract binds to the antibodies and is then detected by addition of enzyme (horseradish peroxidase)-labeled Cry3Bb1 antibody.

After a simple wash step, the results of the assay are visualized with a color development step. Color development increases with increasing Cry3Bb1 sample concentration from 0.1% to 25% YieldGard Rootworm corn, then levels and drops off.

Light color = Low concentration

Darker color = High concentration

How the Kit Performs

The EnviroLogix QualiPlate Kit for Cry3Bb1 Corn is a strictly qualitative (yes/no) assay. Samples are interpreted in comparison with Positive and Negative Control ground corn samples provided in each kit.

Precision

Cry3Bb1-fortified control solutions were repetitively analyzed in different assays on different days. The fortification levels used are roughly equivalent to 0.05% and 0.2% YieldGard Rootworm corn, respectively.

The data is expressed as % CV for both the absorbance (OD) and the Positive Control Ratio (OD of sample divided by the OD of the Positive Control ground corn).

Error Rate

Validation of this QualiPlate Kit involved in-house and beta-site (non-EnviroLogix users) components. Five different in-house operators and five different beta-sites participated. Each corn sample extract was tested in three different QualiPlate Kit manufacturing lots, generating 3 data points per corn sample.

1000-kernel grain samples

0 false positive results out of 360 non-YieldGard Rootworm data points, for a best estimate of false positive rate of 0%.

3 false negative results out of 342 0.1% YieldGard Rootworm data points, for a best estimate false negative rate of 0.88%.

Single seed samples

3 false positive results out of 360 non- YieldGard Rootworm seed data points, for a best estimate false positive rate of 0.83%.

0 false negative results out of 338 YieldGard Rootworm seed data points, for a best estimate false negative rate of 0%.

Single leaf punch samples

0 false positive results out of 360 non-Cry3Bb1 leaf data points, for a best estimate false positive rate of 0%.

5 false negative results out of 360 Cry3Bb1 leaf data points, for a best estimate false negative rate of 1.39%.

NOTE: These are best estimates of expected false positive/false negative rates based upon the data available; actual results may vary.

Materials Not Provided

- PBS/0.05% Tween-20 Wash Buffer (may be purchased in 1L dry packets from Sigma Chemicals, Cat#P-3563, or prepared from salts on site). Store at controlled ambient temperature for up to one week, then discard.
- 1 N Hydrochloric acid (HCl) Stop Solution. Prepare by adding 83 mL of concentrated HCl (36.5-38.0%) to 917 mL of distilled or deionized water; work in a fume hood and use proper protective gear. This reagent may be stored at room temperature for 2 years.
- distilled or deionized water for preparing Wash/Extraction Buffer
- glass bottles or flask plus graduated cylinder with 1 liter capacity for preparation and storage of Wash/Extraction Buffer
- Waring blender model 31BL91 (or equivalent), glass jar adapter (Eberbach # E8495) and 32 oz. glass Mason jars for ground corn samples
- Snap-cap tubes and pestles for extraction of leaf samples (EnviroLogix Cat# ACC 002, 100/package) (optional)
- centrifuge capable of 5000 x g (optional)
- disposable tip, adjustable air-displacement pipettes which will measure 50 and 100 microliters (μL)
- marking pen (indelible)
- tape or Parafilm®
- timer
- microtiter plate reader with 450 nm filter
- wash bottle, or microtiter plate washer
- multi-channel pipette that will measure 50 and 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/Extraction Buffer: Order item # P-3563 from Sigma Chemical Co. (St. Louis, MO), or prepare the equivalent. Store refrigerated when not in use; allow to come to room temperature prior to assay. Use this buffer for the wash step of the assay, and to extract all corn samples.



Prepare Wash /Extraction buffer

Stop Solution: 1 N Hydrochloric acid (HCl). Prepare by adding 83 mL of concentrated HCl (36.5-38.0%) to 917 mL of distilled or deionized water; work in a fume hood and use proper protective gear. This reagent may be stored at room temperature for 2 years.

Positive Control ground corn extract: Extracts of this control must be run in every assay. To extract, add 5 mL of Wash/Extraction Buffer to each tube containing 2 grams of ground Control corn. Cap and shake vigorously by hand or vortex for 20-30 seconds. Let stand at room temperature for 1 hour to extract. Mix again at the end of the hour, then clarify by allowing to settle 10 minutes or by centrifuging 5 minutes at 5000 x g.

Note: It is recommended that the user prepare known negative and positive seed or leaf samples to be run in every assay as controls, in addition to the Positive Control ground corn supplied with the kit.

If running the assay at a later date, or more than one assay per plate, freeze 0.5 mL aliquots of each clarified extract. Thaw just prior to use.

Sample Preparation

Ground Bulk Grain Samples:

Testing of bulk grain for Cry3Bb protein is an indicator of the presence or absence of Cry3Bb1 corn in a given sample. A negative test with this kit is not an indicator of the absence of other genetic modifications.

The test will detect 0.1% Cry3Bb1 corn (one positive kernel in a sample of 1000 kernels). This protocol calls for a small sample to be analyzed (20 to 50 grams). It is essential that this sample be well mixed and representative of the larger bulk. 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.

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

The finer the grind, the faster and more efficient the extraction.

1. For 1000 kernel samples, grind in a 32 ounce "Mason" jar on a blender at high speed for 1 minute. Shake jar to mix, then repeat the grinding a second time.

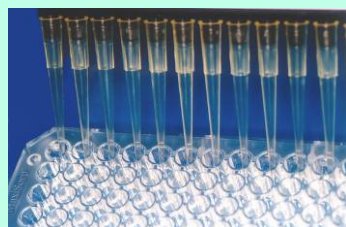
Thoroughly clean the grinding equipment between samples to prevent cross-contamination.

2. Weigh at least 20 grams of ground corn sample into a jar or cup.
3. Add 50 mL of Wash/Extraction Buffer to each 20 gram sample. For all other ground corn sample sizes, add Wash/Extraction Buffer at the rate of 2.5 mL per gram of corn. Cap and shake vigorously by hand or vortex for 20-30 seconds. Let stand at room temperature for 1 hour to extract. Mix again at the end of the hour.
4. For best results, clarify the extracts by centrifuging at 5000 x g for 5 minutes. Alternatively, allow them to settle out for at least 10 minutes. Insert a pipette tip below any floating lipid layer and above the pellet to remove the clarified sample. **NOTE:** Dispensing particles into the test plate can cause false positive results.

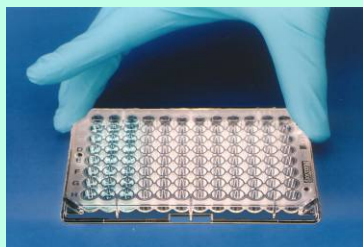




Allow all reagents to reach room temperature before beginning



Add Enzyme-Conjugate, followed immediately by Control and sample extracts to the plate



Mix plate



Bottle Wash method

Single Seed Samples

1. Crush seeds: Seeds may be placed in a re-sealable plastic bag and smashed with a hammer, then transferred to a tube; or, a seed crusher/48-well plate combination may be used (for example Hypure #HSC-100, PerkinElmer, Norton, OH, with Costar plate #3548, Corning Life Sciences, Acton, MA, or equivalent). Check to be sure that all seeds have been crushed. Take extreme care not to cross-contaminate between seed samples.
2. Add 1 mL of Wash/Extraction Buffer to each crushed corn seed. Mix for at least 30 seconds, then allow particles to settle. Dispensing particles into the test plate can cause false positive results.

Single Leaf Punch Samples:

1. Take a single leaf punch of approximately 5 millimeters diameter, using a micro-tube cap or a paper punch. Mash the leaf tissue with a pestle matched to the micro-tube, or with a disposable pipette tip, or a Hypure cutter (HCT-200, PerkinElmer, Norton, OH) in a 96-well plate (Costar #3370, Corning Life Sciences, Acton, MA, or equivalent).
2. Add 0.25 mL of Wash/Extraction Buffer per leaf punch. Mix for at least 30 seconds, then allow particles to settle. Take extreme care not to cross-contaminate between leaf samples. Dispensing particles into the test plate can cause false positive results.

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 plates and reagents at room temperature - do not remove plate from bag with desiccant until it has warmed up).
- Organize all Control and sample extracts, and pipettes so that Step 1 can be performed in 15 minutes or less, using a multi-channel pipette.
- Use the well identification markings on the plate frame as a guide when adding the samples and reagents. For this qualitative assay, duplicate wells of the Wash/Extraction Buffer Blank (BL), user-supplied known-negative control (NC), and the Positive Control (PC), along with 90 sample extracts (S) in single wells may be run on one plate. (See the Qualitative Assay Example Plate Layout - Figure 1A).

1. Add **50 μ L** of **Cry3Bb1 Enzyme Conjugate** to each well, followed immediately by **50 μ L** of **Wash/Extraction Buffer Blank (BL)**, **50 μ L** of **Negative and Positive Control ground corn extracts (PC and NC)** and **50 μ L** of each **sample extract (S)** to their respective wells, as shown in Figure 1A.

NOTE: In order to minimize setup time it is strongly recommended that a multi-channel pipette be used in steps 1, 5, and 7.

2. Thoroughly mix the contents of the wells by moving the plate 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 45 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



Plate Wash option



Complete protocol and
add Stop Solution



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

wells completely with Wash/Extraction Buffer, then shake to empty. Repeat this wash step three times. Alternatively, perform these four washes (300 μ L/well) with a microtiter plate or strip washer. Slap the inverted plate on a paper towel to remove as much liquid as possible.

5. Add **100 μ L** of **Substrate** 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 15 minutes**. If an orbital plate shaker is available shake plate at 200 rpm.

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

8. Add **100 μ L** of **Stop Solution** to each well and mix thoroughly. This will turn the well contents yellow.

NOTE: Read the plate within 30 minutes of the addition of Stop Solution.

How to Interpret the Results

Spectrophotometric Measurement

1. Set the wavelength of your microtiter plate reader to 450 nanometers (nm). (If it has dual wavelength capability, use 600, 630 or 650 nm as the reference wavelength.)
2. Set the plate reader to blank on the Wash/Extraction Buffer Blank wells (this should automatically subtract the mean optical density (OD) of the Blank wells from each control and sample OD). If the reader cannot do this, it must be done manually.

General test criteria:

The mean OD of the BLANK wells should not exceed 0.2.

The mean, blank-subtracted OD of the Positive Control wells should be at least 0.1 and at least 3x greater than the mean, blank-subtracted OD of the Negative Control wells.

The coefficient of variance (%CV) between the duplicate Positive Control wells should not exceed 15%:

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

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 Calculate the Positive Control Ratio

Divide the OD of each sample extract by the mean OD of the Positive Control ground corn extract wells. This number is the "Positive Control Ratio".

How to Interpret the Qualitative Results

Ground corn samples

If the Positive Control Ratio calculated for a sample is less than 0.25, the ground corn contains less than 0.1% YieldGard Rootworm corn.

If the Positive Control Ratio of a sample is greater than or equal to 0.25, the sample contains 0.1% or greater YieldGard Rootworm corn.

NOTE: Ground corn samples containing more than 25% YieldGard Rootworm corn may show decreasing OD's with increasing concentration.

However, the OD's will be much greater than that of a 0.1% YieldGard Rootworm sample. This test is to be used qualitatively only, with yes/no results at 0.1% YieldGard Rootworm corn. For information on testing at different cutoff levels, please contact EnviroLogix Technical Service.

Single Leaf and Seed samples

If the Positive Control Ratio calculated for a sample is less than 0.5, the sample is not YieldGard Rootworm corn.

If the Positive Control Ratio of a sample is greater than or equal to 0.5, the sample is YieldGard Rootworm corn.

These types of samples are by their nature either 100% positive or 100% negative. Any low level positive results from single seed or leaf samples must be due to either some form of sample cross-contamination (flying particles or dust from YieldGard Rootworm corn, corn leaf residue on leaf punch, etc.) or can be caused by transfer of particulate matter from leaf or seed extracts into the assay wells. If there is any question of the latter occurring, re-extraction and re-testing is recommended.

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86
B	NC	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87
C	PC	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88
D	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73	S81	S89
E	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	S90
F	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83	BL
G	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84	NC
H	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85	PC

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 plates from one QualiPlate Kit with reagents or plates from a different QualiPlate 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.
- Cry3Bb 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.
- Observe any applicable regulations when disposing of samples and kit reagents.



**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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