QualiPlate™ Combo Kit for Cry1Ab & Cry3Bb1 Corn

Catalog Number AP 039 NW V50

Highlights:

- *Use a single test well to screen corn seeds or leaf samples for presence/ absence of Cry1Ab and Cry3Bb1*
- *Results for two analytes in three hours*

Contents of Kit:

- *50 Cry1Ab and Cry3Bb1 antibody-coated solid plates*
- *Enzyme Conjugate*
- *S1 Substrate (for Cry3Bb1 results)*
- *S2 Substrate (for Cry1Ab results)*

- ***Note:** To handle bulk packaged Enzyme Conjugate, S1 Substrate and S2 Substrate: into clean containers, pour off 6 mL of Conjugate and 11 mL of each Substrate per plate to be run each day. Use a multiple-channel pipette to dispense. Do not pour excess Substrates back into the reagent bottles.*

Intended Use

The EnviroLogix QualiPlate Combo Kit for Cry1Ab & Cry3Bb1 is designed for the qualitative laboratory detection of the presence or absence of Cry1Ab and Cry3Bb1 protein in corn single leaf or single seed samples, with both analytes measured in the same well of the assay plate. This assay can be used to detect the presence of these proteins in corn products including Yieldgard®, Yieldgard Rootworm, Yieldgard Plus, YieldGard VT Rootworm/RR2™, and YieldGard VT Triple™ as follows:

	YieldGard Rootworm	YieldGard Plus YieldGard Plus/RR2	YieldGard VT Rootworm/RR2	YieldGard VT Triple	YieldGard YieldGard/RR2
Cry3Bb1	•	•	•	•	
Cry1Ab		•		•	•

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 that recognize Cry1Ab and Cry3Bb1 proteins. Any Cry1Ab or Cry3Bb1 protein present in the sample extract binds to the antibodies and is then detected by addition of alkaline-phosphatase-labeled Cry3Bb1 antibody, or horseradish peroxidase-labeled Cry1Ab antibody.

After a simple wash step, the results of the Cry3Bb1 assay are determined via the addition of a *p*-nitrophenyl phosphate S1 Substrate. Once the yellow color develops and is read, the wash step is repeated, and a 3,3',5,5'-tetramethylbenzidine S2 Substrate is added. The Cry1Ab results are determined via the development of the resulting blue color.

Light color = Low concentration
Darker color = High concentration

Non-Specific Interferences

The performance of this QualiPlate Kit when testing single leaf or seed samples will not be adversely affected by the presence of leaf or seed from the following non-transgenic crops:

Barley, canola, cotton, rice, sorghum, soybean, sugar beet, or wheat.

Note that any Cry1Ac-containing cotton **will** cause a positive result in the Cry1Ab portion of this test kit.

Materials Not Provided

- *PBS/0.05% Tween-20, pH 7.4* ***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.*
- *PBS/0.55% Tween-20* ***Extraction Buffer*** *May be prepared by adding 0.5% (5 mL per liter) Tween-20 to already prepared PBS/ 0.05% Tween-20 Wash Buffer. Prepare only enough for a few days' usage. Store refrigerated when not in use; warm to room temperature prior to assay.*
- *distilled or deionized water for preparing above solutions*
- *Positive and Negative Controls –recommended that the user prepare known positive control samples and negative control samples (seed and/or leaf extracts) to run in each assay*
- *EnviroLogix Tissue Extraction Kit (ACC 002) or other suitable equipment for taking and extracting leaf punch samples*
- *equipment for pulverizing seeds or leaves*
- *disposable tip, adjustable air-displacement multi-channel pipettes which will measure 50 and 100 microliters (µL)*
- *marking pen (indelible)*
- *tape or Parafilm®*
- *timer*
- *microtiter plate reader with 405 and 650 or 630 nm filters*
- *wash bottle, or microtiter plate washer*

Sample Preparation

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.

Positive Control Ground Corn extract:
Extracts of this control must be run in every assay. To extract, add 5 mL of Extraction Buffer to the 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.

If running the assay at a later date, or more than one assay per plate, freeze (-20°C) 0.5 mL aliquots of each clarified extract in a non-self-defrosting freezer for up to 6 months. Thaw just prior to use.

Single Seed Samples

1. Crush seeds: Seeds may be placed in a re-sealable plastic bag and smashed with a rubber mallet or hammer, then transferred to a tube for extraction; alternately, a drill-press based machine incorporating a seed cutter/48-well plate combination may be used (for example Hypure #HCT-100, PerkinElmer, Norton, OH, with Costar plate #3548, Corning Life Sciences, Acton, MA, or equivalent). If using this cutting equipment, it is recommended that you cut the seeds 4 times, rotating the seeds between cuttings. If necessary, a metal probe should be used to manually rotate lodged seeds for thorough cutting. Make certain that each seed has been broken into at least 3 pieces.

NOTE: Cry3Bb1 protein is expressed at high concentrations in corn seed compared to Cry1Ab. There is serious potential for cross-contamination with Cry3Bb1 between samples during seed crushing. Use the utmost care to avoid this.

2. Add 0.75 mL of Extraction Buffer to each crushed corn seed. Mix for at least 30 seconds, let stand 1 - 24 hours (overnight extraction should be done at refrigerator temperatures), then mix again. For optimal extraction, shake for the last few minutes on an orbital shaker or other mixer (taking care not to splash extract out of the tubes or wells). Visually examine extracted seed samples for the absence of intact seeds. Well-extracted seeds should result in a white to yellow cloudy extract. Note the presence of any extracts that appear clear and/or colorless – these may not have extracted properly and assay data would be invalid. For best results, extract another sample from the seed lot.

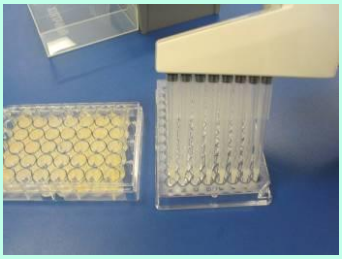
Single Leaf Punch Samples:

1. Take two leaf punches of approximately 5 millimeters diameter or a single punch of 10 mm diameter, using a paper punch or a micro-tube cap. Mash the leaf tissue with a pestle matched to the micro-tube, or beat with beads in a reciprocating shaker to the point of liquefaction of the leaf. The extraction efficiency of whatever method used will vary proportionately with the amount of tissue disruption performed.
2. Add 0.5 mL of Extraction Buffer per sample and macerate again. Assay immediately, or after overnight extraction at refrigerator temperature. For optimal extraction, shake for the last few minutes on an orbital shaker or other mixer (taking care not to splash extract out of the tubes or wells). Use extreme care not to cross-contaminate between leaf samples. Visually examine extracted leaf samples for the absence of intact leaf punches. Well-extracted leaves should result in a green cloudy extract. Note the presence of any extracts that appear clear and/or colorless – these may not have extracted properly and assay data would be invalid. For best results, extract another representative sample.

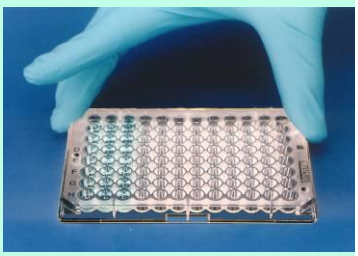
How to Run the Assay

- 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 to guide you when adding the samples and reagents. It is recommended that at least two wells each of Blank (Extraction Buffer) and user-supplied Positive and Negative Controls be run on each plate. Additional quality control samples may be added at the discretion of the user. Sample extracts may be run in either single or duplicate wells. See example of typical assay setup, Figure 1A, on page 4.

Prepare Wash and Extraction buffer



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



Mix

1. Add **50 µL** of **Cry1Ab/Cry3Bb1 Enzyme Conjugate** to each well, followed immediately by **50 µL of Extraction Buffer Blank (BL), 50 µL each of Negative and Positive Control extracts,** and **50 µL** of each **sample/user-prepared control extract (S)** to their respective wells, as shown in Figure 1A. **Caution**: Dispensing particles into the test plate can cause false positive results.

   NOTE: In order to minimize setup time it is strongly recommended that a multi-channel pipette be used in steps 1, 5, and 10.
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 contents!
3. Cover the wells with tape or Parafilm to prevent evaporation and **incubate** at **ambient temperature for 2 hours**.
4. After incubation, carefully remove covering and vigorously shake contents of wells into 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 inverted plate on a paper towel to remove as much liquid as possible.
5. Add **100 µL** of **S1 Substrate** to each well. BE SURE TO USE **S1** SUBSTRATE AT THIS STEP!
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 20 to 30 minutes**. Mix plate manually, or set reader to shake for 2-5 seconds.
8. **Read and record the yellow Cry3Bb1 results using a microtiter plate reader at a wavelength of 405 nanometers.** Set the plate reader to blank on the Extraction Buffer Blank wells (this should automatically subtract the mean optical density (OD) of the Blank wells from each control and sample OD).
9. Wash the plate four times as described in step 4.
10. Add **100 µL** of **S2 Substrate** to each well. BE SURE TO USE **S2** SUBSTRATE AT THIS STEP!
11. Thoroughly mix the contents of the wells as described in step 2. Be careful not to spill the contents!
12. Cover the wells with new tape or Parafilm to prevent evaporation and **incubate** at **ambient temperature for 20 to 30 minutes**. Mix plate manually, or set reader to shake for 2-5 seconds.

Wash Plate

13. **Read and record the blue Cry1Ab results with a microtiter plate reader at a wavelength of 650 or 630 nanometers.** Set the plate reader to blank on the Extraction Buffer Blank wells (this should automatically subtract the mean OD of the Blank wells from each control and sample OD).

How to Interpret the Results

Spectrophotometric Measurement

General test criteria:

The mean OD of the BLANK wells in the Cry1Ab portion of the test should not exceed 0.15. The mean OD of the BLANK wells in the Cry3Bb1 portion of the test should not exceed 0.35.

The mean, blank-subtracted OD of the Positive Control Solution wells should be at least 0.2.

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

$$\%CV = \frac{\text{standard deviation of OD's}}{\text{mean Positive Control OD}} \times 100$$

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.

Read plates in a Plate Reader at the appropriate wavelength:

- *405 nanometers for the Cry3Bb1 test result,*
- *650 OR 630 nanometers for the Cry1Ab test results.*

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".

Interpret the Qualitative Results

Cry3Bb1 & Cry1Ab Results:

If the Positive Control Ratio calculated for a single seed or leaf punch sample in either the Cry3Bb1 or Cry1Ab portion of the test is less than 0.5, the sample does not contain that protein .

If the Positive Control Ratio of a sample in either the Cry3Bb1 or Cry1Ab portion of the test is greater than or equal to 0.5, the sample contains that protein.

Single leaf and seed samples are by their nature either 100% positive or 100% negative, resulting in a clear delineation of color between negative and positive samples. Low level positive results may be due to insufficient extraction, or can be caused by some form of sample cross-contamination (flying particles or dust from corn seed, corn leaf residue on leaf punch, etc.) or by transfer of particulate matter from leaf or seed extracts into the assay wells. Re-extraction and re-testing of questionable samples is recommended.

Contact EnviroLogix Technical Service for assistance with making this test work best with your own samples and extraction equipment/protocols. When positive samples are not as well delineated from negative samples as they should be, any or all of the following alterations to the testing may help: Change the extraction method to one that causes more disruption to the sample; increase the extraction time and the degree of mixing during extraction; increase the Enzyme-Conjugate/sample reaction step time; add time onto either or both of the substrate development steps (in 10 minute increments).

This Kit recognizes the following enhanced crops containing both the biotechnology proteins Cry1Ab and Cry3Bb1: YieldGard Plus, YieldGard VT Rootworm/RR2, and YieldGard VT Triple.

Corn products such as YieldGard, Agrisure™ CB LL, and Agrisure GT CB LL will run positive in the Cry1Ab portion of the assay, while YieldGard Rootworm and SmartStax® will run positive in the Cry3Bb1 portion of the assay. See table on page 1.

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 S1 Substrate or S2 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.
- Do not use a stopping solution of any kind during this assay.
- 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.
- This kit will detect Cry3Bb1 protein found in multiple corn hybrids including YieldGard Rootworm, YieldGard Plus, YieldGard VT Rootworm/RR2, YieldGard VT Triple, and SmartStax. The test does not necessarily distinguish between these corn hybrids.

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