QualiPlate™ Combo Kit for Cry1A & Cry2A

Catalog Number AP 051 NW V10

Highlights:

- *Use a single test well to screen cotton seeds, cotton leaf, or corn leaf samples for the presence/absence of Cry1Ab/Cry1Ac/ Cry1A.105 and Cry2A*
- *Results for two analytes in about 2 hours*

Contents of Kit:

- *10 Cry1A and Cry2A antibody-coated solid plates*
- *Enzyme Conjugate*
- *Positive Control*
- *S1 Substrate (for Cry2A results)*
- *S2 Substrate (for Cry1A 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.*

Prepare Wash and Extraction buffer

Intended Use

The EnviroLogix QualiPlate Combo Kit for Cry1A & Cry2A is designed for the qualitative laboratory detection of the presence or absence of these proteins in cotton seed, cotton leaf, or corn leaf samples (Cry1A.105 is not reliably detected in single corn kernels). Both analytes are measured in the same well of the assay plate. This assay can be used to detect the presence of these proteins in cotton products including Bollgard®, Bollgard II, and WideStrike™, and corn products such as VT Triple PRO™ and SmartStax®.

How the Test Works

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

In the test, sample extracts are added to test wells coated with antibodies that recognize Cry1A and Cry2A proteins. Any Cry1A or Cry2A protein present in the sample extract binds to the antibodies and is then detected by addition of alkaline-phosphatase-labeled Cry2A antibody, or horseradish peroxidase-labeled Cry1A antibody.

After a simple wash step, the results of the Cry2A assay are determined via the addition of *p*-nitrophenyl phosphate S1 Substrate. Once the yellow color develops and is read, the wash step is repeated, and 3,3',5,5'-tetramethylbenzidine S2 Substrate is added. The Cry1A 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: Canola, rice, sorghum, soybean, sugar beet, or wheat.

Sample Preparation

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

Single Cotton Seed Samples

1. Crush seeds: Seeds may be placed in a plastic bag or tube and crushed with a rubber mallet or pliers, then transferred to a tube for extraction; alternately, a drill-press based machine incorporating a seed crusher/48-well plate combination may be used (for example Hypure #HSC-100, PerkinElmer, Norton, OH, with Costar plate #3548, Cottoning 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. If using the seed crusher, dip the crushing prongs in clean water, then shake off the excess prior to crushing. After crushing, slide a piece of paper between the plate and the crushing prongs as you remove them from the wells. These procedures help to prevent seed particles from jumping from one well to the next, reducing the risk of cross-contamination.

Items Not Provided:

- *PBS/0.05% Tween-20* ***Wash Buffer, pH 7.4*** *(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*** *This 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*
- *EnviroLogix Tissue Extraction Kit (ACC 002) or other suitable equipment for taking and extracting leaf punch samples*
- *equipment for pulverizing seeds or leaves*
- *user-supplied controls: seed and/or leaf extracts from known negative and positive samples (optional)*
- *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*

NOTE: Cry2A protein is expressed at high concentrations in cotton seed compared with Cry1A. There is serious potential for cross-contamination with Cry2A between samples during seed crushing. Use the utmost care to avoid this.

2. Add 1 mL of Extraction Buffer to each crushed cotton 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 yellow to brown 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 Corn or Cotton 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 0.35 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.

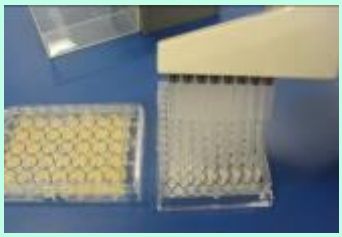
How to Run the Assay

- 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. For this qualitative assay, duplicate wells of the Extraction Buffer blank (BL) and Positive Control, along with 92 sample extracts (S) (including user-supplied controls) in single wells may be run on one plate. (See the Qualitative Assay Example Plate Layout - Figure 1A).

1. Add **50 µL** of **Cry1A/Cry2A Enzyme Conjugate** to each well, followed immediately by **50 µL of Extraction Buffer Blank (BL), 50 µL Positive Control (PC),** 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!



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

Mix

Wash Plate

Read plates in a Plate Reader at the appropriate wavelength:

- *405 nanometers for the Cry2A test result,*
- *650 or 630 nanometers for the Cry1A test results.*

3. Cover the wells with tape or Parafilm to prevent evaporation and **incubate** at **ambient temperature for at least 1 hour**.
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 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 30 minutes**. Mix plate manually, or set reader to shake for 2-5 seconds.
8. **Read and record the yellow Cry2A 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 30 minutes**. Mix plate manually, or set reader to shake for 2-5 seconds.
13. **Read and record the blue Cry1A 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 Cry1A portion of the test should not exceed 0.15. The mean OD of the BLANK wells in the Cry2A portion of the test should not exceed 0.35.

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

The coefficient of variance (CV) between the duplicate Positive Control 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.

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.*

Calculate the Positive Control Ratio

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

Interpret the Qualitative Results

Cry2A Results:

If the Positive Control Ratio calculated for a single seed or leaf punch sample in the Cry2A portion of the test is less than 1.0, the sample does not contain Cry2A.

If the Positive Control Ratio of a sample is greater than or equal to 1.0, the sample contains Cry2A protein.

Cry1A Results:

If the Positive Control Ratio calculated for a single seed or leaf punch sample in the Cry1A portion of the test is less than 0.5, the sample does not contain Cry1A.

If the Positive Control Ratio of a sample is greater than or equal to 0.5, the sample contains Cry1A 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 cotton seed, cotton 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 from 1 hours to 2 hours; add time onto either or both of the substrate development steps (in 10 minute increments).

This Kit recognizes the following enhanced crops containing the biotechnology proteins Cry1A and Cry2A: Bollgard® II, VT Triple PRO™ and SmartStax®.

Other corn and cotton products including Bollgard, WideStrike™, and YieldGard® will run positive in the Cry1A portion of the test.

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87
B	PC	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88
C	S1	S90	S17	S25	S33	S41	S49	S57	S65	S73	S81	S89
D	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	S90
E	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83	S91
F	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84	S92
G	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85	BL
H	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86	PC

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