

Highlights:

- Use a single test well to screen cotton seeds or leaf samples for presence/absence of Cry1Ac and Cry2Ab
- Results for two analytes in about 2 hours

Contents of Kit:

- Cry1Ac and Cry2Ab antibody-coated solid plate
- Enzyme Conjugate
- Positive Control
- Substrate 1 (for Cry2Ab results)
- Substrate 2 (for Cry1Ac results)
- **Note:** To handle bulk packaged Enzyme Conjugate, Substrate 1 and Substrate 2: 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.

Catalog Number AP 051

Intended Use

The EnviroLogix QualiPlate Combo Kit for Cry1Ac & Cry2Ab is designed for the qualitative laboratory detection of the presence or absence of these proteins in cotton 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 cotton products including Bollgard®, Bollgard II, and WideStrike™.

How the Test Works

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

In the test, **cotton** sample extracts are added to test wells coated with antibodies that recognize Cry1Ac and Cry2Ab proteins. Any Cry1Ac or Cry2Ab 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 Cry1Ac antibody.

After a simple wash step, the results of the Cry2Ab assay are determined via the addition of *p*-nitrophenyl phosphate Substrate #1. Once the yellow color develops and is read, the wash step is repeated, and 3,3',5,5'-tetramethylbenzidine Substrate #2 is added. The Cry1Ac 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, corn, rice, sorghum, soybean, sugar beet, or wheat.

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

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 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

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

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.

NOTE: Cry2Ab protein is expressed at high concentrations in cotton seed compared with Cry1Ac. There is serious potential for cross-contamination with Cry2Ab 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 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 Cry1Ac/Cry2Ab 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.



Prepare Wash and Extraction buffer



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



Mix



Wash Plate

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 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 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 # 1** to each well. **BE SURE TO USE SUBSTRATE #1 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.**
8. **Read and record the yellow Cry2Ab 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 μ L of Substrate #2** to each well. **BE SURE TO USE SUBSTRATE #2 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.**
13. **Read and record the blue Cry1Ac 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 Cry1Ac portion of the test should not exceed 0.15. The mean OD of the BLANK wells in the Cry2Ab 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.



Read plates in a Plate Reader at the appropriate wavelength:

- 405 nanometers for the Cry2Ab test result,
- 650 or 630 nanometers for the Cry1Ac test results.

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

Cry2Ab Results:

If the Positive Control Ratio calculated for a single seed or leaf punch sample in the Cry2Ab portion of the test is less than 1.0, the sample does not contain Cry2Ab.

If the Positive Control Ratio of a sample is greater than or equal to 1.0, the sample contains Cry2Ab protein.

Cry1Ac Results:

If the Positive Control Ratio calculated for a single seed or leaf punch sample in the Cry1Ac portion of the test is less than 0.5, the sample does not contain Cry1Ac.

If the Positive Control Ratio of a sample is greater than or equal to 0.5, the sample contains Cry1Ac 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 Cry1Ac and Cry2Ab: Bollgard II.

Cotton products such as Bollgard and WideStrike will run positive in the Cry1Ac 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

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 1 or Substrate 2 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.



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