ENVIROLOGIX™

QuantiPlate™ Kit for Cry2A

Catalog Number AP 005

Highlights:

- *Range of 1 to 10 ppb Cry2Aa in sample extract*
- *Less than 2 hours to run*

Contents of Kit:

- *12 strips of 8 antibody-coated wells each, in plate frame*
- *Cry2A Negative Control*
- *1.0 ppb Cry2Aa Calibrator*
- *5.0 ppb Cry2Aa Calibrator*
- *10 ppb Cry2Aa Calibrator*
- *Cry2A-Enzyme Conjugate*
- *5X Extraction/ Dilution Buffer for Bt Plate Kits*
- *1 packet of Buffer Salts*
- *Substrate*
- *Stop Solution*

Precision

	Recovery (%CV)	OD (%CV)
Intra-Assay		**n=7**
3 ppb	4.4%	4.1%
7 ppb	2.2%	2.1%
Inter-Assay		**n=8**
3 ppb	6.4%	n/a
7 ppb	6.4%	n/a

Intended Use

The QuantiPlate Kit for Cry2A is designed for the semi-quantitative laboratory detection of Cry2Aa endotoxin in cotton leaf tissue samples.

How the Test Works

This kit is a "sandwich" Enzyme-Linked ImmunoSorbent Assay (ELISA). In the test, cotton leaf sample extracts are added to test wells coated with antibodies raised against Cry2Aa toxin. Any Cry2Aa protein present in the sample extract bind to the antibodies, and are then detected by addition of enzyme (horseradish peroxidase)-labeled Cry2 antibody.

After a simple wash step, the results of the assay are visualized with a color development step; color development is proportional to Cry2Aa concentration in the sample extract.

Lighter color = Lower concentration
Darker color = Higher concentration

Limit of Detection

The Limit of Detection (LOD) of this kit is 0.52 parts per billion (ppb) Cry2Aa in cotton leaf extract. The LOD was determined by interpolating an OD equal to three times the background OD from a Cry2Aa standard curve.

Limit of Quantification

The Limit of Quantification (LOQ) of the EnviroLogix Cry2A Plate Kit was validated at 0.5 parts per million (ppm) in cotton leaf. The LOQ was determined by fortifying a population of negative cotton leaf samples at 0.5 ppm Cry2Aa. The mean recovery was 78% with a coefficient of variation [CV, (standard deviation/mean) x 100] of 3.9%.

Precision

Cry2Aa-fortified control solutions were repetitively analyzed both within a single assay, and in different assays on different days. The data is expressed as % CV for both the recovered concentration and for absorbance (OD).

Fortification and Recovery

Eight cotton leaf samples were fortified with Cry2Aa to concentrations ranging from 0.7 ppm to 1.5 ppm. The average recovery was 93%.

Materials Not Provided

- Disposable Tissue Extractors, EnviroLogix Cat. # ACC 002
- distilled or deionized water for preparing Wash Buffer and diluting 5X Extraction/Dilution Buffer– see recipe below
- glass bottles or flasks with 175 mL capacity for storage of 1X Extraction/ Dilution Buffer and 1 liter capacity for Wash Buffer
- test tubes for dilution of sample extracts
- disposable tip, adjustable air-displacement pipettes which will measure 20, 100, 500 and 1000 microliters (µL)

- 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)

Prepare Wash and Extraction Buffers

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 Extraction/Dilution Buffer: To prepare 1X working Extraction/Dilution Buffer, add the entire contents of the bottle of 5X (35 mL) supplied in the kit 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.

Obtain leaf tissue

Grind tissue, add buffer, grind again

Sample Preparation

Sample Extraction:

1. Take 2 leaf punch samples (approximately 10 milligrams each) by snapping the tube cap of the Disposable Tissue Extractor down on the leaf. 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. Use extreme caution to prevent sample-to-sample cross-contamination with plant tissue or exudate.*

   NOTE: If the assay is to be used to quantitate levels of Cry2A toxin in cotton tissue, the weight of each leaf punch sample must be determined and recorded.

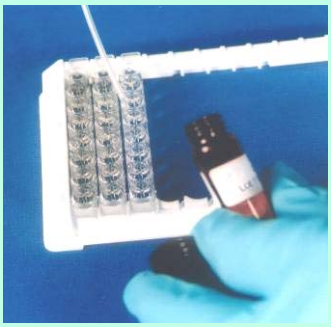
2. Add 0.5 mL of 1X Extraction/Dilution Buffer to the tube.
3. Repeat the grinding step to mix tissue with Extraction/Dilution Buffer. Repeat this protocol for each sample to be tested, using a new tube and pestle for each. Allow the solids to settle in each tube for a few minutes.

Sample Dilution:

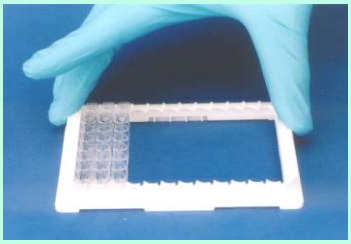
Concentrations of Cry2A toxins will vary from plant to plant. Sample extracts must be diluted at least 1:11 prior to assay, but larger dilutions may be required in order to bring assay results within the range of calibration. Instructions follow for both 1:11 and 1:51 dilution schemes. If a more sensitive assay is required, contact EnviroLogix for technical assistance.

1. For a 1:11 dilution: add 0.5 mL 1X Extraction/Dilution Buffer to dilution tubes labeled for each sample. Add 50 µL sample extract and mix.
2. For a 1:51 dilution: add 1 mL 1X Extraction/Dilution Buffer to dilution tubes labeled for each sample. Add 20 µL sample extract and mix.

Remove unneeded strips



Add calibrators and sample extracts



Mix plate

Incubate

Bottle Wash method

How to Run the Assay

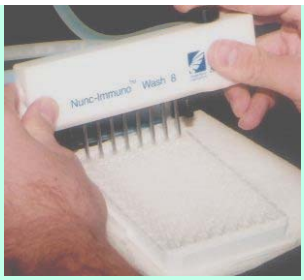
- Read all of these instructions before running the kit.
- Allow all reagents to reach room temperature before beginning (at least 30 minutes with un-boxed strips and reagents at room temperature - do not remove strips from bag with desiccant until they have warmed up).
- Organize all Calibrators and diluted 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 as a guide when adding the samples and reagents. In a qualitative assay, the Negative Control (NC), the lowest calibrator and 46 diluted sample extracts (S) may be run 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 diluted sample extracts (S) may be run in duplicate wells on one plate. (See the Quantitative Assay Example Plate Layout - Figure 1B).

Procedure

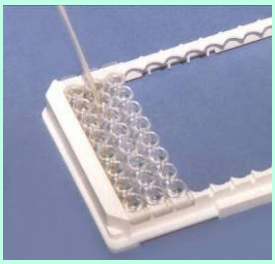
1. Add **100 µL** of **Negative Control, 100 µL** of each **Calibrator,** and **100 µL** of each **diluted sample extract** to their respective wells, as shown in the Example Plate Layouts (Figures 1A and 1B). Follow this same order of addition for all reagents.

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

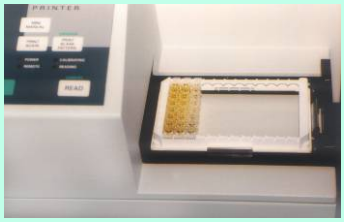
2. Thoroughly mix the contents of the wells by moving the strip holder in a rapid circular motion on the benchtop for a full 20-30 seconds. Be careful not to spill the contents!
3. Cover the wells with tape or Parafilm to prevent evaporation and **incubate at** ambient **temperature for 15 minutes.**
4. Add **100 µL** of **Cry2A-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**.
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. Slap the plate on a paper towel to remove as much water as possible. Alternatively, perform these four washes with a microtiter plate or strip washer (set to 300 µL fill volume).
8. Add **100 µL** of **Substrate** to each well.



Strip Plate Wash option

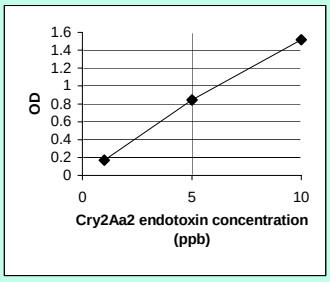


Complete protocol and add Stop Solution



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

Figure 3.
Illustrative standard curve



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.

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.

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 Negative Control wells. If the reader cannot do this, measure and record the optical density (OD) of each well's contents, then subtract the average OD of the Negative Control wells from each of the readings.

   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%.

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

3. For a quantitative Cry2A 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 Cry2A Results" section.

How to Interpret the Semi-Quantitative Results

Compare the OD's of the diluted sample extracts to those of the Calibrators to obtain an estimate of the amount of Cry2A endotoxin in your sample extract, expressed in terms of Cry2Aa reactivity.

How to Calculate the Quantitative Cry2A Results

NOTE: Although Cry2Ab calibrators are not provided with this kit, it is possible to use this kit to quantitate Cry2Ab in cotton samples. To do this, substitute the Cry2Ab calibrator values shown in the table below for the corresponding Cry2Aa calibrator concentration. Use these Cry2Ab concentrations to prepare the standard curve. Interpret results from this standard curve as ppb Cry2Ab.

1. After reading wells, average the OD of each set of calibrators and samples.
2. Graph the mean OD of each Calibrator against its Cry2Aa concentration on a linear scale (see Figure 3).
3. Determine the Cry2Aa concentration of each sample by finding its OD value and the corresponding concentration level on the graph. Multiply the result by the dilution factor incurred during extraction (500 µL ÷ *x* mg leaf tissue) multiplied by the 1:11 or 1:51 dilution of sample extract employed, and divide by 1000. Report results as micrograms Cry2Aa toxin per gram of tissue (ppm).
4. Interpolation of sample concentration is only possible if the OD of the sample falls within the range of OD's of the Calibrators.

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.*
- *The assay has been optimized for use with the protocol provided in the kit. Deviation from this protocol may invalidate the results of the test.*
- *As with all tests, it is recommended that results be confirmed by an alternate method when necessary.*
- *Cry2A endotoxins are proteins which can be degraded by heat and sunlight. Take samples from green, actively growing leaves. Samples that cannot be extracted immediately may be stored frozen for up to 1 week prior to analysis.*
- *Observe any applicable regulations when disposing of samples and kit reagents.*

If the OD of a sample is lower than that of the Low Calibrator (1.0 ppb Cry2Aa), the sample must be reported as less than: (1.0 ppb x dilution factor during extraction x dilution of sample extract employed) ÷ 1000 = *x* ppm Cry2Aa.

If the OD of a sample is higher than that of the High Calibrator (10 ppb Cry2Aa), the sample must be reported as greater than:
(10 ppb x dilution factor during extraction x dilution of sample extract employed) ÷ 1000 = *x* ppm Cry2Aa.

If a concentration must be determined for these high level samples, dilute the sample extract 10-fold more than executed in the original assay in 1X Extraction/Dilution Buffer. Run this dilution in a repeat of the immunoassay. 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.

Kit Cry2Aa Calibrators	Equivalent Cry2Ab Calibrators
Negative Control (NC)	Negative Control (NC)
1.0 ppb Cry2Aa (C1)	2 ppb Cry2Ab (C1)
5.0 ppb Cry2Aa (C2)	10 ppb Cry2Ab (C2)
10 ppb Cry2Aa (C3)	20 ppb Cry2Ab (C3)

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	C0	C0	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	C0	C0	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

Figure 2. Illustrative quantitative calculations

Well contents	OD	Average OD ± sd	% CV	Cry2A Conc. (ppb)
Negative Control	0.053 - 0.053	0.053 ±0.0	0	NA
1.0 ppb Calibrator	0.166* - 0.172	0.169 ±0.004	2.5	NA
5.0 ppb Calibrator	0.847* - 0.840	0.844 ± 0.005	0.6	NA
10 ppb Calibrator	1.510* - 1.520	1.515 ± 0.007	0.5	NA
Sample	0.523* - 0.528	0.526 ± 0.003	0.7	3.2 ppb**

* Figures are after subtraction of Negative Control values.

**Concentration from curve = 3.2 ppb Cry2A, multiplied by 1:11 dilution of sample extract = 35.2 ppb, multiplied by 1:25 dilution during extraction, and divided by 1000 = 0.880 ppm Cry2A in cotton leaf.

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

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