QualiPlate™ Kit for Roundup Ready® Corn Event 603 and Cotton

Catalog Number AP 010 NW V10

Highlights:

- *Will detect 0.1% (1 seed in 1000) of Event 603 corn*
- *Test Roundup Ready corn or cotton seed lot purity in 1 hour*

Contents of Kit:

- *10 antibody-coated 96-well plates*
- *Roundup Ready Enzyme Conjugate*
- *Substrate*

Intended Use

The EnviroLogix QualiPlate Kit for Roundup Ready Corn Event 603 and Cotton is designed for the qualitative laboratory detection of CP4 EPSPS enzyme (CP4) coded for by the Roundup Ready gene in Corn Event 603 grain, leaf, or seed, and cotton leaf and single seed. This test will detect the CP4 enzyme found in 0.1% Event 603 corn (one seed in 1000) and requires 1 hour to run.

NOTE: In corn, this kit can ONLY be used to detect RR Corn Event 603. It will NOT detect RR corn with the Event known as GA21.

How the Test Works

The EnviroLogix QualiPlate Kit for Roundup Ready Corn Event 603 and Cotton is a "sandwich" Enzyme-Linked ImmunoSorbent Assay (ELISA).

In the test, corn or cotton sample extracts are added to test wells coated with antibodies raised against CP4. Any CP4 present in the sample extract binds to the antibodies and is then detected by addition of enzyme (horseradish peroxidase)-labeled CP4 antibody.

After a simple wash step, the results of the assay are visualized with a color development step. Color development increases with increasing CP4 sample concentration.

Lighter color = Low concentration
Darker color = High concentration

How the Kit Performs

The EnviroLogix QualiPlate Kit for Roundup Ready Corn Event 603 and Cotton is a strictly qualitative (yes/no) assay. Samples are interpreted in comparison with Positive and Negative Controls. Instructions for interpreting results based upon these controls start on page 6.

Precision

CP4-fortified control solutions were repetitively analyzed in different assays on different days (inter-assay). The fortification levels used are roughly equivalent to 0.15% and 0.4% Event 603 corn, respectively. The data is expressed as % CV for both the optical density absorbance (OD) and the Positive Control Ratio (OD of sample divided by the OD of the Positive Control ground corn).

	OD (%CV)	**Pos. Ctl. Ratio** (%CV)
	Inter-Assay	**n=33**
0.15%	23.9%	21.1%
0.4%	22.1%	20.8%

Error Rate - Event 603 Corn

Validation of this QualiPlate Kit for corn 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 Plate Kit manufacturing lots, generating 3 data points per corn sample.

1000-kernel seed/grain samples

- 2 false positive results out of 378 non-Corn Event 603 data points, for a best estimate false positive rate of 0.53%.
- 2 false negative results out of 360 0.1% Corn Event 603 data points, for a best estimate false negative rate of 0.55%.

Single seed samples
- 0 false positive results out of 387 non-Corn Event 603 seed data points, for a best estimate 0% false positive rate.
- 0 false negative results out of 366 Corn Event 603 seed data points, for a best estimate 0% false negative rate.

Single leaf punch samples
- 0 false positive results out of 378 non-Corn Event 603 leaf data points, for a best estimate 0% false positive rate.
- 0 false negative results out of 378 Corn Event 603 leaf data points, for a best estimate 0% false negative rate.

IMPORTANT NOTE: The presence of Roundup Ready Soybean in a corn sample WILL cause a positive result in this assay.

Error Rate - Roundup Ready Cotton

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

Single seed samples
- 0 false positive results out of 591 non-Roundup Ready cotton seed data points, for a best estimate 0% false positive rate.
- 7 false negative results out of 555 Roundup Ready cotton seed data points, for a best estimate 1.3 % false negative rate.

Single leaf punch samples
- 13 false positive results out of 1593 non-Roundup Ready cotton leaf data points, for a best estimate 0.8 % false positive rate.
- 0 false negative results out of 567 Roundup Ready cotton leaf data points, for a best estimate 0% false negative rate.

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.
- **Positive Control**. It is recommended that the user prepare a known positive control sample to run in each assay. A ground corn Positive Control may be purchased through EnviroLogix (CON-105, Part #10764).
- disposable tip, adjustable air-displacement pipettes which will measure 50 and 100 microliters (µL), preferably of multi-channel style
- distilled or deionized water for preparing Wash/Extraction Buffers
- glass bottles or flask plus graduated cylinder with 1 liter capacity for preparation and storage of Wash/Extraction Buffer
- Tween® 20 (Sigma cat# P 1379, or equivalent), Sodium tetraborate (Borax, Sigma cat# S 9640, or equivalent, optional) for cotton sample extraction
- Waring laboratory blender (model 31BL91 or equivalent), glass jar adapter (Eberbach # E8495) and 32 oz. glass Mason jars for ground corn samples

Prepare wash buffer and grain extraction solutions

USDA Websites

- **http://www.gipsa.usda.gov/publications/fgis/handbooks/gihbk1_insphb.html** - USDA Grain Inspection Handbook, Book 1, Grain Sampling.
- **http://www.gipsa.usda.gov/fgis/biotech/sample2.htm** - Guidance document entitled Sampling for the Detection of Biotech Grains.
- **http://www.gipsa.usda.gov/fgis/biotech/sample1.htm** - Practical Application of Sampling for the Detection of Biotech Grains.
- **www.gipsa.usda.gov/fgis/biotech/samplingplan1.xls** - This website provides a simple to use Sample Planner (29K Excel Spreadsheet). The planner allows you to enter different assumptions in terms of sample size, number of samples, acceptable quality level and to determine the probability of accepting lots with given concentration levels. It also plots the probabilities in graph form for easy interpretation. Specific data can be saved for documentation and future analyses.

- snap-cap tubes and pestles for extraction of leaf samples (EnviroLogix Cat No. ACC 002 / Part#11213, 100/package)
- centrifuge capable of 5000 x g (optional)
- marking pen (indelible)
- tape or Parafilm®
- timer
- microtiter ELISA plate reader
- wash bottle, or microtiter plate or strip washer
- multi-channel pipette that will measure 50 and 100 µL
- racked dilution tubes for loading samples into the plate with a multi-channel pipette, or the equivalent
- 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.

Cotton Extraction Buffers: Cotton leaf and seed samples may be extracted with either of the following buffers:

PBS-0.55% Tween: Add 0.5 mL Tween 20 to 100 mL Wash/Extraction Buffer. Store refrigerated when not in use; allow to come to room temperature prior to assay.

Borate-Tween: Prepare 0.1 M sodium tetraborate/0.5% Tween 20 (38.1 grams per liter of de-ionized water plus 5 mL Tween 20). Adjust pH to 7.5. Store refrigerated when not in use; allow to come to room temperature prior to assay.

Sample Preparation

Sampling Ground Corn Grain/Seed

This protocol requires that a small sample (20 to 50 grams) be analyzed. It is essential that this sample be well mixed and representative of the larger bulk. The test will detect 0.1% Event 603 corn (one positive kernel in a sample of 1000 kernels).

NOTE: 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. The USDA/GIPSA has prepared several guidance documents to address the issues involved in obtaining representative grain samples from static lots - such as trucks, barges, and railcars - and for taking samples from grain streams.

Sampling plans should be chosen that best meet the needs of both the buyer and seller in terms of acceptable risks. Increasing the number of kernels in the sample and taking multiple samples will increase the likelihood of obtaining representative samples, and maximize the probability of detecting any contamination in the grain lot. For further information on USDA/GIPSA guidelines for obtaining representative samples and assessing detection probabilities for biotech grain, see the websites listed to the left.

Grind and Extract the Samples

Ground Corn Grain/Seed:

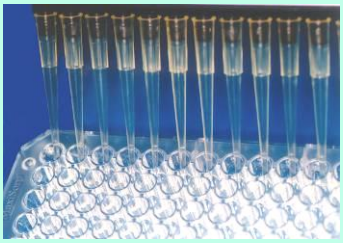
Once representative samples have been obtained from a truck or container, they can be reduced in size using a splitter and uniformly ground and mixed. **The finer the grind, the faster and more efficient the extraction.**

Allow all reagents to reach room temperature before beginning

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 grain sample sizes, add Wash/Extraction Buffer at the rate of 2.5 mL per gram of grain. 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 <u>at least</u> 10 minutes. Insert a pipette tip below any floating lipid layer and above the pellet to remove the clarified sample. Dispensing particles into the test plate can cause false positive results.

Single Corn or Cotton Seed Samples:

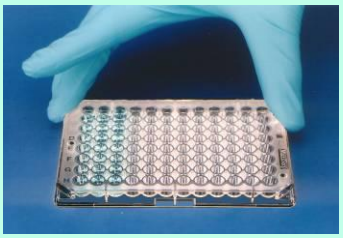
1. Crush seeds: Seeds may be placed in a resealable plastic bag and smashed with a hammer, then transferred to a tube; or, a seed crusher/48-well plate combination may be used (for example Hypure #HSC-100, PerkinElmer, Norton, OH, with Costar plate #3548, Corning Life Sciences, Acton, MA, or equivalent). Check to be sure that all seeds have been crushed. Take extreme care not to cross-contaminate between seed samples. 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.
2. Add 1 mL of Wash/Extraction Buffer to each crushed corn seed; add 1 mL of PBS-0.55% Tween or Borate-Tween to each crushed cotton seed. Mix for at least 30 seconds, then allow particles to settle. Dispensing particles into the test plate can cause false positive results.



Add Conjugate, controls and samples

Single Corn or Cotton 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 corn leaf punch; add 0.25 mL of PBS-0.55% Tween or Borate-Tween to each cotton 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.



Mix plate

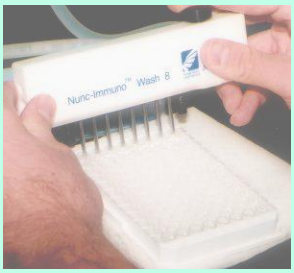
NOTE: It is recommended that the kit user extract known conventional and known CP4-containing samples of the matrix being tested, and run these as negative and positive controls in each assay, along with the required Positive Control ground corn extract.

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.

Incubate

Bottle Wash method



Strip Plate Wash option

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

- 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), user-supplied known-negative control (NC) and Positive Control (PC) extracts, along with 90 sample extracts (S) in single wells may be run on one plate. (See the Qualitative Assay Example Plate Layout - Figure 1).

Procedure

1. Add **50 µL** of **Roundup Ready Enzyme Conjugat**e to each well, followed immediately by **50 µL of Wash/Extraction Buffer Blank (BL)**, **50 µL** of user-supplied **Negative and Positive Controls (PC and NC)** and **50 µL** of each **sample extract (S)** to their respective wells, as shown in the Example Plate Layout (Figure 1).

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 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 **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 wells completely with Wash/Extraction 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 **Substrate** to each well.
6. Thoroughly mix the contents of the wells, as in step 2. Cover the wells with new tape or Parafilm and **incubate** for **15 minutes at ambient temperature**. Use orbital shaker if available.

   CAUTION: Stop Solution is 1.0N Hydrochloric acid. Handle carefully.
7. Add **100 µL** of **Stop Solution** to each well and mix thoroughly. This will turn the well contents yellow.

How to Interpret the Results

Spectrophotometric Measurement

1. Set the wavelength of the 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 0.2 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.

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

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% Event 603 corn.

If the Positive Control Ratio of a sample is greater than or equal to 0.25, the sample contains 0.1% or greater Event 603 corn.

NOTE: Ground corn samples containing more than 25% Event 603 corn may show decreasing OD's with increasing concentration. However, the OD's will be much greater than that of a 0.1% Event 603 sample. This test is to be used qualitatively only, with yes/no results at 0.1% Event 603 corn. For information on testing at different cutoff levels, please contact EnviroLogix' Technical Service.

Single Corn or Cotton Leaf and Seed samples:
If the Positive Control Ratio calculated for a sample is less than 1.0, the sample is not Event 603 corn or Roundup Ready cotton.

If the Positive Control Ratio of a sample is greater than or equal to 1.0, the sample is Event 603 corn or Roundup Ready cotton.

Leaf and seed 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 (stray particles or dust from Event 603 corn or cotton, 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 1. 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 QualiPlate Kit components at 4°C to 8°C (39°F to 46°F) when not in use.
- Do not expose QualiPlate 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.
- The assay has been optimized to be used 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.
- Observe any applicable regulations when disposing of samples and kit reagents.
- Use caution to prevent sample-to-sample cross-contamination with samples, fluids, or disposables.

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LICENSE

EnviroLogix has developed this kit using proprietary reagents as well as reagents licensed from the Monsanto Company.