ENVIROLOGIX™

QualiPlate™ Kit for Cry1F

Catalog Number AP 016

Intended Use

The EnviroLogix QualiPlate™ Kit for Cry1F is designed for the qualitative laboratory detection of Cry1F endotoxin in corn and cotton, seed, leaf and bulk seed/grain samples. Two assay protocols are presented: The High Sensitivity Protocol will detect the Cry1F protein found in 0.17% Herculex I corn or 0.17% WideStrike cotton (1 positive seed in a 600 seed sample) and requires two hours to run. The Rapid Protocol (one hour total) is intended for use in screening individual seed or leaf punch samples for the presence of Cry1F.

How the Test Works

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

In the test, sample extracts are added to test wells coated with antibodies raised against Cry1F protein. Any Cry1F present in the sample extract binds to the antibodies, and is then detected by addition of enzyme (horseradish peroxidase)-labeled Cry1F antibody.

After a simple wash step, the results of the assay are visualized with a color development step; color development is proportional to Cry1F concentration in the sample extract.

Lighter color = Lower concentration
Darker color = Higher concentration

Items Not Provided

- distilled or deionized water for preparing Wash and Extraction Buffer
- Tween-20 (Sigma P-1379, or equivalent) for preparation of Extraction Buffer
- glass bottles or flasks plus graduated cylinder with 1 liter capacity for preparation and storage of Wash and Extraction Buffer
- Waring laboratory blender (model 31BL91 or equivalent), glass jar adapter (Eberbach # E8495) and appropriate size glass Mason jars for ground seed samples
- Snap-cap tubes and pestles for extraction of leaf samples (EnviroLogix Cat# ACC 002, 100/package), optional
- centrifuge capable of 5000 x g, optional
- disposable tip, adjustable air-displacement pipettes which will measure 50 and 100 microliters (µL), preferably of multi-channel configuration
- marking pen (indelible)
- tape or Parafilm®
- timer
- microtiter plate reader or strip reader
- wash bottle, or microtiter plate or strip washer
- racked dilution tubes for loading samples into the plate with a multi-channel pipette (optional)
- orbital plate shaker (optional)

Highlights:

- **High Sensitivity Protocol**
  Can detect as little as 0.17% Herculex™ I corn or WideStrike™ Cotton in a 2 hour assay
- **Rapid Protocol**
  Screens single seed or leaf samples for the presence of Cry1F protein in a 1 hour assay

Contents of Kit:

- *1 antibody-coated plate*
- *Cry1F Positive Control*
- *Cry1F Enzyme Conjugate*
- *1 packet of Buffer Salts*
- *Substrate*
- *Stop Solution*

Prepare wash buffer and extraction solutions

USDA Websites

http://archive.gipsa.usda.gov/ reference-library/handbooks/ grain-insp/ grbook1/bk1.pdf
USDA Grain Inspection Handbook, Book 1, Grain Sampling. This document provides a comprehensive overview of recommended sampling guidelines for static lots and grain streams. It reviews the various types of equipment and strategies that can be used to obtain a representative grain sample from different types of containers.

http://archive.gipsa.usda.gov/ biotech/ sample2.htm
Guidance document entitled Sampling for the Detection of Biotech Grains, which provides important statistical sampling considerations when testing for the presence of biotech grains. It covers the basis for making probability determinations in accepting lots based upon different assumptions with respect to sample size, number of samples, sample preparation, etc.

http://archive.gipsa.usda.gov/ biotech/sample1.htm
Practical Application of Sampling for the Detection of Biotech Grains. This one-page application guide provides a table that gives sample sizes for selected lot concentrations and probability of rejecting the specified concentrations. It also provides a formula for making the calculation for other combinations.

http://archive.gipsa.usda.gov/ 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.

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. If more wash buffer is needed, order item # P-3563 from Sigma Chemical Co. (St. Louis, MO), or prepare the equivalent.

Extraction Buffer:

Add 0.5 mL Tween-20 to 100 mL of prepared Wash Buffer, and stir to dissolve. Store refrigerated when not in use; warm to room temperature prior to assay.

Cry1F Positive Control:

The Positive Control is used as provided in the Rapid Protocol, but must be diluted 1:4 for use in the High Sensitivity Protocol. Prepare this dilution just prior to running the assay: mix 50 µL Cry1F Positive Control with 150 µL Extraction Buffer for each set of duplicate wells to be filled.

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 kit Positive Control.

Ground 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 0.17% Herculex I corn or WideStrike cotton (one positive seed in a sample of 600 seeds). Bulk cottonseed samples should be delinted.

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.

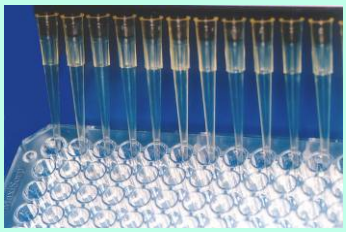
It is the responsibility of the user to ensure proper sampling and thorough mixing prior to analysis. Once representative samples have been obtained from the truck or container, they can be reduced in size using a splitter and uniformly ground and mixed. **The finer the grind, the faster and more efficient the extraction.**

1. For 600 seed samples, grind in a “Mason” jar (32 oz. for corn, 16 oz. for delinted cottonseed) 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 sample into a jar or cup.

Extract grain sample

Centrifuge to clarify grain extract

Leaf punch using microtube cap



Add Extraction Buffer Blank, Negative and Positive Control, and each sample extract to the plate

3. Add 50 mL of Extraction Buffer to each 20 gram corn sample, or 60 mL to each cotton sample. For all other grain sample sizes, add Extraction Buffer at the rate of 2.5 mL per gram of corn or 3.0 mL per gram of cotton. 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. Clarify the extracts by centrifuging at 5000 x g for 5 minutes. Alternatively, allow them to settle out for at least 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.
5. Use the High Sensitivity Protocol to test these sample extracts.

Single Seeds or Leaf Punches:

Individual seeds:

1. Crush seeds: Seeds may be crushed by any number of methods, from hammers or pliers in a bag or tube, to 48-well seed crushers, to bead-beater type grinders. Whatever the method used, take extreme care not to cross-contaminate between seed samples.
2. Add 1 mL of Extraction Buffer to each crushed seed. Mix for at least 30 seconds. For best results, allow to extract for an hour, mixing again at the end of that time. If seeds are thoroughly crushed, this extraction time can be reduced. Allow extracts to settle completely. Dispensing particles into the test plate can cause false positive results.

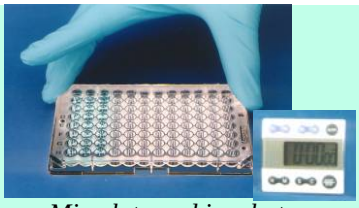
Leaf testing:

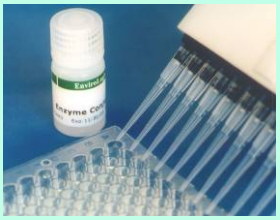
1. Take a single leaf punch of approximately 5 to 10 millimeters diameter, using a paper punch or micro-tube cap. Mash the leaf tissue with a pestle matched to the micro-tube, or disrupt via another method. The extraction efficiency of whatever method used will vary proportionately with the amount of tissue disruption performed.
2. Add 0.25 to 0.5 mL of Extraction Buffer per leaf punch. Mix for at least 30 seconds, and 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.

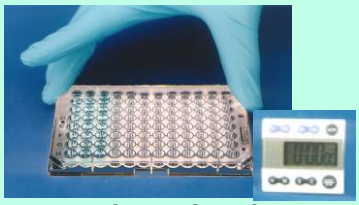
Use the Rapid Protocol to test seed and leaf extracts.

How to Run the Assay

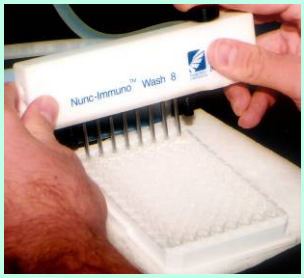
- Read all of these instructions before running the kit.
- Allow all reagents to reach room temperature before beginning (at least 30 minutes with un-boxed plates and reagents at room temperature - do not remove plates from bag with desiccant until they have warmed up).
- Organize all Controls, clarified sample extracts, and pipettes so that Step 1 can be performed in 15 minutes or less. The use of a multi-channel pipette is strongly recommended for all reagent additions.
- Use the well identification markings on the plate frame as a guide when adding the samples and reagents. In a qualitative assay, the Blank (BL), Positive Control (PC) in duplicate wells, and 92 sample extracts (S) in single wells may be run on one plate. (See the Qualitative Assay Example Plate Layout - Figure 1A).
- Choose the assay protocol that fits the testing needs.


Mix plate and incubate


Add Enzyme Conjugate


Mix plate and incubate


Bottle Wash method



Strip Plate Wash option

HIGH SENSITIVITY PROTOCOL

The **High Sensitivity Protocol** will detect 0.17% Herculex I corn or WideStrike cotton in ground grain/seed, and requires two hours of total assay incubation time. Dilute the Cry1F Positive Control 1:4 in Extraction Buffer for this protocol.

1. Add **50 µL** of **Extraction Buffer Blank (BL)**, **50 µL** of diluted **Positive Control (PC)**, and **50 µL** of each **sample and user-prepared control extract (S)** to their respective wells, as shown in the Example Plate Layout (Figure 1A).

   NOTE: In order to minimize setup time it is strongly recommended that a multi-channel pipette be used in steps 1, 5, 8, and 10.

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 **30 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 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 **50 µL Cry1F-enzyme Conjugate** to each well. Thoroughly mix the contents of the wells, as in step 2.
6. Cover the wells with tape or Parafilm to prevent evaporation and incubate at ambient temperature for **1 hour**. If an orbital plate shaker is available, shake plate at 200 rpm.
7. Wash the plate as described in step 4.
8. Add **100 µL** of **Substrate** to each well.
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**. Use orbital shaker if available.

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.

RAPID PROTOCOL

The **Rapid Protocol** is used to screen single seed or leaf samples for presence of Cry1F and requires one hour of total assay incubation time. Use the Cry1F Positive Control without dilution for this protocol.

1. Add **50 µL Cry1F-enzyme Conjugate** to each well. Immediately add **50 µL** of **Extraction Buffer Blank (BL)**, **50 µL** of **Positive Control (PC)**, and **50 µL** of each **sample extract and user-prepared control extract (S)** to their respective wells, as shown in the Example Plate Layout (Figure 1A).

Slap inverted plate on towel to remove as much liquid as possible

Complete protocol and add Stop Solution

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

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 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.

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 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 **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 0.15.
   - The mean, blank-subtracted OD of the Positive Control wells should be at least 0.15.
   - 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 wells. This number is the "Positive Control Ratio".

Interpret the Qualitative Results

Ground corn or cottonseed samples

If the Positive Control Ratio calculated for a sample is less than 0.5, the ground corn contains less than 0.17% Herculex I corn or WideStrike cotton.

If the Positive Control Ratio of a sample is greater than or equal to 0.5, the sample contains 0.17% or greater Herculex I corn or WideStrike cotton.

NOTE: This test is to be used qualitatively only, with yes/no results at 0.17% positive. For information on testing at different cutoff levels, please contact EnviroLogix' Technical Service.

Single leaf and seed samples:

If the Positive Control Ratio calculated for a sample is less than 1.0, the sample does not contain Cry1F at the levels normally found in Herculex I corn or WideStrike cotton.

If the Positive Control Ratio of a sample is greater than or equal to 1.0, the sample is Herculex I corn or WideStrike 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, 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 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	S9	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 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 test well strips from one Plate Kit with reagents or test well strips from a different Plate 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.
- The results generated through the proper use of this kit reflect the condition of the working sample directly tested. Extrapolation as to the condition of the originating lot from which the working sample was derived should be based on sound sampling procedures and statistical calculations which address random sampling effects, non-random seed lot sampling effects, and assay system uncertainty. A negative result obtained when properly testing the working sample does not necessarily mean the originating lot is entirely negative for the analyte or protein in question.
- 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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This Limited Warranty states the entire obligation of EnviroLogix with respect to the Products. If any part of this Limited Warranty is determined to be void or illegal, the remainder shall remain in full force and effect.

This test kit has been validated and approved by Dow AgroSciences for detection of the Cry1F protein expressed in Herculex I corn and WideStrike cotton.