

Highlights:

- **High Sensitivity Protocol**
Can detect as little as 0.17% Herculex™ I corn in a 2.5 hour assay
- **Rapid Protocol**
Screens single seed or leaf samples for the presence of Cry1F protein in a 1.5 hour assay

Contents of Kit:

- 1 antibody-coated solid plate
- 1 vial Negative Control ground corn (2 grams)
- 1 vial Positive Control ground corn (2 grams)
- 1 bottle of Cry1F Enzyme Conjugate
- 1 packet of Wash Buffer Salts
- 1 bottle of Substrate
- 1 bottle of Stop Solution



Prepare wash buffer and extraction solutions

Catalog Number AP 016

Intended Use

The EnviroLogix QualiPlate™ Kit for Cry1F is designed for the qualitative laboratory detection of Cry1F endotoxin in corn grain, seed or leaf samples. Two assay protocols are presented: The High Sensitivity Protocol will detect the Cry1F protein found in 0.17% Herculex I corn (1 positive kernel in 600 conventional kernels) and requires 2.5 hours to run. The Rapid Protocol (1.5 hours total) is intended for use in screening individual seeds or leaf samples for the presence of Cry1F.

How the Test Works

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

In the test, corn 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

Cross-Reactivity

The QualiPlate Kit for Cry1F does not recognize any of the following biotechnology enhanced corn varieties in either of the two protocols:

Corn varieties tested: YieldGard® Corn Borer (Bt11, MON810), Knockout®/NatureGard® (Bt176), LibertyLink® T25, StarLink®, Roundup Ready® (Events GA21 and 603), YieldGard Plus and YieldGard Rootworm (MON 863).

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 16 oz. glass Mason jars for ground corn 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)
- marking pen (indelible)

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.

- tape or Parafilm®
- timer
- microtiter plate reader or strip reader
- wash bottle, or microtiter plate or strip washer
- multi-channel pipette that will measure 50 and 100 μL (optional)
- racked dilution tubes for loading samples into the plate with a multi-channel pipette (optional)
- orbital plate shaker (optional)

Preparation of Solutions

Wash Buffer:

Add the contents of the packet of **Wash 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.

Sample Preparation

Positive and Negative Control ground corn extracts:

Extracts of these controls must be run in every assay. To extract, add 5 mL of Extraction Buffer to each tube containing 2 grams of ground Control corn. 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, then clarify by allowing to settle 10 minutes or by centrifuging 5 minutes at 5000 x g.

The High Sensitivity Protocol requires that the Positive Control ground corn extract be diluted 1:3 in Negative Control ground corn extract (mix 100 μL Positive extract plus 200 μL Negative extract) prior to use. No dilution of the Positive Control ground corn extract is needed for use in the Rapid Protocol.

If you will be running the assay at a later date, or more than one assay per plate, freeze 0.5 mL aliquots of each clarified extract at -20°C or lower in a non self-defrosting freezer. Thaw just prior to use.

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 (one positive kernel in a sample of 600 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



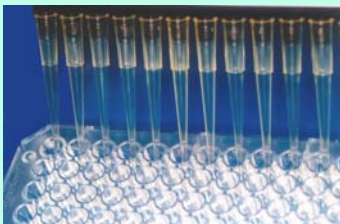
Extract grain sample



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

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 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 Extraction Buffer to each 20 gram sample. For all other grain sample sizes, add 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 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:

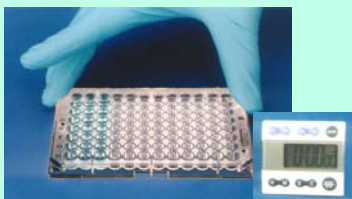
Individual seeds:

1. Crush seeds: Seeds may be placed in a baggie and smashed with a hammer, then transferred to a tube; or a seed cutter/48-well plate combination may be used (for example Hypure #HCT-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 cutter, at least 4 cuttings are recommended, rotating seeds between cuttings. 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 0.75 mL of Extraction Buffer to each crushed/cut corn seed. Mix for at least 30 seconds, then allow particles to settle. Dispensing particles into the test plate can cause false positive results.

Leaf testing:

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) in a 96-well plate (Costar #3370, Corning Life Sciences, or equivalent).
2. Add 0.25 mL of Extraction Buffer per 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.

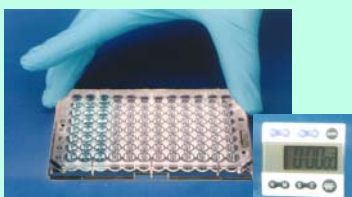
Use the Rapid Protocol to test seed and leaf extracts.



Mix plate and incubate



Add Enzyme Conjugate



Mix plate and incubate



Bottle Wash method



Strip Plate Wash option

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), Negative Control and Positive Control (PC) in duplicate wells, and 90 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.

HIGH SENSITIVITY PROTOCOL

The **High Sensitivity Protocol** will detect 0.17% Herculex I corn in ground grain/seed, and requires 2.5 hours of total assay incubation time. Dilute the Positive Control ground corn extract 1:3 in Negative Control ground corn extract for this protocol.

1. Add **50 μ L** of **Extraction Buffer Blank (BL)**, **50 μ L** of **Negative Control (NC) ground corn extract**, **50 μ L** of **diluted Positive Control (PC) ground corn extract**, and **50 μ L** of each **sample extract (S)** to their respective wells, as shown in the Example Plate Layout (Figure 1A).

NOTE: In order to minimize setup time it is recommended that a multi-channel pipette be used in steps 1, 4, 7, and 9.

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. Add **50 μ L Cry1F-enzyme Conjugate** to each well. Thoroughly mix the contents of the wells, as in step 2.
5. Cover the wells with tape or Parafilm to prevent evaporation and incubate at ambient temperature for **90 minutes**. If an orbital plate shaker is available, shake plate at 200 rpm.
6. 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.
7. Add **100 μ L** of **Substrate** to each well.
8. 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.

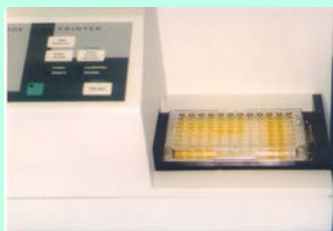
9. Add **100 μ L** of **Stop Solution** to each well and mix thoroughly. This will turn the well contents yellow.



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: 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 and 30 minutes of total assay incubation time. Use the Positive Control ground corn extract without dilution for this protocol.

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

NOTE: In order to minimize setup time it is 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 **1 hour**. 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 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** 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 30 minutes at ambient temperature**. Use orbital shaker if available.

Caution: Stop Solution is 1.0N Hydrochloric acid. Handle carefully.

7. Add **100 μ L 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 0.2.
- 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 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.

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.

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

Single Corn Leaf and Seed samples:

If the Positive Control Ratio calculated for a sample is less than 0.5, the sample does not contain Cry1F at the levels normally found in Herculex I corn.

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

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 Cry1F corn, 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	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 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.



**For Technical Support
Contact Us At:**

Envilab GmbH
Cyriakusstraße 27

47839 Krefeld

Tel: (02151) 5671549

Fax: (02151) 5695602

e-mail:
info@envilab-gmbh.de

website:
www.envilab-gmbh.de



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StarLink is a trademark of Aventis CropScience
LibertyLink is a trademark of Bayer
Herculex is a trademark of Dow AgroSciences LLC
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