ENVIROLOGIX™

QualiPlate™ Kit for Modified Cry3A

Catalog Number AP 050 NW V10

Highlights:

- *Test corn seed lot purity in 2 hours*

Contents of Kit:

- *10 antibody-coated 96-well plates*
- *Positive Control*
- *mCry3A Enzyme Conjugate*
- *1 bottle of Substrate*

Prepare wash buffer and extraction solutions

Intended Use

The EnviroLogix QualiPlate Kit for Modified Cry3A is designed for the qualitative laboratory detection of modified Cry3A protein (mCry3A) in corn leaf, single seed, and ground grain/seed samples. The mCry3A protein is found in products containing the Syngenta corn rootworm event MIR 604, such as Agrisure™ RW.

How the Test Works

This 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 mCry3A. Any mCry3A present in the sample extract binds to the antibodies and is then detected by addition of enzyme (horseradish peroxidase)-labeled mCry3A antibody.

After a simple wash step, the results of the assay are visualized with a color development step. Color development increases with increasing Cry3A sample concentration.

Lighter color = Low concentration
Darker color = High concentration

How the Kit Performs

This kit is a strictly qualitative (yes/no) assay. Samples are interpreted in comparison with the Positive Control provided in each kit. Instructions for interpreting results start on page 4.

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.
- distilled or deionized water for preparing Wash and Extraction Buffers
- Tween® 20 (Sigma cat# P 1379, or equivalent) for preparing 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
- disposable tip, adjustable air-displacement pipettes which will measure 50 and 100 microliters (µL)
- marking pen (indelible)
- tape or Parafilm®
- timer

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 applica-tion guide provides a table that gives sample sizes for selected lot concentrations and probability of rejecting the specified concentra-tions. 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.

- 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 Buffer: Add PBS/0.05% Tween-20 Wash Buffer packet to 1 liter of distilled or deionized water and stir to dissolve. Store refrigerated when not in use; allow to come to room temperature prior to assay. May be ordered in 1-liter size packets, item # P-3563, from Sigma Chemical Co. (St. Louis, MO), or prepare the equivalent. Use this buffer for the wash step of the assay, and to prepare Extraction Buffer.

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

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.

Sample Preparation

NOTE: It is recommended that the kit user extract known conventional and known mCry3A-containing samples of the matrix being tested, and run these as negative and positive controls in each assay, in addition to the kit Positive Control. If ground corn reference material is being used, it is extremely important that ground grain samples be of the same grind consistency as the reference material. This will ensure comparable protein extraction efficiency from both the reference and sample materials.

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 0.2% Agrisure RW corn by weight, based upon testing of a reference sample.

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.

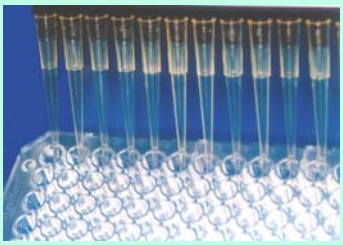
It is the responsibility of the user to ensure proper sampling and thorough mixing prior to analysis. Once representative samples have been obtained from the truck or container, they can be reduced in size using a splitter and uniformly ground and mixed. **The finer the grind, the faster and more efficient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

Allow all reagents to reach room temperature before beginning

1. Weigh at least 20 grams of well mixed ground sample into a jar or cup.
2. Add 50 mL of Extraction Buffer to each 20 gram corn sample. For all other grain sample sizes, add Extraction Buffer at the rate of 2.5 mL per gram of 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.
3. Clarify the extracts by centrifuging at 5000 x g for 5 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 Seed Samples:

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.



Add Controls and samples

Single Corn Leaf Punch Samples:

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.

Mix plate

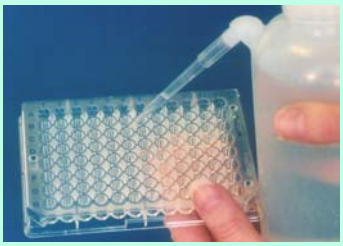
How to Run the Assay

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

Incubate

Procedure

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



Bottle Wash method

Strip Plate Wash option

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, 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. Seal 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 wash step three times. Alternatively, perform these four washes (300µL/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** of **mCry3A Enzyme Conjugate** to each well. 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.
6. Seal 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. 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.
8. Add **100 µL** of **Substrate** to each well.
9. Thoroughly mix the contents of the wells, as in step 2. Seal 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.

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 0.2.
The coefficient of variance (%CV) between the duplicate Positive Control wells should not exceed 15%:

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

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 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 kit with reagents or plates 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.*
- *Use of ground corn standard(s) with this test kit will provide accurate results only if the test samples are ground to the same consistency as that standard.*
- *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.*

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 Grain/Seed samples:

A reference sample of ground MIR604 corn was fortified into 10 conventional corn seed samples at a concentration of 0.2% by weight. The samples were thoroughly ground and mixed, and extracted as described in this Product Insert. The average Positive Control Ratio was 1.14, with a standard deviation of 0.27, and a range from 0.61 to 1.74. To ensure that all 0.2% MIR604 samples are called positive, we recommend interpreting all samples with a Positive Control Ratio of 0.6 or greater (the mean minus 2 standard deviations) as likely (97.7% confidence level) to contain 0.2% or more MIR604 corn by weight.

Single Corn Leaf and Seed samples:

If the Positive Control Ratio calculated for a sample is less than 0.5, the sample is not MIR604 corn.

If the Positive Control Ratio of a sample is greater than or equal to 0.5, the sample is MIR604 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 MIR604 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 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

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