QualiPlate™ Kit for eCry3.1Ab

Catalog Number AP 074 NW V10

Highlights:

Screens single seed or leaf or bulk grain samples for the presence of eCry3.1Ab protein in a 1.5 hour assay

Contents of Kit:

- *10 antibody-coated plates*
- *eCry3.1Ab Positive Control*
- *eCry3.1Ab Antibody Enzyme Conjugate*
- *Substrate*

Prepare wash buffer and extraction solutions

Intended Use

The QualiPlate Kit for eCry3.1Ab is designed for the qualitative laboratory detection of eCry3.1Ab protein at levels typically expressed in genetically modified corn seed, leaf, and bulk seed/grain samples (Event 5307, Agrisure Duracade™). Corn plants derived from transformation Event 5307 contain the gene *ecry3.1Ab* encoding an eCry3.1Ab protein, which is an engineered chimera of modified Cry3A (mCry3A) and Cry1Ab proteins. The kit will detect concentrations of 0.25% or more Event 5307 corn in a ground corn sample.

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 reactive to eCry3.1Ab protein. Any eCry3.1Ab present in the sample extract binds to the antibodies, and is then detected by addition of enzyme (horseradish peroxidase)-labeled eCry3.1Ab antibody.

After a simple wash step, the results of the assay are visualized with a color development step; color development is proportional to eCry3.1Ab concentration in the sample extract.

Lighter color = Lower concentration
Darker color = Higher concentration

Items 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).
- Tween-20 (Sigma P-1379, or equivalent), sodium tetraborate, and sodium hydroxide, for preparation of Extraction Buffer
- 1 N Hydrochloric acid (HCl) for Stop Solution
- distilled or deionized water for preparing Wash and Extraction Buffers
- glass bottles or flasks plus graduated cylinder with 1 liter capacity for preparation and storage of Wash and Extraction Buffer
- Snap-cap tubes and pestles for extraction of leaf samples (EnviroLogix Cat# ACC 002, 100/package), optional
- Waring laboratory blender (model 31BL91 or equivalent), glass jar adapter (Eberbach # E8495) and appropriate size glass Mason jars for ground seed samples
- centrifuge capable of 5000 x *g*
- 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)

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 one Sigma Chemicals, Cat#P-3563, packet of **Buffer Salts** (phosphate buffered saline-0.05% Tween 20, pH 7.4) 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. Store at controlled ambient temperature for up to one week, then discard.

Extraction Buffer, Borate/Tween: For 5 liters of Extraction Buffer (25 mM Borate/0.01% Tween 20, pH 10):

- 47.65 grams Sodium Tetraborate Decahydrate (Borax, CAS# 1303-96-4, Sigma S9640 or equivalent)
- Stir Borax in 4.9 liters of distilled/deionized water until completely dissolved.
- Add 0.5 mL of Tween 20 (CAS# 9005-64-5, Sigma P1379 or equivalent) and stir to mix.
- Calibrate a pH meter, and insert pH probe into stirring solution. Add Sodium Hydroxide solution (2 to 6 Normal) while stirring until pH reaches 10.0. Bring total volume of solution to 5 liters with distilled or deionized water. Extraction Buffer can be stored at controlled room temperature (20 to 25°C/ 68 to 77°F) for up to six months from date of preparation.

Stop Solution: Prepare 1 N Hydrochloric acid (HCl) 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.

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 0.25% eCry3.1Ab corn (one positive seed in a sample of 400 seeds).

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.

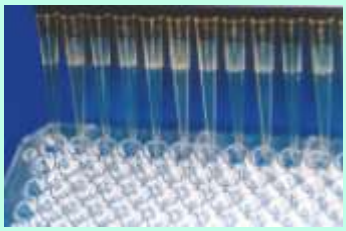
It is the responsibility of the user to ensure proper sampling and thorough mixing prior to analysis. Once representative samples have been obtained from the truck or container, they can be reduced in size using a splitter and uniformly ground and mixed. **The finer the grind, the faster and more efficient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

Extract grain sample

Centrifuge to clarify grain extract

Leaf punch using microtube cap

Add Enzyme Conjugate



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

1. For 400 seed samples, grind in a "Mason" jar (32 oz) on a blender at high speed for 20 seconds. Shake jar to mix and repeat grinding a second time. Make sure sample is thoroughly ground with no large seed pieces remaining. Thoroughly clean the grinding equipment between samples to prevent cross-contamination.
2. Weigh at least 5 grams of ground sample into a jar or cup.
3. Add 12.5 mL of Grain Extraction Buffer to each 5 gram corn sample. For all other grain sample sizes, add Grain 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. Incubate on bench 10 minutes.
4. 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. Run the assay as soon as possible after preparing samples.

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. Allow extracts to settle completely. Dispensing particles into the test plate can cause false positive results.

   CAUTION: eCry3.1Ab protein is expressed at high concentrations in Agrisure Duracade corn seed, so there is serious potential for cross-contamination between samples during seed crushing. Use the utmost care to avoid this. Cleaning the cutting/crushing surfaces with an alcohol-soaked pad between samples is recommended.

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.

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, 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 1).


Mix plate and incubate


Bottle Wash method


Strip / Plate Wash option


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


Complete protocol and add Stop Solution

Procedure

1. Add **50 µL eCry3.1Ab Antibody 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).

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 **60 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 **30 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.

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

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 1.0, the ground corn contains less than 0.25% eCry3.1Ab corn.

If the Positive Control Ratio of a sample is greater than or equal to 1.0, the sample contains greater than or equal to 0.25% eCry3.1Ab corn.

> **NOTE:** This test is to be used qualitatively only, with yes/no results at 0.25% detection limit.

Single leaf and seed samples: If the Positive Control Ratio calculated for a sample is less than 1.0, the sample does not contain eCry3.1Ab at the levels normally found in Agrisure Duracade™ corn.

If the Positive Control Ratio of a sample is greater than or equal to 1.0, the sample does contain eCry3.1Ab.

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 1. 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.
- 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 test kit has been approved by Syngenta for detection of the eCry3.1Ab protein in Agrisure Duracade™ traited corn.