ENVIROLOGIX™

# QualiPlate™ Kit for Enogen™ Corn

***Highlights:***

- **High Sensitivity Protocol**
  *Can detect as little as 1% Enogen™ corn in a 2 hour assay*

***Contents of Kit:***

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

*Prepare Wash and Extraction buffers*

*Catalog Number AP 070*

## Intended Use

The EnviroLogix QualiPlate Kit for Enogen™ Corn is designed for the qualitative laboratory detection of the amylase protein in Enogen™ corn in single seed and bulk grain samples. The Kit is not designed to provide quantitative results. Enogen™ corn derived from transformation Event 3272 contains the transgene *amy797E*, which encodes a synthetic thermostable alpha-amylase protein. The assay will detect the amylase protein found in 1% Enogen™ corn (1 positive grain in a 100 negative grain sample) and requires two hours to run.

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

After a simple wash step, the results of the assay are visualized with a color development step; color development is proportional to amylase 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 Buffers
- Tween-20 (Sigma P-1379, or equivalent), sodium tetraborate, and sodium hydroxide, for preparation of Extraction Buffer
- equipment for pulverizing single seed samples
- Waring laboratory blender (model 31BL91 or equivalent), glass jar adapter (Eberbach # E8495) and appropriate size glass Mason jars for bulk grain samples
- 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 with 450 nm filter
- 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)

***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-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.

**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 the Borax in 4.9 liters of distilled or 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.

# Sample Preparation

Note: It is recommended that the user prepare known negative and positive seed samples to be run in every assay as controls, in addition to the kit Positive Control Solution.

## Bulk Grain:

This protocol requires that a small sample (20 to 40 grams and not exceeding 100 kernels) be analyzed. It is essential that this sample be well mixed and representative of the larger bulk. The test will detect 1% Enogen™ corn (one positive grain in a sample of 100 grains).

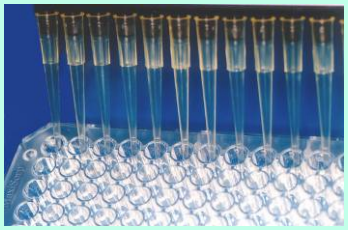
**NOTE:** Thorough mixing of the bulk grain sample and determination of an appropriate sampling plan are critical to the results of this testing, and are the responsibility of the user of this test kit. The USDA/GIPSA has prepared several guidance documents to address the issues involved in obtaining 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.

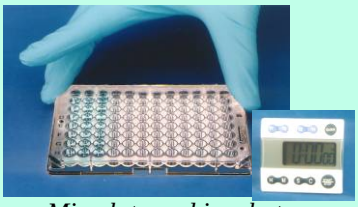
It is the responsibility of the user to ensure proper sampling and thorough mixing prior to analysis. Once representative samples have been obtained from the truck or container, they can be reduced in size using a splitter and uniformly ground and mixed. **The finer the grind, the faster and more efficient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

*Extract grain sample*

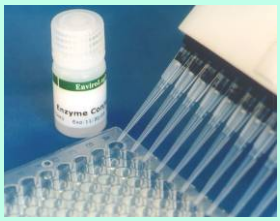
*Centrifuge to clarify grain extract*



*Add Extraction Buffer Blank, Positive Control, and sample extracts to the plate*



*Mix plate and incubate*



*Add Enzyme Conjugate*

1. For 100-grain samples, grind in a 4 oz. "Mason" jar on a blender at high speed for 30 seconds. Shake jar to mix. 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.
3. 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 5 - 10 minutes to extract. Mix again.
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.

### Single 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.

   **CAUTION:** Amylase protein is expressed at high concentrations in corn seed, so there is serious potential for cross-contamination between samples during seed crushing. Cleaning the cutting/crushing surfaces with an alcohol-soaked pad between samples, as well as placing a damp towel over crushed seeds to reduce static charge, are steps recommended to avoid this.
2. Add 1 mL of Extraction Buffer to each crushed seed. Mix for at least 30 seconds. Allow to extract for 15 minutes at ambient temperature. If an orbital shaker is available, shake plate slowly at 75 rpm. Mix sample again for at least 30 seconds. Allow extracts to settle completely. Dispensing particles into the test plate can cause false positive results.

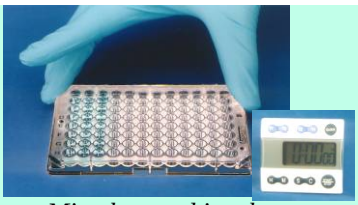
# How to Run the Assay

- 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, clarified sample extracts, and pipettes so that Step 1 can be performed in 10 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).

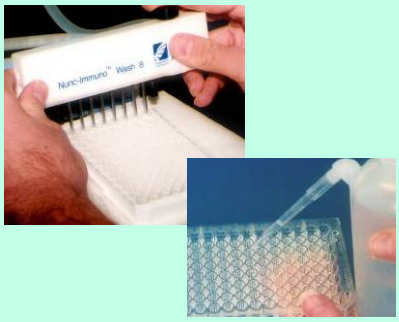
**Procedure**

1. Add **50 µL** of **Extraction Buffer Blank (BL)**, **50 µL** of **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 (Fig. 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!



*Mix plate and incubate*



*Strip Plate Wash or Bottle Wash*

*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*

3. Cover the wells with tape or Parafilm to prevent evaporation and incubate at ambient temperature for **30 minutes**.
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 Enogen 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**.
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**.

**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.

# 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%:

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

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".


*Source: NREL*


*Source: USDA ERS*

## Interpret the Qualitative Results

**Bulk grain samples**

If the Positive Control Ratio calculated for a sample is less than 0.5, the bulk grain contains less than 1% Enogen™ corn.

If the Positive Control Ratio of a sample is greater than or equal to 0.5, the sample contains 1% or greater Enogen™ corn.

**NOTE:** This test is to be used qualitatively only, with yes/no results at 1% Event 3272 corn. For information on testing at different cutoff levels, please contact EnviroLogix' Technical Service. Do not attempt to use the Kit in a quantitative manner.

**Single seed samples:**

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

If the Positive Control Ratio of a sample is greater than or equal to 0.5, the sample contains Enogen™ corn.

Seed samples are by their nature either 100% positive or 100% negative. Any low level positive results from single seed samples must be due to either some form of sample cross-contamination (stray particles or dust, etc.) or can be caused by transfer of particulate matter from 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 for testing sweet corn samples.
- Do not use kit components after the expiration date.
- Do not use reagents from one Plate Kit with reagents from a different Kit.

*Source: USDA EERE*

*Source: NREL*

- 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:**

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## LIMITED WARRANTY

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This test kit has been validated and approved by Syngenta for detection of the amylase protein in Enogen™ corn.