QualiPlate™ Kit for LibertyLink® PAT/*bar*

Catalog Number AP 013

Highlights:

- *High Sensitivity Protocol detects the PAT enzyme from the* bar *gene (PAT/*bar*) found in 0.1% StarLink corn*
- *Rapid Protocol screens individual seeds or leaf samples for the presence of PAT/*bar

Contents of Kit:

- *1 antibody-coated 96-well plate*
- *PAT/*bar *Enzyme Conjugate*
- *1 packet of Buffer Salts*
- *Substrate*
- *Stop Solution*

Intended Use

The QualiPlate Kit for LibertyLink PAT/*bar* is designed for the qualitative laboratory detection of phosphinothricin acetyl transferase enzyme (PAT) coded for by the bar gene in grain, leaf, or seed. Two assay protocols are presented: The High Sensitivity Protocol will detect the PAT enzyme from the *bar* gene (PAT/*bar*) found in 0.1% StarLink® corn (by weight) and requires 2.5 hours to run. The Rapid Protocol (one hour total) is intended for use in screening individual seeds or leaf samples for the presence of PAT/*bar*. LibertyLink PAT from the *pat* gene, at concentrations present in T25 corn, is not detected in either format.

How the Test Works

This QualiPlate Kit is a "sandwich" Enzyme-Linked ImmunoSorbent Assay (ELISA). In the test, **corn** or **cotton** sample extracts are added to test wells coated with antibodies raised against PAT from the *bar* gene. Any residues present in the sample extract bind to the antibodies, and are then detected by addition of enzyme (horseradish peroxidase)-labeled PAT/*bar* antibody.

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

Lighter color = Lower concentration
Darker color = Higher concentration

How the Kit Performs

The High Sensitivity Protocol will detect 0.1% StarLink corn grain. Samples are judged to contain more or less PAT/*bar* enzyme than 0.1% StarLink standard by comparing sample absorbances to the absorbance of a 0.1% StarLink sample extract (prepared by the user).

The Rapid Protocol is a strictly qualitative test, in which seed and leaf samples are screened for the presence or absence of the PAT/*bar* enzyme at the levels commonly seen in commercially modified crops. It is recommended that the user prepare extracts from known conventional and known PAT/*bar* expressing plant tissues to run in each assay as negative and positive controls.

Materials not Provided

- distilled or deionized water for preparing Wash/Extraction Buffer
- glass bottles or flask plus graduated cylinder with 1 liter capacity for preparation and storage of Wash/Extraction Buffer
- test or centrifuge tubes for extraction of grain
- Tissue Extraction Kit: snap-cap tubes and pestles for extraction of leaf samples (EnviroLogix Cat. No. ACC 002, 100/package)
- 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®

Prepare Wash/Extraction Buffer

Preparation of Solutions

Wash/Extraction 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 extraction buffer is needed, order item # P-3563 from Sigma Chemical Co. (St. Louis, MO), or prepare the equivalent.

- timer
- 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)

Sample Preparation

Note: PAT/*bar* protein is not stable in solution. Extraction should be performed in 30 minutes or less. Do not extract overnight.

High Sensitivity Protocol for Ground Grain/Seed

This protocol requires that a small sample be analyzed. It is essential that this sample be well mixed and representative of the larger bulk. The test will detect 0.1% StarLink corn, containing PAT/*bar* (one kernel in a sample of 1000 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 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 on page 3.

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. Weigh 5 grams of ground corn sample into a 15 mL capacity vial or tube.
2. Add 12.5 mL of Wash/Extraction Buffer to each 5 gram sample. For all other grain sample sizes, add Wash/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 to extract.
3. The extracted samples/controls must be clarified by centrifuging the extract 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.

Rapid Protocol for Screening 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 crusher/48-well plate combination may be used (for example Hypure #HSC-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.
2. Add 0.5 mL of Wash/Extraction Buffer to each crushed corn seed, or 1 mL to each crushed cotton seed. Mix for at least 30 seconds, then allow particles to settle.

Reference 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.

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, Norton, OH) in a 96-well plate (Costar #3370, Corning Life Sciences, Acton, MA, or equivalent).
2. Add 0.25 mL of Wash/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.

NOTE: It is recommended that the kit user extract known conventional and known PAT/*bar*-containing samples of the matrix being tested, and run these as Negative and Positive Controls (NC and PC) in each assay.

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 Control and 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.
- Use a disposable-tip air-displacement pipette and a clean pipette tip to add each Calibrator and sample extract to the wells. Conjugate, Substrate, and Stop Solution may be added in the same manner; alternatively, use a repeating pipette with a disposable tip on the end of the Combitip for these three reagents.
- Use the well identification markings on the plate frame to guide you when adding the samples and reagents. In a qualitative assay, the Blank (BL), Negative Control (NC), and the Positive Control (PC) and 90 sample extracts (S) may be run in single wells on one plate. (See the Qualitative Assay Example Plate Layout - Figure 1A).

HIGH SENSITIVITY PROTOCOL: The High Sensitivity Protocol will detect 0.1% StarLink corn (by weight) in ground grain/seed, and requires 2.5 hours of total assay incubation time.

Procedure

1. Add 50 µL of PAT/bar-enzyme Conjugate to each well, followed immediately by 50 µL of Wash/Extraction Buffer Blank (BL), 50 µL of the centrifuged 0.1% StarLink ground corn and Negative ground corn extracts (PC and NC), and 50 µL of each centrifuged 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 2 hours. 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

Allow all reagents to reach room temperature before beginning

Leaf punch

Extract sample

Centrifuge to clarify sample extract
(High Sensitivity protocol only)

completely with Wash/Extraction 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.
7. Caution: Stop Solution is 1.0N Hydrochloric acid. Handle carefully.
8. 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 less sensitive (able to detect 1% StarLink corn) but only requires one hour of total assay incubation time.

Procedure

1. Add 50 µL of PAT/bar-enzyme Conjugate to each well, followed immediately by 50 µL of Wash/Extraction Buffer Blank (BL), 50 µL of the user-prepared Positive and Negative Control extracts (PC and NC), and 50 µL of each sample extract (S) to their respective wells, as shown in the Example Plate Layout (Figure 1A).

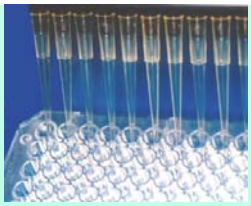
NOTE: In order to minimize setup time it is recommended that a multi-channel pipette be used in steps 1, 5, and 7.

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 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/Extraction 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.

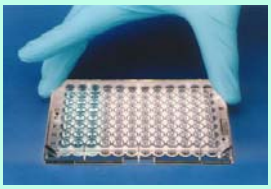
Caution: Stop Solution is 1.0N Hydrochloric acid. Handle carefully.

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.



Add Conjugate, controls and samples



Mix plate

Incubate

Wash plate

Read plates in a Plate Reader 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 Wash/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.

Note: The mean OD of the BLANK wells should not exceed 0.2.

Interpret the Results

For leaf and seed screening, compare the OD's of sample extracts to OD's of extracts from known leaf or seed samples (conventional and PAT/*bar*-expressing varieties). Note: very low positive results in a seed-screening assay may be due to cross-contamination during crushing. Truly positive seeds will result in OD readings typically greater than 0.500.

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 Plate Kit components at 4°C to 8°C (39°F to 46°F) when not in use.
- Do not expose Plate 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 test wells.
- The assay has been optimized to be used 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 extreme caution to prevent sample-to-sample cross-contamination with samples, fluids, or disposables.

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