Roundup Ready ® CP4 EPSPS

PathoScreen kit for the detection of CP4 EPSPS protein
Peroxidase Label
Catalog number: PSP 74000

List of contents

Lot Number	Item	96 wells	288 wells	480 wells	4800 wells
_____	Antibody-coated 96-well microtiter plates	1 plate	3 plates	5 plates	50 plates
_____	Peroxidase enzyme conjugate	0.150 ml	0.350 ml	0.550 ml	5.5 ml
_____	TMB substrate	25 ml	40 ml	60 ml	550 ml
_____	Positive control	1 vial	1 vial	1 vial	5 vials
_____	Negative control	Sold separately	Sold separately	Sold separately	Sold separately
_____	Nonfat dried milk	5 g	2 X 5 g	3 X 5 g	3 X 25 g
	The above items should be stored at 4° C.				
_____	Tween-20	15 ml	30 ml	30 ml	30 ml
_____	PBST buffer	3 pouches	5 pouches	7 pouches	3 X 110 g
	The above items should be stored at room temperature.				

Materials required but not provided

Airtight plastic container (for incubation of microplate)
Paper towels
Distilled water
Micropipettes
Sterile micropipette tips
Graduated cylinder
Balance 1-500 gms
Grinding equipment:
- Blender (Osterizer® Sunbeam Corporation, Model No.,1-800-597-5978)
- Blender jars 1000ml, Nalgene (“Mason” type, Fisher Scientific, Catalog No. 2115-1000)
- Blender blade pack assembly (Factory Services Inc., Catalog No. OC-DUX, 1-800-237-8699)
- Threaded bottom cap (Factory Services Inc., Catalog No. OJN)
- Plastic extraction bottles 1000ml
- Sample tube rack
- Microfuge tubes
- Sample extraction bags (Agdia Catalog No. ACC 00930)

Storage

Store all kit components at the recommended temperature to assure their full shelf life. Each ELISA plate pouch contains desiccant packet. Keep the plate sealed in the pouch with the desiccant and store in the refrigerator. Make sure the foil pouch remains intact with no holes or tears.

Intended use

This kit is intended for seed quality purposes to determine the presence of the CP4 EPSPS protein in seed and leaves of corn, cotton, soybean and other crops. The expression of CP4 EPSPS transgenic protein in plants results in Roundup® herbicide resistance. Roundup® is a broad spectrum herbicide used to control weeds.

Currently this test is approved for use in cotton and corn. Using this test system, you can reliably detect 1 transgenic CP4 EPSPS seed in 1000 seeds (0.1%) and 1 transgenic CP4 EPSPS leaf in 100 leaves (1%) of cotton and corn.

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Test principle

This test is a double-antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA). The anti-CP4 EPSPS antibody is coated to the testwells of a microplate. Extracted samples are added to the antibody coated testwells. If CP4 EPSPS protein is present in the sample, the coated antibody on the microplate captures the protein. The plate is then washed to remove all unbound material. An enzyme conjugate, consisting of anti-CP4 EPSPS antibody chemically linked to an enzyme, is added to detect any captured protein. The antibody portion of the conjugate will bind to captured protein on the plate. The plate is washed again to remove any unbound conjugated antibody. Finally, a substrate is added to the plate. If any enzyme is present a color will be produced signifying the presence of CP4 EPSPS protein. The color reaction is usually read with a spectrophotometer, or may be scored visually.

Precautions

Prevent direct skin and eye contact with, or ingestion of, kit components. Obtain medical attention in case of accidental ingestion of kit components. Always wash hands thoroughly after using the kit. Please read these instructions carefully before performing the test.

Limitations

Prepare only the amount of 1X buffers needed for the day. Dilute only the amount of enzyme conjugate necessary at the time of each test run. Do not store 1X buffers.

Preparing for the test

Familiarize yourself with the kit components. Check that all components are present in the kit (refer to content list on page 1).

Prepare buffers

Prepare PBST wash buffer, MEB sample extract buffer, and ECM conjugate buffer according to the instructions on the back page. Prepare just enough buffers for one day.

Prepare testwells

If less than a full 96-well plate is used, remove any unused strips and seal them in the foil pouch with the desiccant and store at 4C. Using a permanent marker, number the strips in case a strip becomes separated from the frame.

Fold a paper towel to fit inside an airtight plastic box. Lay the folded paper towel in the bottom of the box, and then put in just enough water to wet the paper towel. Keeping the plate in this humid box during incubation steps will help prevent samples from evaporating.

Make a copy of the loading diagram and record the locations of your samples and controls.

Prepare samples

Leaves, seedlings, or seeds must be ground and diluted in MEB sample extraction buffer. For best results, samples should be diluted in MEB buffer according to the ratios listed in the table below. After samples have been ground in buffer, let the extract sit for at least 30 seconds.

Crop	LEAF to MEB buffer ratio (weight/volume)	SEED to MEB buffer ratio (weight/volume)
Corn	1:10	1:2
Cotton	1:20	1:20
Soybean	1:20	1:20

Sample grinding in Agdia sample extraction bags

Leaf extraction

For leaf samples use Agdia's disposable sample extraction bags, a clean mortar and pestle, or any other grinding device to help extract samples.

Individual leaves

A simple method for grinding a single leaf sample is by using Agdia's special sample extraction bags (Catalog No. ACC 00930). Use only one sample per bag and be sure to label each bag. Add the appropriate volume of buffer to an empty bag. A recommended 1:20 dilution, would require a 0.15 g leaf sample and 3 ml of buffer. Place the sample between the mesh linings of the pouch. Rub the pouch with a pen to completely crush the sample and to mix the contents uniformly.

Cork borer and leaf disc

Multiple leaves

For composite leaf samples (up to 100 leaves), taking a representative leaf disc or leaf punch is recommended. Stack the leaves on a clean surface and using a No. 2 cork borer (Fisher Scientific Catalog No. 07-845C) punch through the leaves to produce 100 leaf discs. Dislodge the discs from the cork borer with a clean metal wire, weigh and transfer the discs into Agdia's disposable sample extraction bags and extract in buffer according to the recommended ratios. The weight of the discs varies with the growing conditions, age, and variety of the plant. Determine the average weight of the leaf discs and add the appropriate volume of buffer.

Crop	LEAF to MEB buffer ratio (weight/volume)	Approximate weight of 100 discs	Volume of MEB Buffer
Corn	1:10	0.2 grams	2 ml
Cotton	1:20	0.2 grams	4 ml
Soybean	1:20	0.1 grams	2 ml

Seed extraction

Single seeds

Single seeds can be crushed with a seed crusher or hammer. Determine the average weight of the seed and add the appropriate volume of MEB buffer. Let the extract sit for at least 30 seconds before testing with the ELISA.

Note:
It is very important to clean all the grinding equipment between the samples. Wash the equipment with detergent, rinse well, and completely dry with paper towel. Wiping the grinding device and work area with 20% methanol is also recommended between samples

Multiple seeds

For seed samples to be tested at 0.1% sensitivity level, it is recommended to use Osterizer® blender with "Mason" type jars to accommodate 1000 seeds. However, depending on the sample size other devices like coffee grinders, ball mill, other blenders, or seed crusher may be used to grind the samples. The guidelines provided are optimized for Osterizer® blender with "Mason" type jars.

Put the seed sample in a dry "Mason" jar and assemble the blade attachment. Grind the seed at high speed for about 45-60 seconds or until all the seeds are ground to a powder. Remove the jar from the blender and tap to collect all the powder. Shake the jar to mix and check for any unground seed.

Transfer the ground powder to a container and weigh the specified amount (sub sample) from the following table to a 500 ml disposable bottle. Add the buffer at the specified ratio, close the lid and shake the bottle for 10-15 seconds. Let the extract sit for at least 30 seconds before testing with the ELISA. Use only the supernatant (top layer of liquid) for testing.

Crop	SEED to MEB buffer ratio (weight/volume)	Sub sample weight	Volume of MEB Buffer
Corn	1:2	50 grams	100 ml
Cotton	1:20	20 grams	400 ml
Soybean	1:20	20 grams	400 ml

Prepare controls

Reconstitute the bottle of lyophilized positive control and negative control with 2.0 ml MEB sample extract buffer. The concentration of the reconstituted control is about 1% CP4 EPSPS seed.

Make control aliquots

After preparing the positive and negative control, divide them into aliquots, each sufficient for one use. Dispense aliquots into tubes that can be securely capped. Dispense 120 µl if one well is used for positive control or 220 µl if two wells are used per test. Each aliquot should be sufficient for the tests to be run plus a small additional volume to assure easy dispensing.

Note:
Do not refreeze controls

Control aliquots must be stored frozen (-20C freezer or household freezer). Do not thaw until just before use. At the time of each test run, remove from storage only the aliquots that will be used. Allow the tubes to thaw, then mix the contents thoroughly. At the time you add sample extracts to testwells, add the same volume of negative and positive control to the appropriate control wells.

Test Procedure

1. Dispense samples/controls	Following your loading diagram, dispense 100 µl of each prepared sample into the appropriate testwells of the ELISA plate. Add 100 µl of positive and negative control into the appropriate testwells.
2. Incubate plate	Set the plate inside the humid box and incubate for 1 hour at room temperature or overnight in the refrigerator (4˚C).
3. Wash plate	When the incubation with the sample is complete, wash the plate. While squeezing the long sides of the frame to hold the strips in place, use a quick flipping motion to empty the contents of the wells into a sink or waste container. Fill all the wells to overflowing with 1X PBST wash buffer, and then quickly empty them again. Repeat 6-7 times. If using an automatic plate washer, please be sure that the machine is set to properly wash the flat bottomed plates of this kit.
4. Soak plate	Fill each well with 1X PBST wash buffer and allow to sit for 3 minutes. Empty the wells with a quick flipping motion. Then hold the frame upside down and tap firmly on a folded paper towel to remove the remaining drops of buffer from the wells.
5. Dilute enzyme conjugate **Note:** **Always prepare enzyme conjugate within 10 minutes before use.**	The peroxidase enzyme conjugate is concentrated and must be diluted with ECM buffer before use. The total volume of ECM buffer required depends on the number of testwells used. 100 µl will be needed for each testwell, plus a small additional volume for liquid handling. Dilute only the amount of peroxidase enzyme conjugate needed for your test run. For example, if the dilution given on the label is 1:100, and you are preparing 10 ml of working enzyme conjugate, you should first measure 10 ml of ECM conjugate buffer, then add 100 µl of peroxidase enzyme conjugate. Mix the enzyme conjugate thoroughly.
6. Add enzyme conjugate	Dispense 100 µl of prepared enzyme conjugate per well.
7. Incubate plate	Set the plate inside the humid box and incubate one hour at room temperature.
8. Wash plate	When the second incubation is complete, wash the plate. While squeezing the long sides of the frame to hold the strips in place, use a quick flipping motion to empty the contents of the wells into a sink or waste container. Fill all the wells to overflowing with 1X PBST wash buffer, and then quickly empty them again. Repeat 6-7 times. If using an automatic plate washer please be sure that the machine is set to properly wash flat bottomed plates.

9. Soak plate	Fill each well with 1X PBST wash buffer and allow to incubate for 3 minutes. Remove the plate from the humid box. Empty the wells with a quick flipping motion. Then hold the frame upside down and tap firmly on a folded paper towel to remove the remaining drops of buffer from the wells.
10. Add substrate solution	Add 100 µl of the TMB substrate solution into each well of the plate.
11. Incubate plate	Incubate the plate for 20 minutes in a humid box.
12. Measure color	Measure the optical density of the testwells on a plate reader at 650 nm or visually. Wells in which a blue color develops indicate positive results. Wells that remain clear or very light blue indicate negative results. If either control well does not show the appropriate color, disregard results.

Interpreting results

Data gathered from validation tests performed by several operators in different labs on a variety of cotton lines was used to determine the following positive and negative cutoff O.D. values.

Optical Density	Test Result
Greater than 0.3	Positive
Less than 0.1	Negative
Between 0.1 – 0.3	Indeterminate result, requires more analysis

To interpret samples in the indeterminate OD range and to perform a more discriminating analysis of your data, perform the following analysis:

Sort all of the data from a single microplate into a series of increasing OD values. Plot the OD values either as a histogram or x, y scatter plot with no x axis input. From the plots determine visually the OD value of the high end of the apparent negative sample population. Compute the average [Avg] and standard deviation [SD] for the apparent negative population from the sorted data.

Then, positive sample OD (ODpos) should be > [Avg] + 4 X [SD].

After computing an ODpos threshold, check the ODpos determined above for consistency with the generated histogram and with known samples.

Test Performance

This test shows no cross-reaction with Bt-Cry1Ab, Bt-Cry1Ac, Bt-Cry1C, Bt-Cry1F, Bt-Cry2A, Bt-Cry3A, or Bt-Cry9C.

A total of 1750 transgenic CP4 EPSPS seed (0.1%) and leaf samples (1%) and 1750 conventional CP4 EPSPS seed and leaf samples were tested by several operators using 3 separate plate and antibody conjugate lots of product. The results showed no false positives or false negatives.

Technical Service

If you have any questions about using this kit, please contact Agdia, Inc. Monday - Friday by phone at 1-800-622-4342, 574-264-2014, or by e-mail at info@agdia.com.

Preparing Buffers

PBST wash buffer

Dilute the 20X PBST wash buffer to working (1X) dilution with distilled water.

or

Prepare 1X buffer by dissolving PBST buffer powder in water.

Buffer powder 10.0 g
Distilled water 1.0 liter

MEB sample extract buffer

Calculate the volume of MEB buffer needed based on the number of samples and controls and the amount required to extract and dilute each sample.

Add to 100 ml of 1X PBST:

Nonfat dried milk 0.4 g
Tween 20 0.5 g

Stir slowly at room temperature until dissolved.

ECM conjugate buffer

The volume of ECM buffer required depends on the number of testwells used, with 100 µl needed per testwell, plus a small additional volume for easy dispensing.

Add to 25 ml of 1X PBST:

Nonfat dried milk 0.1 g.

Stir slowly at room temperature until dissolved.

Date _____ Test _____

Test performed by _____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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