Reagent Set

DAS ELISA, Alkaline phosphatase label for BBWV-1,2 and WMV
Broad bean wilt virus-1,2 and Watermelon mosaic virus
Catalog numbers: SRA 46202, SRA 54001

List of contents

Lot number	Item	96 wells	500 wells	1000 wells	5000 wells
_____	Capture antibody	0.150 ml	0.275 ml	0.525 ml	2.525 ml
_____	Alkaline phosphatase enzyme conjugate	0.150 ml	0.275 ml	0.525 ml	2.525 ml
	The above items should be stored at 4° C.				
_____	Tween 20	15 ml	30 ml	2 X 30 ml	250 ml
_____	General extract buffer 3 (GEB3) powder	24 g	48 g	2 X 48 g	3 X 176 g
_____	96-well microtiter plates, strip or solid	1 strip	5 solid	10 solid	50 solid
	The above items can be stored at room temperature.				

Materials required, but not provided

- Carbonate Coating Buffer (formulation on page 6)
- PBST Wash Buffer (formulation on page 6)
- ECI Buffer (formulation on page 6)
- PNP Substrate Buffer (formulation on page 6)
- PNP Substrate Tablets (ACC 00404)
- Buffer packs containing the above required items can be purchased from Agdia (ACC 00555)
- Distilled or purified water
- Paper towels
- Micropipette
- Micropipette tips
- Sample grinding device such as:
    - Agdia sample mesh bag (ACC 00930)
    - Agdia tissue homogenizer (ACC 00900)
    - Mortar and pestle
- Airtight container for incubations

Limitations

Expiration: This test should be used within 1 year of purchase.

Storage: Test results may be weak or the test may fail if storage instructions are not followed properly.

Dilutions: Read all labels carefully prior to preparing solutions to assure proper antibody concentrations. All antibody dilutions have been optimized for the greatest possible sensitivity and specificity based on available isolates and hosts. Using dilutions other than those listed can lead to potential false positives or false negatives.

Sample Dilution: ELISA performance is very dependent on the proper sample (tissue weight in g: buffer volume in ml) dilution.

Buffers: Do not store 1X buffers from day to day. Buffers should be warmed to room temperature prior to use. General extract buffer 3 (GEB3) and ECI buffer must be used to give optimal results. Not using these buffers can result in higher background and false positive reactions.

Tissue reaction: Plant tissue interactions with ELISA tests vary greatly between plant species and even varieties. Some healthy tissues can cause a tissue reaction resulting in an elevated or high O.D. value. A known healthy sample of the same kind of plant should be used with this test to rule out any tissue reactions.

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Precautions

Prevent direct skin and eye contact with, or ingestion of, product components. Obtain medical attention in case of accidental ingestion of kit components. Always wash hands thoroughly after using this product.

Technical Service

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

Test Principle

This test system uses a direct, double antibody sandwich protocol known as DAS ELISA. Specific antibodies are coated to the testwells of a microplate. Next, samples are added to the microplate. If the target analyte is present in the sample, it is bound by the antibodies and captured on the microplate during the incubation period. After incubation, the plate is washed to remove unbound sample. The alkaline phosphatase enzyme conjugate is added and binds to any captured target analyte. After incubation the plate is washed to remove any unbound conjugate. This final binding creates a sandwich of the target analyte between the two specific antibodies. PNP substrate (pNPP) is added to the microplate. If the alkaline phosphatase conjugate is present a yellow color will be produced indicating the presence of the target analyte. Buffer wells and negatives should remain colorless. The color reactions can be observed visually or measured with a spectrophotometer.

Coat testwells of ELISA plate

1. Prepare humid box

Prepare a humid box by lining an airtight container with a wet paper towel. Keeping testwells in a humid box during incubation will help prevent evaporation.

2. Prepare capture antibody

Note: 96 testwell reagents have a different dilution ratio than larger reagent sets. Please read label on capture antibody bottle for appropriate dilution ratio.

Note: All antibodies and enzyme conjugates should be prepared in a container made of a material such as polyethylene or glass that does not readily bind antibodies. Do not use polystyrene.

The capture antibody is provided as a concentrated solution and must be diluted with carbonate coating buffer before use. The recommended antibody to buffer ratio is given on the label.

Prepare the volume of carbonate coating buffer needed for the test. You will need 100 µl of carbonate coating buffer for each test well you are using. A full plate will require about 10 ml. Then, add the appropriate volume of concentrated capture antibody to the carbonate coating buffer at the dilution on the label.

Example 1: If the dilution given on the bottle of concentrated capture antibody is 1:200, and you are preparing 10 ml of capture antibody solution, you should mix 10 ml of carbonate coating buffer with 50 µl of the concentrated capture antibody. Mix the prepared capture antibody solution thoroughly and use immediately.

Example 2: If the dilution given on the bottle of concentrated capture antibody is 1:100, and you are preparing 10 ml of capture antibody solution, you should mix 10 ml of carbonate coating buffer with 100 µl of the concentrated capture antibody. Mix the prepared capture antibody solution thoroughly and use immediately.

3. Coat plate

Pipette 100 µl of the prepared capture antibody into each well.

4. Incubate plate

Incubate the plate in a humid box for 4 hours at room temperature or overnight in the refrigerator (4°C). Do not store coated plates longer than 24 hours. If long term storage is desired, contact Agdia about postcoat buffers.

5. Wash plate

Empty the wells into a sink or waste container. Fill the wells completely with 1X PBST, and then quickly empty them again. Repeat 2 times.

Hold the plate upside down and tap firmly on a folded paper towel to remove excess liquid.

Note: Use freshly coated plates immediately.

General extract buffer (GEB3)

GEB3 is used to dilute and extract samples. Refer to the sample extraction guidelines below for appropriate sample to buffer ratios.

Buffer powder	48 g
Distilled or Purified water	1000 ml (1 liter)
Tween 20	20 ml or 20 g

To make 1000 ml of GEB3 sample extract buffer, first make a smooth slurry by adding a small amount of water to 48 g of powder. Then while mixing, add Tween-20 to the slurry. Add water to bring the final volume to 1 liter. Stir for 30 minutes.

Agdia recommends preparing only as much buffer as is needed for one day. Those who store buffers outside of this recommendation are advised to add sodium azide (Sigma S-2002) to 1X liquid buffers at a rate of 0.2 g per liter (0.02%).

Grind and dilute samples

When possible, select samples showing symptoms. Leaf and stem tissue is often used in ELISA testing. In some cases, composites of up to ten leaves per testwell can be used to make testing more economical. However, too many plant samples per well can reduce the sensitivity of the test.

Use Agdia's general extract buffer 3 (GEB3) to grind and dilute samples.

Grind plant tissue in sample extraction buffer at a 1:10 ratio (tissue weight in g: buffer volume in ml). You will need 100 µl of diluted sample extraction per testwell, plus an additional amount to assure easy dispensing. You can use Agdia's sample mesh bags (ACC 00930), Agdia's tissue homogenizer (ACC 00900), a mortar and pestle, or other grinding devices to grind samples.

Test Procedure

1. Dispense samples

Following your loading diagram, dispense 100 µl of prepared sample into sample wells. Dispense 100 µl of positive control into positive control wells, and dispense 100 µl of extraction buffer into buffer wells.

2. Incubate plate

Set the plate inside the humid box and incubate for 2 hours at room temperature or overnight in the refrigerator (4°C).

3. Prepare enzyme conjugate

Note: 96 testwell reagents have a different dilution ratio than larger reagent sets. Please read label on enzyme conjugate bottle for appropriate dilution ratio.

Note: Always prepare enzyme conjugate within 10 minutes before use.

The bottle of enzyme conjugate is supplied as a concentrate and must be diluted with 1X ECI buffer before use. The recommended conjugate to diluent ratio is given on the label. Dispense the appropriate volume of prepared 1X ECI buffer into a dedicated container. You will need 100 µl of diluent for each testwell you are using. Then, add the enzyme conjugate according to the dilution given on the labels.

Example 1: If the dilution given on bottle of concentrated enzyme conjugate is 1:200, and you are preparing 10 ml of enzyme conjugate solution, you should first dispense 10 ml of 1X ECI buffer. Then, add 50 µl of the concentrated enzyme conjugate to the 1X ECI buffer.

Example 2: If the dilution given on bottle of concentrated enzyme conjugate is 1:100, and you are preparing 10 ml of enzyme conjugate solution, you should first dispense 10 ml 1X ECI buffer. Then, add 100 µl of the concentrated enzyme conjugate to the 1X ECI buffer.

After adding the enzyme conjugate, mix thoroughly. It is important to mix the enzyme conjugate solution well.

4. Wash plate

When the sample incubation is complete, wash the plate. Use a quick flipping motion to dump the wells into a sink or waste container without mixing the contents.

Fill all the wells completely with 1X PBST, and then quickly empty them again. Repeat 7 times.

After washing, hold the frame upside down and tap firmly on a folded paper towel to remove all droplets of wash buffer.

Inspect the testwells. All wells should be free of plant tissue. If tissue is present repeat the wash step and tap firmly on a paper towel.

5. Add enzyme conjugate

Dispense 100 µl of prepared enzyme conjugate per well.

6. Incubate plate

Incubate the plate in the humid box for 2 hours at room temperature.

7. Prepare PNP solution

Each PNP tablet (ACC 00404) will make 5 ml of PNP solution, at a concentration of 1 mg/ml, about enough for five 8-well strips.

About 15 minutes before the end of the above incubation step, measure 5 ml of room temperature 1X PNP buffer for each tablet you will be using. Then, without touching the tablets, add the PNP tablets to the buffer.

Note: Do not touch the PNP tablets or expose the PNP solution to strong light. Light or contamination could cause background color in negative wells.

8. Wash plate

As before, wash the plate 8 times with 1X PBST.

Inspect the wells looking for the presence of air bubbles. Tap firmly on the paper towel to remove remaining wash buffer and any air bubbles. If air bubbles are still present they may be broken with a clean pipette tip.

9. Add PNP substrate

Dispense 100 µl of PNP substrate into each testwell.

10. Incubate plate

Incubate the plate for 60 minutes. Plates should be protected from direct or intense light.

11. Evaluate results

Examine the wells by eye, or measure on a plate reader at 405 nm. Air bubbles which are present at the time of reading can alter results, if in the light path. Agdia recommends that bubbles be eliminated prior to reading.

Wells in which color develops indicate positive results. Wells in which there is no significant color development indicate negative results. Test results are valid only if positive control wells give a positive result and buffer wells remain colorless.

Results may be interpreted after more than 60 minutes of incubation as long as negative wells remain virtually clear.

Interpreting sample results

Setting one general positive-negative ELISA threshold that applies to all users is generally not possible. The samples, environment and test goals may be different for each user. Interpretation of results can also be done in several ways.

Agdia tries to design most ELISA so that when all negative samples are below 0.1 O.D., then samples greater than 0.2 O.D. are positive. With this simple guideline, samples that give ELISA values between 0.1 and 0.2 O.D. are borderline and should be repeated or handled as borderline samples. Users able to demonstrate consistent performance with this ELISA may find it possible to select and validate a value between 0.1 and 0.2 for the positive threshold.

We recommend referring to the publication below for guidance in setting positive-negative ELISA thresholds.

Sutula, Chester L., Gillett, Jerri M., Morrissey, Susan M., and Ramsdell, Donald C. Interpreting ELISA data and establishing the positive-negative threshold. Plant Disease. 70:722-726.

Buffer Formulations

Carbonate Coating buffer (1X)

Dissolve in distilled water to 1000 ml:

Sodium carbonate (anhydrous)	1.59 g
Sodium bicarbonate	2.93 g
Sodium azide	0.2 g

Adjust pH to 9.6. Store at 4° C.

PBST Buffer (Wash Buffer) (1X)

Dissolve in distilled water to 1000 ml:

Sodium chloride	8.0 g
Sodium phosphate, dibasic (anhydrous)	1.15 g
Potassium phosphate, monobasic (anhydrous)	0.2 g
Potassium chloride	0.2 g
Tween-20	0.5 g

Adjust pH to 7.4

ECI Buffer (1X)

Add to 1000 ml 1X PBST:

Bovine serum albumin (BSA)	2.0 g
Polyvinylpyrrolidone (PVP) MW 24-40,000	20.0 g
Sodium azide	0.2 g

Adjust pH to 7.4. Store at 4° C.

PNP Buffer (1X)

Dissolve in 800 ml distilled water:

Magnesium chloride hexahydrate	0.1 g
Sodium azide	0.2 g
Diethanolamine	97.0 ml

Adjust pH to 9.8 with hydrochloric acid. Adjust final volume to 1000 ml with distilled water. Store at 4° C.

Date _____ Test _____

Test performed by _____

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B												
C												
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