PathoScreen® Kit
DAS ELISA, alkaline phosphatase label
Catalog numbers: PSA 22001, PSA 23203 and PSA 98700
ToRSV, ArMV and PDV

List of contents

<table>
<thead>
<tr>
<th>Lot number</th>
<th>Item</th>
<th>96 wells</th>
<th>288 wells</th>
<th>480 wells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Antibody–coated 96-well microtiter plates</td>
<td>1</td>
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<td></td>
<td>Alkaline phosphatase enzyme conjugate</td>
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<td>0.150 ml</td>
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<td>RUB6 enzyme conjugate diluent, 1X</td>
<td>11 ml</td>
<td>33 ml</td>
<td>55 ml</td>
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<td>PNP substrate tablets, 5 mg each</td>
<td>12 ml</td>
<td>12 ml</td>
<td>25 ml</td>
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<td></td>
<td>PNP substrate buffer, 5X concentrate</td>
<td>12 ml</td>
<td>12 ml</td>
<td>25 ml</td>
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<tr>
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<td>Positive control (if available)</td>
<td>1</td>
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<td>1</td>
</tr>
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</table>

The above items should be stored at 4°C

|            | PBST wash buffer, 20X concentrate, 50 ml         | 3        | 5         | 7         |
|            | Tween-20                                         | 15 ml    | 30 ml     | 30 ml     |
|            | General extract buffer 3 (GEB3)                  | 24 g     | 48 g      | 48 g      |

The above items should be stored at room temperature.

Materials required, but not provided

- Distilled or purified water
- Paper towels
- Micropipette
- Micropipette tips
- Lyophilized negative control can be purchased from Agdia
- 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.

Buffers: Do not store 1X buffers from day to day. Buffers should be warmed to room temperature prior to use. Buffer formulations on page 6 are for reference only.

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.

Buffers: General extract buffer 3 (GEB3) and RUB6 enzyme conjugate diluent must be used to give optimal results. Not using these buffers can result in higher background and false positive reactions.

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

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.
Preparing for the test
Familiarize yourself with the kit components. Check that all components are present in the kit.

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.

Prepare buffers

PBST wash buffer
Prepare PBST wash buffer by diluting one 20X pouch of PBST wash buffer with 950 ml of distilled water.

General extract buffer (GEB3)
Prepare only the amount of 1X buffers needed for the day.

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 or until completely dissolved.

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%).
PNP substrate buffer

PNP substrate buffer is used to dilute PNP substrate tablets. The volume of 1X PNP buffer required depends on the number of testwells used. You will need 100 µl of prepared PNP substrate buffer for each testwell you are using. To estimate the volume needed, prepare 1 ml for each 8 well strip used. A full plate will require about 10 ml.

To prepare 10 ml of working PNP substrate buffer, mix 2 ml of 5X PNP buffer concentrate with 8 ml of distilled water.

Note: PNP substrate tablets will not be added at this time. Tablets will be added prior to completion of the enzyme conjugate incubation.

Prepare controls

Reconstitute lyophilized positive control and lyophilized negative control with 2.0 ml general extract buffer 3 (GEB3) per bottle.

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. If you will be using a control in one well each time you run the test, prepare 120 µl aliquots. If you will be using a control in two wells, prepare 220 µl aliquots. Each aliquot should be sufficient for the tests to be run plus a small additional volume to assure easy dispensing.

Control aliquots must be stored frozen (-20°C 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 and 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.

Do not refreeze controls.

Prepare testwells

If you will be using less than a full 96-well plate, remove any unused strips and seal them in the foil pouch with the desiccant. Using a permanent marker, number the strips in case a strip becomes separated from the frame.

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 samples from evaporating.

Make a copy of the loading diagram and record the locations of your samples and controls. We recommend that you use a buffer well and positive control well on each plate each time you run the test.
Grind and dilute samples

When possible, select samples showing symptoms. Leaf tissue is often used in ELISA testing. Stem, seed, and other tissue can also be tested. 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 extract 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. If you are using a mortar and pestle, wash and rinse it thoroughly between 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 general extract buffer into buffer wells. If you will be using a negative control, dispense 100 µl into negative 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: Always prepare enzyme conjugate within 10 minutes before use.

The bottle of alkaline phosphatase enzyme conjugate is supplied as a concentrate and must be diluted with RUB6 buffer before use. The recommended conjugate to buffer ratio is given on the label. Dispense the appropriate volume of prepared RUB6 buffer into a dedicated container. You will need 100 µl of buffer for each testwell you are using. To estimate the volume needed, prepare 1 ml for each 8-well strip used. A full plate will require about 10 ml. Then, add the alkaline phosphatase enzyme conjugate according to the dilution given on the labels.

Example: If the dilution given on bottle of concentrated alkaline phosphatase enzyme conjugate is 1:100, and you are preparing 10 ml of enzyme conjugate solution, you should first dispense 10 ml RUB6 buffer. Then add 100 µl of the concentrated enzyme conjugate to the RUB6 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 solution

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

Sample interpretations should be performed on a case-by-case basis. The samples, environment, and end goals are different for each user and test. Some common methods for setting a threshold include twice the mean O.D. of negative (healthy) controls or the mean of negative (healthy) controls + 3 standard deviations.

These methods depend heavily on how well the negative (healthy) control matches the sample population in regards to variety, growing conditions, tissue type and age.

Buffer Formulations

Concentrated buffers are included with your kit. Formulations provided below are for reference only.

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

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.
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Test performed by _________________________________