

Document Control Number WI-B-T-4-3	WORK INSTRUCTION USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number Original
Effective Date: 2-29-2008	Detection of Plum Pox Virus using Agdia PPV ELISA	Page 1 of 11

This work instruction describes detection of Plum Pox Virus (PPV) using the Enzyme-Linked Immunosorbent Assay (ELISA) kit from Agdia Inc. (Elkhart, IN). The ELISA detects five (5) known PPV subgroups: PPV-C, PPV-D, PPV-EA, PPV-M and PPV-W in leaves, fruit and flowers.

This new work instruction replaces the **retired WI-B-T-4-1 Supplemental Instructions for ELISA for Plum Pox Virus** work instruction from May, 2007. **This new instruction now includes ELISA test information taken directly from Agdia's PPV ELISA manufacturer instruction, which we have inserted in blue font.** It is expected that you will use this new work instruction to run your PPV ELISA testing.

I. Reference

<http://www.agdia.com/doc/m243.pdf>

II. Introduction to PPV DAS ELISA

Agdia's PPV ELISA is a Double Antibody Sandwich (DAS) ELISA. Testwells of a microtiter plate are coated with antibodies specific to PPV. Samples are added to the microtiter plate. If PPV is present in the sample, it is bound by the antibodies and captured on the microtiter plate. After sample incubation, the plate is washed to remove any unbound sample. A PPV-specific polyclonal antibody conjugated to alkaline phosphatase is added and binds to any captured PPV. After a brief incubation the plate is washed to remove any unbound conjugate. PNP substrate is added to the microtiter plate. If the alkaline phosphatase conjugate is present a color will be produced signifying the presence of PPV. The color reactions can be measured with a spectrophotometer or observed visually.

III. Equipment, Materials and Reagents

A. Equipment

1. Homogenizer: Homex 6 homogenizer (Bioreba #400004 or 400005) or hand grinder tissue homogenizer with circular bearings (Agdia #ACC 00900, Bioreba #400010) (Hand grinder can also be attached to drill press)
2. ELISA plate reader (Spectrophotometer), capable of reading 405 nm (any vendor)
3. Dedicated calibrated pipettors (P10, P50, P200, P1000, 8 or 12 channel multi-channel)
4. Plate washer bottle (Agdia #ACC 00520)
5. Magnetic stirrer plate and magnetic bars (any vendor)
6. Analytical balance, capable of weighing 0.3 to 33 grams (any vendor)

B. Materials

1. **PPV ELISA Kit (Agdia, #SRA 31505, available as 50, 500, 1000 or 5000 test wells)**

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Effective Date: 2-29-2008	Detection of Plum Pox Virus using Agdia PPV ELISA	Page 2 of 11

2. PPV-positive control strip (Agdia #SPC 31505) (*Note: some positive control strips are included with the ELISA kit*)
3. Negative control (Agdia #LNC 31505) (*Note: Order if no healthy Prunus plant is available*)
4. Buffer pack, alkaline Phosphatase, DAS/Compound (Agdia # ACC 00113) contains carbonate coating buffer, PNP substrate and buffer, and PBST wash buffer (*These items not included with the ELISA kit and are sold separately.*)
5. Extraction bags (mesh) bags: 12 x 14 cm, (Agdia #ACC 00930, Bioreba #430100, Neogeneurope.com ,#05-002)
6. Airtight container for incubations (i.e.: plastic box with airtight lid)
7. Sealing film: SealPlate™ (i.e: LabSource #P98-241)
8. Sterile filter (barrier) pipette tips for the corresponding pipettors
9. Wide orifice tips (i.e: LabSource #P23-149) for loading ground plant samples
10. 1.5ml disposable micro centrifuge tubes (any vendor)
11. Disposable multi-channel solution basins (i.e: LabSource #P53-611)
12. Plastic transfer pipettes (*optional*)
13. Ice
14. Gloves
15. Paper Towels
16. Basin to collect sap runoff
17. Bleach to treat sap runoff
18. Distilled or deionized water

IV. ELISA kit Limitations and Notes

- Expiration: The ELISA test kit 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.
- Sample Dilution: ELISA performance is very dependent on the proper sample dilution.
- Sampling: PPV virus in infected trees can be unevenly distributed in the tree and will be at lower concentrations in the summer as temperatures increase. These factors can limit the ability of detecting the virus. Sampling from throughout the tree canopy, selecting symptomatic tissue, and testing in spring and early summer will increase the chances of detecting the virus.
- Please read all reagent and antibodies labels before preparing reagents or antibody dilutions. The antibodies may have different dilution ratio in different reagent sets.

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Effective Date: 2-29-2008	Detection of Plum Pox Virus using Agdia PPV ELISA	Page 3 of 11

- Antibody/conjugate solutions should be prepared according to the manufacturer's instruction in glass or polyethylene containers/tubes (lower protein binding capacity) 10 min prior to use.
- All incubations should be done using a humid box (line an air-tight container with wet paper towels).
- Always use aseptic techniques when opening and removing reagents from vials and bottles. Keep the plate covered except when adding reagents, washing or reading. Always use different pipette tips for each reagent.
- We hand wash our ELISA plates. You have the option of using an automatic plate washer for every wash step after the sap wash, which should be done by hand.

V. Leaf Samples

- Collected samples should be processed as soon as possible. Field samples can be stored at 4°C up to seven days prior to processing. Longer storage will decrease the amount of virus detected due to degradation of the virus in the aging plant tissue.
- **Samples that are partially decayed (have a brown appearance or are moldy) should not be tested due to potentially decreased absorbance values. These materials must be recollected.**
- **If you are brought samples and they arrive warm to the touch, they will need to be placed in at 4°C for several hours prior to handling. Our experience has shown that the titer will be diminished if you do not chill the samples first to stop degradation.**
- We strongly recommend wearing gloves when handling samples and changing them as often as possible. We realize that changing gloves between samples in high throughput situations is not practical. In those situations, we recommend changing gloves between sets of samples, based on the samples loaded on each plate. **We strongly suggest wearing gloves when handling a suspect positive sample.**
- Samples for PPV need to be kept cold (4°C) for virus integrity; however, leaf samples that are to be ELISA tested should not be stored in a freezer.

VI. ELISA procedure

A. Coating an ELISA plate with PPV-antibody solution

1. Prepare humid box

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

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Effective Date: 2-29-2008	Detection of Plum Pox Virus using Agdia PPV ELISA	Page 4 of 11

2. Determine the number of plates you need to coat based on the number of samples being tested. You can load 45 samples and 3 controls on each plate (duplicate wells loaded).

3. Prepare capture antibody

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 by dilution of 10X stock with distilled H₂O (1ml 10X carbonate buffer to 9 ml dH₂O for example). You will need 100 µl of carbonate coating buffer for each test well you are using. A full plate will require about 10 ml.

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.

4. Coat plate(s)

Pipette 100 µl of the prepared capture antibody into each well. Use the SealPlate™ film (recommended) to prevent plate evaporation.

5. Incubate plate(s)

Incubate the plate in a humid box for 4 hours at room temperature RT or overnight in the refrigerator (4°C).

Note: Do not store coated plates longer than 24 hours. If long term storage is desired, contact Agdia about postcoat buffers.

Prepare GEB4 buffer and samples during this period of time (see sections B and C).

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Effective Date: 2-29-2008	Detection of Plum Pox Virus using Agdia PPV ELISA	Page 5 of 11

6. Wash plate(s)

Empty the plate 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.

B. General extract buffer 4 (GEB4)

GEB4 is used to dilute and extract samples. Prepare fresh GEB4 buffer (see recipe below) after coating the ELISA plate, before handling the samples.

Buffer powder	33 g
Distilled water	1000 ml (1 liter)
Tween 20	20 ml or 20 g

1. Shake the bottle containing dry GEB4 powder to mix well. Weigh specified amount of powder, then transfer to a beaker containing a magnetic bar and place on a stir plate.
2. To make 1 liter of GEB4 sample extract buffer, add about 50 ml of water to the powder and mix into a smooth slurry. While mixing, slowly add the remaining volume of water. Add Tween 20 to the solution. Stir for 30 minutes.
3. Chill the grinding buffer on ice for a minimum of 30 minutes so it is ice cold prior to use.

Note: 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%).

C. Sample and Control Preparation

1. Sample and tissue control preparation

Note: If healthy and/or PPV-infected tissue controls are available, please prepare them with the samples, following the preparation procedure below.

- a. Label a set of grinding bags and a set of 1.5 ml microcentrifuge tubes for the number of samples. Place tubes in ice prior to use.
- b. A laboratory sample should consist of no more than 8 leaves. **Stack the leaves one on top of another. Unlike Agdia's instructions, tear a portion of the leaves nearest the petiole end, along the mid rib on one side of the leaf, leaving the other side available for confirmation testing if necessary.** Leaves can also be cut with a single-

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Effective Date: 2-29-2008	Detection of Plum Pox Virus using Agdia PPV ELISA	Page 6 of 11

edged razor blade. If cork borers or scissors are used they must be decontaminated to prevent sample to sample contamination that could effect subsequent testing. (Do not use the petiole and do not tear off the entire bottom portion of the stacked leaves as this may be needed for confirmation testing.)

- c. Weight 0.3 to 0.5g of each sample and place in the corresponding mesh bag. For Agdia's grinding bags, place plant sample between the mesh layers. Keep bags with samples on ice until all samples are prepared.
- d. Add pre-chilled GEB4 grinding buffer to each bag in 1:10 ratio (tissue weight in g: buffer volume in ml). *Example: A sample weighing 0.5 grams requires 5 ml of GEB4 buffer.* (Chilled buffer helps to prevent virus degradation.)
- e. You will need 100 µl of the ground sample per test well x two test wells per sample, plus an additional amount to assure easy dispensing. (We recommend a min. of 300 µl per sample or control.)
- f. Grind tissue samples well using a tissue homogenizer grinding device. We recommend keeping bags with ground samples on ice at an angle of 45° to facilitate sap collection in one corner. Please note that the Agdia grinding bags have no mesh in one of the corners: a convenient place to gather the sap.
- g. After all samples and controls are ground you should transfer plant sap using plastic transfer pipette from the bag into a pre-labeled and chilled 1.5ml tube. Keep tubes on ice.
Note: We transfer ground sap into labeled tubes because we use this same sap for Immunocapture of the virus for RT-PCR (see WI-B-T-2-4).
- h. All processed samples should be loaded into a pre-coated plate within 1-2 hours of grinding. **Ground samples should NOT BE stored overnight for loading the next day.** Storage of ground samples can lower the absorbance readings due to sample degradation.

2. PPV-positive strip control

Before opening the container of control strips, let the container warm at room temperature for 15 minutes. This maintains the shelf life of the strips. Do not allow the container to remain open. Keep tightly sealed between uses. We recommend that you take the container with the positive control strips out of the cold storage just before washing the plate, prior to loading the samples.

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Effective Date: 2-29-2008	Detection of Plum Pox Virus using Agdia PPV ELISA	Page 7 of 11

Each control strip requires 500 µl of GEB4 buffer. Dispense the required amount of GEB4 sample extract buffer into a microcentrifuge tube or other container. Dip the pad end of the strip into the buffer and let it sit for 5 minutes. Use the strips to stir the buffer before using.

The control strip can only be used once, after which it should be discarded. Do not store the positive control solution. It should be discarded after one day.

3. Negative control preparation

Please use the manufacturer's instruction provided with the negative control to prepare and store the negative lyophilized ELISA control.

4. Grinding buffer control

Each plate must include at least two test wells of the GEB4 buffer that was used for sample preparation.

D. Loading the plate(s)

1. Prepare a loading diagram for each plate. Each plate must include one positive and at least two negative control(s) (purchased negative control and/or fresh healthy tissue, and GEB4 buffer control).
2. Using a coated plate (after step VI-A-6), load 100µl of each sample or control using wide orifice tips. Each sample and control is loaded into 2 duplicate wells, adjacent to each other, to improve the accuracy of the assay. Change tips between each sample/control.
3. Positive controls should be loaded away from the samples and the negative control so no drift occurs when washing the plate. We recommend loading positive controls in the rightmost column. (You can put buffer wells in between.)
4. If you are not using certified plates, we recommend loading buffer in the outside (edge) wells to avoid 'edge effect'. This is less critical when using certified plates.
5. Cover the plate with SealPlate™, place in the humid box and incubate at 4°C for at least 16 hours or 2 hours at RT. (The cover helps prevent the sap from drying out or spilling.)

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Effective Date: 2-29-2008	Detection of Plum Pox Virus using Agdia PPV ELISA	Page 8 of 11

E. Washing the plate to remove plant sap (1X PBST)

When it is time to wash the sap out of the plate, if you did the overnight incubation then remove the plate from 4°C. We recommend letting it warm up a few minutes so sap is easier to wash out.

Begin washing but **don't start counting the washes until after the green sap is no longer visible**. Hold the plate(s) upside down over a basin in order to catch the sap/buffer runoff. Treat this runoff with diluted (10%) household bleach for 20-30 minutes to deactivate potentially positive sap before it is released down the drain.

To hand wash plates, hold the plate at a 45° angle, with column twelve at the lowest end, to prevent contamination from the positive control well. Use a specialized wash bottle with an 8-opening squirt head containing 1X PBST. Wash out the wells in column 12, then column 11, column 10, and so on, to avoid contaminating adjacent rows. Tap the plate on paper toweling several times to remove the excess moisture.

F. Prepare enzyme conjugate

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

1. The alkaline phosphatase enzyme conjugate is supplied as a concentrate and must be diluted with RUB3 enzyme conjugate diluent before use. The recommended conjugate to buffer ratio is given on the label.
2. Dispense the appropriate volume of RUB3 enzyme conjugate diluent into a dedicated container. You will need 100 µl of diluent for each test well. Then, add the alkaline phosphatase enzyme conjugate according to the dilution given on the label.

Example 1: If the dilution given on bottle of concentrated alkaline phosphatase enzyme conjugate is 1:200, and you are preparing 10 ml of enzyme conjugate solution, you should first dispense 10 ml of RUB3 buffer. Then, add 50 µl of the concentrated enzyme conjugate to the RUB3 buffer.

Example 2: 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 RUB3 buffer. Then, add 100 µl of the concentrated enzyme conjugate to the RUB3 buffer.

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

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Effective Date: 2-29-2008	Detection of Plum Pox Virus using Agdia PPV ELISA	Page 9 of 11

4. Add 100 µl of the enzyme conjugate into each test well. Cover the plate with SealPlate™, place in the humid box and incubate for 2 hours at RT.

G. PNP substrate incubation and plate reading

Prepare PNP substrate 10 -15 min before the end of the incubation in step F-5.

Do not touch the PNP tablets or solution, or place your fingers inside the reagent reservoirs. Phosphatase contaminants from your fingers will utilize the PNP substrate and develop color before addition to the test plate. Do not expose PNP substrate to strong light. Light or contamination could cause background color in negative wells.

1. Prepare a working solution of the substrate buffer by adding 1 ml of 5X substrate buffer to 4 ml of dH₂O or double the volumes for a whole plate in a plastic tube (i.e.: 15 ml falcon tube with screw-on cap) wrapped with aluminum foil. Add 2 PNP tables (can use a forceps) for 10ml buffer (1 tablet for 5ml buffer). Tablets will dissolve faster if the tube is placed on a shaker at slow motion for several minutes.
2. Wash the plate to remove the enzyme conjugate 8 times with 1X PBST. Tap the plate on paper towel several times to remove the excess moisture. Inspect each well for presence of air bubbles. Use a clean pipette tip to break any existing air bubbles.
3. Dispense 100 µl of the PNP substrate into each test well, place plate in a humid box in dark (in a drawer for example) and incubate for 1 hour at RT. (Do not cover the plate with SealPlate™ at this step.)
4. Before the end of the incubation turn on and set up your ELISA reader at 405nm. Wipe the bottom of the plate with paper towel to remove evaporation or moisture and make sure there are no bubbles in the test wells because that can interfere with readings. (If bubbles are observed, gently break the bubble using a clean pipette tip, one tip per well.) Any remaining bubbles should be noted on the loading diagram. Read each plate and either save and/or print the Optical Densities (ODs).
5. Results may be interpreted after more than 60 minutes of incubation as long as negative wells remain virtually clear.

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Effective Date: 2-29-2008	Detection of Plum Pox Virus using Agdia PPV ELISA	Page 10 of 11

VII. Results interpretation

1. Determine the background on each plate by averaging the ODs of two wells of negative control (or healthy Prunus, if available). This background = 'A'.
2. Then determine your threshold (cut-off), "B", by multiplying 'A' x 2.5 = 'B'.

Positive Control Readings

The averaged positive control OD must be positive (OD >B) for the ELISA run to be valid.

The PPV positive control strip should result in OD of 1.0 to 2.0. (Infected plants used as positive controls often result in higher ODs, e.g.: 3.0 to 4.0+)

If a positive control fails to reach expected OD values then none of the ELISA results on that plate are valid and all the samples loaded on that plate must be retested using newly ground sap.

Make note of variations in positive controls between plates or between days. Lower than expected values can indicate an improperly conducted test that could invalidate test data, or an improperly performing positive control that also invalidates the test. (A poorly-performing positive control that is determined to not be due to operator error should be reported to Agdia, especially if the kit is new.)

Negative Control and Buffer Control Readings

The negative control and buffer wells cannot have significant color or the test is invalid. The average negative control OD should be <B (colorless).

Note: The buffer control is used to determine the background which can be subtracted from all the wells.

Sample Readings

1. Determine each sample's OD value by averaging the ODs from the samples duplicated wells.
2. Interpret each sample result by determining if the average sample OD is less than, equal to or greater than "B".
 - Any sample that is >B is considered **suspect positive**.
 - Any sample that is < B is considered negative.

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Effective Date: 2-29-2008	Detection of Plum Pox Virus using Agdia PPV ELISA	Page 11 of 11

- Any sample that is = B is considered borderline and should be retested using a new subsample and/or collect and test a new sample.

VIII. Reporting an ELISA positive result

Positive sample test results should be reported immediately to the USDA-APHIS-PPQ National Plum Pox Virus Operations Director, Don Albright, phone 717-241-0705 (e-mail: donald.i.albright@aphis.usda.gov) and Stephen Poe, Program Manager, phone 301-734-8899 (e-mail: stephen.r.poe@aphis.usda.gov).

Remaining leaf tissue from a sample suspected as positive for PPV should not be discarded until it's retested and/or it is determined the sample should be forwarded. (Samples that are determined to test negative should be autoclaved after the test results are reviewed and approved as negative.)

IX. ELISA Trouble shooting

Problems with ELISA kits should be forwarded to your Agdia representative. Provide them with the kit name, lot number, expiration date and your OD readings.

There are many factors that can cause an ELISA to produce inadequate results. See Agdia's website: http://www.agdia.com/faq/ELISA_Problems.shtml

Document Revision History

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Original	Original	2-29-2008	To baseline the work instruction.

Approved by: **SIGNATURES ON FILE**

Date: 2-29-2008

Renee DeVries, NPGBL Quality Manager

Laurene Levy, NPGBL Technical Manager

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