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Diagnostics
Diagnostic

PM 7/125 (1) ELISA tests for viruses

Specific scope

This Standard describes the most commonly used ELISA tests in virology and how to perform these tests for detection and/or identification of plant viruses.[1]

Specific approval and amendment

Approved as an EPPO Standard in 2015-09.

1. Introduction

Enzyme Linked Immuno Sorbent Assay (ELISA) is a technique commonly used to detect and identify viruses (Clark & Adams, 1977; Clark, 1981). This is convenient for screening of large numbers of samples. There are several types of ELISA tests (Koenig & Paul, 1982; Cambra *et al.*, 1991, 2011) and the most widely used are:

(i) Double antibody sandwich ELISA (DAS-ELISA),
(ii) Double antibody sandwich indirect ELISA (DASI-ELISA; also named triple antibody sandwich-TAS),
(iii) Plate Trapping Antigen ELISA (PTA direct and indirect ELISA),
(iv) Tissue print-ELISA, also named Direct tissue blot immunoassay-DTBIA (direct-ELISA).

2. General instructions

Instructions to perform the different types of ELISA tests are given in Appendix 1. Negative and positive controls should be included in each series of tests. Details on positive and negative controls and their preparation are provided in Appendix 2. Examples of buffers and substrates are presented in Appendix 3.

2.1. Antibodies

Antibodies (immunoglobulins) or recombinant antibody fragments are critical for the performance of ELISA tests. Only validated polyclonal, monoclonal or recombinant antibodies specific to the target should be used (see validation procedures described in PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostics*). As stated in PM 7/98, a change of lots/batches of reagent may influence the performance of a test. In such cases, verification of the performance of the reagent should be carried out by comparison with the reagent previously used, by testing appropriate controls or according to the guidance provided. Additional specificity data may need to be produced, especially when using ELISA with polyclonal antibodies.

The coloured final product of any ELISA reaction is produced through an enzyme (usually alkaline phosphatase) conjugated or fused to specific conventional antibodies, antibody fragments or recombinant antibodies or is produced through a reaction based on the biotin/streptavidin-enzyme conjugated system. The coloured final product is semi-quantitatively detected by an ELISA reader.

2.2. Preparation of the samples

The tests should preferably be performed on fresh plant extracts or by direct tissue-print or squash of fresh sections of plant material immobilized on membranes. Depending on the type of virus (e.g. particle stability), it may be possible to use freeze-dried or frozen plant material or extracts. Information regarding the possibility of bulking depends on several factors including the virus concerned, the type of material to be tested, and the antiserum and needs to be validated. This information is provided when available in pest-specific Diagnostic Protocols.

2.2.1. Plant extracts preparation

The tissue/buffer ratio for the preparation of the extract is determined depending on the type of tissue and is about 1/10 w/v (usually 0.2 g of fresh plant material in 2 mL).

[1] Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

The extract for ELISA is prepared by grinding plant tissue in an appropriate buffer (see Appendix 3). Grinding can be done using a pestle and mortar, or in plastic bags using rollers or specific tools or apparatus (e.g. Homex machine, Bioreba®), or in appropriate tubes using specific homogenizers (e.g. Polytron®, Kinematica®) or any equivalent method. The remainder of the extract is stored at approximately 4°C until testing is completed.

This type of extract is commonly used for DAS- and DASI (or TAS)-ELISA.

For other ELISA tests, the plant extract is filtered through a layer of muslin or centrifuged (for 5 min, at approximately 2000 *g* preferably at 4°C) or if this is not available the plant material is allowed to settle. Depending on the type of ELISA performed the supernatant is used to coat the wells of the ELISA plate in the test.

2.2.2. Sample preparation for tissue-print (without plant extract preparation)

For immobilization of samples for tissue print on commercial nitrocellulose membranes clean cuts on tender shoots, leaf petioles, fruit peduncles or flowers should be made. The freshly made sections should be pressed carefully against the membrane. The trace (print) is left to dry for a few minutes. For routine testing at least two prints from different sections per selected shoot or peduncle should be performed and one per leaf petiole or flower. Another option for viruses not restricted to the vascular area is to squash the material on the membrane. For immobilization for squashes the plant material is gently pressed on the membrane and the trace is left to dry as above.

2.2.3. Plates

Usually polystyrene plates of 96 wells flat bottom or equivalent appropriate immuno modules with 8, 12 or 16 wells (e.g. PolySsorp® or equivalent) are used It should be noted that the type of plates can influence the OD values obtained.

3. Feedback on this Standard

If you have any feedback concerning this Standard, please send it to diagnostics@eppo.int.

4. Standard revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press this will also be marked on the website.

5. Acknowledgements

This protocol was originally drafted by Mariano Cambra (IVIA-Protection and Biotechnology Center, 46113 Moncada, Valencia, Spain) and Yuri Shneider (All-Russian Plant Quarantine Center, Bykovo, Russia). It was reviewed by the Panel on Diagnostics in Virology and Phytoplasmology.

6. References

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Appendix 1 – Instructions to perform the most common ELISA tests

A Description of the tests

For commercial kits it is recommended to follow the instructions of the supplier.

Indications are given on possible temperature for incubation. Plates can also be incubated at other temperatures in order to influence the rate of the reaction and such changes should be validated.

The volume in any step of ELISA might be reduced down to 100 µL according to the sample volume, but given current sensitivity of ELISA tests 200 µL is recommended.

Depending on the dynamic of the reaction, the matrix, the target, readings of the plates may begin 30 min after the addition of the substrate and be continued at appropriate intervals (usually up to 120 min).

i. DAS-ELISA

(1) Prepare the appropriate dilution of antibodies, antibody fragments or recombinant antibodies in carbonate coating buffer pH 9.6 (Appendix 3). Add 200 µL to each well of plate. Borders of the plates should be avoided for better repeatability. However, some techniques are available to prevent border effect such as covering the plate with a film or plastic cover to avoid evaporation,

and incubation in a humid chamber. In such cases border wells can be used. Incubate at 37°C for 4 h or at 4°C overnight.

(2) Wash the wells usually three times (e.g. using a wash bottle or a washing apparatus) with PBS-Tween (Appendix 3).

(3) Add 200 µL of each sample (plant extract, see 2.2.1) to two plate wells and the following controls:
- at least two wells of positive controls;
- at least two wells of negative controls of healthy plant extracts;
- at least two wells of negative controls of extraction buffer ("blanks").

Incubate approximately 4 h at 37°C or overnight at 4°C.

(4) Wash as before (2).

(5) Prepare the appropriate dilution of the specific antibodies, antibody fragments or recombinant antibodies conjugated or fused with alkaline phosphatase (or linked with other enzyme or biotin) in conjugate buffer (Appendix 3). Add 200 µL to each well. Incubate at 37°C for 2–4 h (according to the instructions of the protocol for enzymes or for biotin/streptavidin system).

(6) Wash as before (2).

(7) Prepare a fresh solution of 1 mg mL^{-1} p-nitrophenyl phosphate in alkaline phosphatase substrate buffer (Appendix 3). For other enzymes or for biotin/streptavidin follow the recommendations of the commercial kit. Add 200 µL of this solution to each well. Incubate in the dark at room temperature (approximately 20°C) or in an incubator at 37°C covering the plate and read absorbance at 405 nm (for other enzyme-substrate combinations or systems follow the recommendation of the supplier of the kit) at regular intervals within 120 min, or following the instructions of the supplier. Note that temperature influences the substrate conversion and the resulting OD values.

ii. DASI-(TAS-)ELISA

(1) Prepare the appropriate dilution of polyclonal antibodies in carbonate coating buffer (Appendix 3). Add 200 µL to each well of a plate. Borders of the plates should be avoided for better repeatability. However, some techniques are available to prevent border effect such as covering the plate with a film or plastic cover to avoid evaporation, and incubation in a humid chamber. In such cases border wells can be used. Incubate at 37°C for 4 h or overnight at 4°C.

(2) Wash the wells usually three times (e.g. using a wash bottle or a washing apparatus) with PBS-Tween (Appendix 3).

(3) Add 200 µL of each sample (plant extract, see 2.2.1) to two plate wells and the following controls:
- at least two wells of positive controls;
- at least two wells of negative controls of healthy plant extracts;
- at least two wells of negative controls of extraction buffer ("blanks").

Incubate for approximately 4 h at 37°C or overnight at 4°C.

(4) Wash as before (2).

(5) Prepare the appropriate dilution of the specific antibodies (preferably mouse monoclonal antibodies or antibodies raised in a different animal species from the one used to produce the polyclonal antibodies of step 1) in conjugate buffer (Appendix 3) and add 200 µL to each well. Incubate at 37°C for approximately 2 h.

(6) Wash as before (2).

(7) Prepare the appropriate dilution of the anti-species antibodies (when using specific mouse monoclonal antibodies: anti-mouse immunoglobulins) conjugated with alkaline phosphatase (or linked with another enzyme) in conjugate buffer (Appendix 3). Add 200 µL to each well. Incubate at 37°C for approximately 2 h.

(8) Wash as before (2).

(9) Prepare a solution of 1 mg mL^{-1} p-nitrophenyl phosphate in alkaline phosphatase substrate buffer (Appendix 3). For other enzymes or systems follow the recommendations of the commercial kit. Add 200 µL to each well. Incubate in the dark at room temperature (approximately 20°C) or in an incubator at 37°C covering the plate and read absorbance at 405 nm (for other enzyme-substrate combinations follow the recommendation of the supplier of the kit) at regular intervals within 120 min.

iii. PTA-ELISA (direct and indirect)

(1) Add 200 µL of each sample (plant extract, see 2.2.1) to two plate wells and the following controls:
- at least two wells of positive controls;
- at least two wells of negative controls of healthy plant extracts;
- at least two negative wells of controls of extraction buffer ("blanks"). Borders of the plates should be avoided for better repeatability. However, some techniques are available to prevent border effect such as covering the plate with a film or plastic cover to avoid evaporation, and incubation in a humid chamber. In such cases border wells can be used. Incubate at 37°C for approximately 4 h or overnight at 4°C.

(2) Wash the wells usually three times (e.g. using a wash bottle or a washing apparatus) with PBS-Tween (Appendix 3). Washing too strongly with some washing apparatus can affect the coating result and, consequently, the sensitivity, especially in detection.

(3) Add 200 µL of blocking buffer (Appendix 3) to each test well and incubate at 37°C for 1 h.

(4) Wash as before (2) or knock out the blocking buffer.

(5) Prepare the appropriate dilution of specific antibodies, antibody fragments or recombinant antibodies in conjugate buffer (Appendix 3). For validated commercially available antibodies use the recommended working

dilutions. When the specific recombinant antibodies are already fused with an enzyme or biotin and after the incubation and washing steps (6 and 7) directly proceed with step number 10.

(6) Add 200 µL to each well and incubate for 2 h at 37°C.
(7) Wash as before (2).
(8) Prepare the appropriate dilution of the conjugate (immunoglobulins anti-animal species used to raise the antibodies used in step 5, also called secondary antibodies, usually alkaline phosphatase conjugated, but other enzymes or biotin may also be used) in conjugate buffer. Add 200 µL to each well and incubate for 1 h at 37°C.
(9) Wash as before (2).
(10) Prepare a fresh solution of 1 mg mL^{-1} p-nitrophenyl phosphate in alkaline phosphatase substrate buffer (Appendix 3). For other enzymes or for the biotin/streptavidin system follow the supplier's instructions in the commercial kit. Add 200 µL of this solution to each well. Incubate in the dark at room temperature (approximately 20°C) or in an incubator at 37°C and read absorbance at 405 nm (for other enzyme-substrate combinations or systems follow the recommendation of the supplier of the kit) at regular intervals within 120 min.

Note: for DAS-, DASI- and PTA-ELISA the reaction can be delayed by storing at 4°C or temporarily stopped by freezing. It can be permanently stopped by adding 50 µL per well of sodium hydroxide solution (1M).

iv. Tissue print-ELISA or Direct tissue blot immunoassay

Use the nitrocellulose membrane provided in some commercially available kits or cut a rectangular nitrocellulose sheet.

(1)
  (a) Prepare smooth, freshly cut pieces of plant tissues (tender shoots, leaf petioles, fruit peduncles or flowers) and press them onto a nitrocellulose membrane to obtain a tissue-print.
  (b) Directly squash symptomatic plant material onto the nitrocellulose membrane. Include positive and negative tissue prints as controls. Let the tissue-prints, squashes or blots dry for at least 5 min at room temperature. Printed membranes can be mailed, for example, to another laboratory at ambient temperature.
  (c) The membranes can be kept for several months in a dark and dry place (in an hermetic box) at approximately 4°C.
(2) Block the nitrocellulose membrane using a solution of 1% bovine serum albumin (BSA) in PBS (Appendix 3). Add enough solution to cover the membrane in an appropriate container. The blocking step can be done overnight at 4°C or for 1 h at room temperature. Remove the BSA solution without washing.
(3) Prepare the appropriate dilution of specific antibodies or recombinant antibodies conjugated or fused with alkaline phosphatase in PBS (Appendix 3). Add enough solution to cover the membrane in an appropriate container. Incubate for 2 h at room temperature under slight agitation (100 rpm).
(4) Remove the conjugate and subsequently wash the membrane three times with PBS-Tween (Appendix 3), with 5 min washing steps, while shaking (100 rpm).
(5) Prepare a solution of precipitating substrate for alkaline phosphatase (e.g. NBT + BCIP; Appendix 3) in substrate buffer or distilled water, following the supplier's recommendations. Cover the membrane with this solution in an appropriate container and incubate at room temperature for 10–15 min. Stop the reaction by washing under tap water, dry on filter paper, and observe final purple-violet-coloured precipitates using a low power (×5 to ×10) magnification stereo microscope or a magnifying glass.

B Interpretation of the test reading

Verification of the controls

Negative ELISA readings in positive control wells/print or dot indicate that the test has not been performed correctly or that it has been inhibited. Positive ELISA readings in blanks and/or negative control wells/print or dot indicate that cross-contamination or non-specific antibody binding has occurred. In such cases the test should be performed again with the appropriate modifications.

Interpretation

(i) Interpretation for ELISA (except tissue print)

There are different possible options for interpreting ELISA test results and in particular to establish a threshold. Further information is provided in Sutula *et al.* (1986).

The following procedure is recommended, however, it is recognized that in particular when the negative control of healthy plant is not the same as the plant to be tested, the laboratory should adjust and validate the calculation of the threshold, or confirm positive results by another method.

ELISA test negative

The ELISA test is considered negative if the average optical density (OD) value from duplicate sample is

- less than 0.1 or;
- is $< 2\times$ OD of that in the negative control of healthy plant extracts.

ELISA test positive

Usually the ELISA test is considered positive if the average OD value from each of the duplicate sample wells is

- $\geq 2\times$ OD of that in the negative control of healthy plant extracts.

Note that when using polyclonal antibodies, it is essential that the negative controls are as similar as possible to the matrix tested in the same plate.

The test should be repeated when duplicate wells differ by more than 50% OD value. In critical cases, for samples that give a reaction close to the threshold of e.g. 2× OD of that in the negative control of healthy plant extracts or when matrix effects cannot be excluded, it is recommended that another test (different source of antibody or another method) is used.

Other procedures for interpretation are in use involving consideration of standard deviations (average of healthy controls + 3× standard deviation).

Note: It is recognized that visual reading of plates is a common practice for some routine laboratories, however it is not recommended in this Standard.

(ii)Interpretation for Tissue print ELISA

The ELISA test is negative if there is no coloured precipitate in the sample print, squash or dot.

The ELISA test is positive, if there is purple-violet-coloured precipitate in the sample print, squash or dot. For some viruses restricted to the phloem tissues, the observation of precipitates should occur in the vascular area only.

Appendix 2 – Positive and negative controls

Commercial kits

When using a commercial ELISA kit, a negative control from a healthy plant of a similar matrix prepared in the same extraction buffer should be included in addition to the negative controls provided in the kit.

A positive control should preferably be added in addition to the positive control provided in the kit (see below).

Aliquots of positive controls should be prepared to prevent repeated freezing and thawing.

Controls should be stored as recommended by the supplier.

In-house controls

Positive controls

Naturally infected tissue or extracts (maintained by lyophilization or freezing at below −20°C) should be used whenever possible. Aliquots of positive controls should be prepared to prevent repeated freezing and thawing.

Prepare a positive control of the target organism in extraction buffer (Appendix 3).

For Tissue print-ELISA, positive controls previously immobilized on membranes can be used.

Two wells or prints should be prepared per positive control.

Negative controls

Healthy plant extract should be used as negative control. The healthy plant should whenever possible be the same species and the same variety and the same plant part at the same growth stage as the samples to be analysed. Aliquots/extracts of the same host plant which previously tested negative for the target virus can be used as negative controls. For Tissue print-ELISA, healthy controls previously immobilized on membranes can be used.

At least two wells or prints should be prepared per negative control.

Appendix 3 – Examples of buffers and substrates for the ELISA test

Carbonate coating buffer, pH 9.6	
Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled water	1000 mL
Dissolve ingredients and check pH 9.6	
10× Phosphate buffered saline (PBS), pH 7.2 (dilute 1:10 before use)	
NaCl	80.0 g
KCl	2.0 g
$NaH_2PO_4{\cdot}2H_2O$	2.0 g
$Na_2HPO_4{\cdot}12H_2O$	29.0 g
Distilled water	1000 mL
PBS-Tween (PBST)	
10× PBS	100 mL
10% Tween 20	0.5 mL
Distilled water	900 mL
General Extraction Buffer	
Polyvinylpyrrolidone (PVP)	20 g
Ovalbumin	2 g
Sodium sulphite (anhydrous)	1.3 g
PBS-Tween	1000 mL
Blocking buffer (PBSTM)	
Non-fat dried milk powder (preferably from a certified supplier)	5 g
PBST	1000 mL
Conjugate buffer	
Bovine serum albumin (BSA)	0.2 g
PBST	100 mL
Alkaline phosphatase substrate buffer, pH 9.8	
Diethanolamine	100 mL
Distilled water	900 mL
Mix and adjust to pH 9.8 with concentrated HCl	
Just before use add para nitrophenyl phosphate (pNPP) to reach a final concentration of 1 mg mL^{-1}	
Precipitating substrate buffer for alkaline phosphatase	
NBT + BCIP (18.75 mg mL^{-1} NBT (Nitro blue tetrazolium chloride) and 9.4 mg mL^{-1} BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) in 67% DMSO (v/v) or SIGMA FAST™ BCIPP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets from Sigma Aldrich	