Diagnostics
Diagnostic

ELISA tests for plant pathogenic bacteria

Specific scope

Enzyme Linked Immuno Sorbent Assay (ELISA) tests for bacteria may be used for screening of large numbers of samples as an alternative to immunofluorescence (IF) in certain cases (see PM 7/97 *Indirect Immunofluorescence test for plant pathogenic bacteria*). They can also be used as part of the identification of pure cultures. This standard describes how to perform an ELISA test for detection and/or identification in bacterial diagnostics using: (i) Indirect; (ii) Double antibody sandwich-DAS; (iii) Double antibody sandwich indirect-DASI also named triple antibody sandwich-TAS; and (iv) Direct tissue-print, squash or colony-dot.

Specific approval and amendment

Approved as an EPPO Standard in 2010–09

General instructions

Instructions to perform the different tests are given in Appendix 1. Antisera 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 or according to the guidance provided by standard PM 7/98. Additional specificity data may need to be produced, especially when using ELISA with polyclonal antibodies, as described in EU Directive 2006/63/CE (EU, 2006).

The tests can be performed on fresh plant material (tissue pieces or macerates or comminuted material), on fresh or frozen sample extracts, on fresh or frozen bacterial cell suspensions, or on bacterial exudates. If these have been stored at −20°C with glycerol, the glycerol should be removed by the addition of PBS buffer, centrifugation at about 10°C for 15 min at 7000 ***g***, and re-suspension of the pellet in an equivalent volume of PBS. 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.

As the sensitivity of ELISA for detection of plant pathogenic bacteria is usually relatively low (about 10^5–10^6 cfu mL^{-1}), because of the lower coating ability of bacteria to the plates as compared to viruses and some other problems (Alvarez, 2004), a preceding enrichment step may be performed before ELISA (Indirect, DAS or DASI formats) to improve the sensitivity of the detection while maintaining the specificity. This is especially necessary when using specific monoclonal antibodies (López *et al.*, 2003). This step is unnecessary for identification of pure cultures. The enrichment should be performed using the most appropriate conditions of incubation (e.g. temperature, oxygen requirements, shaking and media) specified in the pest-specific diagnostic protocol. Enrichment can fail due to the presence or development of other microorganisms and should be avoided when this is predictable.

Acknowledgements

This test description was originally drafted by M.M. López (IVIA, Spain) and co-reviewed by J. Janse (NAK, The Netherlands).

References

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EU (2006) Commission Directive 2006/63/CE of 14 July 2006 amending annexes II to VII to Council Directive 98/57 on the control of *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* *Official Journal of the European Communities*, **206**, 36–106.

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Appendix 1 – Instructions to perform ELISA tests

Description of the test

(i) Indirect ELISA

(1) Use 200 µL (sample amount might be reduced down to 100 µL according to the sample volume) aliquots of prepared sample extract, macerate or bacterial suspension (according to the instructions of the protocol) in 1.5–2.0 mL microvials.
(2) Add an equal volume of double-strength coating buffer (Appendix 3) and vortex.
(3) Apply an equivalent volume of the sample to two wells of a microtitre plate with good coating characteristics (e.g. Nunc-Polysorp or equivalent) and avoid the borders of the plate for better repeatability. Some techniques are available to prevent border effect such as covering the plate with transparent film to avoid evaporation, in such cases border wells can be used. Incubate at 37°C for 4 h or overnight at 4°C. Prepare positive and negative controls in the same way. It is also necessary to include negative controls of extraction buffer and of the enrichment medium when appropriate.
(4) Wash the wells gently (e.g. using a wash bottle) three times with PBS-Tween (Appendix 3), as washing too strongly with some washing apparatus can affect the coating result and, consequently, the sensitivity, especially in detection.
(5) Prepare the appropriate dilution of specific antibodies in the appropriate blocking buffer (Appendix 3). For validated commercially available antibodies use the recommended working dilutions.
(6) Add 200 µL to each well and incubate for 2 h at 37°C.
(7) Wash as before (4).
(8) Prepare the appropriate dilution of the conjugated, second specific antibodies. (usually alkaline phosphatase conjugated, but other enzymes may also be used) in the appropriate blocking buffer. Add 200 µL to each well and incubate for 1 h at 37°C.
(9) Wash as before (4).
(10) Prepare a fresh solution of 1 mg mL^{-1} p-nitrophenyl phosphate in alkaline phosphatase substrate buffer (Appendix 3). For other enzymes follow the recommendations of the commercial kit. Add 200 µL of this solution to each well. Incubate in the dark at room temperature and read absorbance at 405 nm (or at the recommended wavelength for other enzyme-substrate reactions) at regular intervals within 120 min, or as indicated in the protocol.

(ii) DAS ELISA

(1) Prepare the appropriate dilution of antibodies in carbonate coating buffer pH 9.6 (Appendix 3). Add 200 µL to each well of a microtitre plate with good coating characteristics (e.g. Nunc-Polysorp or equivalent) and avoid the borders of the plate for better repeatability. Some techniques are available to prevent border effect such as covering the plate with transparent film to avoid evaporation, in such cases border wells can be used. Incubate at 37°C for 4 h or at 4°C overnight.
(2) Wash the wells gently (e.g. using a wash bottle) with PBS-Tween (Appendix 3), as washing too strongly with some washing apparatus can affect the coating result and, consequently, the sensitivity, especially in detection.
(3) Add 200 µL of each sample (plant extract, macerate or bacterial suspension) to two wells previously enriched or treated if necessary. Include two wells per plate of positive and negative controls. It is also necessary to include negative controls of extraction buffer and of the enrichment medium used. Incubate 4 h at 37°C or overnight at 4°C.
(4) Wash as before (2).
(5) Prepare the appropriate dilution of the specific antibodies conjugated with alkaline phosphatase (or linked with other enzyme) in PBS. Add 200 µL to each well. Incubate at 37°C for about 2 h.
(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 follow the recommendations of the commercial kit. Add 200 µL of this solution to each well. Incubate at room temperature 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, or as indicated in the protocol.

(iii) DASI ELISA

(1) Prepare the appropriate dilution of polyclonal antibodies in carbonate coating buffer pH 9.6. Add 200 µL to each well of a microtiter plate with good coating characteristics and avoid the borders of the plate for better repeatability. Some techniques are available to prevent border effect such as covering the plate with transparent film to avoid evaporation, in such cases border wells can be used. Incubate at 37°C for 4 h or overnight at 4°C.
(2) Wash the wells gently (e.g. using a wash bottle) with PBS-Tween (Appendix 3) as washing too strongly with some washing apparatus can affect the coating result and, consequently, the sensitivity, especially in detection.
(3) Add 200 µL of each sample (plant extract, macerate or bacterial suspension) to two wells previously enriched or treated if necessary. Include two wells per plate for both the positive and negative controls. It is also necessary to include negative controls of extraction buffer and of the enriched medium used. Incubate for 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 monoclonal) in PBS pH 7.2 (Appendix 3) plus 0.5% bovine serum albumin (BSA) and add 200 µL to each well. Incubate at 37°C for about 2 h.

(6) Wash as before (2).
(7) Prepare the appropriate dilution of the anti-species antibodies (when using monoclonals: anti-mouse immunoglobulins) conjugated with alkaline phosphatase (or linked with another enzyme) in PBS. Add 200 µL to each well. Incubate at 37°C for about 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 follow the recommendations of the commercial kit. Add 200 µL to each well. Incubate at room temperature 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, or as indicated in the protocol.

(iv) Tissue print, squash or colony dot ELISA

Use a nitrocellulose membrane with tables printed by the manufacturer, or cut a rectangular nitrocellulose sheet and draw (using a ruler and pencil) a table with 12 columns and 8 rows on each. Mark the cell rows A–H and the columns 1–12. An ELISA microplate provides a good template.

(1) (a) Prepare smooth, freshly cut pieces of symptomatic plant tissues and press them onto a nitrocellulose membrane to obtain a tissue-print. (b) Directly squash symptomatic plant material or bacterial exudate onto the nitrocellulose membrane. (c) Directly dot the nitrocellulose membrane with a colony or a suspension of the target bacterium. Include as controls positive and negative tissue prints, squashes or pure cultures (as bacterial growth or in suspension), respectively. Let the tissue-prints, squashes, colony dots or blots dry for at least 10 min at room temperature. The imprinted or blotted membranes can be stored in the dark at room temperature for long periods (more than one year) and are durable enough to be mailed, for example, to another laboratory.
(2) Block the nitrocellulose membrane using a solution of 1% bovine serum albumin (BSA) in PBS pH 7.2 (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 the alkaline phosphatase conjugated specific antibodies in PBS 0.01 M, pH 7.2. Add enough solution to cover the membrane in an appropriate container. Incubate for 2 hours at room temperature under slight agitation (100 rpm).
(4) Remove the conjugate and subsequently wash the membrane three times with PBS-Tween (Appendix 3), or with another appropriate buffer, 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) magnification stereo microscope or a magnifying glass.

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 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 for Indirect ELISA, DAS ELISA or DASI ELISA

Interpretation of ELISA tests for identification (pure cultures)

The ELISA test is considered negative if the average absorbance or optical density (OD) reading from duplicate sample wells is <2× OD of that in the negative sample control well, provided that the OD for the positive controls are all above 1.0 (after 120 min incubation with the substrate) and are greater than twice the OD obtained for negative sample extracts.

The ELISA test is considered positive if the average OD reading from each of the duplicate sample wells is ≥2× OD in the negative sample extract well, provided that the OD readings in all negative control wells are <2× those in the positive control wells.

It is recommended that the test is repeated for samples that give a reaction just below the limit of the threshold.

Interpretation of ELISA tests for detection (plant material)

For detection interpretation the OD value of the negative sample extract well should be the basis for determining the thresholds of detection (background) minus the OD of the substrate well.

The positive result is determined on a case-by-case basis depending on the pest and the matrix.

It is recommended that the test is repeated for samples just below the limit of the threshold.

Interpretation for tissue print, squash or dot ELISA

The ELISA test is negative if there is no coloured precipitate in the sample print or dot, provided that the positive control is positive and the negative control is negative.

The ELISA test is positive, if there is purple–violet-coloured precipitate in the sample print or dot, provided that the positive control is positive and the negative control is negative.

Negative ELISA readings in the positive control(s) indicate that the test has not been performed correctly or that problems such as inhibition of antibody-antigen or precipitation occurred. Positive ELISA readings in the negative control indicate a non-specific reaction of the plant material or cross-contaminations.

Appendix 2 – Positive and negative controls

When using a commercial ELISA kit, the following controls should be added in addition to the positive and negative controls provided in the kit:

- a positive control of the same matrix, inoculated or spiked with the target bacterium for tests used for detection or of the target bacterial suspension for tests used for identification
- a negative control from healthy plant, buffer only and, when appropriate, the enrichment buffer for identification, or of the same matrix, in the appropriate buffer for detection.

These positive and negative controls should be checked (preferably in advance) with the same antibodies following the appropriate ELISA procedure.

Controls should be stored as recommended by the supplier, either refrigerated for a few days or at temperatures below −16°C for longer periods.

Positive controls

Prepare separate positive controls of the homologous strain or any other reference strain of the target organism, suspended in healthy host plant extract, as specified below, and in PBS buffer (Appendix 3). It is recommended that reference strains are used as positive controls to avoid misinterpretations due to cross-reactions. Reference strains are commercially available from, e.g.: National Collection of Plant Pathogenic Bacteria (NCPPB), FERA, York, UK; Culture Collection of the Plant Protection Service (PD), Wageningen, the Netherlands; or Collection Française de Bactéries Phytopathogènes (CFBP), INRA Station Phytobactériologie, Angers, France. Naturally infected tissue (maintained by lyophilization or freezing at below −16°C) should be used whenever possible.

Two wells or prints should be prepared per positive controls.

Negative controls

Healthy plant extract (for detection) or a suspension of the heterologous bacterial species (for identification) should be used as negative controls. The healthy plant should be the same species and preferably the same variety and the same plant part at the same growth stage for better comparison with the samples to analyse. Aliquots/extracts of the same host plant which previously tested negative for the target bacterium can be used as negative controls.

Positive and negative controls should be prepared as described below:

(1) Obtain a 48-h culture of a virulent strain of the target bacterium on Nutrient Agar medium or another suitable general medium and suspend in PBS buffer (Appendix 3) to obtain a cell density of approximately 10^8 cells mL^{-1}. This is usually obtained by a faintly turbid suspension equivalent to an optical density of 0.1 at 600 nm.

(2) Prepare sterile 1.5 mL microvials with 900 µL of the plant extract previously tested negative for the target bacterium. Transfer 100 µL of the 10^8 suspension of the bacterium to the first microvial. Vortex. Establish three 10-fold dilutions by further diluting in the next microvials. Transfer 100 µL of suspension buffer to the microvials that do not contain bacteria suspension. Vortex. The microvials which contain bacterial suspension can be used as positive controls and to evaluate the sensitivity of the protocol. The microvials which do not contain bacteria suspension can be used as negative controls.

(3) The presence and quantification of the target bacterium in the control samples should be first confirmed by the appropriate ELISA test.

(4) Prepare aliquots of 100 µL in sterile 1.5 mL microvials thus obtaining repeats of each control sample.

(5) Dilute the contaminated suspensions appropriately to establish levels of contamination of 10^8, 10^7 and 10^6 cells mL^{-1}. Apply a measured standard volume of each of these dilutions and of the negative control on the wells. Whenever possible apply a naturally infected sample and its two 10-fold dilutions.

Appendix 3 – Buffers and substrates for the ELISA test

Double-strength carbonate coating buffer, pH 9.6.	
Na_2CO_3	3.18 g
$NaHCO_3$	5.86 g
Distilled water	1000 mL
Dissolve ingredients and check pH.9.6	
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
NaH_2PO_4 .$2H_2O$	4.0 g
Na_2HPO_4.$12H_2O$	27.0 g
Distilled water	1000 mL
PBS-Tween	
10× PBS	50 mL
10% Tween 20	0.5 mL
Distilled water	950 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	
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.	