Diagnostics
Diagnostic

Indirect immunofluorescence test for plant pathogenic bacteria

Specific scope

This standard describes how to perform an indirect immunofluorescence test (IF) for plant pathogenic bacteria.[1]

Specific approval and amendment

Approved as an EPPO Standard in 2009–09.

Instructions to perform the IF test are given in Appendix 1. A validated source of antiserum or antibodies to the target organism should be used. If validation data are not provided by the supplier of the reagent, then the laboratory should generate these. The titre of the antiserum/antibodies should be determined for each batch. The titre is defined as the highest dilution at which optimum reaction occurs when testing a suspension containing 10^5–10^6 cells per mL of a reference strain of the target organism and using an appropriate dilution of antibody conjugate labelled with a fluorescent dye (e.g. fluorescein or rhodamin) according to the manufacturer's recommendations. The recommended reference strain should preferably be the homologous strain, this is the strain used to produce the antiserum/antibody. Crude polyclonal antisera should, where available, have a minimum titre of 2000. During routine testing, the antiserum/antibodies should be used at a working dilution (WD) close to or at the minimum titre.

The IF test for detection should be performed on freshly-prepared sample extracts. The IF test for identification should be performed on suspensions of pure cultured isolates. If required, the IF test can be successfully performed on extracts stored below −68°C under glycerol (10–25% v/v). Glycerol can be removed from the extract by adding and mixing an equal volume of suspension buffer (Appendix 2) to the extract volume, followed by centrifugation for 15 min at approximately 7000 *g* and re-suspension of the pellet in a volume of suspension buffer equal to the original volume of the extract.

Control slides should be prepared separately according to Appendix 3. The antiserum/antibodies control (= positive control) should be prepared with the homologous strain or another reference strain of the target organism suspended in sample extract which tested negative for the target organism. Cells in suspension buffer, prepared according to Appendix 3, can be used optionally. For this purpose frozen or freeze-dried extracts spiked with the selected strain can be used.

Extract from naturally infected tissue, maintained by lyophilisation or freezing below −16°C, should be used where possible as an additional control on the same slide. This will allow observation of cell morphology in natural infections which can be quite different from cell morphology on culture medium used to prepare spiked extracts.

Aliquots of sample extract which tested negative for the target organism should be used as process control (= negative control) (Appendix 3).

The IF test can also be performed to identify pure cultures of presumptive isolates of the target organism. A suspension of approximately 10^6 cells per mL in IF-buffer (Appendix 2) is prepared and used.

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

Acknowledgements

This test description was originally drafted by Müller P (JKI, DE), Janse J (Dutch General Inspection Service, NL) based on the EU Council Directive on the control of *Ralstonia solanacearum* (EU, 1998) and Commission Directive 2006/63/CE of 14 July 2006 amending Annexes II to VII to Council Directive 98/57/EC on the control of *Ralstonia solanacearum* (Smith) Yabuuchi *et al. Official Journal of the European Communities* (EU, 2006).

References

EU (1998) Council Directive 98/57/EC of 20 July 1998 on the control of *Ralstonia solanacearum.* Annex II-test scheme for the diagnosis, detection and identification of *Ralstonia solanacearum. Official Journal of the European Communities* No. **L235**, 8–39.

EU (2006) Commission Directive 2006/63/CE of 14 July 2006 amending Annexes II to VII to Council Directive 98/57/EC on the control of *Ralstonia solanacearum* (Smith) Yabuuchi *et al. Official Journal of the European Communities* no. **L206**, 36–106.

Appendix 1 – Instructions to perform an IF test

Use multiwell microscope slides with preferably 10 windows of at least 6 mm diameter.

Test control material in an identical manner to the sample(s).

1. Preparation of test slides

Prepare the test slides using one of the following procedures:

1.1. For plant sample extracts with relatively little starch/debris sediment:

Pipette a measured standard volume (15 µL is appropriate for 6 mm window diameter – scale up volume for larger windows) of a 1/100 dilution of the sample extract onto the first window of the IF slide.

Subsequently pipette a similar volume of undiluted sample extract (1/1) onto the remaining windows on the row. The second row can be used as duplicate or for a second sample[2] as presented in Fig. 1A.

1.2 For other sample extracts:

Prepare decimal dilutions (1/10, 1/100) of plant extract in suspension buffer. Pipette a measured standard volume (15 µL is appropriate for 6 mm window diameter – scale up volume for larger windows) of the sample extract and each dilution onto a row of windows. The second row can be used as duplicate or for a second sample[2] as presented in Fig. 1B.

2. Fixation of bacterial cells

Dry the droplets at ambient temperature or by warming (maximum temperature 45°C). Fix the bacterial cells to the slide either by flaming, heating (15 min at a maximum temperature of 60°C), by covering the windows with ethanol (>95%) for 3 min or according to specific instructions from the suppliers of the antibodies.

Slides should be preferably be used as soon as possible but if necessary fixed slides may be stored frozen in a desiccated box (up to a maximum of 3 months) prior to further testing.

3. IF-procedure

3.1 According to test slide preparation in 1.1

Prepare a set of twofold dilutions of the antibody in IF buffer (Appendix 2). The first well should have ½ of the titre (*T*/2), the others ¼ of the titre (*T*/4), ½ of the titre (*T*/2), the titre (*T*) and twice the titre (2*T*).

[2]Take care to avoid cross contamination.

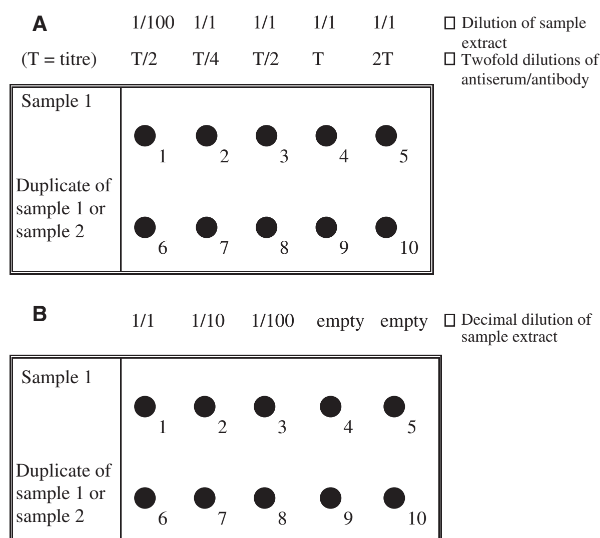

Fig. 1 (A) Dilutions of sample extract, preparation of the test slide according to 1.1 and 3.1. (B) Working dilution of antiserum/antibody, preparation of the test slide according to 1.2 and 3.2.

3.2 According to test slide preparation in 1.2

Prepare the working dilution (WD) of the antibody (close to or at the titre) in IF buffer (Appendix 2).

3.3 Arrange the slides on moist tissue paper. Cover each test window completely with the antibody dilution(s). The volume of antibody applied on each window should be at least the volume of extract applied.

The following procedure should be carried out in the absence of specific instructions from the suppliers of the antibodies.

3.4 Incubate the slides on moist paper under a cover for 30 min (±10 min) at ambient temperature (18–25°C).

3.5 Shake the droplets off each slide and rinse carefully with IF buffer. Wash by submerging for approximately 5 min in IF buffer–Tween (Appendix 2) and subsequently in IF buffer. Avoid causing aerosols or droplet transfer that could result in cross-contamination. Carefully remove excess moisture by blotting gently.

3.6 Arrange the slides on moist paper. Cover the test windows with the dilution of FITC conjugate used to determine the titre. The volume of conjugate applied on the windows should be at least the volume of antibody applied.

3.7 Incubate the slides on moist paper under a cover for 30 min (±10 min) at ambient temperature (18–25°C).

3.8. Shake the droplets of conjugate off the slide. Rinse and wash as before (3.5).

Carefully remove excess moisture.

3.9 Pipette 5–10 µL of 0.1 M phosphate-buffered glycerol (Appendix 2) or a commercial anti-fading mountant on each window or distribute a sufficient amount across the slide, apply a coverslip and avoid exposure of the slides to excess light.

4. Reading the IF test

4.1 Examine test slides on an epifluorescence microscope with filters and light source suitable for excitation of FITC, under oil, glycerol or water immersion at a magnification of 500–1000. Scan windows across two diameters and around the perimeter. For samples showing no or low numbers of cells observe at least 40 microscope fields.

Check the positive control slide first. Cells should be bright fluorescent and the cell wall completely stained at the determined antibody titre or working dilution. The IF test should be repeated if the staining is aberrant.

4.2 Observe for bright fluorescing cells with characteristic morphology of the target organism in the test windows of the test slides. The fluorescence intensity should be equivalent to the positive control strain at the same antibody dilution. Cells with incomplete staining or with weak fluorescence should be disregarded.

If any contamination is suspected the test should be repeated. This may be the case when all slides in a batch show positive cells due to the contamination of buffer or if positive cells are found (outside of the slide windows) on the slide coating.

4.3 There are several problems inherent to the specificity of the immunofluorescence test. Background populations of fluorescing cells with atypical morphology and cross reacting saprophytic bacteria with size and morphology similar to the target organism may occur in the plant or seed sample.

4.4 Consider only fluorescing cells with typical size and morphology at the determined antiserum/antibody titre or at the working dilution.

5. Interpretation of the IF reading

The IF test is positive when fluorescing morphologically typical cells are detected in the pellet suspension or in the sample extract. However, in any case a conclusive result of a latently infected sample cannot be given on the basis of a positive IF test alone. A positive IF result implies a suspected occurrence of the target organism in this sample. The pellet suspension or the sample extract require further analysis with other tests to confirm or refute the IF test result.

The detection threshold of the IF test is usually between 10^3 and 10^4 cells per mL of pellet suspension or sample extract. Usually, IF test positive results at the detection threshold are produced by cross reactive bacterial cells. Calculated contamination levels at the detection threshold should therefore be given careful consideration when testing for latent infections. The IF test at the detection threshold may be regarded as positive and expert judgement is required to decide on what further analysis is required.

It should be noted that cell counting is relevant for the detection of latent populations of the target organisms in samples but not if the IF-test is applied on suspensions of pure cultures, or extracts of infected host tissue (as samples or as controls).

6. Determination of contamination level in an IF test

6.1 Count the mean number of typical fluorescent cells per field of view (c).

6.2 Calculate the number of typical fluorescent cells per microscope slide window (C).

$$C = c \times S/s$$

where S = surface area of window of multispot slide and s = surface area of objective field.

$$s = \pi i2/4G2\mathrm{K}2$$

where i = field coefficient (varies from 8 to 24 depending upon ocular type), K = tube coefficient (1 or 1.25), G = magnification of objective (100×, 40× etc.).

6.3 Calculate the number of typical fluorescent cells per mL of re-suspended pellet (N).

$$N = C \times 1000/y \times \mathrm{F}$$

where y = volume of re-suspended pellet on each window and F = dilution factor of re-suspended pellet.

Appendix 2 – Buffers

Suspension buffer (10 mM phosphate buffer, pH 7.2)

This buffer is used both for resuspension of sample extracts following concentration to a pellet by centrifugation and further dilution of any sample extract

$Na_2HPO_4.12H_2O$	2.7 g
$NaH_2PO_4.2H_2O$	0.4 g
Distilled water	1.0 L

Dissolve ingredients, check pH and sterilise by autoclaving at 121°C for 15 min

IF-Buffer [10 mM phosphate buffered saline (PBS), pH 7.2]

This buffer is used for dilution of antibodies

$Na_2HPO_4.12H_2O$	2.7 g
$NaH_2PO_4.2H_2O$	0.4 g
NaCl	8.0 g
Distilled water	1.0 L

Dissolve ingredients, check pH and sterilise by autoclaving at 121°C for 15 min

IF-buffer–Tween

This buffer is used to wash slides

Add 0.1% Tween 20 to the IF buffer

Phosphate buffered glycerol, pH 7.6

This buffer is used as a mountant fluid on the windows of IF slides to enhance fluorescence

$Na_2HPO_4.12H_2O$	3.2 g
$NaH_2PO_4.2H_2O$	0.15 g

Glycerol	50 mL
Distilled water	100 mL

Anti-fading mountant solutions are commercially available e.g. Vectashield® (Vector Laboratories, Peterborough, GB) or Citifluor® (Leica, Karlsruhe, DE)

Appendix 3 – Preparation of positive and negative control extracts and slides

Prepare separate positive control slides of the homologous strain or any other reference strain of the target organism, suspended in plant extract, as specified below, and optionally in buffer. Naturally infected tissue (maintained by lyophilisation or freezing at a temperature below −16°C) should be used where possible as a similar control on the same slide. As negative controls, aliquots of sample extract which previously tested negative for the target organism can be used. Standard reference strains are recommended for use as positive controls or during optimisation of tests to avoid misinterpretation due to cross-reactions. Reference strains are commercially available from e.g.:

a. National Collection of Plant Pathogenic Bacteria (NCPPB), Central Science Laboratory, York (GB)

b. Culture Collection of the Plant Protection Service (PD), Wageningen (NL).

c. Collection Française de Bactéries Phytopathogènes (CFBP), INRA Station Phytobactériologie, AngersFrance (FR).

1. Produce by incubation for an appropriate period (generally 48-h) a culture of a virulent strain of the target organism on Nutrient Agar medium or another suitable general medium and suspend in suspension buffer (Appendix 2) to obtain a cell density of approximately 10^8 cells per mL. This is usually obtained by a faintly turbid suspension equivalent to an optical density of approximately 0.15 at 600 nm. Process plant material, previously tested negative for the target organism, as usual and resuspend the pellet in 10 mL suspension buffer (Appendix 2).

2. Prepare 10 sterile 1.5 mL microvials with 900 µL of the resuspended pellet (after centrifugation of plant extract) or undiluted plant tissue extract. Transfer 100 µL of the suspension of the target organism to the first microvial. Vortex. Establish decimal levels of contamination by further diluting in the next four microvials. Transfer 100 µL of suspension buffer to the five non contaminated microvials. The five contaminated microvials can be used as positive controls. The five non-contaminated microvials can be used as negative controls. Label the microvials accordingly.

3. The presence and quantification of the target organism in the control samples should be first confirmed by IF.

4. Prepare aliquots of 100 µL in sterile 1.5 mL microvials thus obtaining nine repeats of each control sample. Label the microvials accordingly. Store at a temperature below −16°C until use.

5. An IF-control slide is prepared as follows: Dilute the contaminated suspensions appropriately to establish levels of contamination of approximately 1×10^6, approximately 1×10^4 and approximately 5×10^3 cells per mL. Apply a measured standard volume of each dilution and of the negative control on the control slide (15 µL is appropriate for 6 mm window diameter – scale up volume for larger windows; but it should be the same as for the sample to be tested). In addition apply (if available) naturally infected sample extract and two 10-fold dilutions thereof on the slide. The 1×10^6 dilution step and the naturally infected sample extract could be used as negative conjugate controls (NC). Preferably apply the controls on the slide as shown in the Fig. 2.

Perform assays on positive and negative control samples with each series of test samples. The target organism must be detected in the positive controls and not in any of the negative controls.

Control slide					
	● NC 1.10^6	● 1.10^6	● 1.10^4	● 5.10^3	● negative
	● NC naturally infected	● naturally infected	● naturally infected 1:10	● naturally infected 1:100	● empty

NC: negative conjugate controls

Fig. 2 Application of the positive and negative controls on the control slide.