International Rules for Seed Testing 2025

Validated Seed Health Testing Methods

7-029: Detection of *Pseudomonas syringae* pv. *pisi* in *Pisum sativum* (pea) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2024 in Cambridge, United Kingdom

Effective from 1 January 2025

Validation reports

See References. Copies are available by e-mail from the ISTA Secretariat at ista.office@ista.ch.

Please send comments, suggestions or reports of problems relating to this method to the ISTA Seed Health Committee, c/o ISTA Secretariat.

Disclaimer

Whilst ISTA has taken care to ensure the accuracy of the methods and information described in this method description, ISTA shall not be liable for any loss or damage, etc. resulting from the use of this method.

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during weighing out of ingredients. It is assumed that persons carrying out this test are in a laboratory suitable for carrying out microbiological procedures and familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic techniques. Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Note on the use of the translations

The electronic version of the International Rules for Seed Testing includes the English, French, German and Spanish versions. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.

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7-029: Detection of *Pseudomonas syringae* pv. *pisi* in *Pisum sativum* (pea) seed

Host: *Pisum sativum* L.

Pathogen(s): *Pseudomonas syringae* pv. *pisi* (Sack.) Young, Dye & Wilkie

Prepared by: International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg)

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Revision history

Version 1.0, 2014-01-01
Version 1.1, 2017-01-01: Method: steps 6.1 & 6.2 modified; Reporting results revised
Version 1.2, 2021-01-01: Sample size revised
Version 1.3, 2024-01-01: Sample size, Methods and General methods revised
Version 1.4, 2025-01-01: Host, Materials, Methods and General methods revised

Background

Pseudomonas syringae pv. *pisi* (*P. syringae* pv. *pisi*), causal organism of bacterial blight on pea seeds (Grondeau *et al.*, 1993), is a seed-borne (Hollaway *et al.*, 2007) and seed-transmitted (Grondeau *et al.*, 1993; 1996; Roberts *et al.*, 1992, 1996) bacterial pathogen. Several studies on the characterisation of *P. syringae* pv. *pisi* (Grondeau *et al.*, 1996; Elvira-Recuenco & Taylor, 2001) and distinction between the pv. *syringae* and pv. *pisi* (Malandrin & Samson, 1998) have been conducted for identification purposes and for the development of tests for the *P. syringae* pv. *pisi* detection on pea seed (Lyons & Taylor, 1990; Fraaije *et al.*, 1993). The serological assays can not provide information on the bacterium's viability and pathogenicity (Schaad, 1982). Therefore, the available methods in use by seed health laboratories are based on seed wash-dilution-plating assays on semi-selective media (Fraaije *et al.*, 1993; Grondeau *et al.*, 1993; Mohan & Schaad, 1987) and confirmation of suspect colonies by a pathogenicity test (Grondeau *et al.*, 1992; Malandrin & Samson, 1998).

This method for the detection of *P. syringae* pv. *pisi* on untreated pea seeds has been validated in a comparative test organised by the International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg) with results of seven laboratories. It includes a seed wash-dilution-plating on the KBBCA and SNAC semi-selective media, optional biochemical tests on suspect colonies and a pathogenicity test for their confirmation. The biochemical tests allow for a reduced number of *P. syringae* pv. *pisi* suspects to be confirmed and subsequently for reduced time and labour in the pathogenicity test. The two pathogenicity test methods provide the user with a higher flexibility to operate in different laboratory conditions.

Treated seed

Seed treatments may affect the performance of this test. This method was not validated on treated seed. (Definition of treatment: any process, physical, biological or chemical, to which a seed lot is subjected, including seed coatings. See 7.2.3.)

Sample size

The sample size (total number of seeds to be tested) and subsample size depend on intended use, the maximum acceptable infection level and the analytical sensitivity of the method. The minimum recommended working sample size is 5000 seeds and the maximum subsample size must be 1000 seeds.

Materials

Reference material: known strain of *Pseudomonas syringae* pv. *pisi* or standardised reference material

Plates of KBBCA medium: 90 mm Petri dishes (6 plates of each medium per subsample + controls)

Plates of SNAC medium: 90 mm Petri dishes (6 plates of each medium per subsample + controls)

Polythene bags or containers: with sterile saline (0.85 % NaCl) for soaking of seeds (2.5 ml per gram of seed)

Cold room or refrigerator: operating at 4 ±3 °C

Dilution bottles: containing 4.5 ml of sterile saline (2 per subsample). Other volumes may be acceptable; see General methods

Automatic pipettes: check accuracy and precision regularly

Sterile pipette tips

Sterile bent glass rods (or equivalent)

70 % ethanol or an equivalent disinfecting product

Balance: capable of weighing to the nearest 0.001 g

Incubator: operating at 28 ±2 °C

UV lamp (365 nm): to check fluorescence

Materials for oxidase tests: 1 % aqueous N,N-dimethyl paraphenylene diamine oxalate solution or ready-to-use tests

Pea seedlings: susceptible to all races of the pathogen for pathogenicity test (e.g. 'Kelvedon Wonder')

Module/growth chamber: capable of operating/maintaining temperature at 20 ±2 °C)

Greenhouse: capable of operating/maintaining temperature at 20–25 °C

Sample preparation

This can be done in advance of the assay.

It is vital to exclude any possibility of cross-contamination between seed samples. It is therefore essential to disinfect all surfaces, containers, hands, etc. both before and after handling each sample. This can be achieved by swabbing/spraying equipment and gloved hands with 70 % ethanol.

1. Count the number of seeds in a known weight. Estimate the thousand-seed weight (TSW) as:
   TSW = (weight of seeds / number of seeds) × 1000
2. Based on the estimated TSW, weigh out subsamples of the required size into new, clean polythene bags or containers.

Methods

Critical control points are indicated by CCP.

1. Extraction

1.1 Suspend seeds of each subsample in sterile saline in a polythene bag or container. The volume of the sterile saline should be adjusted according to the number of seeds used (2.5 ml per gram of seeds).

1.2 Soak the subsamples overnight (18–24 h) at 4 °C (±3 °C) under agitation.

2. Dilution and plating

2.1 Shake by hand the polythene bags or containers to obtain a homogenous extract before dilution.

2.2 Prepare two tenfold dilution series from each seed extract. Pipette 0.5 ml of the extract into 4.5 ml of sterile saline and vortex to mix (10^{-1} dilution). Pipette 0.5 ml of the 10^{-1} dilution into another 4.5 ml of sterile saline and vortex to mix (10^{-2} dilution)(see General methods).

2.3 Pipette 100 µl of each dilution and the undiluted seed extract onto two plates of each of the semi-selective media (KBBCA and SNAC) and spread over the surface with a sterile bent glass rod or equivalent (see General methods).

2.4 Incubate inverted plates at 28 ±2 °C and examine after 4–5 days.

3. Positive control (culture or reference material)

3.1 Prepare a suspension of a known strain of *Pseudomonas syringae* pv. *pisi* in sterile saline or reconstitute standardised reference material according to the supplier's instructions.

3.2 Dilute the suspension sufficiently to obtain dilutions containing approximately 10^{-2} to 10^{-4} colony-forming units (cfu) per millilitre. This may require up to seven ten-fold dilutions from a turbid suspension.

3.3 Pipette 100 µl of appropriate countable dilutions onto plates of each of the semi-selective media (KBBCA, SNAC) and spread over the surface with a sterile bent glass rod or equivalent (see General methods).

3.4 Incubate plates with the sample plates.

4. Sterility check

4.1 Prepare a dilution series from a sample of the extraction medium (i.e. sterile saline) containing no seeds, and plate on each of the semi-selective media as for samples.

5. Examination of the plates

5.1 Examine sterility check and recovery of positive control on both semi-selective media (KBBCA, SNAC)(CCP).

5.2 Examine the sample plates for the presence of typical *P. syringae* pv. *pisi* colonies by comparison with the positive control plates. If necessary, estimate the number of cfu.

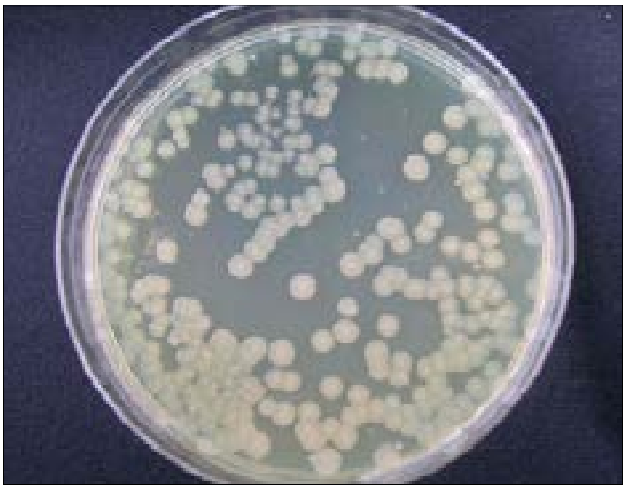

Figure 1. Plate of KBBCA medium after 4 days of incubation at 28 ±2 °C showing typical colonies of *P. syringae* pv. *pisi*.

Figure 2. Plate of SNAC medium after 4 days of incubation at 28 ±2 °C showing typical colonies of *P. syringae* pv. *pisi* that are levan positive.

5.3 After 4 days on KBBCA, *P. syringae* pv. *pisi* colonies are creamy and half-translucent (Fig. 1).

5.4 After 4 days on SNAC, *P. syringae* pv. *pisi* colonies are circular, white to transparent, mucoid, dome-shaped and levan positive. (Fig. 2).

5.5 The colony size and colour can differ within a sample.

5.6 Record the presence of suspect colonies (see General methods). If necessary, estimate the number of cfu of suspect and other colonies.

6. Confirmation/identification of suspect colonies

6.1 Pick up at least six suspect colonies, if present, per subsample grown on KBBCA medium and subculture on sectored plates of SNAC medium (CCP).

6.2 Pick up at least six suspect colonies, if present, per subsample grown on SNAC medium and subculture on sectored plates of KBBCA medium (CCP).

6.3 Repeat with the positive control colonies. Subculture on a sectored plate of SNAC medium two colonies grown on KBBCA and subculture on a sectored plate of KBBCA medium two colonies grown on SNAC.

6.4 Incubate sectored plates at 28 ±2 °C for 2–3 days.

6.5 Check colonies subcultured on SNAC medium for levan production. *P. syringae* pv. *pisi* colonies are levan positive (Fig. 2). Compare with the positive control.

6.6 Check colonies subcultured on KBBCA medium for blue fluorescence, under UV light and/or for the typical morphology (optional step; it can decrease the number of suspect colonies). There is a variation in the genus and some *P. syringae* pv. *pisi* produce a blue fluorescent pigment under UV light whereas others do not (Fig. 3). As both types of pathogen colonies may be present, it is necessary to make a comparison with a positive control strain on the same media.

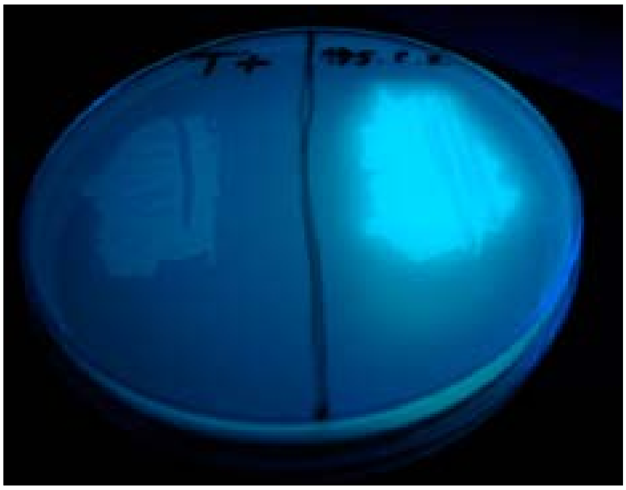

Figure 3. Fluorescent and non-fluorescent *P. syringae* pv. *pisi* isolates under UV light.

6.7 Identify suspect colonies subcultured on both media with an oxidase test (optional step; it can decrease the number of suspect colonies). Use ready-to-use tests (e.g. Bactident Oxidase Merck, 1.13300.0001) or put a drop of 1 % aqueous N,N-dimethyl paraphenylene diamine oxalate solution on a filter paper. Add quickly

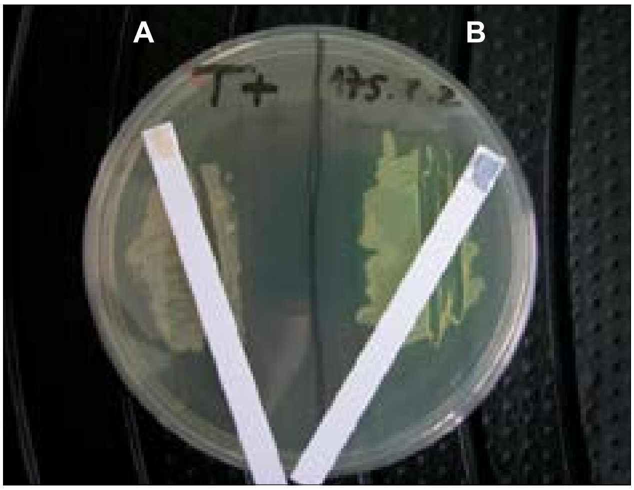

Figure 4. Oxidase-negative positive control isolate (A) and oxidase positive non *P. syringae* pv. *pisi* isolate (B).

Figure 5. Typical greasy lesions on the stem of a pea seedling 'Kelvedon Wonder', 9 days after inoculation following pathogenicity assay Option 1.

a smear from a suspect bacterial colony on the filter paper and make a bacterial emulsion. *P. syringae* pv. *pisi* colonies are oxidase negative (no cytochrome C oxidase): no red staining (Fig. 4). Compare to the positive control.

6.8 Record results for each subcultured colony.

6.9 All oxidase-negative, typical fluorescent or non-fluorescent colonies on KBBCA and all oxidase-negative colonies that produce levan on SNAC are considered suspect colonies.

6.10 Confirm the identity of all the suspect colonies by a pathogenicity assay on pea seedlings of known susceptibility (CCP).

7. Pathogenicity assay (option 1)

7.1 Germinate seeds of a pea cultivar known to be susceptible to all races of *P. syringae* pv. *pisi* (e.g. 'Kelvedon Wonder') in a wet blotter paper. Roll the paper with the seeds and place it in a plastic bag. Incubate the closed bag at room temperature (18–20 °C) for 2–4 days to allow for seed germination. Make sure to germinate enough seeds for all the suspect colonies that will be tested.

7.2 Prepare a suspension in sterile demineralised water of 24–48 h suspect bacterial culture on KBBCA and SNAC and dilute to a concentration of 10^8 cfu/ml.

7.3 Repeat with a 24–48 h positive control culture to get a concentration of 10^8 cfu/ml (CCP).

7.4 Cut the root tips of 2-day-old germinated pea seeds and incubate 3 seeds in each bacterial suspension for 15 min.

7.5 Repeat with incubation of 3 pea seeds in sterile demineralised water to serve as negative control.

7.6 Remove seeds from bacterial suspension and sow them in a labelled potting substrate or equivalent. Incubate at 20 ±5 °C) with 12 h light/12 h dark or 16 h light/8 h dark and 100 % saturating humidity.

7.7 Examine seedlings for typical greasy lesions on stems and leaflets after 5–9 days (Fig. 5). Compare with positive and negative controls (CCP).

7.8 Record the suspect colonies as positive if greasy lesions are observed.

8. Pathogenicity assay (option 2)

8.1 Grow seedlings of a pea cultivar known to be susceptible to all races of *P. syringae* pv. *pisi* (e.g. 'Kelvedon Wonder') in small pots or containers with potting soil at 20–25 °C with sufficient light until the 2 true leaves stage (approx. 8–10 days after sowing).

8.2 Prepare a suspension in sterile demineralised water of a 24–48 h suspect bacterial culture grown on KBBCA and SNAC and dilute to a concentration of 10^6 cfu/ml (CCP).

8.3 Repeat with a 24–48 h positive control culture to get a concentration of 10^6 cfu/ml (CCP).

8.4 Inject each bacterial suspension with a syringe and needle in the stem of at least 2 pea seedlings (2 seedlings per suspect colony).

8.5 Repeat injection with sterile demineralised water in the stem of 2 pea seedlings to serve as negative control.

8.6 Incubate the inoculated seedlings at 20 ±5 °C with saturating humidity.

8.7 Examine seedlings for extended greasy lesions from the point of inoculation after 5–9 days. Compare with positive and negative controls (CCP).

8.8 Record the suspect colonies as positive if greasy lesions are observed.

General methods

Preparation of ten-fold dilution series: Each dilution should be prepared by pipetting 0.5 ml (±5 %) from a well-mixed seed extract or previous dilution into a universal bottle (screw-capped) or similar container containing 4.5 ml (±2 %) of sterile diluent and then vortexing to mix prior to the next dilution step. A new sterile pipette tip should be used for each dilution step. Pipettes should be checked regularly for accuracy and precision and re-calibrated as necessary. It is acceptable to prepare ten-fold dilutions using other volumes provided that the laboratory can demonstrate that the required accuracy and precision can be achieved.

Plating of dilutions: This should be done as soon as possible after dilutions have been prepared and certainly within 30 min. Working from the highest (most dilute) dilution to the undiluted extract, 0.1 ml is pipetted onto the centre of a surface-dry, labelled agar plate. The liquid should then be spread evenly over the entire surface of the medium with a bent glass rod. If care is taken to work from the highest to the lowest dilution (or undiluted extract) a single pipette tip and a single bent glass rod can be used for each sample. Ensure that all liquid has been absorbed by the agar before inverting and incubating plates. If necessary allow plates to dry under a sterile air-flow in a microbiological safety cabinet or laminar flow hood.

Sectored plates: Using a laboratory marker pen, draw lines on the base of a standard 90 mm plate (Petri dish) to divide it into six equal sectors. Subculture single colonies from dilution plates and make a single zigzagged streak within a single sector on the plate. Take care to leave sufficient space between each isolate to ensure the growth does not coalesce. Thus six suspect colonies can be subcultured to each sectored plate. Separate plates should be used for each sample/subsample. If the purity of subcultured isolates is doubtful, they should be further streaked out on whole plates.

Reporting results: The result of a seed health test should indicate the scientific name of the pathogen detected and the test method used. When reported on an ISTA Certificate, results are entered under ‘Other Determinations’.

The report must indicate the number of seeds tested.

In the case of a negative result (pathogen not detected in any subsample), the results must be reported as ‘not detected’.

In the case of a positive result, the report must indicate the number of positive subsamples out of the total number tested. The number of cfu can be indicated.

Recording of dilution plates: Record the results for all dilution plates. The most accurate estimate of bacterial numbers should be obtained from spread plates with a total number of between 30 and 300 colonies. However, this may be further complicated depending on the relative numbers of suspect pathogen colonies and other colonies. To minimise effort, start recording with the highest dilution (most dilute) and count the number of suspect colonies and the number of other colonies. If the total number of colonies on a plate greatly exceeds 300 there is little value in trying to make a precise count if a more reliable count has already been obtained from a more dilute plate. In this case, it is sufficient to record the number of colonies as ‘m’ (many) if they are still separate or ‘c’ (confluent) if they have run together.

Quality assurance

A record should be kept of the date and results of pipette calibration checks.

It is essential that operators have received appropriate training and use automatic pipettes correctly.

Critical control points (CCP)

- Dilution plates prepared from positive control isolate(s) or reference material, should give single colonies with typical morphology (Step 5.1).
- The numbers of colonies on dilution plates prepared from the positive control isolate(s) or reference material should be similar on both media (Step 5.1).
- Numbers of bacteria on dilution plates should be consistent with the dilution (i.e. should decrease approx. tenfold with each dilution) (Step 5.1).
- There should be no growth on dilution plates prepared as a sterility check (Step 5.1).
- Due to the potential for non-pathogenic isolates to be present in seed lots together with pathogenic isolates, it is essential to subculture, if present, at least the minimum number of suspect colonies specified (two per subsample and per semi-selective medium)(Steps 6.1, 6.2) and to test all *Pseudomonas*-like subcultured isolates for pathogenicity (Step 6.10).
- In Pathogenicity Test option 2, the bacterial suspension of suspect and positive control colonies must not have a concentration higher than 10^6 cfu/ml (Steps 8.2 and 8.3). If the concentration exceeds the 10^6 cfu/ml, then the risk of not having typical symptoms on

seedlings increases and in this case the test will not be considered accurate.

- Positive control isolates (Steps 7.3 and 8.3) and inoculations with sterile demineralised water (Steps 7.5 and 8.5) should be included in every pathogenicity test.
- The positive control isolate should give typical symptoms (Steps 7.7 and 8.7) and the negative control should give no symptoms in the pathogenicity test.
- The activity units per gram of some antibiotics may vary between batches. It may be necessary to adjust the weight or volume added to ensure that the final number of units per litre of medium is consistent (KBBCA and SNAC media).

Media and solutions

Sterile saline

Sodium chloride (NaCl): 8.5 g
Distilled/deionised water: 1000 ml

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add 1000 ml of distilled/deionised water.
3. Dissolve and dispense into the final containers.
4. Autoclave at 121 °C, 15 psi for 15 min.

Storage

Provided containers are tightly closed, may be stored for several months before use.

KBBCA medium

Proteose peptone (e.g. #3 Difco): 20.0 g
Glycerol: 10.0 g
K_2HPO_4: 1.5 g
$MgSO_4$ anhydrous: 0.73 g
H_3BO_3: 1.5 g
NaOH (1N): 2.0 ml
Agar: 15 g
Distilled/deionised water: 1000 ml
Cycloheximide[a]: 100.0 mg
Cephalexin monohydrate[b]: 40.0 mg

[a, b] Added after autoclaving. Antibiotic amounts for guidance only (CCP).
[a] Dissolve 500 mg of cycloheximide in 10 ml 70 % ethanol. Add 1 ml/l.
[b] Dissolve 800 mg of cephalexin monohydrate in 10 ml 70 % ethanol. Add 1 ml/l.

Filter sterilise when the antibiotics are dissolved in water rather than 70 % ethanol.

Note: Nystatin could be used as an alternative for cycloheximide to control fungi. Dissolve 350 mg of nystatin in 10 ml 70 % ethanol, add 1 ml to cool medium.

Preparation

1. Weigh out all ingredients except the antibiotics into a suitable container.
2. Add 1000 ml of distilled/deionised water.
3. Stir to dissolve.
4. Autoclave at 121 °C, 15 psi for 15 min.
5. Prepare the antibiotic solutions and filter sterilise as appropriate.
6. Allow the medium to cool to approximately 50 °C before adding the antibiotic solutions.
7. Mix the molten medium gently to avoid air bubbles and pour the plates (18 ml per 90 mm plate).
8. Leave the plates to dry in a laminar flow bench or similar before use.

Storage

Store prepared plates inverted in polythene bags at 4–8 °C and use within four weeks of preparation to ensure activity of antibiotics.

SNAC medium

Tryptone: 5.0 g
Peptone: 3.0 g
NaCl: 5.0 g
Sucrose: 50.0 g
H_3BO_3: 10 ml (0.1 g/ml)
Agar: 15 g
Distilled/deionised water: 1000 ml
Cephalexin monohydrate[a]: 80.0 mg
Nystatin[b]: 35.0 mg

[a, b] Added after autoclaving. Antibiotic amounts for guidance only (CCP).
[a] Dissolve 800 mg of cephalexin monohydrate in 10 ml 70 % ethanol. Add 1 ml/l.
[b] Dissolve 350 mg of nystatin in 10 ml 70 % ethanol. Add 1 ml/l.

Filter sterilise when antibiotics are dissolved in water rather than 70 % ethanol.

Preparation

1. Weigh out all ingredients except the agar and antibiotics into a suitable container.
2. Add 1000 ml of distilled/deionised water.
3. Stir to dissolve.
4. Autoclave at 121 °C, 15 psi for 15 min.
5. Prepare the antibiotic solutions.
6. Allow medium to cool to approximately 50 °C and add the antibiotic solutions.
7. Mix the molten medium gently to avoid air bubbles and pour the plates (18 ml per 90 mm plate).
8. Leave the plates to dry in a laminar flow bench or similar before use.

Storage

Store prepared plates inverted in polythene bags at 8 ±2 °C) and use within four weeks of preparation to ensure activity of antibiotics.

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