International Rules for Seed Testing 2024

Validated Seed Health Testing Methods

7-019b: Detection of *Xanthomonas campestris* pv. *campestris* in disinfested/disinfected *Brassica* spp. seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2023 in Verona, Italy

Effective from 1 January 2024

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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7-019b: Detection of *Xanthomonas campestris* pv. *campestris* in disinfested/disinfected *Brassica* spp. seed

Host: *Brassica* spp.
Pathogen(s): *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson

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: Materials – numbers of Petri dishes for media deleted; Methods steps 2.5 & 2.6 revised; Reporting results revised
Version 1.2, 2018-01-01: Reference to the addition of TaqMan assay as a third option for suspect screening in method 7-019a
Version 1.3, 2021-01-01: Safety precautions added; Sample size revised
Version 1.4, 2024-01-01: Sample size, Methods and General methods revised

Background

The ISTA Rule 7-019 has been developed and validated for the detection of *Xanthomonas campestris* pv. *campestris* (*Xcc*) on *Brassica* spp. untreated seed. Hot water treatment (HWT) and related proprietary treatments against *Xcc* are a common practice to treat *Brassica* spp. seed lots found to be pathogen positive. To monitor the efficacy of such treatments, the seed lots are retested for viable cells of *Xcc*. The ISTA Rule 7-019 involves only seed soaking for pathogen extraction. This relatively mild extraction of the pathogen from whole seeds does not allow for the detection of internally located *Xcc* cells which might have survived the treatment. To facilitate detection, seed must be ground to extract the internally located cells that have a better chance of surviving the treatment than bacteria located on the seed surface. A modification of the ISTA Rule 7-019, involving wet grinding of the disinfested/disinfected seed, has been developed and validated in a comparative test between eight laboratories organised by the International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg). Wet grinding strongly enhances the extraction and detection of viable *Xcc* located internally in disinfested/disinfected seed. Other changes to the ISTA Rule 7-019 consist of the use of phosphate buffered saline (PBS), rather than saline, as well as a larger ratio of buffer to the seed. This avoids a reduction of the *Xcc* recovery due to a suboptimal pH, especially with certain proprietary treatments (Koenraadt *et al.*, 2007). Other changes include concentration of the seed extract by centrifugation, a longer incubation time of the plated extracts to increase the sensitivity of the assay, modifications of the semi-selective media mCS20ABN and mFS, and modification of the pathogenicity test.

Safety precautions

Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Ethidium bromide

Ethidium bromide is carcinogenic. If possible, use an alternative chemical e.g. Gel Red™ (Biotium). Use ethidium bromide according to safety instructions. It is recommended to work with solution instead of powder. Some considerations are mentioned below.

- Consult the Material Safety Data Sheet on ethidium bromide before using the chemical.
- Always wear personal protective equipment when handling ethidium bromide. This includes wearing a lab coat, nitrile gloves and closed toe shoes.
- Leave lab coats, gloves, and other personal protective equipment in the lab once work is complete to prevent the spread of ethidium bromide or other chemicals outside the lab.
- All work with ethidium bromide is to be done in an 'ethidium bromide' designated area in order to keep ethidium bromide contamination to a minimum.

Ultraviolet light

Ultraviolet (UV) light must not be used without appropriate precautions. Ensure that UV protective eyewear is utilised when working with ethidium bromide.

Treated seed

This test method is suitable for seed that has been treated using physical (hot water) or chemical (chlorine) or proprietary processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the reliability of the assay. This test method has not been validated for seed treated with protective chemicals or biological substances.

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 sample size should be 30 000 seeds and the maximum subsample size should be 10 000 seeds.

Materials

Reference material: known strain of *Xanthomonas campestris* pv. *campestris* (*Xcc*) or standardised reference material
Plates of mFS medium: 90 mm Petri dishes
Plates of mCS20ABN medium: 90 mm Petri dishes
Plates of YDC (yeast dextrose chalk) medium: for subculture (at least 1 per subsample)
Conical flasks: with sterile PBS (0.05 M phosphate) plus Tween™ 20 (0.02 % v/v) for soaking of seeds (25 ml per 1,000 seeds)
Orbital shaker
Grinder: e.g. Ultra Turrax with S25N-25G dispersion tool or equivalent
Filter bags: e.g. bag filter model P 400 ml (Interscience, France) or universal extraction bag model with synthetic intermediate layer (Bioreba, Switzerland) or filter extraction bags (Neogen Europe, Scotland) for filtering coarse particles from extracts
Centrifuge: e.g. capable of providing centrifugal force of 5000× *g*
Dilution bottles: containing 4.5 ml of sterile PBS (0.05 M phosphate) plus Tween™ 20 (0.02 % v/v) (1 per subsample). Other volumes may be acceptable, see General methods
Automatic pipettes: check accuracy and precision regularly
Sterile pipette tips
Sterile bent glass rods
70 % ethanol: for disinfection of surfaces, equipment
Balance: capable of weighing to the nearest 0.001 g
pH meter: capable of being read to the nearest 0.01 pH unit
Incubator: capable of operating at 28–30 °C)
***Brassica* spp. seedlings:** susceptible to all races of the pathogen for pathogenicity test (e.g. *B. oleracea* 'Wirosa')

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 PBS (0.05 M phosphate) plus Tween™ 20 (0.02 % v/v) in a conical flask or equivalent container. The volume should be adjusted according to the number of seeds used (25 ml per 1000 seeds)(CCP**).**
1.2 Shake for 2.5 h at room temperature (20–25 °C) on an orbital shaker set at 100–125 rpm.
1.3 Grind the seeds with a grinder until all seeds are completely ground. This point should be reached in at most 2 min of grinding. If not, select alternative grinding equipment. Depending on the type of grinder used, disinfect properly between subsamples and samples to avoid any cross-contamination (CCP).
2. Dilution and plating
2.1 Filter coarse particles from the seed extract, using a bag filter model P 400 ml (InterScience, France), universal extraction bag model with synthetic intermediate layer (Bioreba, Switzerland) or filter extraction bags (Neogen Europe, Scotland) (Fig. 1).

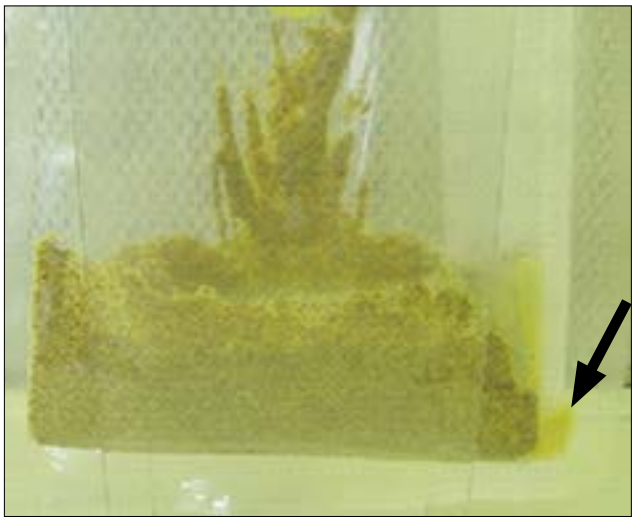

Figure 1. Extracts of ground cabbage seeds in a lateral filter bag. The lateral filter is used to remove coarse particles from the crude seed extract (see arrow).

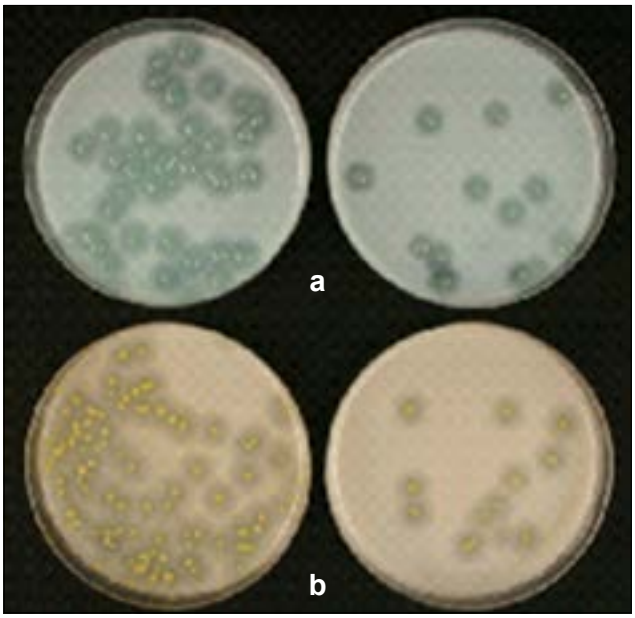

Figure 2. Plates of mFS (**a**) and mCS20ABN (**b**) after 5 days of incubation at 28 °C showing typical colonies of *Xanthomonas campestris* pv. *campestris* surrounded by zones of starch hydrolysis.

2.2 Transfer 3.5 ml of the filtered seed extract to a tube and keep the samples on ice. This 3.5 ml sample must be used to prepare a tenfold dilution (use 0.5 ml) and a tenfold concentration (use 2 ml). The remaining sample (1 ml) must be used to plate the undiluted seed extract.

2.3 Prepare a tenfold dilution (10^{-1} dilution) from the filtered seed extract. Pipette 0.5 ml of the extract into 4.5 ml of sterile PBS (0.05 M phosphate) plus Tween™ 20 (0.02 % v/v) and vortex to mix (10^{-1} dilution)(see General methods).

2.4 Prepare a tenfold concentrated extract (10^{1} concentration) by centrifugation of 2 ml sample for 5 min at 5000× *g*. Carefully remove the supernatant and re-suspend the pellet in 200 µl of sterile PBS (0.05 M phosphate) plus Tween™ 20 (0.02 % v/v).

2.5 Pipette 100 µl of the tenfold dilution (10^{-1} dilution) and then the undiluted seed extract onto plates of each of the selective media (mFS and mCS20ABN) and spread over the surface with a sterile bent glass rod (see General methods).

2.6 Pipette 100 µl of the tenfold concentrated seed extract (10^{1} concentration) onto plates of each of the selective media (mFS and mCS20ABN) and spread over the surface with a sterile bent glass rod (see General methods).

2.7 Incubate plates at 28–30 °C upside down and examine after 4–6 days **(**CCP**).**

3. Positive control (culture or reference material)

3.1 Prepare a suspension of a known strain of *Xcc* in sterile PBS (0.05 M phosphate) plus Tween™ 20 (0.02 % v/v) or reconstitute standardised reference material according to the supplier's instructions.

3.2 Dilute the suspension sufficiently to obtain dilutions containing approx. 10^{-2} to 10^{-4} cfu/ml. This may require up to seven tenfold dilutions from a turbid suspension.

3.3 Pipette 100 µl of appropriate countable dilutions onto plates of each of the selective media (mFS, mCS20ABN) and spread over the surface with a sterile bent glass rod.

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. PBS plus Tween™ 20), containing no seeds, and plate on each of the media as for samples.

5. Examination of the plates

5.1 Examine sterility check and positive control plates (CCP).

5.2 Examine the sample plates for the presence of typical *Xcc* colonies by comparison with the positive control plates.

5.3 On mFS after 4–6 d, *Xcc* colonies are small, pale green, mucoid and surrounded by a zone of starch hydrolysis. This zone appears as a halo that may be easier to see with a black background (Fig. 2a).

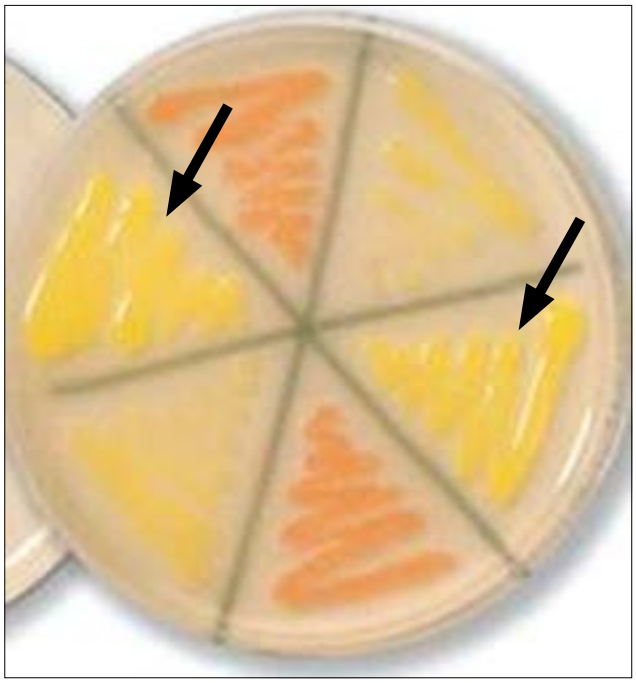

Figure 3. Typical yellow mucoid growth of isolates of *Xanthomonas campestris* pv. *campestris* on a sectored plate of YDC after 3 days at 28 °C. Only suspect cultures are indicated by arrows.

Figure 4. Cabbage leaves 7 days post-inoculation with *Xanthomonas campestris* pv. *campestris*. Typical symptoms are black veins, wilting and chlorosis. The lower left leaf was used as a negative control.

5.4 After 4–6 d on mCS20ABN, *Xcc* colonies are yellow, mucoid and surrounded by a zone of starch hydrolysis (Fig. 2b).

5.5 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 Subculture suspect colonies to sectored plates of YDC. To avoid the potential for cross-contamination of isolates, use a new sectored plate for each subsample. The precise numbers of subcultured colonies will depend on the number and variability of suspect colonies on the plate: if present at least six suspect colonies should be subcultured per subsample (CCP).

6.2 Subculture the positive control isolate to a sectored plate for comparison.

6.3 Incubate sectored plates for 3–4 d at 28–30 °C.

6.4 Compare appearance of growth with positive control. On YDC, *Xcc* colonies are yellow and mucoid (Fig. 3) (CCP).

6.5 Confirm the identity of isolates by pathogenicity on *Brassica* seedlings of known susceptibility or by polymerase chain reaction (PCR) (CCP).

6.6 Record results for each colony subcultured.

7. Pathogenicity assay

7.1 Grow seedlings of a *Brassica* cultivar known to be susceptible to all races of *Xcc* (e.g. 'Wirosa'; see Vicente *et al.*, 2001) at 20–30 °C (±2 °C) in small pots or modules until at least 2–3 true leaf stage.

7.2 Scrape a small amount of bacterial growth directly from a 24–48 h YDC culture (e.g. sectored plate) with a sterile cocktail stick or insect pin.

7.3 Inoculate the secondary veins of the first two true leafs by stabbing with the cocktail stick or insect pin.

7.4 Inoculate 2–4 plants per isolate.

7.5 Inoculate seedlings with the positive control isolate and stab with a sterile cocktail stick or insect pin as a negative control (CCP).

7.6 Grow on plants at 20–30 °C.

7.7 Examine plants for the appearance of typical progressive V-shaped, yellow/necrotic lesions with blackened veins after 10–14 days (Fig. 4). Symptoms may be visible earlier depending on temperature and the aggressiveness of the isolate. Compare with positive control (CCP). It is important to discriminate between the progressive lesions caused by the vascular pathogen *Xcc* and the limited dark necrotic lesions at the inoculation site caused by leaf spot *Xanthomonas*, classified as *X. c.* pv. *raphani* (see Kamounv *et al.*, 1992; Alvarez *et al.*, 1994; Tamura *et al.*, 1994; Vicente *et al.*, 2001; Roberts *et al.*, 2004).

8. PCR test (CCP)

8.1 Follow PCR option 1, PCR option 2 or PCR option 3 described in the ISTA Rule 7-019a to confirm the suspect isolates.

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 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.

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 total number between 30 and 300 colonies. However this may be further complicated depending on the relative numbers of suspect pathogen and other colonies. In order to minimise effort, start recording with the highest dilution (most dilute) and count the number of suspect 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 which 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.

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.

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)

- Dry grinding of seed followed by the addition of an extraction buffer has found to be inadequate (Step 1.1).
- In case an Ultra Turrax grinder is used to grind the seed, the grinder should be run in hot water and/or 70 % ethanol and subsequently in sterile water to prevent any cross-contamination between subsamples. To achieve complete sterilisation between samples the S25N-25G dispersion tool of the grinder has to be autoclaved or disassembled and the parts immersed in 70 % ethanol (Step 1.3).
- The time between grinding and plating of the corresponding suspensions must be kept under 1 hour (Step 1.3).
- Due to the exposure to harsh conditions during the seed treatment the initial multiplication time of *Xcc* cells is longer than for *Xcc* cells from untreated seeds or cells of the positive control. To obtain a similar colony size therefore a longer incubation is required when testing treated seed (Step 2.7).
- There should be no growth on dilution plates prepared as a sterility check (Step 5.1).

- Dilution plates prepared from positive control isolate(s) or reference material, should give single colonies with typical morphology (Step 5.1).
- Numbers of bacteria on dilution plates should be consistent with the dilution (i.e. should decrease approx. ten-fold in the 10^{-1} dilution)(Step 5.5).
- Due to the potential for non-pathogenic isolates to be present in seed lots together with pathogenic isolates, it is essential to subculture (Step 6.1), if present, at least the minimum number of suspect colonies specified (six per subsample) and to test all *Xanthomonas*-like subcultured isolates for pathogenicity or in PCR test (Step 6.5).
- The positive control isolate(s) or reference material should give colonies with typical morphology on YDC (Step 6.4).
- Positive control isolates should be included in every pathogenicity test (Step 7.5).The positive control isolates should give typical symptoms in the pathogenicity test (Step 7.7).
- The CCP of the PCR option 1, PCR option 2 and PCR option 3 are described in ISTA Rule 7-019a (Step 8).
- The source of starch used in the selective media is critical for observation of starch hydrolysis. Verify that each new batch of starch gives clear zones of hydrolysis with reference cultures of *Xcc* (mFS and mCS20ABN media).
- The activity per gram (g) 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 (mFS and mCS20ABN media).
- Prepare antibiotics stock solutions and other supplements in water, or in 50 % or 70 % ethanol. Antibiotics solutions and other supplements prepared in distilled/deionised water must be filter sterilised with a 0.2 µm bacterial filter. Alternatively it is possible to add the amount of powder to autoclaved distilled/deionised water. Solutions prepared in ethanol need no sterilisation (mFS and mCS20ABN media).
- The activity of neomycin against some strains of *Xcc* is known to be affected by pH. It is essential that the pH of the medium is less than 6.6 (mCS20ABN medium, Step 3).

Media and solutions

PBS (0.05 M phosphate) with Tween™ 20 (0.02 % v/v)(pH 7.2–7.4)

Compound	Amount for 1 l
Sodium chloride (NaCl)	8.0 g
Na_2HPO_4	5.75 g
KH_2PO_4	1.0 g
Tween™ 20	0.2 ml
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 final containers.
4. Autoclave at 121 °C, 15 psi for 15 min.
5. Add 0.2 ml of sterile Tween™ 20 per 1 l after autoclaving.

Storage

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

mCS20ABN medium

Compound	Amount for 1 l
Soya peptone	2.0 g
Tryptone (BD Bacto™ Tryptone)	2.0 g
KH_2PO_4	2.8 g
$(NH_4)_2HPO_4$	0.8 g
$MgSO_4 \cdot 7H_2O$	0.4 g
L-Glutamine	6.0 g
L-Histidine	1.0 g
D-Glucose (dextrose)	1.0 g
Soluble starch (Merck 1252)(CCP)	25.0 g
Agar (BD Bacto™ Agar)	18.0 g
Distilled/deionised water	1000 ml
Nystatin[a] (10 mg/ml 50 % ethanol)	35 mg (3.5 ml)
Neomycin sulphate[b] (20 mg/ml distilled/ deionised water)	40 mg (2.0 ml)
Bacitracin[c] (50 mg/ml 50 % ethanol)	100 mg (2.0 ml)

[a–c] Added after autoclaving. Antibiotics amounts for guidance only (CCP).
[a] Dissolve 100 mg nystatin in 10 ml 50 % ethanol. Add 3.5 ml/l.
[b] Dissolve 200 mg neomycin sulphate (770 U/mg) in 10 ml sterile distilled/deionised water. Add 2.0 ml/l
[c] Dissolve 500 mg bacitracin,(60 U/mg) in 10 ml 50 % ethanol. Add 2.0 ml/l.

Preparation

1. Weigh out all the ingredients except the antibiotics into a suitable container.
2. Add 1000 ml of distilled/deionised water.
3. Dissolve and check pH which should be 6.5, adjust if necessary (important CCP).
4. Autoclave at 121 °C, 15 psi for 15 min.
5. Prepare antibiotic solutions and filter sterilise as appropriate.
6. Allow the medium to cool to approx. 50 °C and add antibiotic solutions.
7. Mix thoroughly but gently by inversion/swirling to avoid air bubbles and pour plates (18 ml per 90 mm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

Storage

Store the prepared plates inverted in polythene bags at 4 ±2 °C and use within four weeks of preparation to ensure activity of antibiotics.

Depending on the source of starch, prestorage in the refrigerator for several days before use may result in more easily visible zones of starch hydrolysis.

mFS medium

Compound	Amount for 1 l
K_2HPO_4	0.8 g
KH_2PO_4	0.8 g
KNO_3	0.5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
Yeast extract	0.1 g
Methyl Green (1 % aq.)	1.5 ml
Soluble starch (Merck 1252)(CCP)	25.0 g
Agar (BD Bacto™ Agar)	15.0 g
Distilled/deionised water	1000 ml
Nystatin[a] (10 mg/ml in 50 % ethanol)	35 mg (3.5 ml)
D-Methionine[b] (1 mg/ml 50 % ethanol)	3 mg (3.0 ml)
Pyridoxine HCl[c] (1 mg/ml 50 % ethanol)	1 mg (1 ml)
Cephalexin[d] (20 mg/ml 50 % ethanol)	50 mg (2.5 ml)
Trimethoprim[e] (10 mg/ml 70 % ethanol)	30 mg (3 ml)

[a–c, d, e] Added after autoclaving. Antibiotics amounts for guidance only (CCP).
[a] Dissolve 100 mg nystatin in 10 ml 50 % ethanol. Add 3.5 ml/l.
[b] Dissolve 10 mg D-methionine in 10 ml 50 % ethanol. Add 3.0 ml/l.
[c] Dissolve 10 mg pyridoxine HCl in 10 ml 50 % ethanol. Add 1 ml/l.
[d] Dissolve 200 mg cephalexin in 10 ml 50 % ethanol. Add 2.5 ml/l.
[e] Dissolve 100 mg trimethoprim in 10 ml 70 % ethanol. Add 3 ml/l.

Preparation

1. Weigh out all the ingredients except antibiotics, pyridoxine HCl and D-methionine into a suitable container.
2. Add 1000 ml of distilled/deionised water.
3. Dissolve and check pH which should be 6.5, adjust if necessary (important CCP).
4. Autoclave at 121 °C, 15 psi for 15 min.
5. Prepare the antibiotic, pyridoxine HCl and D-methionine solutions and filter sterilise as appropriate.
6. Allow medium to cool to approx. 50 °C before adding the antibiotics, pyridoxine HCl and D-methionine solutions.
7. Mix the molten medium gently to avoid air bubbles and pour plates (18 ml per 90 mm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

Storage

Store the prepared plates inverted in polythene bags at 4 ±2 °C and use within four weeks of preparation to ensure activity of antibiotics.

Depending on the source of starch, pre-storage in the refrigerator for several days before use may result in more easily visible zones of starch hydrolysis.

Yeast dextrose chalk (YDC) agar

Compound	Amount for 1 l
Agar (BD Bacto™ Agar)	15.0 g
Yeast extract	10.0 g
$CaCO_3$ (light powder)	20.0 g
D-Glucose (dextrose)	20.0 g
Distilled/deionised water	1000 ml

Preparation

1. Weigh out all ingredients into a suitable oversize container (i.e. 250 ml of medium in a 500 ml bottle/flask) to allow swirling of medium just before pouring.
2. Add 1000 ml of distilled/deionised water.
3. Steam to dissolve.
4. Autoclave at 121 °C, 15 psi for 15 min.
5. Allow medium to cool to approx. 50 °C.
6. Swirl to ensure even distribution of $CaCO_3$ and avoid air bubbles, and pour plates (22 ml per 90 mm plate).
7. Leave plates to dry in a laminar flow bench or similar before use.

Storage

Store prepared plates inverted in polythene bags at room temperature.

Prepared plates can be stored for several months provided they do not dry out.

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Reproducibility: dispersion = 1
Repeatability: dispersion = 1
Detection limits: 1.5 cfu/ml (theoretical, P = 0.95)