International Rules for Seed Testing
2017

Validated Seed Health Testing Methods

7-021: Detection of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* in *Phaseolus vulgaris* (bean) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2016, Tallinn, Estonia

Effective from 1 January 2017
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 and German versions. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.
**7-021: Detection of Xanthomonas axonopодis pv. phaseoli and Xanthomonas axonopодis pv. phaseoli var. fuscans in Phaseolus vulgaris (bean) seed**

**Host:** Phaseolus vulgaris L.

**Pathogen(s):** Xanthomonas axonopodis pv. phaseoli (Smith) Vauterin, Hoste, Kersters & Swings, syn. X. campestris pv. phaseoli (Smith) Dye; Xanthomonas axonopodis pv. phaseoli var. fuscans Vauterin, Hoste, Kersters & Swings, syn. X. campestris pv. phaseoli var. fuscans (Burkholder) Starr & Burkholder

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

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**Revision history**

Version 1.0, 2006-02-22
Version 2.0, 2006-08-18
Version 2.1, 2010-01-01: Editorial change to Critical control points
Version 2.2, 2012-01-01: Notice regarding review of pathogenicity test
Version 2.3, 2013-01-01: Definition of sample size
Version 3.0, 2014-01-01: New pathogenicity assay and PCR test; common name of host added
Version 3.1, 2017-01-01: Materials: numbers of Petri dishes for media deleted; Method: steps 8.1 & 8.5 modified; Reporting results revised; CCP (k) modified

**Background**

This method is derived from the validation studies carried out by ISTA in 2003 and 2011, in collaboration with the International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg) (Sheppard & Remeeus, 2005). For routine testing of bean seed a combination of two complementary semi-selective media, Milk Tween™ agar (MT) and X. campestris pv. phaseoli agar (XCP1), is recommended with a pathogenicity test to confirm suspect isolates.

The two media, XCP1 and MT, have been chosen for their ease of use and selectivity for X. axonopodis pv. phaseoli. In addition both media can be used to detect both X. axonopodis pv. phaseoli and X. axonopodis pv. phaseoli var. fuscans. Although initially the morphology of fuscans and non-fuscans strains of X. axonopodis pv. phaseoli appear to be similar on the media after a longer incubation the fuscans colonies are distinguished by a distinct brown pigmentation. A further advantage of MT medium is that it can also be used for identifying other bacterial seed-borne pathogens of beans, e.g. *Pseudomonas savastanoi* pv. *phaseolicola* and *Pseudomonas syringae* pv. *syringae*.

In 2010 in the USA and France conflicting data were obtained with the new ISTA method. Research in France (GEVES and INRA) and in the Netherlands (Naktuinbouw) showed that some isolates that were responsible for positive results were causing symptoms in the pathogenicity assay but were not identified as Xap based on molecular methods (genetic bacterial fingerprinting in the Netherlands and pathogen specific PCRs in France). Therefore it was concluded that the pathogenicity assay used in theISTA method, a crucial step in the Xap test, is not reliable enough.

A new pathogenicity assay was developed at INRA to allow a reliable characterization of the aggressiveness of X. axonopodis pv. phaseoli wild type strains and mutants (Darsonval et al., 2009). A comparison study of the new pathogenicity test and primers specific for X. axonopodis pv. phaseoli fuscans and non-fuscans isolates (Audy et al., 1994; Boureau et al., 2012) was carried out as a collaboration between ISTA, ANSES, INRA and ISHI-Veg. This study showed that the new pathogenicity test and Audy et al. 2009.
al., (1994) primers were good confirmation tools and that Diaggene (Boureau et al., 2012) primers gave good results but their use did not improve sensitivity of the method.

Two options are proposed for confirmation of suspect isolates:

**Option 1:** Pathogenicity assay, for laboratories not equipped or experienced with PCR. In this case, CCP must be followed and target and non-target controls added (X. vesicatoria, Xap, water). This option is also valuable and less time consuming when few suspect isolates have been detected but requires a growth chamber or greenhouse equipped for high relative humidity (RH).

**Option 2:** PCR test with Audy et al. (1994) primers. This option can be used for laboratories experienced and equipped for PCR, when a short delay is needed for obtaining results and/or a high number of suspect isolates have been detected.

**Safety precautions**

**Ethidium bromide**

Ethidium bromide is carcinogenic. Use ethidium bromide according to safety instructions. It is recommended to manipulate 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 utilized when visualizing ethidium bromide.

**Treated seed**

Seed treatments may affect the performance of this test. It must only be performed on untreated seed.

**Sample and subsample size**

The sample (total number of seeds tested) and subsample size to be tested depends on the desired tolerance standard (maximum acceptable percentage of seeds infested) and detection limit (theoretical minimum number of pathogen propagules per seed which can be detected). The minimum recommended sample size is 5000 seeds. In any case the maximum subsample size should be 1000 seeds.

**Materials**

**Reference material:** a known strain of *fuscans* or non- *fuscans* types of *X. axonopodis* pv. *phaseoli* (positive control) and of *X. vesicatoria* (negative control) or standardized reference material

| Plates of milk Tween™ agar medium: 90 mm Petri dishes |  |
| Plates of XCP1 medium: 90 mm Petri dishes |  |
| Plates of yeast dextrose chalk (YDC) agar medium: |  |
| for subculture (at least 1 per subsample) |  |
| Polythene bags or containers of sterile saline (0.85% NaCl) plus Tween™ 20 (0.02%): |  |
| for soaking seeds (volume (mL) required is equivalent to 2.5 × TSW (g)) |  |
| Dilution bottles: containing 4.5 mL of sterile saline (2 per subsample). Other volumes may be acceptable; see General methods. |  |
| 70% ethanol: for disinfection of surfaces, equipment |  |
| Incubator operating at 28 ± 2 °C |  |
| Balance: capable of weighing to the nearest 0.001 g |  |
| Automatic pipettes: check accuracy and precision regularly |  |
| Bean seedlings: susceptible to all races of the pathogen for pathogenicity test (e.g. ‘Michelot’, ‘Contender’) |  |
| Growth chamber: capable of operating at 28 °C or greenhouse, with high humidity (95% RH), with quarantine status |  |
| Milli Q and chemicals: for PCR preparation |  |
| Sterile microtubes: 1.5 mL; 0.2 mL |  |
| Microlitre pipettes: e.g. Gilson, Finn, with sterile filtered tips (1–1000 µL) |  |
| Conventional thermocycler |  |
| Electrophoresis equipment: 1.5–2% agarose gels |  |
DNA visualizing system: BET or analogue reagent, UV imaging apparatus

PCR primers (Audy et al., 1994):
p7X4e: 5’ ggcaacacgctaaacctagc 3’
p7X4e: 5’ cgcgaagcagctcctgag 3’

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. before and after handling each sample. This can achieved by swabbing/spraying equipment and gloved hands with 70 % ethanol.

If the submitted sample is received in several packets, these should be combined by emptying into a new, clean polythene bag and mixing by hand to give a composite sample.

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.

Method

Critical control points are indicated by CCP.

1. Extraction
1.1 Suspend seeds in sterile saline plus Tween™ 20 (0.02 % v/v) in a polythene bag or container. The volume of saline required in mL should be equivalent to 2.5 × TSW (g); e.g. TSW = 300 g, therefore volume of saline required is 2.5 × 300 = 750 mL (Olivier & Remeeus, 2004).
1.2 Soak subsamples overnight (16–18 h) at 5 °C (±4 °C).

2. Dilution and plating
2.1 Shake containers or polythene bags to obtain a homogenous extract before dilution.
2.2 Prepare a tenfold dilution series from the seed extract. Pipette 0.5 mL of the extract into 4.5 mL of sterile saline and vortex to mix (10¹ dilution). Pipette 0.5 mL of the 10¹ dilution into another 4.5 mL of sterile saline and vortex to mix (10² dilution)(see General methods).

3. Positive control (culture or reference material)
3.1 Prepare a suspension of a known strain of X. axonopodis pv. phaseoli, fuscans and non-fuscans, in sterile saline or reconstitute standardised reference material according to the supplier’s instructions.
3.2 Dilute suspension sufficiently to obtain dilutions containing approximately 10² to 10⁶ cfu/mL. 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 selective media (MT and XCP1) 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. saline 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 recovery of positive control on semi-selective medium (CCP).
5.2 Examine the sample plates for the presence of typical X. axonopodis pv. phaseoli colonies by comparison with the positive control plates.
5.3 After 4–5 d on MT, X. axonopodis pv. phaseoli colonies are yellow distinguished by two zones of hydrolysis; a large clear zone of casein hydrolysis and a smaller milky zone of Tween™ 80 lysis (Fig. 1a, b). The fuscans of X. axonopodis pv. phaseoli colonies produce a brown diffusible pigment. If not visible after 4 d incubate for an additional day. Often the fuscans type colonies show Tween™ 80 lysis.
5.4 After 4–5 d on XCP1, X. axonopodis pv. phaseoli colonies are yellow, glistening and surrounded by a clear zone of starch hydrolysis (Fig. 2b). The fuscans of X. axonopodis pv. phaseoli colonies produce a brown diffusible pigment after 5 d of incubation (Fig 2a). Often the fuscans type colonies show Tween™ 80 lysis.
5.5 The colony size and colour can differ within a sample.
5.6 Estimate the number of suspect and other colonies (see General methods).
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 colonies subcultured will depend on the number and variability of suspect colonies on the plate: if present, at least six colonies should be subcultured per subsample (CCP).

6.2 Subculture the positive control isolate to a sectored plate for comparison (Fig. 3).

6.3 Incubate sectored plates for 24–48 h at 28 ±2 °C.

6.4 Compare appearance of growth with positive control. On YDC X. axonopodis pv. phaseoli colonies are yellow and mucoid in appearance (Fig. 3)(CCP).

6.5 Confirm the identity of isolates by pathogenicity test on bean seedlings of known susceptibility (option 1) or by PCR (option 2).

6.6 Record results for each colony subcultured.

7. Pathogenicity (CCP): Option 1
(Darsonval et al., 2009)

7.1 Grow seedlings of a bean cultivar known to be highly susceptible to Xap (e.g. ‘Flavert’ or ‘Michelet’) at 20–30 °C in small pots until the first trifoliate leaf stage (approximately 16 days after sowing).

7.2 Make a 10⁷ cfu/mL (CCP) suspension in distilled/deionized water of a culture obtained after growth (24 or 48 h), 28 °C on YDC (i.e. sectored plate).

7.3 Inoculation: dip first trifoliate leaf for 30 s in a container containing inoculum (beaker)(Fig. 4).

7.4 The number of plants which should be inoculated is 3 plants per suspect isolate.

Figure 1. Xanthomonas axonopodis pv. phaseoli colonies on MT plates after 4 d indicated by a large clear zone of casein hydrolysis (a) and a smaller milky zone of Tween™ 80 lysis (b).

Figure 2. Xanthomonas axonopodis pv. phaseoli colonies, fuscans (a) and non-fuscans (b), on XCP1 plates, showing a clear zone of starch hydrolysis and fuscans on XCP1 showing a milky zone, after 4 d.
7.5 Inoculate plants with one positive *X. apisalate*, and 2 negative controls: *X. vesicatoria* and distilled/deionized water.

7.6 Incubate at 28 °C, 16 h light, 95 % RH; 25 °C, 8 h dark, 95 % RH (CCP).

7.7 Record symptoms from 5 to 11 days (depending on when symptoms begin) after inoculation. Compare with positive and negatives controls. Typical Xap symptoms are water-soaked spots. Necrosis of lesions can develop and in case of very aggressive isolates lead to death of tissues (Fig. 5a–d). No lesions occur on negative controls (Fig. 6).

8. Polymerase Chain Reaction (PCR): Option 2
(Audy et al., 1994)

8.1 Make a slightly turbid cell suspension at 10⁷ cfu/mL (OD₆₀₀ nm approximately 0.05) in 1.0 mL sterile distilled/deionized water from the suspect colonies cultured on YDC medium, and the positive and negative controls (CCP). Transfer 1 mL of sterile distilled/deionized water in a separate Eppendorf tube to use as negative process control. Boil the suspension for 5 min at 95 °C for DNA extraction. Store at –20 °C until identification (CCP).

8.2 Use the following *X. axonopodis pv. phaseoli* specific pair of primers from Audy et al. (1994) that will give a product of 800bp:

- p7X4c: 5′ ggcaacaccgatcctaaacagg 3′
- p7X4e: 5′ cgccggaagac gat cctcgaag 3′

8.3 Prepare the reaction mixture (page 7-021-18, adapted by INRA)(CCP). Carry out the PCR reactions in 0.2 mL thin-walled PCR tubes in a final volume of 20 µL (16 µL reaction mixture + 4 µL boiled bacterial suspension).

8.4 PCR profile: An initial 3 min incubation at 94 °C followed by 35 cycles of 1 min at 94 °C, 2 min at 72 °C. A final 10 min incubation at 72 °C and infinity at 12 °C (CCP). During each amplification run, in addition to the positive and negative controls extracted in 8.1, a PCR negative control (DNA extract replaced by molecular biology grade water) is added.

8.5 Fractionate 10 µL of the PCR products, the negative process control and water (negative PCR control) by gel electrophoresis in a 1.5 % agarose gel in 1× Tris-acetate EDTA (TAE buffer)(CCP). Include a 100 bp ladder. Stain with ethidium bromide in a bath and rinse in water.
7‑021: Xanthomonas axonopodis pv. phaseoli on Phaseolus vulgaris (Bean)

8.6 Analyse the amplification products for a *X. axonopodis* pv. *phaseoli* specific product of 800 bp (CCP) = positive identification of *X. axonopodis* pv. *phaseoli*, no band = negative identification (Fig. 7).

In case of a positive identification of *X. axonopodis* pv. *phaseoli*, as a low risk of false positive result is present (Audy *et al.* (1994) primers detect *X. axonopodis* pv. *dieffenbachiae* which are not supposed to be present on bean seeds), a pathogenicity test can be performed as complementary information.
General methods

1. **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.

2. **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.
3. 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.

4. 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.

5. 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 should indicate the mean number of pathogen propagules (cfu) per seed and the number of positive subsamples out of the total number tested.

Quality assurance

General

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)

a) Dilution plates prepared from positive control isolate(s) or reference material, should give single colonies with typical morphology (Step 5.1 and 6.4).
b) 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). Note that recovery of the fuscans type X. axonopodis pv. phaseoli is in general lower on MT than on XCP1.
c) Numbers of bacteria on dilution plates should be consistent with the dilution (i.e. should decrease approximately ten-fold with each dilution)(Step 5.1).
d) There should be no growth on dilution plates prepared as a sterility check (Step 5.1).
e) Due to the potential for non-pathogenic isolates to be present in seedlots together with pathogenic isolates, it is essential to subculture if present, at least the minimum number of suspect colonies specified (six per subsample)(Step 6.1), and to test all Xanthomonas-like subcultured isolates in pathogenicity or PCR test (Step 6.5).
f) Positive control isolates should be included in every pathogenicity test (Step 7.6).
g) The positive control isolate should give typical symptoms in a pathogenicity test (Step 7.8).
h) The quality of milk powder is vital to develop the hydrolysis of milk in MT medium. Two milk sources that work well are Oxoid and Sigma brands.
i) The activity (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 (MT and XCP1 media).
j) For pathogenicity assay, a concentration of $10^7$ cfu/mL must be used as lower concentrations can lead to false negative results and higher concentrations to false
positive results. The humidity during incubation must be very high (minimum 95%) to obtain water-soaked lesions. If the humidity is too low, necrotic lesions can develop without any visible water-soaked spot, making more difficult the interpretation of the result.

k) Positive and negative control isolates, negative process control and negative PCR control should be included in every PCR test (Step 8.1).

l) DNA extracted by boiling cannot be stored for a very long time. If stored at -20 °C, positive and negative controls stored in the same conditions as suspect isolates’ DNA must be used.

m) The preparation of PCR mixture (Step 8.1, 8.4), and the preparation of agarose gel for electrophoresis (Step 8.5) should be adapted to available material and equipment of individual laboratories testing for of X. axonopodis pv. phaseoli under the condition that results will be validated on PCR controls.

**Media and solutions**

**Sterile saline**

(van Vuurde et al., 1989)

**Sodium chloride (NaCl):** 8.5 g

**Distilled/deionized water:** 1000 mL

**Preparation**

1. Weigh out all ingredients in section A into a suitable container.
2. Add 1000 mL of distilled/deionized water.
3. Dissolve and dispense into final containers.
4. Autoclave at 121 °C, 15 p.s.i. for 15 min.
5. For extraction of seeds, add 0.2 mL of sterile Tween™ 20 per 1000 mL.

**Storage**

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

**Milk Tween™ agar medium**

(adapted from Goszczynska & Serfontein, 1998)

**Section A**

**Proteose peptone no. 3:** 10.0 g

**CaCl₂:** 0.25 g

**Tyrosine:** 0.5 g

**Agar:** 15.0 g

**Distilled/deionized water:** 500 mL

**Section B**

**Skim milk powder (Oxoid, Sigma) CCP:** 10.0 g

**Distilled/deionized water:** 500 mL

**Section C**

**Tweent™ 80:** 10.0 mL

**Section D**

**Nystatin:** 40 mg (1 mL)

**Cephalexin:** 80 mg (1 mL)

**Vancomycin:** 10 mg (1 mL)

**Preparation**

1. Weigh out all ingredients in section A into a suitable container.
2. Add 500 mL of distilled/deionized water.
3. Dissolve ingredients.
4. In a separate container, dissolve skim milk powder in 500 mL distilled/deionized water.
5. Separately prepare 10 mL Tween™ 80.
6. Sterilize preparations from section A, skim milk solution (section B) and Tween™ 80 (section C) separately at 121 °C, 15 p.s.i. for 15 min.
7. After sterilisation, of all components aseptically add sterilised skim milk preparation and sterilised Tween™ 80 to sterilised ingredients in section A.
8. Prepare antibiotic solutions (section D).
9. Allow medium to cool to approximately 50 °C and add antibiotics.
10. Mix gently to avoid air bubbles and pour plates 22 mL per 90 mm plate.
11. Leave plates to dry in laminar flow bench or similar before use.
Antibiotics

Amounts for guidance only (CCP).

- Dissolve 400 mg nystatin in 10 mL 70 % ethanol.
- Dissolve 800 mg cephalxin in 10 mL 70 % ethanol.
- Dissolve 100 mg vancomycin in 10 mL 70 % ethanol.

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

Note: Cycloheximide can be used as an alternative for nystatin to control fungi. Dissolve 500 mg of cycloheximide in 10 mL 70 % ethanol, add 1 mL to cool medium.

Storage

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

### XCP1 medium

(adapted from McGuire et al., 1986)

**Section A**
- KBr: 10.0 g
- CaCl₂: 0.25 g
- Soluble potato starch: 10.0 g
- Peptone: 10.0 g
- Agar: 15.0 g
- Crystal violet (1 % aqueous): 0.15 mL
- Distilled/deionized water: 1000 mL

**Section B**
- Tween™ 80: 10.0 mL

**Section C**
- Nystatin*: 40 mg (1 mL)
- Cephalexin*: 10 mg (1 mL)
- Fluorouracil*: 3 mg (1 mL)
- Tobramycin*: 0.16 mg (1 mL)

*–d Added after autoclaving

Preparation

1. Weigh out all ingredients in section A into a suitable container.
2. Add 1000 mL of distilled/deionized water.
3. Dissolve ingredients.
4. Add crystal violet.
5. Sterilise 121 °C, 15 p.s.i. for 15 min.
6. Sterilise 10 mL Tween™ 80 separately (section B) at 121 °C, 15 p.s.i. for 15 min.
7. Aseptically add Tween™ 80 to ingredients in section A.
8. Prepare antibiotic solutions.
9. Allow medium to cool to approximately 50 °C and add antibiotics.
10. Mix gently to avoid air bubbles and pour plates (22 mL per 90 mm plate).
11. Allow plates to dry in laminar flow bench or similar before use.

Antibiotics

Amounts for guidance only (CCP).

- Dissolve 400 mg nystatin in 10 mL 70 % ethanol.
- Dissolve 100 mg cephalxin in 10 mL 70 % ethanol.
- Dissolve 30 mg fluorouracil in 100 mL 70 % ethanol.
- Dissolve 16 mg tobramycin in 100 mL 70 % ethanol.

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

Note: Cycloheximide can be used as an alternative to nystatin to control fungi. Dissolve 500 mg of cycloheximide in 10 mL 70 % ethanol, add 1 mL to cool medium.

Storage

Store prepared plates inverted in polythene bags at 4–8 °C and use within two 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.
Yeast dextrose chalk (YDC) agar

(Wilson et al., 1967)

Agar (BD Bacto™ Agar): 15.0 g  
Yeast extract: 10.0 g  
CaCO₃ (light powder): 20.0 g  
D-Glucose (dextrose): 20.0 g  
Distilled/deionized water: 1000 mL

Preparation

1. Weigh out all ingredients into a suitable oversize container (e.g. 250 mL of medium in a 500 mL bottle/flask) to allow swirling of medium just before pouring.
2. Add 1000 mL of distilled/deionized water.
3. Steam to dissolve.
4. Autoclave at 121 °C, 15 p.s.i. for 15 min.
5. Allow medium to cool to approximately 50 °C.
6. Swirl to ensure even distribution of CaCO₃ 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 8–20 °C. Prepared plates can be stored for several months provided they do not dry out.

Example of reaction mixture preparation for PCR (Audy primers)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial concentration</th>
<th>Final concentration</th>
<th>Volume (µL) in 20 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>10.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Promega Go Taq Buffer*</td>
<td>5×</td>
<td>1×</td>
<td>4</td>
</tr>
<tr>
<td>dNTP</td>
<td>2.5 mM each</td>
<td>0.2 mM</td>
<td>1.6</td>
</tr>
<tr>
<td>p7X4c</td>
<td>20 µM</td>
<td>0.15 µM</td>
<td>0.15</td>
</tr>
<tr>
<td>p7X4e</td>
<td>20 µM</td>
<td>0.15 µM</td>
<td>0.15</td>
</tr>
<tr>
<td>Go Taq polymerase</td>
<td>5 U/µl</td>
<td>0.02 U/µl</td>
<td>0.08</td>
</tr>
<tr>
<td>DNA</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Concentrated PCR reaction buffer (with MgCl₂), from GoTaq DNA polymerase [Promega] (laboratories using a buffer without MgCl₂ will have to add this salt to a final concentration of 1.5 mM).

Example for visualization of PCR products

Tris-acetate EDTA (TAE) Buffer 1×

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-acetate EDTA (TAE 50×)</td>
<td>20 mL</td>
</tr>
<tr>
<td>Distilled/deionized water</td>
<td>QSP 1000 mL</td>
</tr>
</tbody>
</table>

1.5 % agarose gel for electrophoresis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-acetate EDTA (TAE) 1×</td>
<td>300 mL</td>
</tr>
<tr>
<td>Agarose</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>

Preparation

1. Make sure that the gel tray is clean and dry before use. Use the gel caster. Place the gel comb(s) in position in the gel tray.
2. Weigh out the desired amount of agarose and place in an Erlenmeyer flask with a measured amount of electrophoresis buffer, e.g. for a 300 mL gel add 4.5 g of agarose and 300 mL of 1× TAE buffer to a 500 mL flask. The larger flask ensures that the agarose will not boil over.
3. Dissolve the agarose in a microwave oven. All the grains of agarose should be dissolved and the solution clear.
4. Allow the medium to cool down to approx. 60 °C and pour the gel caster.
5. After the gel is completely set, carefully remove the gel comb(s).
6. Remove the gels and place them in the electrophoresis unit.
7. The same electrophoresis buffer used in the gel must also be used for the running buffer.

Note: The amount of 1.5 % agarose gel for electrophoresis to be prepared depends on the available electrophoresis apparatus of a laboratory.
References


Validation reports