International Rules for Seed Testing 2024

Validated Seed Health Testing Methods

7-019a: Detection of *Xanthomonas campestris* pv. *campestris* and *Xanthomonas campestris* pv. *raphani* in *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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7-019a: Detection of *Xanthomonas campestris* pv. *campestris* and *Xanthomonas campestris* pv. *raphani* in *Brassica* spp. seed

Host: *Brassica* spp.

Pathogen(s): *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson and *Xanthomonas campestris* pv. *raphani*

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Revision history

Version 1.0, 2003-05-13
Version 2.0, 2004-08-06 (Koenraadt *et al*., 2004)
Version 3.0, 2006-07-05
Version 3.1, 2010-01-01: Editorial change – correction of autoclaving pressures
Version 4.0, 2013-01-01: Addition of PCR test; definition of sample size (Grimault *et al.* 2012)
Version 4.1, 2014-01-01: Renumbered 7-019a
Version 5.0, 2015-01-01 (Sato *et al*., 2013)
Version 5.1, 2017-01-01: Materials – numbers of Petri dishes for media deleted
Version 6.0, 2018-01-01: Addition of pre-screening methods and addition of TaqMan assay for identification of suspect colonies (Bruinsma *et al*., 2018)
Version 6.1, 2020-01-01: Revision of title, improved explanation of method under Background and editorial changes to method description
Version 6.2, 2021-01-01: Safety precautions added; Sample size and Methods revised
Version 6.3, 2022-01-01: Correction of temperature for DNA extraction in Methods
Version 6.4, 2024-01-01: Sample size, Methods and General methods revised

Background

This method includes a pre-screen and assays to detect and identify suspect colonies, and to confirm the viability and pathogenicity of *Xanthomonas campestris* pv. *campestris* (*Xcc*) and *Xanthomonas campestris* pv. *raphani* (*Xcr*) in untreated *Brassica* seed. Dilution plating for detection and the bioassay for confirmation are mandatory steps in the method as together they show the presence, viability and pathogenicity of the target pathogens.

The original method was based on assays published by Franken *et al*. (1991) and in the 2nd edition of Working Sheet No. 50 in the *ISTA Handbook of Seed Health Testing* (Schaad & Franken, 1996). The method was published as an ISTA Rule in 2004, based on the method for the detection of *Xcc*, *Xcr* and *X. campestris* pv. *armoraciae* on untreated cabbage seeds, developed and validated by the International Seed Health Initiative for Vegetables (ISF/ISHI-Veg). Following a publication by Fargier and Manceau (2007) stating that their study did not 'support the existence of another leaf spot disease caused by *X. campestris* pv. *armoraciae*', the method was restricted to detecting *Xcc* and *Xcr*.

Version 4.0 of the method, published in 2013, included the option of identifying suspect colonies using one of two gel-based polymerase chain reactions (PCRs)

that distinguished between *Xcc* and *Xcr*. In Version 5.0, approved in 2015, the recipes for the semi-selective media mCS20ABN and mFS were modified to improve performance and safety.

The latest version of the method includes a choice of two assays (a seed-extract PCR and a bio-PCR) as an optional 'pre-screen'. The aim of the pre-screen is to identify seed lots that are not infected with the target pathogens. A negative pre-screen result indicates the seed lot is negative and therefore, the end of the test. A positive pre-screen result is indicative of a 'suspect' lot that needs to be investigated further using the dilution plating assay. It also replaces the gel-based PCR assays used for colony identification by a TaqMan qPCR assay. See process flow chart below:

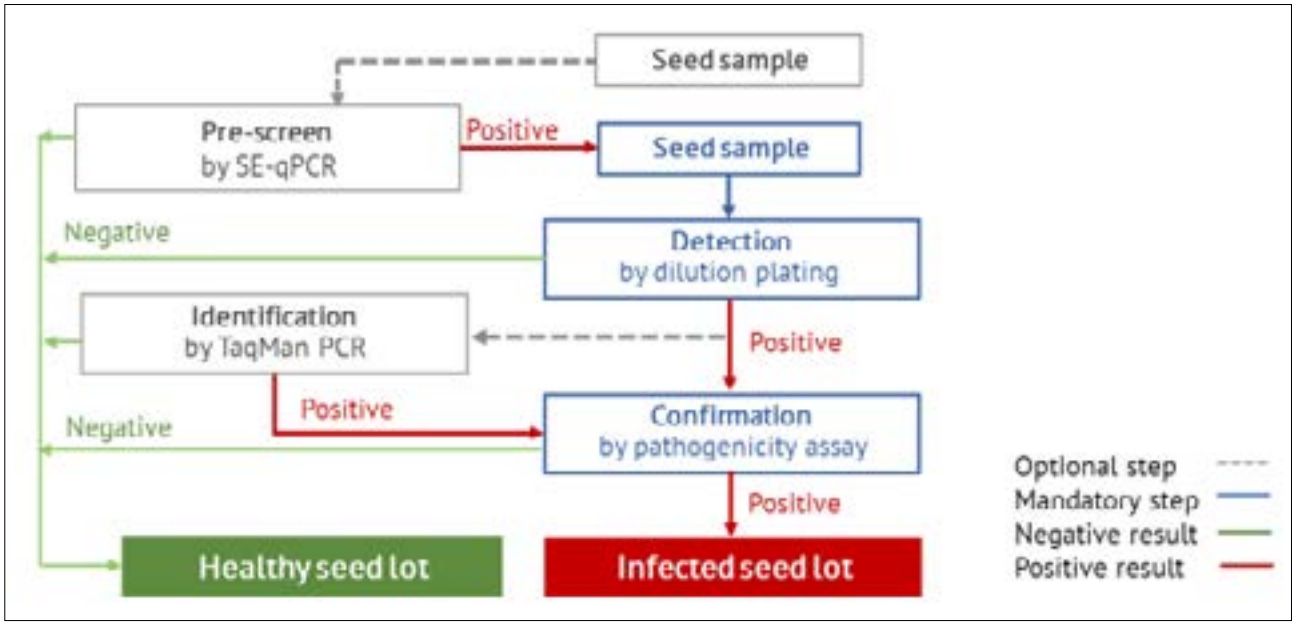

Figure 1. Process flow diagram explaining method assays and decisions taken depending on intermediate results until final result, for detection of *Xanthomonas campestris* pv. *campestris* and *Xanthomonas campestris* pv. *raphani* in *Brassica* seeds.

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

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

Note: *Brassica* seed subjected to a physical treatment, for example hot water, is regarded as treated seed.

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*), *X. campestris* pv. *raphani* (*Xcr*) or standardised reference material. Known *Acidovorax cattleyae* or *A. citrulli* isolate used as Internal Amplification Control (IAC) for pre-screening PCR.
Plates of mFS medium: 90 mm Petri dishes
Plates of mCS20ABN medium: 90 mm Petri dishes
Plates of yeast dextrose chalk (YDC) agar: for subculture (at least 1 per subsample)
Conical flasks or equivalent: containing sterile saline (0.85 % (w/v) NaCl) plus Tween™ 20 (0.02 % (v/v); 20 µl per 100 ml) for soaking of seeds (10 ml per 1000 seeds)
Dilution bottles: containing 4.5 ml of sterile saline (2 per subsample). Other volumes may be acceptable; see General methods
70 % (v/v) ethanol: for disinfection of surfaces, equipment
Incubator: operating at 28–30 °C
Balance: capable of weighing to the nearest 0.001 g
pH meter: capable of reading to the nearest 0.01 pH unit
Automatic pipettes: check accuracy and precision regularly
Brassica seedlings: susceptible to all races of the pathogen (e.g. *B. oleracea* 'Wirosa') for pathogenicity test
Orbital shaker
Sterile pipette tips
Sterile bent glass rods
DNA isolation: DNA isolation kit e.g. Qiagen DNeasy Blood and Tissue kit

Real-time PCR equipment and/or PCR thermal cycler
Controls for PCR: The following positive and negative controls should be included in the SE-PCR.
- Positive Extraction Control (PEC) – *Acidovorax cattleyae*/*A. citrulli* spike (Step 2.1.3, also serves as Internal Amplification Control (IAC));
- Negative Process Control (NPC) – seed extract from healthy seeds (Step 2.1.2);
- Positive Amplification Control (PAC) – *Xcc* DNA;
- No Template Control (NTC) – PCR reaction mix with water (Step 2.4.5) and negative seed or buffer as a NPC. The controls should give the expected (absence of) amplification in the PCR test;
- Inclusion of a positive process control (positive seed sample) is recommended.

For SE-PCR

PCR primer set (Köhl *et al*., 2011):
XCC-F: 5' GTG.CAT.AGG.CCA.CGA.TGT.TG 3'
XCC-R: 5' CGG.ATG.CAG.AGC.GTC.TTA.CA 3'
XCC-Pr: 5' FAM-CAA.GCG.ATG.TAC.TGC.GGC.CGT.G-NFQ-MGB 3'
PCR primer set (Berg *et al*., 2006):
DLH153-F: 5' GTA.ATT.GAT.ACC.GCA.CTG.CAA 3'
DLH154-R: 5' CAC.CGC.TCC.AGC.CAT.ATT 3'
P7: 5' VICrepl-ATG.CCG.GCG.AGT.TTC.CAC.G-BHQ1 3'
PCR primer set *Acidovorax cattleyae* (Koenraadt *et al*., 2014):
Acat 2-F: 5' TGT.AGC.GAT.CCT.TCA.CAA G 3'
Acat 2-R: 5' TGT.CGA.TAG.ATG.CTC.ACA.AT 3'
Acat1-Pr: 5' Texas Red-CTT.GCT.CTG.CTT.CTC.TAT.CAC.G- BHQ2 3'

or *(additional alternative option)*

PCR primer set *Acidovorax avenae* subsp. *citrulli* (Sudarshana, 2010):
Contig21 F: 5' ACC GAA CAG AGA GTA ATT CTC AAA GAC 3'
Contig21 R: 5' GAG CGT GAT GGC CAA TGC 3'
Contig21-Pr: 5' FAM/CAT +CG+C TT+G AGC AG+C AA/3IABkFQ 3'

For bio-PCR

PCR primers (Berg *et al*., 2006):
DLH153: 5' GTA.ATT.GAT.ACC.GCA.CTG.CAA 3'
DLH154: 5' CAC.CGC.TCC.AGC.CAT.ATT 3'
PCR primers (Rijlaarsdam *et al*., 2004):
Zup2309: 5' AAA.TCA.GGG.GGA.TGC.GGT.GG 3'
Zup2310: 5' TCC.GGC.CAG.GGT.CGA.TAC.AGT.G 3'

PCR option 1 for suspect isolate confirmation

PCR primers (Berg *et al*., 2005):
DLH120: 5' CCG.TAG.CAC.TTA.GTG.CAA.TG 3'
DLH125: 5' GCA.TTT.CCA.TCG.GTC.ACG.ATT.G 3'
PCR primers (Rijlaarsdam *et al*., 2004):
Zup2309: 5' AAA.TCA.GGG.GGA.TGC.GGT.GG 3'
Zup2310: 5' TCC.GGC.CAG.GGT.CGA.TAC.AGT.G 3'

Universal primers (adapted from Eden *et al*., 1991):
1052F: 5' GCA.TGG.TTG.TCG.TCA.GCT.CGT. 3'
BacR: 5' TAC.GGC.TAC.CTT.GTT.ACG.ACT.T 3'
Agarose electrophoresis equipment

PCR option 2 for suspect isolate confirmation

PCR primers (Berg *et al*., 2005):
DLH120: 5' CCG.TAG.CAC.TTA.GTG.CAA.TG 3'
DLH125: 5' GCA.TTT.CCA.TCG.GTC.ACG.ATT.G 3'
PCR primers (Rijlaarsdam *et al*., 2004):
Zup2311: 5' GCA.AAG.CCC.TCG.TTC.ACG.CAT 3'
Zup2312: 5' GGT.GGT.GTG.GCC.GCT.CTT.CTC.AT 3'
Universal primers (adapted from Eden *et al*., 1991):
UpBacF: 5' TAC.GGC.TAC.CTT.GTT.ACG.ACT.T 3'
UpBacR: 5' GAA.GAG.TTT.GAT.CCT.GGC.TCA.G 3'
Agarose electrophoresis equipment

PCR option 3 for suspect isolate confirmation

PCR primer set (Köhl *et al*., 2011):
XCC-F: 5' GTG.CAT.AGG.CCA.CGA.TGT.TG 3'
XCC-R: 5' CGG.ATG.CAG.AGC.GTC.TTA.CA 3'
XCC-Pr: 5' FAM-CAA.GCG.ATG.TAC.TGC.GGC.CGT.G-NFQ-MGB 3'
PCR primer set (Berg *et al*., 2006):
DLH153-F: 5' GTA.ATT.GAT.ACC.GCA.CTG.CAA 3'
DLH154-R: 5' CAC.CGC.TCC.AGC.CAT.ATT 3'
P7: 5' VICrepl-ATG.CCG.GCG.AGT.TTC.CAC.G-BHQ1 3'
PCR primer set (Wu *et al*., 2008)
Wu-F: 5' CAA.CGC.GAA.GAA.CCT.TAC.C 3'
Wu-R: 5' ACG.TCA.TCC.CCA.CCT.TCC 3'
Wu-P1: 5' Texas Red -ACG.ACA.ACC.ATG.CAC.CAC.CTG-BHQ2 3'
Wu-P2: 5' Texas Red- ACG.ACA.GCC.ATG.CAG.CAC.CT-BHQ2 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., both before and after handling each sample. This can achieved by swabbing/spraying equipment and gloved hands with 70 % (v/v) 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.

Methods

Critical control points are indicated by CCP.

1. Extraction
1.1 Suspend each subsample of seeds in pre-chilled (2–4 °C) sterile saline plus Tween™ 20 (0.02 % v/v) in a conical flask or equivalent. The volume of saline should be adjusted according to the number of seeds used (10 ml per 1000 seeds).
1.2 Shake for 2.5 h at room temperature (20–25 °C) on an orbital shaker set at 100–125 rpm.
1.3 Shake the flasks to mix just before further processing.

For optional pre-screening SE-PCR, proceed with Step 2; for optional pre-screening with bio-PCR, proceed with Step 3; for dilution plating without pre-screening, proceed with Step 4.

2. Pre-screening SE-PCR
2.1 Spiking of samples
2.1.1 Transfer 10 ml of seed extract from each subsample into a 15 ml centrifuge tube.
2.1.2 Include a seed extract from healthy seeds or buffer without seeds as a Negative Process Control (NPC) (CCP).
2.1.3 Add a positive extraction control (PEC) spike to all subsamples for SE-PCR (e.g. 100 µl of *A. cattleyae* or *A. citrulli* stock OD600 = 0.6) (CCP).
2.2 Concentration of bacteria
2.2.1 Centrifuge sample tubes for 5 min at 1200× *g* (slow spin at room temperature). Carefully transfer supernatant to a clean 15 ml tube and discard the pellet.

Note: The pellet is not always stable. Take the tubes carefully out of the centrifuge.

2.2.2 Centrifuge the supernatant for 20 min at a minimum of 3400× *g* (fast spin at room temperature).

Note: The pellet is not always stable. Take the tubes carefully out of the centrifuge.

2.2.3 Carefully decant as much supernatant as possible without disturbing the pellet.
2.2.4 Use the pellet for the isolation of DNA.
2.3 DNA isolation
2.3.1 Resuspend bacterial pellet in lysis buffer and extract the bacterial DNA as described in the manual of the DNA extraction kit (e.g. DNeasy Blood and Tissue kit).
2.4 TaqMan PCR

Note: Work on ice where possible and minimise the amount of time that probes are exposed to light.

2.4.1 Use the *Xcc* primer sets from Köhl *et al*. (2011) and from Berg *et al*. (2006). Fluorophore labels are flexible.

2.4.2 Also include the appropriate primers and probes for the spike used. For detection of the spike *A. cattleyae*, use the primer set by Koenraadt *et al*. (2014). For detection of the spike *A. citrulli* use the primer set by Sudarshana (2010).

2.4.3 Prepare TaqMan PCR mix.

2.4.4 Test each DNA extraction from each subsample in duplicate.

2.4.5 Use *Xcc* DNA as Positive Amplification Control (PAC) and nucleic acid free water as No Template Control (NTC) (CCP).

2.4.6 Carry out PCR reaction in a final volume of 25 µl (20 µl reaction mixture and 5 µl sample).

2.4.7 Cover the plate after addition of DNA.

2.4.8 Perform TaqMan PCR according to the PCR conditions.

2.4.9 Analyse the results. Cut-off values need to be determined in each laboratory. *A. cattleyae* is preferably set between Ct 28 and 32. *A. citrulli* Ct-value is set <35. Ct-value of *A. cattleyae* or *A. citrulli*, or another spike should be as expected.
A cut-off value of 35 was used for the validation study. Ct-value below cut-off for primer set Köhl *et al*.(2011): *Xcc* detected. Ct-value primer set Berg *et al*.(2006) below cut-off: *Xcc* or *Xcr* detected.

Interpretation & decisions – SE-PCR

Köhl TaqMan	Berg TaqMan	qPCR Result	Follow-up
Positive	Positive	Expected result for *Xcc*	Dilution plating
Negative	Positive	Expected result for *Xcr*	Dilution plating
Positive	Negative	Inconclusive	Dilution plating
Negative	Negative	Expected result for non-*Xcc*/*Xcr*	No follow up: no *Xcc*/*Xcr* DNA detected

3. Optional pre-screening bio-PCR

3.1 Enrichment

3.1.1 Pipette 100 µl of the seed extract from each sample onto two plates of the selective medium (mFS) and spread over the surface with a sterile bent glass rod or sterile spreader.

3.1.2 Prepare control plates.

3.1.2.1 PCR control plates: spread one plate with 100 µl of a dilution (approximatively 10^4 cfu/ml) of a control strain of *Xcc* and one plate with a dilution (approximatively 10^4 cfu/ml) of a control strain of *Xcr*.

3.1.2.2 Spiking control plates (PEC): Add 500 µl of the control strains, diluted to 10^5 cfu/ml, to a tube containing 4.5 ml seed extract for each sample. Spread 100 µl of each spiked seed extract on one plate of selective medium (mFS).

3.1.3 Incubate for 48 h at 28 °C.

3.2 Washing

3.2.1 Pipette 3 ml sterile saline solution (0.85 %) on each plate, use a sterile spreader to suspend the bacterial colonies. Pipette 1 ml of the bacterial suspensions into sterile microtubes suitable for centrifugation.

3.2.2 Pipette 2 × 1 ml of the sterile saline solution into sterile microtubes to be used as a negative bio-PCR control (NPC) (CCP).

3.3 DNA extraction

3.3.1 Centrifuge the suspensions for 5 min at 5000× *g* and remove the supernatant.

3.3.2 Add 500 µl of 0.5 M NaOH and resuspend the pellet by pipetting.

3.3.3 Incubate at 65 °C for 10 min.

3.3.4 Add 5 µl of the lysed bacterial suspension to 495 µl of 20 mM Tris HCl and mix gently with the pipette.

3.4 SYBR Green PCR

3.4.1 Use the primers from Berg *et al*. (2006) and from Rijlaarsdam *et al*. (2004).

3.4.2 Prepare SYBR Green PCR mix.

3.4.3 Use *Xcc* DNA as Positive Amplification Control (PAC) and nucleic acid free water as Non Template Control (NTC) (CCP).

3.4.4 Carry out PCR reaction in a final volume of 15 µl (13 µl reaction mixture and 2 µl sample).

3.4.5 Cover the plate after addition of DNA.

3.4.6 Perform SYBR Green PCR according to the PCR conditions.

3.4.7 Analyse the results. Cut-off values need to be determined in each laboratory. A sample is declared positive if the Ct-value of one or both of the PCRs is less than 35 cycles (Ct<35) and the PCR product is detected at the same melting temperature as the positive controls (±1.5 °C).

Interpretation & decisions – bio-PCR

Rijlaars-dam	Berg	SYBR PCR Result	Follow-up
Positive	Positive	Expected result for *Xcc*	Dilution plating
Negative	Positive	Expected result for *Xcr*	Dilution plating
Positive	Negative	Inconclusive	Dilution plating
Negative	Negative	Expected result for non-*Xcc*/*Xcr*	No follow up: no *Xcc*/*Xcr* DNA detected

4. Dilution and plating

4.1 Prepare two serial tenfold dilutions from the 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).

4.2 Pipette 100 µl of each dilution and the undiluted seed extract onto plates of each of the selective media (mFS, mCS20ABN) and spread over the surface with a sterile bent glass rod (see General methods).

4.3 Incubate plates at 28–30 °C and examine after 3–4 d.

4.4 Positive control (culture or reference material)

4.4.1 Prepare a suspension of a known strain of *Xcc*/*Xcr* in sterile saline or reconstitute standardised reference material according to the supplier's instructions.

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

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

4.4.4 Incubate plates with the sample plates.

4.5 Sterility check

4.5.1 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.

4.6 Examination of the plates

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

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

4.6.3 On mFS after 3–4 d, *Xcc*/*Xcr* 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). Colonies may show marked variation in size and may be visible on mFS after 3 d; if not, incubate for an additional day.

4.6.4 After 3–4 d on mCS20ABN, *Xcc*/*Xcr* colonies are pale yellow, mucoid and surrounded by a zone of starch hydrolysis (Fig. 2b). Colonies may show marked variation in size. Depending on the number of colonies present, it may be easier to evaluate plates after 3 d, before coalescence of starch hydrolysis zones which can make it more difficult to identify suspect colonies.

4.6.5 Incubation of the plates at 4 °C for several hours before recording may result in sharper zones of starch hydrolysis with some starch sources.

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

5. Confirmation/identification of suspect colonies

5.1 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).

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

5.3 Incubate sectored plates for 24–48 h at 28–30 °C.

5.4 Compare appearance of growth with positive control. On YDC, *Xcc*/*Xcr* colonies are pale yellow and mucoid/fluidal (Fig. 3).

5.5 Confirm the identity of isolates by pathogenicity on *Brassica* seedlings (step 6) of known susceptibility or by using one of the three PCR options (steps 7, 8 or 9) (CCP).

5.6 Record results for each colony subcultured.

6. Pathogenicity

6.1 Grow seedlings of a *Brassica* cultivar known to be susceptible to all races of *Xcc*/*Xcr* (e.g. cabbage 'Wirosa', see Vicente *et al.*, 2001) in small pots or modules until at least 3–4 true leaf stage.

6.2 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.

6.3 Inoculate six of the major veins at a point near the leaf edges on the two youngest leaves by stabbing with the cocktail stick or insect pin.

6.4 The number of plants which should be inoculated will depend on the variability of the cultivar and experience of the operator, but 1–3 plants per isolate should usually be sufficient. It is better to inoculate more isolates with one plant per isolate than fewer isolates with three plants per isolate.

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

6.6 Grow on plants at 20–25 °C.

6.7 Examine plants for the appearance of typical progressive V-shaped, yellow/necrotic lesions with blackened veins after 10–14 d (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* (often classified as either *X. c.* pv. *armoraciae* or *Xcr*, see Fargier & Manceau, 2007; Kamoun *et al.*, 1992; Alvarez *et al.*, 1994; Tamura *et al.*, 1994; Vicente *et al.*, 2001; Roberts *et al.*, 2004).

7. Polymerase chain reaction (PCR) option 1

7.1 Make a slightly turbid cell suspension ($OD_{600\ nm}$ approximately 0.05) in 1.0 ml sterile saline from the suspected cultures on YDC medium and the positive control. In addition a non-suspect isolate should be used as a negative control (CCP). Centrifuge bacterial suspensions for 5 min at 8000 rpm. Discard the supernatant and resuspend the pellet with 500 µl of 0.5 M NaOH. Incubate for 10 min at 100 °C by shaking at 1000 rpm. Dilute 5 µl of solution into 495 µl of 20 mM Tris HCl, pH 8, and vortex. Suspensions can be stored at –20 °C until identification.

7.2 Use the *Xcc* specific pair of primers from Rijlaarsdam *et al.* (2004) that will give a product of 370 bp and the specific pair of primers from Berg *et al.* (2005) that will give a product of 619 bp.

7.3 Universal bacterial primers should be used to validate the PCR reaction. Use the pair of primers adapted from Eden *et al.* (1991) that will give a product of 441 bp.

7.4 Prepare the reaction mixture (CCP). Carry out PCR reactions in 0.2 ml thin-walled PCR tubes in a final volume of 20 µl (17 µl reaction mixture + 3 µl bacterial suspension).

7.5 PCR profile: An initial 3 min incubation at 95 °C followed by 6 cycles of 40 s at 95 °C, 40 s at 63 °C with a touchdown of 1 °C per cycle, 40 s at 72 °C followed by 29 cycles of 40 s at 95 °C, 40 s at 58 °C, 40 s at 72 °C. A final 5 min incubation at 72 °C and infinity at 10 °C (CCP).

7.6 Fractionate 10 µl of the PCR products, the negative process control and sterile water (negative PCR control) by gel electrophoresis in 1× Tris acetate EDTA (TAE buffer) (CCP). Include a 100 bp ladder. Stain with ethidium bromide and rinse in water.

7.7 Analyse the amplification products for a *Xcc* specific product of 370 bp/619 bp and a universal product of 441 bp (CCP; Fig. 5). Two bands (441 bp/619 bp) = positive identification of *X. campestris*, suspected presence of *X. c.* pv. *armoraciae* or *Xcr*; two bands (370 bp/441 bp) = indeterminate PCR result, pathogenicity test must be carried out to confirm the suspect isolate; one band (universal) = negative identification; no bands = bacterial template absent, repeat reaction.

8. Polymerase chain reaction (PCR) option 2

8.1 Make a slightly turbid cell suspension ($OD_{600\ nm}$ approximately 0.05) in 1.0 ml sterile saline from the suspended cultures on YDC medium and the positive control. In addition, a non-suspect isolate should be used as a negative control (CCP). Centrifuge bacterial suspensions for 5 min at 8000 rpm. Discard the supernatant and resuspend the pellet with 500 µl of 0.5 M NaOH. Incubate for 10 min at 65 °C by shaking at 1000 rpm. Dilute 5 µl of solution into 495 µl of 20 mM Tris HCl, pH 8, and vortex. Suspensions can be stored at –20 °C until identification.

8.2 Use the *Xcc* specific pair of primers from Rijlaarsdam *et al.* (2004) that will give a product of 445 bp and the specific pair of primers from Berg *et al.* (2005) that will give a product of 619 bp.

8.3 Universal bacterial primers should be used to validate the PCR reaction. Use the pair of primers adapted from Eden *et al.* (1991) that will give a product of 1511 bp.

8.4 Prepare the reaction mixture (CCP). Carry out PCR reactions in 0.2 ml thin-walled PCR tubes in a final volume of 25 µl (24 µl reaction mixture + 1 µl bacterial suspension).

8.5 PCR profile: An initial 5 min incubation at 94 °C followed by 4 cycles of 1 min at 94 °C, 1 min at 65 °C with a touchdown of 1 °C per cycle, 1 min at 72 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C. A final 10 min incubation at 72 °C and infinity at 10 °C (CCP).

8.6 Fractionate 10 µl of the PCR products, the negative process control and sterile water (negative PCR control) by gel electrophoresis in 1× Tris acetate EDTA (TAE buffer) (CCP). Include a 100 bp ladder. Stain with ethidium bromide and rinse in water.

8.7 Analyse the amplification products for a *Xcc* specific product of 445 bp/619 bp and a universal product of 1511 bp (CCP; Fig. 6). Two bands (619 bp/1511 bp) = positive identification of *Xanthomonas campestris*, suspected presence of *X. c.* pv. *armoraciae* or *Xcr*; two bands (445 bp/1511 bp) = indeterminate PCR result, pathogenicity test must be carried out to confirm the suspect isolate; one band (universal) = negative identification; no bands = bacterial template absent, repeat reaction.

9. Quantitative polymerase chain reaction (qPCR) option 3

9.1 Make a slightly turbid cell suspension ($OD_{600\ nm}$ approximately 0.05) in 1.0 ml sterile saline from the suspected cultures on YDC medium and the positive control. In addition, a non-suspect isolate should be

used as a negative control (CCP). Centrifuge bacterial suspensions for 5 min at 8000 rpm. Discard the supernatant and resuspend the pellet with 500 µl of 0.5 M NaOH. Incubate for 10 min at 65 °C by shaking at 1000 rpm. Dilute 5 µl of solution into 495 µl of 20 mM Tris HCl, pH 8, and vortex. Suspensions can be stored at –20 °C until identification.

9.2 Use the *Xcc* specific pair primers and probes from Köhl *et al*. (2011) and from Berg *et al*. (2006).

9.3 Universal primers should be used to validate the qPCR reaction. Use the primers and probes from Wu *et al*. (2008).

9.4 Prepare the reaction mixture (CCP). Carry out the qPCR reaction in 0.2 ml thin-walled PCR tubes in a final volume 25 µl (20 µl reaction mixture + 5 µl bacterial suspension).

9.5 Carry out qPCR reactions in a real-time PCR instrument according to following qPCR profile: an initial 10 min incubation at 95 ºC followed by 40 cycles of 15 s at 95 ºC and 30 s at 60 ºC.

9.6 Determine Ct-values; Ct-values of positive control should consistently be lower than 30. The cut-off Ct-value of the internal amplification control (IAC) should be below 35, and the expected range is to be determined by the user based on experimental data.
In the case of universal bacterial primers, positive reactions may occur in non-template controls (NTC) due to the presence of residual DNA in Taq enzyme reagents. The IAC Ct-values from reactions on suspect isolates should indicate at least a 10-fold higher concentration of bacterial DNA than the IAC Ct-values from the NTC reactions; the difference between Ct-values should be more than 3.3.

Interpretation & decisions – suspect confirm[n].

Köhl TaqMan	Berg TaqMan	qPCR Result	Follow-up
Positive	Positive	Expected result for *Xcc*	Pathogenicity test for confirmation
Negative	Positive	Expected result for *Xcr*	Pathogenicity test for confirmation
Positive	Negative	Inconclusive	Pathogenicity test for confirmation
Negative	Negative	Expected result for non-*Xcc*/*Xcr*	Negative – no follow-up, isolate is not *Xcc*/*Xcr*

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 10-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 diluted) 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)

- Dilution plates prepared from positive control isolate(s) or reference material, should give single colonies with typical morphology (Step 4.6.1).
- The numbers of colonies on dilution plates prepared from the positive control isolate(s) or reference material should be similar on both media (Step 4.6.1).
- Numbers of bacteria on dilution plates should be consistent with the dilution (i.e. should decrease approx. 10-fold with each dilution) (Step 4.6.1).
- There should be no growth on dilution plates prepared as a sterility check (Step 4.6.1).
- 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 (six per subsample) (Step 5.1), and to test all *Xanthomonas*-like subcultured isolates for pathogenicity or by PCR test (Step 5.5).
- The positive control isolate(s) or reference material should give colonies with typical morphology on YDC (Step 5.4).
- Positive control isolates should be included in every pathogenicity test (Step 6.5).
- The positive control isolate should give typical symptoms in the pathogenicity test (Step 6.7).
- Positive and negative control isolates should be included in every PCR test (Steps 7.1, 8.1, 9.1).
- The preparation of the PCR mixture (Steps 7.4, 8.4, 9.4), the amplification PCR program (Steps 7.5, 8.5, 9.5) and the preparation of agarose gel for electrophoresis (Steps 7.6, 8.6, 9.6) should be adapted to available material and equipment of individual laboratories testing for *Xcc* under the condition that results will be validated by PCR controls. Validation studies showed that PCR results were more dependent on laboratory conditions than on PCR protocol when different PCR mixes, amplification products and agarose gels were used in laboratories.
- The positive control isolate should give the expected amplification product in the PCR test (steps 7.7, 8.7, 9.7).
- 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 (units/mg) 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).
- 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 (see Media and solutions, mCS20ABN medium, Step 3).
- Prepare antibiotics stock solutions and other supplements in distilled/deionised water, or in 50 % (v/v) or 70 % (v/v) ethanol. Antibiotics stock 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 preparation of PCR mixture and the PCR conditions (Steps 2.4.3 and 2.4.8 for SE-PCR; Steps 3.4.2 and 3.4.6 for bio-PCR; and Steps 9.4 and 9.5 for suspect confirmation) should be adapted to available material and equipment of individual laboratories under the condition that results will be validated by PCR and process controls.

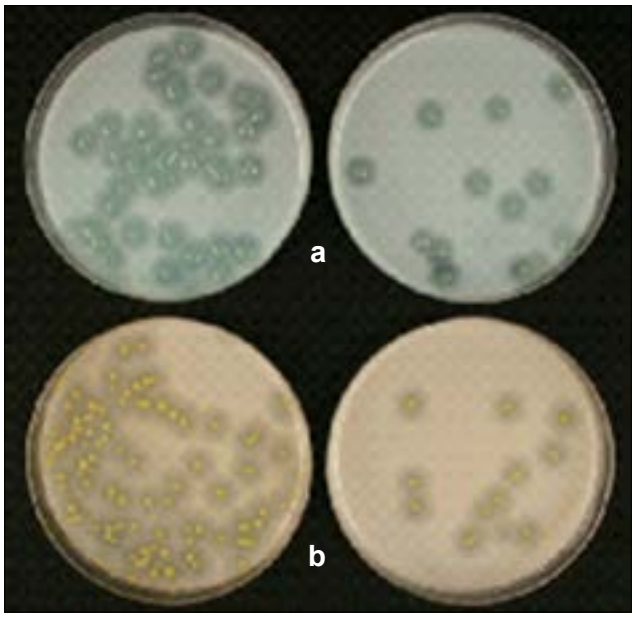

Figure 2. Plates of mFS (**a**) and mCS20ABN (**b**) after 5 days of incubation at 28 °C showing typical colonies of *Xanthomonas campestris* pv. *campestris* surrounded by zones of starch hydrolysis.

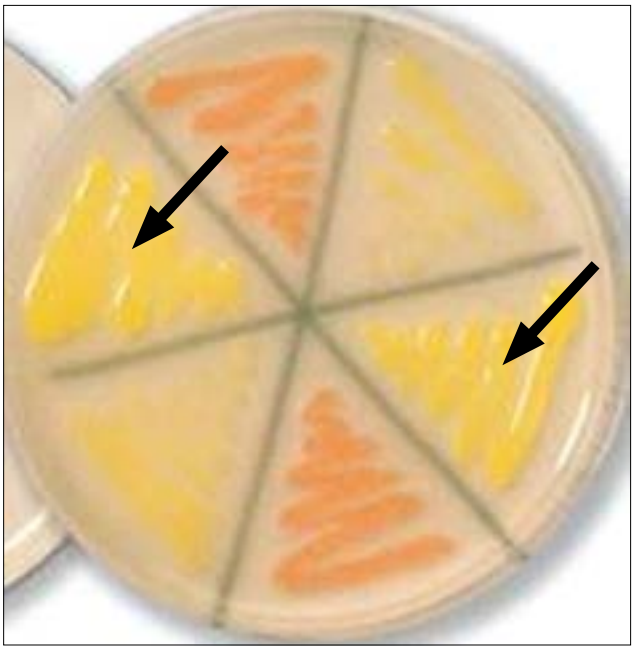

Figure 3. Typical yellow mucoid growth of isolates of *Xanthomonas campestris* pv. *campestris* on a sectored plate of YDC after 3 days at 28 °C. Only suspect cultures are indicated by arrows.

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.

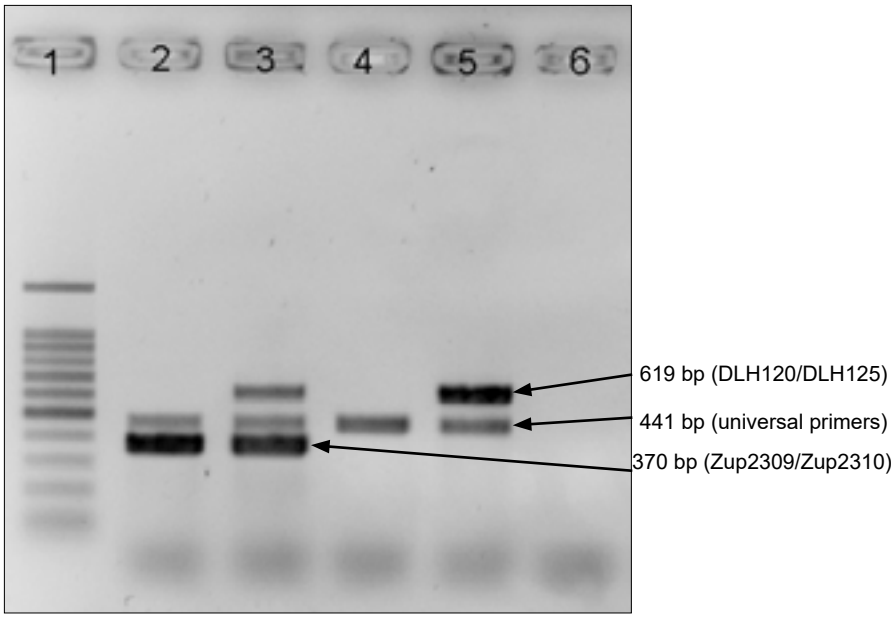

Figure 5. Agarose gel showing *Xanthomonas campestris* and *Xanthomonas campestris* pv. *campestris* amplification products using primer set combinations of PCR option 1. **1:** 100 bp ladder. **2:** Two bands (370 bp/441 bp) – indeterminate PCR result. **3:** Three bands (441 bp/619 bp/370 bp) – positive sample with *Xanthomonas campestris* pv. *campestris* (*Xcc*) (with or without *Xca*/*Xcr*). **4:** One band (441 bp) – negative sample, no *Xanthomonas campestris* (*Xc*). **5:** Two bands (441 bp/619 bp) – positive sample with *Xanthomonas campestris* (*Xca*/*Xcr* suspected presence). **6:** Water (negative PCR control) – no reaction.

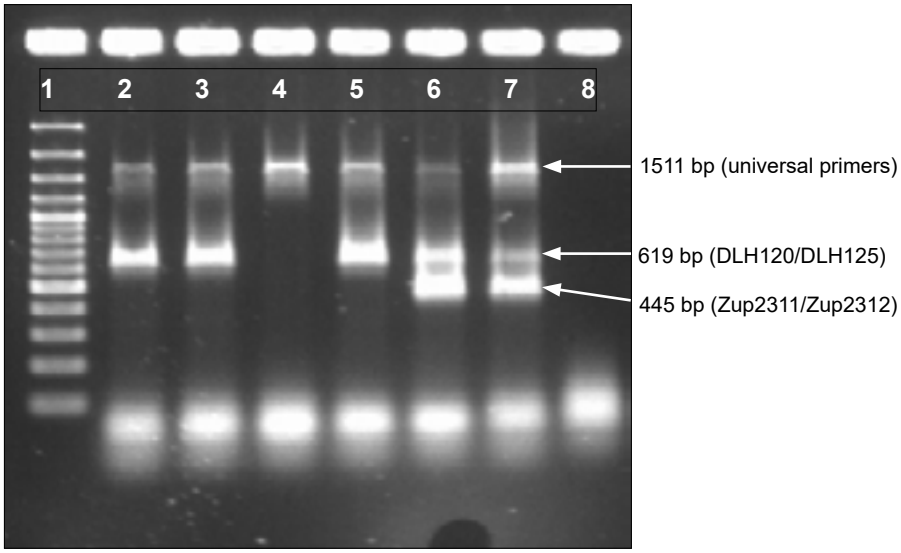

Figure 6. Agarose gel showing *Xanthomonas campestris* and *Xanthomonas campestris* pv. *campestris* amplification products using primer set combinations of PCR option 2. **1:** 100 bp ladder. **2, 3, 5:** Two bands (619 bp/1511 bp) – positive sample with *Xanthomonas campestris* (*Xca*/*Xcr* suspected presence). **4:** One band (1511 bp) – negative sample, no *Xanthomonas campestris* (*Xc*). **6, 7:** Three bands (445 bp/619 bp/1511 bp) – positive sample with *Xanthomonas campestris* pv. *campestris* (*Xcc*) (with or without *Xca*/*Xcr*). **8:** Water (negative PCR control) – no reaction.

Media and solutions

Sterile saline

Compound	Amount/l	Amount/500 ml
Sodium chloride (NaCl)	8.5 g	4.25 g
Distilled/deionised water	1000 ml	500 ml

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add 1000 ml (or 500 ml) of distilled/deionised water.
3. Dissolve and dispense into final containers.
4. Autoclave at 121 °C, 15 psi for 15 min.
5. For extraction of seeds, add 20 µl of sterile Tween™ 20 per 100 ml after autoclaving.

Storage

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

mCS20ABN medium

Compound	Amount/l	Amount/ 500 ml
Soya peptone	2.0 g	1.0 g
Tryptone (BD Bacto™ Tryptone)	2.0 g	1.0 g
KH_2PO_4	2.8 g	1.4 g
$(NH_4)_2HPO_4$	0.8 g	0.4 g
$MgSO_4 \cdot 7H_2O$	0.4 g	0.2 g
L-Glutamine	6.0 g	3.0 g
L-Histidine	1.0 g	0.5 g
D-Glucose (dextrose)	1.0 g	0.5 g
Soluble starch (Merck 1252)(CCP)	25.0 g	12.5 g
Agar (BD Bacto™ Agar)	18.0 g	9.0 g
Distilled/deionised water	1000 ml	500 ml
Nystatin[a] (10 mg/ml in 50 % ethanol)	35 mg (3.5 ml)	17.5 mg (1.75 ml)
Neomycin sulphate[b] (20 mg/ml distilled/deionised water)	40 mg (2.0 ml)	20 mg (1.0 ml)
Bacitracin[c] (50 mg/ml in 50 % ethanol)	100 mg (2.0 ml)	50 mg (1.0 ml)

[a–c] Added after autoclaving. Antibiotic amounts for guidance only (CCP).

[a] Dissolve 100 mg nystatin in 10 ml 50 % (v/v) ethanol. Add 3.5 ml/l (1.75 ml/500 ml).

[b] Dissolve 200 mg neomycin sulphate (770 U/mg) in 10 ml sterile distilled/deionised water. Add 2.0 ml/l (1.0 ml/500 ml).

[c] Dissolve 500 mg bacitracin (60 U/mg) in 10 ml 50 % (v/v) ethanol. Add 2.0 ml/l (1.0 ml/500 ml).

Preparation

1. Weigh out all ingredients except antibiotics into a suitable container.
2. Add 1000 ml (or 500 ml) of distilled/deionised water.
3. Dissolve and check pH which should be 6.5, adjust if necessary (CCP).
4. Autoclave at 121 °C, 15 psi for 15 min.
5. Prepare antibiotic solutions and filter sterilise as appropriate.
6. Allow medium to cool to approximately 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 prepared plates inverted in polythene bags at 4 °C and use within 4 weeks of preparation to ensure activity of antibiotics.

Depending on the source of starch, pre-storage of plates in the refrigerator for at least 4 days before use may result in more easily visible zones of starch hydrolysis.

mFS medium

(Schaad *et al*., 1989)

Compound	Amount/l	Amount/ 500 ml
K_2HPO_4	0.8 g	0.4 g
KH_2PO_4	0.8 g	0.4 g
KNO_3	0.5 g	0.25 g
$MgSO_4 \cdot 7H_2O$	0.1 g	0.05 g
Yeast extract	0.1 g	0.05 g
Methyl Green (1 % aq.)	1.5 ml	0.75 ml
Soluble starch (Merck 1252) (CCP)	25.0 g	12.5 g
Agar (BD Bacto™ Agar)	15.0 g	7.5 g
Distilled/deionised water	1000 ml	500 ml
Nystatin[a] (10 mg/ml 50 % ethanol)	35 mg (3.5 ml)	17.5 mg (1.75 ml)
D-Methionine[b] (1 mg/ ml 50 % ethanol)	3 mg (3.0 ml)	1.5 mg (1.5 ml)
Pyridoxine HCl[c] (1 mg/ ml 50 % ethanol)	1 mg (1 ml)	0.5 mg (0.5 ml)
Cephalexin[d] (20 mg/ ml 50 % ethanol)	50 mg (2.5 ml)	25 mg (1.25 ml)
Trimethoprim[e] (10 mg/ml 70 % ethanol)	30 mg (3 ml)	15 mg (1.5 ml)

[a–c, d,e] Added after autoclaving. Antibiotic amounts for guidance only (CCP).
[a] Dissolve 100 mg nystatine in 10 ml 50 % (v/v) ethanol. Add 3.5 ml/l (1.75 ml/500 ml).
[b] Dissolve 10 mg D-methionine in 10 ml 50 % (v/v) ethanol. Add 3.0 ml/l (1.5 ml/500 ml).
[c] Dissolve 10 mg pyridoxine HCl in 10 ml 50 % (v/v) ethanol. Add 1 ml/l (0.5 ml/500 ml).
[d] Dissolve 200 mg cephalexin in 10 ml 50 % (v/v) ethanol. Add 2.5 ml/l (1.25 ml/500 ml).
[e] Dissolve 100 mg trimethoprim in 10 ml 70 % (v/v) ethanol. Add 3 ml/l (1.5 ml/500 ml).

Preparation

1. Weigh out all ingredients except antibiotics, pyridoxine HCl and D-methionine into a suitable container.
2. Add 1000 ml (or 500 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 antibiotics, pyridoxine HCl and D-methionine solutions and filter sterilise as appropriate.
6. Allow medium to cool to approximately 50 °C before adding 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 prepared plates inverted in polythene bags at 4 °C and use within 4 weeks of preparation to ensure activity of antibiotics.

Depending on the source of starch, prestorage of plates in the refrigerator for at least 4 days before use may result in more easily visible zones of starch hydrolysis.

Yeast dextrose chalk (YDC) agar

Compound	Amount/l	Amount/500 ml
Agar (BD Bacto™ Agar)	15.0 g	7.5 g
Yeast extract	10.0 g	5.0 g
$CaCO_3$ (light powder)	20.0 g	10.0 g
D-Glucose (dextrose)	20.0 g	10.0 g
Distilled/deionised water	1 000 ml	500 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 (or 500 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 approximately 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.

0.5 M NaOH

Compound	Amount/l
NaOH	20 g
Distilled/deionised water	1000 ml

Preparation

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

1 M Tris HCl, pH 8

Compound	Amount/l
Tris base	121.1 g
Distilled/deionised water	1000 ml

Preparation

1. Weigh out ingredient into a suitable container.
2. Add 800 ml of distilled/deionised water.
3. Dissolve and dispense into final containers.
4. Check the pH with a pH meter and adjust if necessary.
5. Autoclave at 121 °C, 15 psi for 15 min and dilute to 20 mM.

Examples of reaction mixture preparations for PCR

Primer set combinations and volumes for triplex TaqMan PCR (SE-PCR)

Preferably the triplex TaqMan PCR, as described in this method, is used. If the PCR machine used is not fit for a triplex including Texas Red, alternatively two duplex (target and spike) reactions can be performed.

PCR mix triplex qPCR	For 1 reaction (in µl)	Final concentration
PCR grade H_2O	9.95	
PerfeCta Multiplex qPCR ToughMix	5.0	1×
10 µM XCC F	0.75	0.3 µM
10 µM XCC R	0.75	0.3 µM
10 µM XCC Pr	0.5	0.2 µM
10 µM DLH153-F	0.75	0.3 µM
10 µM DLH154-R	0.75	0.3 µM
10 µM P7	0.5	0.2 µM
10 µM Acat 2-F or 10 µM contig21-F	0.4	0.16 µM
10 µM Acat 2-R or 10 µM contig21-R	0.4	0.16 µM
10 µM Acat 1-Pr or 10 µM contig21-Pr	0.25	0.1 µM
Subtotal	20.0	
Sample	5.0	
Total	**25.0**	

PCR conditions for triplex TaqMan PCR

PCR conditions			
Step	Temperature	Duration	Remarks
hold	95 °C	2' 00"	
	95 °C	0' 15"	5 °C/sec
40 cycles	60 °C	0' 48"	5 °C/sec

20 mM Tris HCl	
Trizma base	1.211 g
Distilled/deionised water	500 ml

Preparation

1. Weigh out ingredient into a suitable container.
2. Add 500 ml of distilled/deionised water.
3. Dissolve and dispense into final containers.
4. Check the pH with a pH meter and adjust to pH 8.0 if necessary.
5. Autoclave at 121 °C, 15 psi for 15 min.

DLH and Zup primer volumes for SYBR Green PCR (bio-PCR)

Mix DLH	Initial conc.	Volume (µl)	Final conc.
PCR grade H_2O		4.5	
DLH153	20 µM	0.5	0.66 µM
DLH154	20 µM	0.5	0.66 µM
Quantitect SYBR Green PCR mix	2×	7.5	1×
DNA (extract)		2.0	
Total		**15.0**	

Mix Zup	Initial conc.	Volume (µl)	Final conc.
PCR grade H_2O		4.5	
Zup2309	20 µM	0.5	0.66 µM
Zup2310	20 µM	0.5	0.66 µM
Quantitect SYBR Green PCR mix	2×	7.5	1×
DNA (extract)		2.0	
Total		**15.0**	

PCR conditions for SYBR Green PCR

Step	Temperature	Duration	Remarks
Denaturation	95 °C	15 min	
6 cycles:			
Denaturation	94 °C	10 sec	–1 °C/cycle
Hybridisation	63 °C	15 sec	
Elongation	72 °C	30 sec	
34 cycles:			
Denaturation	94 °C	10 sec	
Hybridisation	58 °C	15 sec	
Elongation	72 °C	30 sec	
Melt curve	72–95 °C		

Primer set combinations and volumes for PCR option 1 (suspect isolate confirmation)

Compound	Final concentration	Volume (µl) in 20 µl
Sterile water		0.04
Green Go Taq Buffer 5×	1	4
$MgCl_2$ (25 mM)	1.2 mM	0.96
dNTP (2 mM each)	0.2 mM	2
Zup2309 (5 µM)	0.2 µM	0.8
Zup2310 (5 µM)	0.2 µM	0.8
DLH120 (5 µM)	0.6 µM	2.4
DLH125 (5 µM)	0.6 µM	2.4
1052F (1 µM)	0.085 µM	1.7
BacR (1 µM)	0.085 µM	1.7
Taq polymerase (5 U/µl)	0.05 U/µl	0.2
DNA		3

Primer set combinations and volumes for PCR option 2 (suspect isolate confirmation)

Compound	Final concentration	Volume (µl) in 25 µl
Sterile water		15.9
Taq buffer 10× (including 15 mM $MgCl_2$)	1 (1.5 mM)	2.5
dNTP (5 mM each)	0.2 mM	1
Zup2311 (20 µM)	0.8 µM	1
Zup2312 (20 µM)	0.8 µM	1
DLH120 (20 µM)	0.8 µM	1
DLH125 (20 µM)	0.8 µM	1
UpBacF (20 µM)	0.16 µM	0.2
UpBacR (20 µM)	0.16 µM	0.2
Taq polymerase (5 U/µl)	0.04 U/µl	0.2
DNA		1

Primer set combinations and volumes for PCR option 3 (suspect isolate confirmation)

Pipetting scheme duplex 1		
Berg–Wu duplex	Final concentration	Volume (µl) in 25 µl
DLH153 (10 µM)	0.5 µM	1.25
DLH154 (10 µM)	0.5 µM	1.25
P7 (10 µM, FAM)	0.2 µM	0.5
Wu-F (10 µM)	0.2 µM	0.5
Wu-R (10 µM)	0.2 µM	0.5
Wu-P1 (TexRed, 10 µM)	0.2 µM	0.5
Wu-P2 (TexRed, 10 µM)	0.2 µM	0.5
PerfeCta Multiplex ToughMix (5×)*	1×	5.0
MQ		10.0
Template DNA		5.0
Total		**25.0**

*or equivalent

Pipetting scheme duplex 2		
Köhl–Wu duplex	Final concentration	Volume (µl) in 25 µl
Xca-F (10 µM)	0.2 µM	0.5
Xca-R (10 µM)	0.2 µM	0.5
Xca-Pr (10 µM, Vic)	0.2 µM	0.5
Wu-F (10 µM)	0.2 µM	0.5
Wu-R (10 µM)	0.2 µM	0.5
Wu-P1 (TexRed, 10 µM)	0.2 µM	0.5
Wu-P2 (TexRed, 10 µM)	0.2 µM	0.5
PerfeCta Multiplex ToughMix (5×)*	1×	5.0
MQ		11.5
Template DNA		5.0
Total		**25.00**

*or equivalent

Examples for visualisation of PCR products

Tris acetate EDTA (TAE) buffer 1×

Compound	Amount/l
Tris acetate EDTA (TAE 50×)	20 ml
Distilled/deionised water	1000 ml

1.5 % (w/v) agarose gel for electrophoresis

Compound	1 agarose gel (25 × 15 cm)	Amount/l
Tris acetate EDTA (TAE 1×)	300 ml	1000 ml
Agarose	4.5 g	15.0 g

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.
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.

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Reproductability: dispersion : 1
Repeatability: dispersion : 1
Detection limits: 15 cfu/ml (theoretical, P = 0.95)