Method for the Detection of *Xanthomonas* spp. in Tomato seed

Crop:	Tomato *(Lycopersicon esculentum* L. now *Solanum lycopersicum)*
Pathogen:	*Xanthomonas euvesicatoria (Xe), Xanthomonas vesicatoria (Xv), Xanthomonas perforans (Xp) and Xanthomonas gardneri (Xg).* (See references 1 and 2 for an account of the reclassification of *Xanthomonas* species)
Revision history:	Version 5, July 2017

Sample and sub-sample size

The recommended minimum sample size is 10,000 seeds, with a maximum sub-sample size of 10,000 seeds.

Principle

- Extraction from the seed of externally and internally located bacteria
- Isolation of viable *Xanthomonas* bacteria by dilution plating seed extract on two different semi-selective media
- Confirmation of suspect bacterial colonies by morphology on a non-selective medium, followed by a PCR and by a pathogenicity assay

Restrictions on Use

- This test method is suitable for untreated seed.
- This test method is suitable for seed that has been treated using physical or chemical (calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection provided that any residue, if present, does not influence the assay. It is the responsibility of the user to check for such inhibition by analysis, sample spiking, or experimental comparisons.
- The ability to recover *Xanthomonas spp.* on plates can be influenced by the presence of other microorganisms. It is the responsibility of the user to check for such antagonism by analysis, sample spiking, or experimental comparisons.
- This test method has not been validated for seed treated with protective chemicals or biological substances. If a lab chooses to test treated seed using this method, it is the lab's responsibility to empirically determine (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on the method results.

Validation

This method has been peer reviewed by ISHI members and experts outside of ISHI. Aspects of this method have been validated by an ISHI laboratory and/or ISHI validation testing.

The method has also been approved by the US National Seed Health System (NSHS) as a Standard B (see http://seedhealth.org/seed-health-testing-methods/).

Note: The section **Validation** has been updated. Previously, a PCR-method to identify the bacteria was added in Section 4 in addition to a new section **Method Execution**.

Method Execution

Following the best practices described by ISHI-Veg for the reliable use of molecular techniques and dilution-plating assays in seed health testing (see http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg/) is strongly recommended to ensure process standardization and valid results.

Method description

1. Extraction of bacteria from the seed

1.1. Put every sub-sample into a sterile stomacher bag. Add sterile seed extraction buffer to each bag at a ratio of 4 ml of buffer to 1 g of seed. (v:w). Incubate overnight (minimum 14 hours) at 4°C, and macerate for at least 4 minutes in a stomacher machine.

2. Isolation on semi-selective media

2.1. Filter coarse particles from the required volume of extract using a filter bag (Bagfilter® or Bagpage® from Interscience, France or an extraction bag with synthetic intermediate layer from Bioreba, Switzerland or Neogen Europe, Scotland). Alternately, use a stomacher bag with filter.

2.2. Prepare a 10-fold dilution series (to 10^{-2}) of the seed extract in sterile seed extraction buffer. For sub-samples larger than 5,000 seeds also prepare a 10-fold concentrated extract. Centrifuge the filtered extract with a time and speed sufficient to pellet bacteria (e.g. for 5 minutes at 5,000 g). Remove the supernatant carefully and re-suspend the pellet in 1/10 of the original centrifuged volume of sterile seed extraction buffer.

2.3. Plate 0.1 ml of the concentrated (if applicable), the undiluted and diluted extracts on the two semi-selective media.

2.4. Prepare a 10-fold dilution series of a suspension of a pure culture of a known *Xanthomonas* reference strain in sterile seed extraction buffer. Plate 0.1 ml of the dilutions on each medium to give at least one plate of 30-300 colonies on each medium for use as reference plates (see section 2.6).

2.5. Incubate the plates at 28 °C for 4-7 days.

2.6. Check recovery and morphology of the Xanthomonas reference strain on both media.

- Examine the sample plates for the presence of colonies with typical *Xanthomonas* morphology by comparison with the reference strain. Record the number of these suspected colonies as well as of the other colonies and indicate when growth inhibition of *Xanthomonas* by the other colonies could have occurred.
- After 4-7 days of incubation on mTMB (Figure 1) or CKTM (Figure 2) *Xanthomonas* colonies are yellow, slightly mucoid, mounded and circular in shape. *Xanthomonas* utilizes Tween and in 3-7 days a white crystalline halo usually forms around the yellow colony. Some strains of *Xanthomonas* form only a weak halo or only clear the medium under the colony (not visible).
- The colony size and color can differ within a sample.

2.7. If present, select at least 6 suspected colonies per medium per sub-sample for further identification on Yeast extract-dextrose-$CaCO_3$ (YDC) medium.

3. Identification by morphology on YDC medium

3.1. Transfer suspected colonies as well as the reference strain onto YDC medium.

3.2. Incubate YDC plates at 28 °C for 2-3 days.

3.3. Determine whether transferred colonies have typical *Xanthomonas* morphology by comparison with the reference strain and record which of the isolates are still suspected to be *Xanthomonas*.

- On YDC *Xanthomonas* is pale yellow and mucoid (Figure 3).

3.4. If present, select suspected isolates for further identification with PCR and the pathogenicity assay. Select, if present, at least 6 YDC-suspected isolates from all the sub-samples combined. The selection should reflect the distribution of suspected colonies over sub-samples and/or semi-selective media.

4. Identification by PCR

Note: the following instructions on template preparation and PCR conditions are guidelines. The performance of both tests must be verified using Xe, Xv, Xp, Xg and non-target strains.

Both PCR-tests are needed. They are complimentary for some isolates and together cover the entire tomato and pepper *Xanthomonas* bacterial spot collection that was tested in validation studies.

4.1. Prepare a slightly turbid cell suspension (with an OD_{600nm} of approximately 0.05) in sterile distilled water from each suspect colony on YDC medium and the positive control strains. Cultures should not be older than 5 days after plating. Heat the suspensions for 10 min at 95–100°C. The suspensions can be stored at -20°C until the PCR analysis. Two PCRs (Sections 4.2 and 4.3) are performed for each suspect isolate.

4.2. AFLP derived Taqman® PCR

4.2.1. Use the following specific primers and probes. The probes should be used with the nucleic acid sequences exactly as indicated below; the probe fluorophores and quenchers, however, may be modified:

Primer XEF:	5'-CTCGCTCATCAAAGTGATAACGCC
Primer XER:	5'-GGGCTTGGCAGGAACGGC
Probe XEFAM:	5'-FAM-TCCGGCGAGGCAATGCGCTATAGCT-BHQ1-3'
Primer XVF:	5'-GTGCCGTTGAAATACTTGCTAGCAG
Primer XVR:	5'-CACGCTACGGGCCGCAA
Probe XVFAM:	5'-FAM-TTCGCACCGCGGGCCCTGTTCT-BHQ1-3'
Primer XPF:	5'-GTCGTGTTGATGGAGCGTTCCC
Primer XPR:	5'-CCGTCTGCTACACGACTTCCGA
Probe XPFAM:	5'-FAM-TCTCCCACACCGCGATAGGATTGACAGTAGA-BHQ1-3'
Primer XGF:	5'-ACCTGCTCCACAACGCGCTC
Primer XGR:	5'-GCTTGAATCTGTTTTTTCATTGGGATG
Probe XGFAM:	5'-FAM-TCCCATCAATAGTTGCTGCGCTATAGCTTTTCT-BHQ1-3'

4.2.2. Use Internal Amplification Control (IAC) primers, such as the following universal bacterial primers (adapted from 8), to validate the PCR reaction:

Wu Forward:	5'-CAACGCGAAGAACCTTACC-3'
Wu Reverse:	5'-ACGTCATCCCCACCTTCC-3'
Wu Probe 1:	5'-VIC-ACGACAACCATGCACCACCTG-QSY-3'
Wu Probe 2:	5'-VIC-ACGACAGCCATGCAGCACCT-QSY-3'

4.2.3. Carry out PCR reactions in a real-time PCR instrument. An example of reagent compositions and reaction conditions is described in Appendix 1A.

4.2.4. Determine Ct values; Ct values of positive controls should consistently be lower than 30. The cut-off Ct value of the internal amplification control 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.

4.3. **XopD Taqman® PCR**

4.3.1. Use the following specific primers and probe. The probe should be used with the nucleic acid sequence exactly as indicated below; the probe fluorophore and quencher, however, may be modified:

Primer XDF	5'-TCGACGGCACCTTCGACTACG-3'
Primer XDR	5'-CTGGAGCTTGCTCCGCTTGG-3'
Probe XDFAM	5'-FAM-CCTCATCAGGGATCGTCTTGCCCAAGC-BHQ1-3'

4.3.2. Use Internal Amplification Control (IAC) primers, such as the following universal bacterial primers (adapted from 8), to validate the PCR reaction:

Wu Forward:	5'-CAACGCGAAGAACCTTACC-3'
Wu Reverse:	5'-ACGTCATCCCCACCTTCC-3'
Wu Probe 1:	5'-VIC-ACGACAACCATGCACCACCTG-QSY-3'
Wu Probe 2:	5'-VIC-ACGACAGCCATGCAGCACCT-QSY-3'

4.3.3. Carry out PCR reactions in a real-time PCR instrument. An example of reagent compositions and reaction conditions is described in Appendix 1B.

4.3.4. Determine Ct values; Ct values of positive controls should consistently be lower than 30. The cut-off Ct value of the internal amplification control 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.

4.3.5. Combine results of both PCRs (sections 4.2 and 4.3). If both specific PCRs result in a negative reaction and the IACs are positive, the suspect colony is considered not to be one of the four Xanthomonas species Xv, Xe, Xp or Xg. If one or both PCRs result in a positive reaction, the pathogenicity assay should be performed to reach a final conclusion about the identity of the suspect colony. Possible PCR outcomes and the conclusions are summarized in the table below.

Table 1: Interpretation of PCR-results

AFLP derived Taqmans	XopD Taqman	qPCR Result	Follow-up
Positive	Positive	Expected positive result	Pathogenicity test needed for confirmation
Negative	Positive	Inconclusive*	Pathogenicity test needed for confirmation
Positive	Negative	Inconclusive*	Pathogenicity test needed for confirmation
Negative	Negative	Expected negative result	Negative PCR-result, isolate is not one of the four *Xanthomonas* species

*See note in introduction of Section 4.

5. Identification by pathogenicity assay

5.1. Grow seedlings of a known susceptible tomato cultivar (e.g. Cal Ace) under suitable conditions until the 2-3 true leaf stage (approx. 3-4 weeks after sowing).

5.2. Transfer a small quantity of the selected colonies (in Section 3.4.) to a culture tube with 5-10 ml sterile distilled water. The colonies must not be more than three days old.

5.3. Adjust the inoculum to 10^8 cfu per ml by visual means (to cloudiness) or optical density methods (absorbance of 0.1 to 0.2 at 600 nm).

5.4. Infiltrate a leaflet of an assay plant with the suspension by gently forcing the liquid into the underside of the leaflet using a sterile syringe without a needle.

5.5. Infiltrate a leaflet with the pathogenic isolate (positive control) and blank water (negative control).

5.6. Incubate the inoculated plants at 90 – 100 % humidity and at 27 – 32 °C with 8 – 12 h light per day.

5.7. Observe the plants daily and record the day that water-soaked lesions appear. *Xanthomonas* will develop a water-soaked lesion in 48 – 96 hours. Non-pathogenic bacteria will produce a hypersensitive reaction in 24 hours or will not develop a lesion at all.

Buffers and media

- Use de-ionized water
- Autoclave buffers and media at 121 °C, 115 psi for 15 min.
- Especially the activity of antibiotics (units/mg) is crucial for the recovery of *Xanthomonas*. The purity of antibiotics and therefore its activity can vary per batch. Compare the purity of the old and new batch and the recovery of the target pathogen as well.
- Antibiotics are not stable in time. Therefore, add antibiotics at relative low temperature (<50 °C) and store plates before use in polythene bags at 4 °C in the dark. Use plates within a month to maintain the selectivity of the media.

Seed extraction buffer (pH 7.4) per liter

Compound	Amount per liter
Na_2HPO_4	7.75 g
KH_2PO_4	1.65 g
Tween 20	0.2 ml
$Na_2S_2O_3$ [1]	0.5 g

[1] Recommended when seeds have been treated with hypochlorite

mTMB (modified Tween Medium B) per liter (3)

Compound	Amount per liter
Bacto Peptone	10.0 g
H_3BO_3 (boric acid)	0.1 g
KBr	10.0 g
$CaCl_2$ anhydrous	0.25 g
Bacto agar	15.0 g
Tween 80[1,2]	10.0 ml
Cephalexin[2]	65 mg
5-Fluorouracil[2]	12 mg
Tobramycin sulphate[2]	0.2 mg
Nystatin[2,3]	35 mg

[1] Autoclave separately

[2] Added after autoclaving

[3] Use 100 mg/L cycloheximide, instead of nystatin, when fungal growth on the selective media is not completely inhibited by 35 mg/L nystatin

CKTM medium per liter (5, 6)

Compound	Amount per liter
Soya Peptone	2.0 g
Tryptone	2.0 g
Glucose	1.0 g
L-Glutamine	6.0 g
L-Histidine	1.0 g
$(NH_4)_2HPO_4$	0.8 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.4 g
$CaCl_2$ anhydrous	0.25 g
Bacto agar	15.0 g
Tween 80[1,2]	10.0 ml
Cephalexin[2]	65 mg
5-Fluorouracil[2]	12 mg
Tobramycin sulphate[2]	0.4 mg
Bacitracin[2]	100 mg
Neomycin sulphate[2]	10 mg
Nystatin[2,3]	35 mg

[1] Autoclave separately

[2] Added after autoclaving

[3] Use 100 mg/L cycloheximide, instead of nystatin, when fungal growth on the selective media is not completely inhibited by 35 mg/L nystatin

YDC (Yeast extract Dextrose $CaCO_3$) medium per liter (4, 7)

Compound	Amount per liter
Yeast extract	10.0 g
$CaCO_3$	20.0 g
D-glucose (Dextrose)	20.0 g
Agar	15.0 g

References

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5. Sijam, K., Chang, C.J. and R.D. Gitaitis (1991) An agar medium for the isolation and identification of *Xanthomonas campestris* pv. *vesicatoria* from seed. *Phytopathology* **81**: 831-834.
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8. Wu Y.D., Chen L.H., Wu X.J., Shang S.H., Lou J.T., Du L.Z. and Y.Z. Zheng (2008) Gram-Stain-Specific-Probe-Based Real-Time PCR for Diagnosis of Bacterial Neonatal Sepsis. *Journal of Clinical Microbiology* **46(8)**: 2316-2319.

Figure 1. Colonies of *Xanthomonas* on mTMB medium

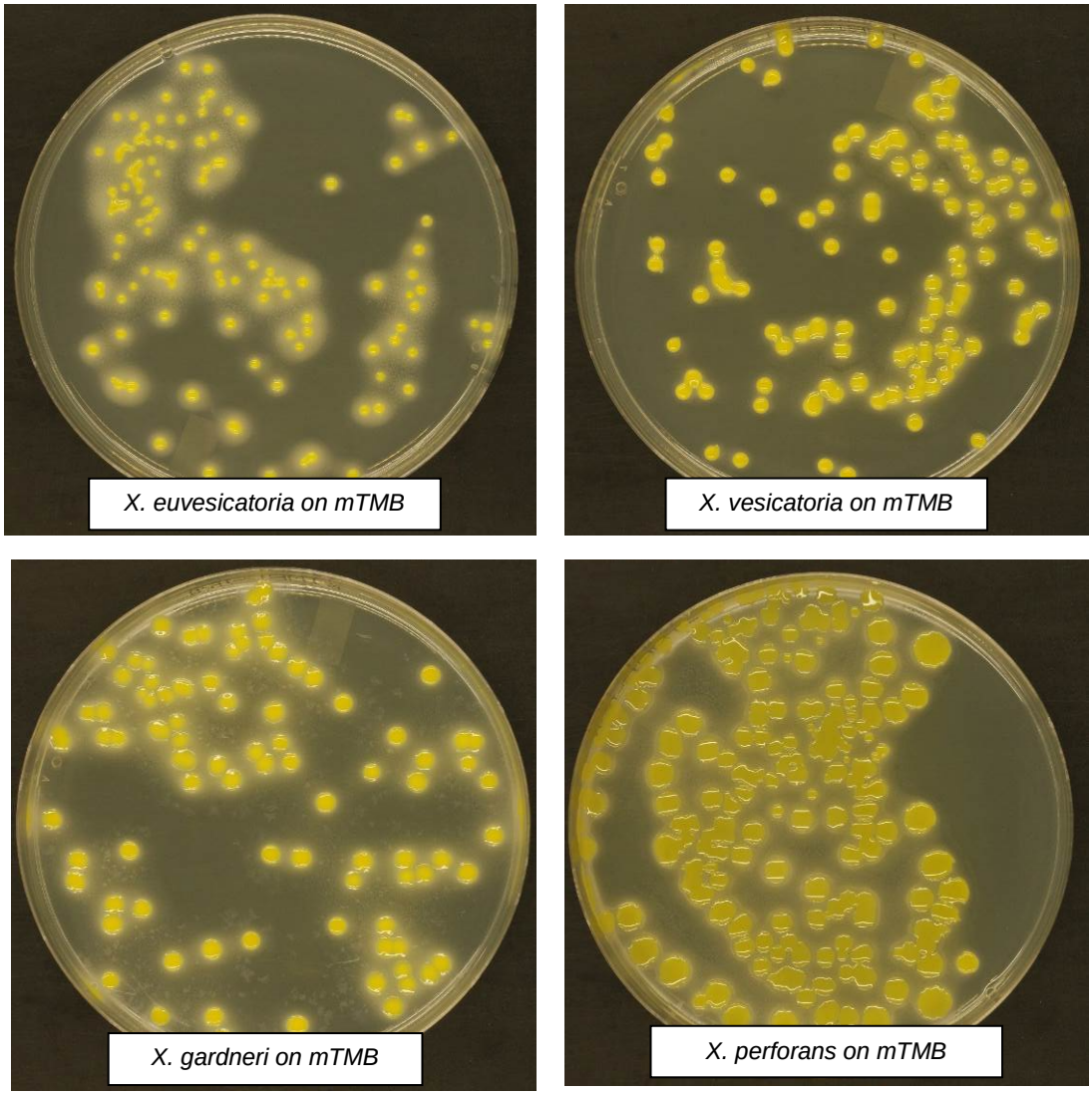


Figure 2. Colonies of *Xanthomonas* on CKTM medium

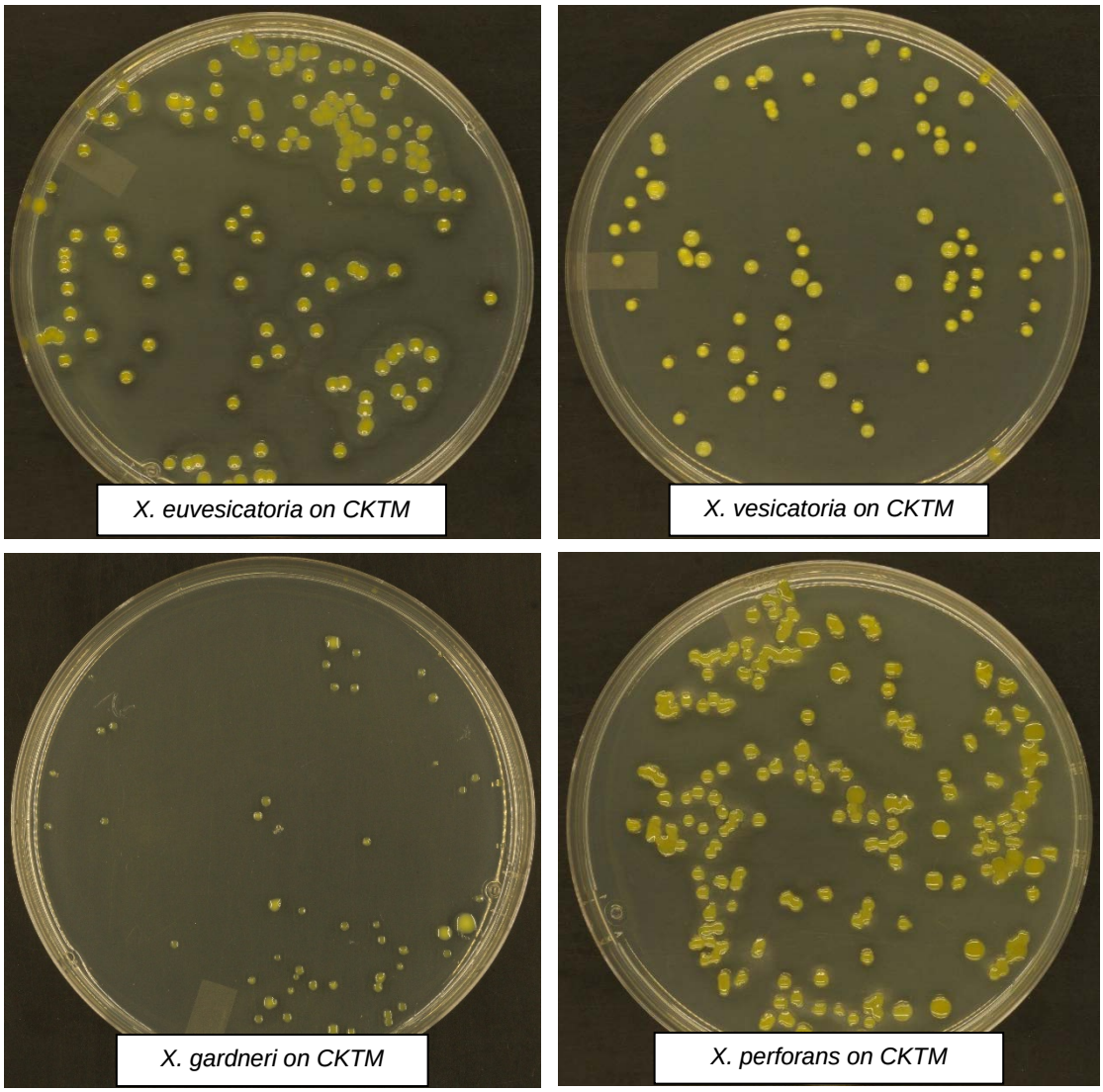
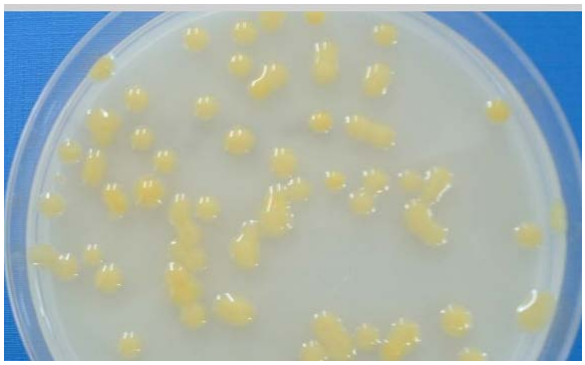


Figure 3. Colonies of *Xanthomonas* on YDC medium



Appendix 1

A. Preparation of Reaction Mixture for AFLP derived Taqman® PCR (Section 4.2.3)

Reagent	Final concentration	Volume (µl) in 15 µl
Taqman probe mastermix 2X[1]	1X	7.50
Water		1.42
XEF (20µM)	0.40µM	0.30
XER (20µM)	0.40µM	0.30
XVF (20µM)	0.40µM	0.30
XVR (20µM)	0.40µM	0.30
XPF (20µM)	0.40µM	0.30
XPR (20µM)	0.40µM	0.30
XGF (20µM)	0.40µM	0.30
XGR (20µM)	0.40µM	0.30
XEFAM (5µM)	0.04µM	0.12
XVFAM (5µM)	0.04µM	0.12
XPFAM (5µM)	0.04µM	0.12
XGFAM (5µM)	0.04µM	0.12
WuF (10µM)	0.20µM	0.30
WuR (10µM)	0.20µM	0.30
WuProbe1 (10µM)	0.20µM	0.30
WuProbe2 (10µM)	0.20µM	0.30
Bacterial suspension		2.00

[1] During the validation studies two mastermixes were tested: Qiagen Rotorgene probe 2X and Applied Taqman Universal Mastermix II. PCR reactions were done in a real-time PCR instrument in a final volume of 15 µl (13 µl reaction mixture + 2 µl bacterial suspension). PCR conditions used with the Rotogene probe mix: 5 min incubation at 95°C followed by 35 cycles of 10 s at 95°C and 15 s at 60°C.

B. Preparation of Reaction Mixture for XopD TaqMan® PCR (Section 4.3.3)

Reagent	Final concentration	Volume (µl) in 15 µl
Taqman probe mastermix 2X[1]	1X	7.50
Water		3.58
XDF(20µM)	0.40µM	0.30
XDR(20µM)	0.40µM	0.30
XDFAM(5µM)	0.04µM	0.12
WuF (10µM)	0.20µM	0.30
WuR (10µM)	0.20µM	0.30
WuProbe1 (10µM)	0.20µM	0.30
WuProbe2 (10µM)	0.20µM	0.30
Bacterial suspension		2.00

[1] During the validation studies two mastermixes were tested: Qiagen Rotorgene probe 2X and Applied Taqman Universal Mastermix II. PCR reactions were done in a real-time PCR instrument in a final volume of 15 µl (13 µl reaction mixture + 2 µl bacterial suspension). PCR conditions used with the Rotogene probe mix: 5 min incubation at 95°C followed by 35 cycles of 10 s at 95°C and 15 s at 60°C.