International Rules for Seed Testing 2027

Validated Seed Health Testing Methods

7-034: Detection of *Fusarium oxysporum* f.sp. *lycopersici* in *Solanum lycopersicum* (tomato) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2026 in Calgary, Canada

Effective from 1 January 2027

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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7-034: Detection of *Fusarium oxysporum* f.sp. *lycopersici* in *Solanum lycopersicum* (tomato) seed

Host: *Solanum lycopersicum* L.

Pathogen(s): *Fusarium oxysporum* f.sp. *lycopersici* (Saccardo) Snyder & Hansen, syn. *F. bulbigenum* var. *lycopersici* (Bruschi) Wollenweber & Reinking; *F. lycopersici* D.Bruschi

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Revision history

Version 1.0, 2026-01-01: New method
Version 1.1, 2027-01-01: Flowchart in Methods revised

Background

Fusarium oxysporum f.sp. *lycopersici* (Saccardo, 1886; Wollenweber and Reinking, 1935) is the causal agent of *Fusarium* wilt in *Solanum lycopersicum*. The first report of the disease was in Italy in the late 1880s. Since then, the disease has been reported worldwide and is of high economic importance (CABI Crop Protection Compendium).

F. oxysporum f.sp. *lycopersici* (*Fol*) is a soil-borne fungus which can survive as dormant chlamydospores in soil for extended periods of time. The fungus can infect and parasitise the root cortex of a wide range of crop and weed species (Katan, 1971). On tomato, chlamydospores germinate in the presence of small feeder roots and infect the root cortex. The fungus then penetrates the vascular system, invading the xylem (Beckman, 1987). The seed-borne nature of *Fol* has long been suspected on the basis of circumstantial evidence, but Elliott and Crawford (1922) were the first to demonstrate this method of transmission experimentally. They showed conclusively the seed-borne nature of the fungus by recovering *Fol* from seed from infected plants in Arkansas, USA, and proving that isolates were pathogenic on tomato. Approximately 3.5 % of the seeds from fruits produced on heavily infected plants contained the pathogen. Other workers have confirmed the seed-borne nature of the pathogen (Kendrick, 1944; Walker, 1971).

Symptoms of *Fusarium* wilt of tomato often occur on mature plants after flowering and at the beginning of fruit set. Initial symptoms can appear as slight wilting on part of the plant. Chlorotic symptoms begin to appear on one side of a leaf, then all leaflets become yellow on one-half of the leaf. As symptoms progress, wilting is often associated with one side of the plant. Wilt symptoms are more commonly observed during the warmest part of the day. Cutting longitudinal sections into the xylem at the base of the stem reveals a dark-brown to red discoloration. As the disease becomes more severe, vascular discoloration can be observed further up the stem, even extending into the vascular bundles of the petioles (Walker, 1971; Jones, 1991). When seedlings are infected, in addition to the symptoms described on mature plants, they can appear stunted with periodic wilting resulting in crop and economic losses.

Incubation of tomato seeds on agar plates has been used in seed health laboratories for many years for the detection and morphological identification of *F. oxysporum* sp. As a species, *F. oxysporum* includes morphologically indistinguishable pathogenic as well as non-pathogenic strains. It is important to test tomato seeds for the presence of *F. oxysporum* and to discriminate between pathogenic and non-pathogenic strains, to reduce seedling loss. Currently, determination of the host-specific pathogenic *formae speciales* is carried out by testing the fungus for pathogenicity. This bioassay is effective but is highly time-consuming and laborious (Katan *et al*., 1991; Katan *et al*., 1997) Therefore, molecular identification by quantitative polymerase chain reaction (qPCR) using two independent *Fol*-specific TaqMan assays, is included in the protocol to assess whether or not a bioassay is needed to determine pathogenicity. The primers and probes described in this method are derived from a next-generation sequencing (NGS) project in which effector profiles were identified that have the ability to distinguish between *formae speciales* of *F. oxysporum* (van Dam *et al*., 2016, 2017, 2018). Execution of the validated method is carried out according to the process flow diagram shown under Methods.

Treated seed

This method has not been validated for the detection of *F. oxysporum* f.sp. *lycopersici* on treated seed. (Definition of 'treatment': any process, biological or chemical, to which a seed lot is subjected, including seed coatings. See 7.2.3.)

Sample size

The sample size (total number of seeds tested) and subsample size depend on the desired tolerance standard (maximum acceptable number of seeds infested) and detection limit (theoretical minimum number of pathogen propagules per seed which can be detected). The minimum sample size should be 400 seeds.

Materials

Reference material: known strain of *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) or standardised reference material to use as positive process control (PPC); known strain of *F. oxysporum* f.sp. other than *Fol* to serve as negative process control (NPC) in TaqMan qPCR

Plates of potato dextrose agar with streptomycin (PDA+): 90 mm sterile Petri dishes (one per 20 seeds)

70 % (v/v) ethanol: for disinfection of surfaces, equipment

Incubator: operating at 25 ±2 °C darkness (incubation of samples and growing of reference material)

Balance: capable of weighing to the nearest 0.001 g

pH meter: capable of reading to the nearest 0.01 pH unit

Automatic pipettes (sterilised): check accuracy and precision regularly

Tomato seedlings: susceptible to all races of the pathogen (e.g. tomato 'Marmande verte' or 'Moneymaker'; see CPVO or UPOV guidelines) for pathogenicity test

Autoclave

Sterile distilled/deionised water

Steriliser

Forceps

Bürker-Türk counting chamber (haemocytometer)

Microscope: capable of ×200 and ×400 magnification

Plastic bags

Steamed potting medium

Trays, cups and cover

Growth chamber: operating at 20 ±2 °C, 80 % RH

0.5 M NaOH: used in crude DNA isolation of suspect colonies

Thermoshaker: up to 100 ±2 °C

Vortexer

Sterile inoculation loop

Real-time PCR equipment and/or PCR thermal cycler

Controls for PCR: the following positive and negative controls should be included in the confirmation PCR

- PPC – a *Fol* reference isolate grown on PDA+
- NPC – a *F. oxysporum* f.sp. reference isolate other than that of *Fol* (e.g. *F. oxysporum* f.sp. *melonis*) grown on PDA+
- No template control – PCR reaction mix with 0.5 M NaOH (see Methods, Step 3.1); the controls should give the expected (absence of) amplification in the PCR test

Real-time PCR primer and probe sequences for suspect isolate confirmation using TaqMan-qPCR

***Fol*-specific primers and probe**

PCR primers and probe *Fol* SIX2

Fol SIX2 F:5'TTC.TAC.AGG.GCG.TCA.ATG.GT3'

Fol SIX2 R:5'GCA.ACG.CCG.TTT.GAA.TAA.GC3'

Fol SIX2 Pr:5'FAM-TGG.CCT.ATG.TTG.ACC.ATG.TCC.GAT.GT-BHQ1 3'

PCR primers and probe *Fol* SIX5

Fol SIX5 F:5'CCT.CAT.AGT.GAT.TGG.GCA.ATT.GG3'

Fol SIX5 R:5'GCG.AAT.CGT.GGA.CCA.GGT.TT3'

Fol SIX5 Pr:5'VICrepl-TCC.AGC.ATA.TTG.GTT.TGC.TGA.CCG.TCA-BHQ1 3'

Generic *Fusarium* elongation factor primers and probe

EF1α Fgen

EF1α Fgen F:5'CGC.TGA.GCT.CGG.TAA.GGG3'

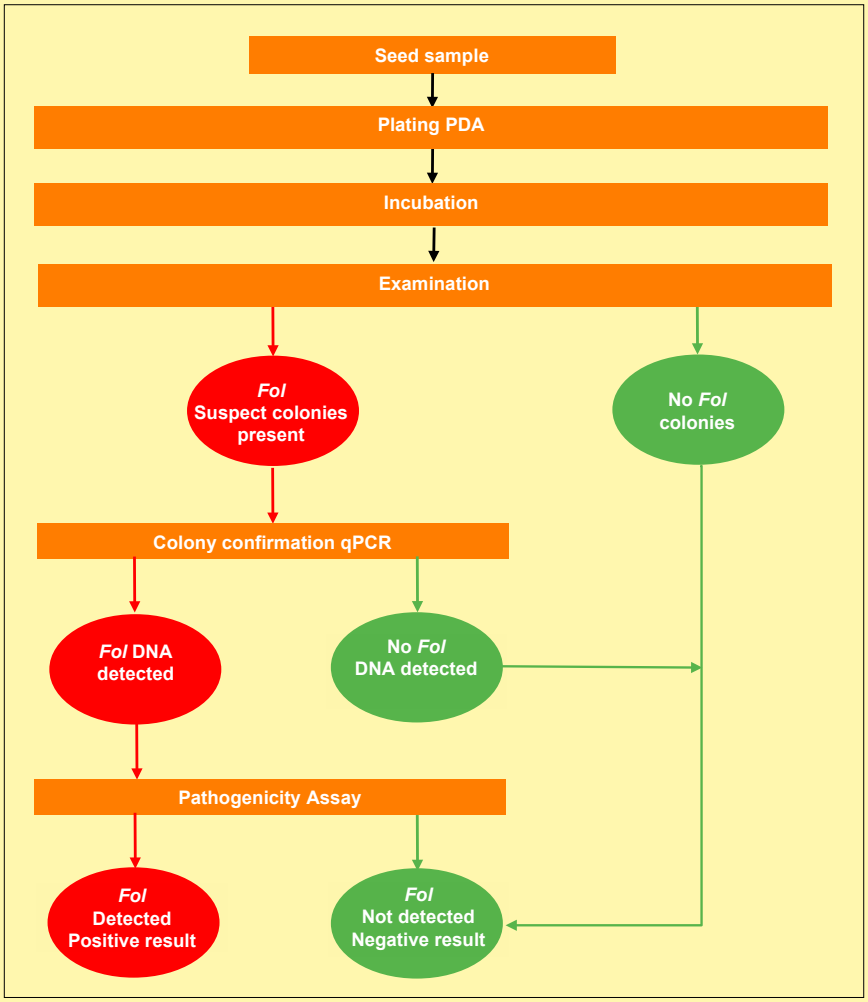
EF1α Fgen R:5'CCA.GAG.AGC.AAT.ATC.GAT.GGT.GA3'

EF1α Fgen Pr:5'Texas Red-ACG.CCT.GGG.TTC.TTG.ACA.AGC.TCA-BHQ2 3'

Sample preparation

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

The seeds must be analysed without any surface disinfection, as *Fol* may be present on the seed coat as well as inside the seed.



Methods

Note: All the steps described here are important and should be followed as written. Critical control points are indicated by 'CCP'.

1. Detection method

1.1 Plating: Aseptically place a maximum of 20 seeds onto the surface of each PDA+ plate, evenly spaced around the perimeter of the plate.

1.2 Include a sterility check by positioning an open PDA+ plate in the cabinet where the PDA+ plates with seeds are to be placed, prior to execution, then close the plate after all seeds have been processed.

1.3 Positive and negative control (reference material): Aseptically plate a reference culture on media.

1.4 Incubation: 5 days at 25 ±2 °C in darkness.

2. Examination (CCP)

2.1 After 5 days, examine the sterility check and control plates. If the sterility check is compromised by an overgrowth of fungi, yeasts and/or bacteria, evaluate if this effect is also present on the sample plates. The test should be restarted in this case. The positive and negative control should show as expected. If either of them shows false positive or false negative results or is due to overgrowth not to be evaluated, the test should be restarted.

2.2 Examine each seed by naked eye for colonies typical for *Fol* (Fig. 1). *Fol* is a fast-growing fungus and appears as a fluffy cushion surrounding the seed. Colours found for *Fol* range from white, to pink to dark purple; other colours like orange are also known but this is predominantly associated with the PDA+ brand used.

2.3 Record the percentage of presence of suspect colonies (See General methods and Fig. 1).

3. Confirmation/identification of suspect colonies by qPCR

3.1 With a sterile inoculation loop cut a small piece (0.5 × 0.5 cm) from the *Fol* suspect colonies on the PDA+ plate and transfer to a safe-lock microtube containing 1 ml of 0.5 M NaOH. The precise number of suspect colonies to be confirmed by TaqMan qPCR depends on the number and variability of suspects growing from the seeds: If present, at least six suspect colonies should be tested per sample; test isolates should

preferably be selected from different PDA+ plates (CCP). For eventual use in the pathogenicity test also transfer another piece of each suspect to a PDA+ plate and incubate for 7 days (25 ±2 °C).

3.2 Follow the same procedure for the reference culture (positive control) and a non-target reference control (negative control) (CCP).

3.3 Vortex the suspect cultures thoroughly for 20 s per tube and transfer the liquid phase by pipette to a new safe-lock tube, leaving the agar residue in the tube.

3.4 Heat the suspect samples (and controls) for 10 min at 100 ±5 °C then store the crude DNA on ice.

3.5 Confirm the identity of the suspects by TaqMan qPCR.

3.6 Use both of the *Fol*-specific pairs of primers and probes SIX2 and SIX5. Although described in a multiplex format in this protocol, simplex execution is also a possibility.

3.7 A universal fungal assay should be used to validate the TaqMan qPCR reaction. Use the primers and probes for EF1α Fgen which are *F. oxysporum*-generic for the elongation factor.

3.8 Prepare the reaction mixture as described in 'Primer set combinations and volumes for triplex TaqMan qPCR' (Media and solutions) (CCP). Carry out the TaqMan qPCR reaction in 0.2 ml white well PCR tubes in a final volume of 25 µl (20 µl reaction mixture + 5 µl crude fungal DNA).

3.9 Carry out qPCR reactions in a real-time PCR instrument according to the following qPCR profile: an initial 10 min incubation at 95 °C followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C (CCP).

3.10 Determine Ct-values; the Ct-values of the positive control should consistently be lower than 30. The cut-off Ct-value of the internal isolation control (IIC), the EF gene of *F. oxysporum*, should be below 35, and the expected range is to be determined by the user based on experimental data.

Interpretation & decisions – suspect confirmation

SIX2 TaqMan	SIX5 TaqMan	EF1α TaqMan (internal isolation control)	qPCR result	Follow-up
Positive	Positive	Positive	Expected result for *Fol*	Pathogenicity test for confirmation
Negative	Positive	Positive	Expected result for *Fol*	Pathogenicity test for confirmation
Positive	Negative	Positive	Expected result for *Fol*	Pathogenicity test for confirmation
Negative	Negative	Positive	Expected result for non-*Fol Fusarium* isolates	Negative – no follow-up, isolate is not *Fol*
Negative	Negative	Negative	Fungi other than *Fusarium*	Negative – no follow-up, isolate is not *Fol*

4. Pathogenicity test

4.1 Grow seedlings of a tomato cultivar known to be susceptible to all races of *Fol* (e.g. 'Marmande verte'; options detailed in UPOV guidelines 'Assessing distinctness in disease resistance characteristics', TWV/58/ (upov.int)) in small pots or modules until the first true leaf is just unfolding (24 ±2 °C for 12 days).

4.2 Prepare spore suspensions of all the isolates and reference isolates functioning as positive process controls (PPC) by transferring a 0.5 × 0.5 cm cube of isolate material grown on PDA+, four times into 100 ml of V8 broth (see Media and solutions) in a conical flask. Incubate the spore suspensions for 4–7 days at room temperature at 125 rpm on a shaking platform.

4.3 After incubation, filter the spore suspensions through cheesecloth and count the spore concentration using a cell counter (e.g. Bürker-Türk counting chamber or haemocytometer) under a suitable microscope.

4.4 Dilute the suspensions to a concentration of $1*10^6$ spores/ml. (A minimum of 20 ml spore suspension is needed.)

4.5 Remove the seedlings and dip the roots of three to six plants per isolate for 15 min in the spore suspension before planting them in a suitable potting medium. Plant multiple isolates together with negative control plants in a square box (see Fig. 2). Include a box with positive reference controls of the susceptible and resistant tomato varieties.

4.6 Initial evaluation according to the rules indicated in the table below should be after 14 days (24 ±2 °C day/night), and second and final scoring after 21 days. Check whether the positive controls show typical *Fol* symptoms before continuing with the sample evaluation (CCP).



Figure 1. Typical white to purple *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) colonies growing out of tomato seed on potato dextrose agar plus streptomycin (PDA+) (Millipore).

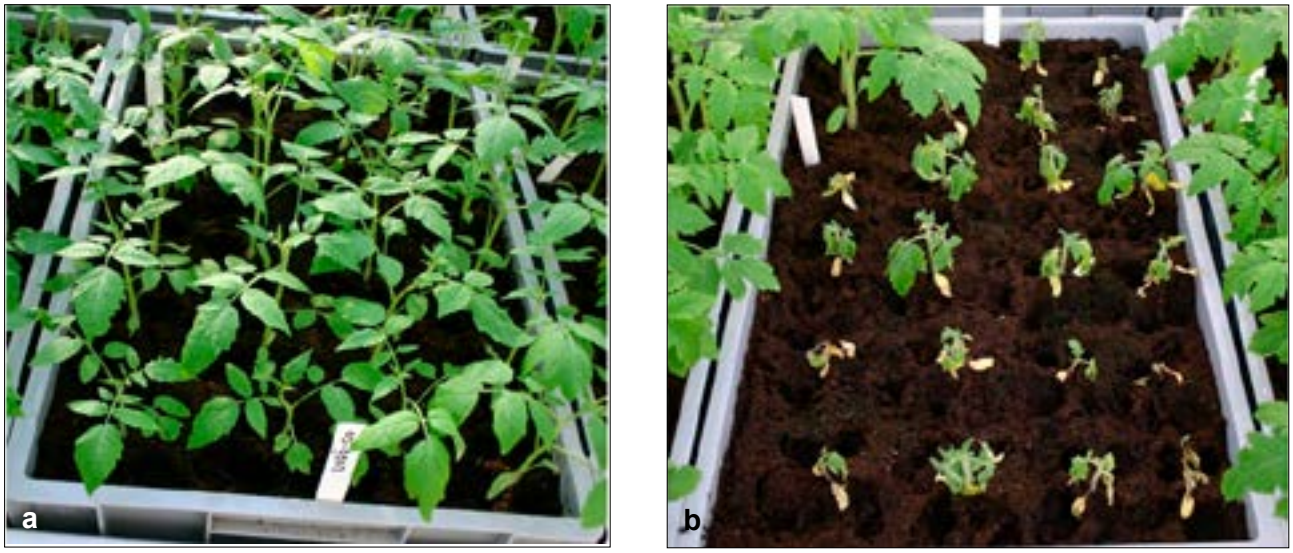


Figure 2. Test set-up for conducting the *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) pathogenicity experiment (picture taken after 14 days): negative control (**a**) and positive control (**b**).

Interpretation & decisions – pathogenicity test

Class	Symptom description	Visual result	Result
0	Healthy compared to non-inoculated control		Negative: most plants in classes 0 and 1; at most 10% of plants can be observed in classes 2 and 3
1	Healthy compared to non-inoculated control, with brown vessels above cotyledons (observed when plant stem is cut just above cotyledons)		
2	Growth reduction of more than 50 % and/or yellowing and/ or wilting of cotyledons and/ or leaves		Positive: most plants in classes 2 and 3; at most 10% of plants can be observed in classes 0 and 1
3	Nearly dead: strong growth reduction with plants looking dwarfed (there can be necrosis but not always) or dead		

Note: If all plants fall in class 0, or if all plants fall in classes 2 and 3, it is not necessary to cut the plants. In case of variety or control with different levels of symptoms, cut the plants to check for presence of strongly discoloured brown vessels above the cotyledons. If no brown vessels are observed, or are found *below* the cotyledons, the plant belongs to class 0. If brown vessels are present *above* the cotyledons, the plant is classified as a class 1 plant.

General methods

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.

For the detection method and suspect confirmation by qPCR:

In the case of a negative result (pathogen not detected), the results must be reported as 'not detected'.

In the case of a positive result, the report must indicate 'detected' and the percentage of infected seeds should be included.

For the pathogenicity test:

In the case of a negative result (absence of symptoms on all seedlings), the results must be reported as 'not pathogenic'.

In the case of a positive result (presence of symptoms on at least one of the seedlings), the results must be reported as 'pathogenic'.

Quality assurance

Specific training

This test should only be performed by persons who have been trained in basic *F. oxysporum* identification, or under the direct supervision of someone already trained, and who is trained in the execution of a TaqMan qPCR and has extensive experience with the execution of the pathogenicity test.

A record should be kept of the date and results of pipette calibration checks. It is essential that operators have received appropriate training and are able to use automatic pipettes correctly.

Critical control points (CCP)

- Preparation of plates: The source of agar may influence the results. The level of available nutrients may vary from manufacturer to manufacturer. When a new batch of agar is used, check the quality using a reference lot with a known infection level, or a reference isolate and measure sustainability of isolate. Pay particular attention to the growth characteristics of reference isolates.
- The positive control isolate(s) or reference material should give structures with typical morphology on PDA+.
- Due to the potential for non-pathogenic isolates to be present in seed lots together with pathogenic isolates, it is essential to subculture at least the minimum number of suspect colonies specified (six per subsample), and to test all *Fol*-like isolates by TaqMan qPCR test and/or by pathogenicity test.
- Positive and negative control isolates should be included in every PCR test.
- The preparation of the PCR mixture and the PCR amplification cycle should be adapted to the available material, reagents and equipment of individual laboratories testing for *Fol*, 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 TaqMan qPCR test.
- Positive control isolates should be included in every pathogenicity test (see 'Controls for PCR' under Materials).
- The positive control isolate should give typical symptoms in the pathogenicity test.

Media and solutions

Potato dextrose agar + streptomycin

Potato dextrose agar: 39 g (Millipore; follow manufacturer's instructions if using a different brand)
Distilled/deionised water: 1000 ml
Streptomycin sulphate*: 50–130 mg
*added after autoclaving

Use 50–130 mg of streptomycin sulphate, depending on the level of saprophytic bacterial contamination commonly encountered.

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add distilled/deionised water to 1000 ml.
3. Dissolve ingredients completely in water by stirring.
4. Check pH and adjust to 5.7 using NaOH or HCl.
5. Autoclave at 121 °C and 15 psi for 15 min.
6. Cool the medium to ~45 °C before adding streptomycin sulphate.
7. Pour 18–20 ml of the molten agar into 90 mm Petri dishes and allow to solidify before use.

Storage

Store prepared plates inverted in polythene bags at 4–8 °C and use within 1 month of preparation.

V8 broth

V8 Vegetable Juice: 1000 ml

Preparation

1. Distribute the V8 Vegetable Juice evenly by weight in four swingout buckets. (4 × 250 ml).
2. Centrifuge for 20 min, 4000 RCF at 4 °C and transfer supernatant to a new swingout bucket.
3. Repeat centrifugation and transfer supernatant to a sterile flask or tubes.
4. Dilute the stock solution to a 10 % solution with sterile water.

Storage

Store stock solution at −20 °C. A 10 % solution can be stored at room temperature, or preferably at 4–7 °C.

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. Mix to dissolve NaOH and dispense into final containers.
4. Autoclave at 121 °C and 15 psi for 15 min.

Reaction mixture for PCR

Primer set combinations and volumes for triplex TaqMan qPCR

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

PCR mix triplex qPCR	For 1 reaction (in µl)	Final concentration
PCR grade H_2O	9.0	
PerfeCta Multiplex qPCR ToughMix	5.0	1×
10 µM SIX2 F	0.75	0.3 µM
10 µM SIX2 R	0.75	0.3 µM
10 µM SIX2 Pr	0.5	0.2 µM
10 µM SIX5 F	0.75	0.3 µM
10 µM SIX5 R	0.75	0.3 µM
10 µM SIX5 Pr	0.5	0.2 µM
10 µM EF1α F	0.75	0.3 µM
10 µM EF1α R	0.75	0.3 µM
10 µM EF1α Pr	0.5	0.2 µM
Subtotal	20.0	
Sample	5.0	
Total	**25.0**	

PCR conditions for triplex TaqMan qPCR

Step	Temperature	Duration (min)	Remarks
hold	95 °C	2' 00"	
40 cycles	95 °C	0' 15"	3.5 °C/s
	60 °C	0' 48"[a]	3.5 °C/s

[a] plus 12 s reading

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