

Republic of Latvia

Cabinet

Regulation No. 365

Adopted 29 May 2007

## **Procedures for the Combating and Limiting of the Spread of Potato Ring Rot**

*Issued pursuant to Section 5, Clause 13  
of the Plant Protection Law*

### **I. General Provisions**

1. These Regulations prescribe the procedures for the combating and limiting of the spread of potato ring rot.
2. Potato ring rot is caused by a quarantine organism *Clavibacter michiganensis* subsp. *sepedonicus* (hereinafter – organism).
3. In order to detect potato ring rot, the State Plant Protection Service (hereinafter – the Service) shall:
  - 3.1. regularly examine tubers and plants of *Solanum tuberosum* L. potatoes;
  - 3.2. take samples of tubers and plants of *Solanum tuberosum* L. seed potatoes and potatoes intended for other use in the storage sites and send them for laboratory analyses;
  - 3.3. examine samples of *Solanum tuberosum* L. potatoes visually by cutting tubers. If visual signs of potato ring rot are detected, samples of the potatoes shall be taken and sent for laboratory analyses;
  - 3.4. diagnose the organism pursuant to the laboratory methods referred to in Annex 1 to these Regulations;
  - 3.5. use the enrichment media referred to in Annex 2 to these Regulations for isolation and growing of the organism;
  - 3.6. use the buffers referred to in Annex 3 to these Regulations for laboratory analyses;
  - 3.7. detect the contamination of the organism in accordance with Annex 4 to these Regulations;
  - 3.8. perform laboratory analyses in accordance with Annexes 5, 6 and 7 to these Regulations; and
  - 3.9. apply and control the measures for the limiting of the spread and combating the potato ring rot (hereinafter – phytosanitary measure).
4. Persons may not store or multiply purified cultures of the organism, except for the cases referred to in regulatory enactments regarding trials or introduction and movement of harmful

organisms, plants, plant products and objects, which have come into contact with them, intended for scientific purposes and varietal selection.

5. In order to limit the spread of potato ring rot, a person who grows potatoes shall restore seed potatoes with certified seed potatoes every year in the amount of 25% from the area to be planted.

6. A person who grows potatoes shall keep documents that confirm the purchase of certified seed potatoes.

## **II. Specification of Phytosanitary Measures**

7. If the Service detects visual signs of potato ring rot during the examination of *Solanum tuberosum* L. potatoes or the first positive testing result has been acquired in accordance with the immunofluorescence (IF) test referred to in Annex 1 to these Regulations, the Service shall take a decision regarding application of the following phytosanitary measures:

7.1. it is prohibited to move from a farm and to plant at a farm potatoes of such a batch or cargo, from which the sample was taken, until all the necessary laboratory tests referred to in these Regulations are completed. If there is no risk of the spread of the organism, a person shall perform only the measures referred to in Sub-paragraphs 14.2.1, 14.2.2 and 14.2.3 of these Regulations;

7.2. a person is prohibited from moving other plants of the nightshade family (*Solanaceae*) (hereinafter – host plant) from his or her farm; and

7.3. measures are performed in order to determine the possible cause of occurrence of the organism.

8. If in subsequently performing the laboratory tests referred to in Annex 1 to these Regulations, the organism is not detected in a sample, the Service shall cancel the specified phytosanitary measures.

9. In order to determine the place of origin of the organism, the Service shall examine the potato batches, which have been related to the potatoes referred to in Paragraph 7 of these Regulations.

10. The Service shall confirm the potato ring rot if the organism is detected in at least two of the different laboratory tests referred to in Annex 1 to these Regulations.

11. If the organism is detected, the Service shall take a decision regarding application of phytosanitary measures in accordance with Paragraphs 12, 13, 14 and 15 of these Regulations.

12. In order to specify the spread of contamination and mark the contaminated area (field, growth site and farms, which are in any way related to the contaminated potatoes) in the plan of a plot of land, the following shall be specified as contaminated:

12.1. the cargo or batch of *Solanum tuberosum* L. potato tubers or their plants, from which the sample was taken (hereinafter – contaminated potatoes);

12.2. the warehouse or part thereof, agricultural machinery and the vehicle, which has been in contact with the contaminated potatoes;

12.3. other objects, including packaging, which have been in contact with the contaminated potatoes;

- 12.4. the field and growth site where the batch of contaminated potatoes was obtained;  
and  
12.5. the field and growth site where potato samples were taken during the vegetation period of the potatoes.

13. In order to specify the spread of contamination and mark the contaminated area in the plan of a plot of land, the following shall be specified as possibly contaminated:

- 13.1. all tubers of potatoes or their plants, which are grown or are located in the contaminated area;  
13.2. plots of land, growing sites and premises that are related to potato tubers or plants, which have been specified as contaminated, including growing sites, which use agricultural machinery, vehicles and premises together with the growing site where the contaminated potatoes have been detected;  
13.3. potato tubers and their plants, which have been grown in the sites referred to in Sub-paragraph 13.2 of these Regulations or have been there during specification of the contamination;  
13.4. production sites or premises where activities involving potatoes, which have been moved from the sites or premises referred to in Sub-paragraphs 13.1 and 13.2 of these Regulations, are performed;  
13.5. equipment, agricultural machinery, vehicles, warehouses and other objects, including packaging, which may have come into contact with the contaminated potatoes within the time period of the previous 12 months;  
13.6. potato tubers and their plants, which have been in contact with warehouses or other objects prior to cleaning and disinfection thereof;  
13.7. potato tubers and their plants, which are related to the origin of the contaminated batch of seed potatoes or have been obtained at the same place as the contaminated potatoes because the spread of contamination is possible when multiplying plants; and  
13.8. the growing sites referred to in Sub-paragraph 13.7 of these Regulations.

14. The Service shall determine the following phytosanitary measures:

- 14.1. prohibit planting contaminated and potentially contaminated potatoes;  
14.2. entrust to perform one of the following measures:  
14.2.1. to destroy the contaminated potatoes under the supervision of the Service;  
14.2.2. to perform industrial processing of the contaminated potatoes by immediately delivering them directly to a processing undertaking, which has the appropriate equipment for waste disposal, equipment for the disinfection of the warehouse and vehicles at departure. This requirement shall be applied if the processing undertaking agrees to process the contaminated potatoes;  
14.2.3. after thermal treatment, which destroys the organism, contaminated potatoes may be used as animal feed;  
14.2.4. to deliver the contaminated potatoes to the site of waste disposal where there is no risk that the pathogen might get into the environment;  
14.2.5. to burn the contaminated potatoes;  
14.2.6. to destroy the waste of contaminated potatoes and the hard waste, which is related to the contaminated potatoes, at the site for waste disposal where there is no risk that the organism might get into the environment, or to burn, or to perform other phytosanitary measures if the risk of the spread of the organism is eliminated;  
14.2.7. to destroy the liquid waste related to the contaminated potatoes under the supervision of the Service;

14.2.8. to use the potentially contaminated potatoes for other purposes (for example, in food or to send directly for sale at sales points) under the supervision of the Service. Potato tubers shall be delivered to the sales point in packaging, and they shall not be re-packaged. Potatoes intended for planting shall be packaged only after cleaning and disinfection of the packaging site;

14.2.9. to destroy the potentially contaminated plants under the supervision of the Service; and

14.2.10. to perform industrial processing of the potentially contaminated potatoes by immediately delivering them in packaged form directly to a processing undertaking, which has the appropriate equipment for waste disposal and where the disinfection of vehicles at departure is possible.

15. The Service shall entrust a grower of potatoes to select and perform one of the following phytosanitary measures in the growing sites that have been specified as contaminated:

15.1. one of the following phytosanitary measures shall be applied in fields, which have been specified as contaminated, according to the intensity of use of the field:

15.1.1. if a phytosanitary measure is applied four years after detection of the contamination:

15.1.1.1. to destroy all wintered potatoes and other host plants of the organism in the referred period of time;

15.1.1.2. it shall not be allowed to plant potato tubers, plants thereof, sow seeds thereof, as well as to grow beets and other host plants of the organism for the subsequent three years after detection of the contamination, until there are no wintered potatoes in the field for at least two subsequent vegetation periods;

15.1.1.3. it shall be allowed to plant potatoes in the fourth year in order to obtain them for food, processing or animal feed if wintered potatoes and host plants have not been found during at least two preceding vegetation periods prior to planting. Potatoes planted in the fourth year shall be examined after harvesting in accordance with Paragraph 3 of these Regulations;

15.1.1.4. it shall be allowed to plant potatoes for acquisition of seed or for acquisition of potatoes intended for other purposes (food, processing, animal feed) in the current growth year of potatoes, taking into account crop rotation. The referred to potatoes shall be examined after harvesting in accordance with Paragraph 3 of these Regulations;

15.1.2. if a phytosanitary measure is applied five years after detection of the contamination:

15.1.2.1. to destroy all wintered potatoes and other host plants of the organism in the referred period of time;

15.1.2.2. to arrange and maintain bare fallow, pasture, which is frequently mowed and pastured low or intensely, during the first four vegetation periods;

15.1.2.3. it shall be allowed to plant potatoes for acquisition of seed or for acquisition of potatoes intended for other purposes (food, processing, animal feed) in the fifth growth year of potatoes if wintered potatoes and host plants have not been found during at least two previous vegetation periods. The referred to potatoes shall be examined after harvesting in accordance with Paragraph 3 of these Regulations;

15.2. in other fields, which are located in the contaminated area where wintered potatoes and host plants of the organism have not been found and which the Service has examined, the crop rotation and the following conditions shall be observed:

15.2.1. potato tubers, plants thereof shall not be planted, seeds thereof or host plants of the organism shall not be sown during the first vegetation period after detection of contamination. The Service shall entrust to destroy the wintered potatoes or allow to plant

only certified seed potatoes for acquisition of potatoes intended for other purposes (for example, food, processing, animal feed) if all wintered potatoes and host plants of the organism are destroyed and beets have not been grown in the preceding year;

15.2.2. during the second vegetation period after detection of contamination it shall be allowed to plant certified seed potatoes or potatoes, in which the Service has not detected the organism, for usage in food or acquisition of seed, except for the growing sites, which have been recognised as contaminated;

15.2.3. during the third growth year of potatoes after detection of contamination it shall be allowed to plant certified seed potatoes or potatoes, which have been grown from certified seed potatoes, for usage in food or acquisition of seed;

15.2.4. the Service shall entrust to destroy wintered potatoes and host plants of the organism and supervise the fulfilment of the measure during each of the vegetation periods referred to in Sub-paragraphs 15.2.1 and 15.2.2 of these Regulations; and

15.3. it is prohibited to plant potato tubers and plants thereof, as well as host plants of the organism in covered areas, except cases if certified seed potatoes, micro-tubers or meristem plants, which have been examined at the Service, are used for potato growing, as well as a complete change of growing substrate and cleaning and disinfection of covered areas and equipment has been performed.

16. The Service shall entrust a person under the supervision thereof:

16.1. to cleanse and disinfect the contaminated or potentially contaminated warehouses, agricultural machinery, vehicles and other objects, to destroy or disinfect the packaging, which has come into contact with the contaminated or potentially contaminated potatoes, immediately after detection of contamination, as well as after the subsequent vegetation period. Such objects shall not be regarded contaminated or potentially contaminated after disinfection;

16.2. to perform cleaning, as well as where appropriate disinfection, of any agricultural machinery and warehouses related to potato growing, the covered areas immediately after detection of contamination and during each subsequent vegetation period until the first allowed year of growing potatoes in fields, which were specified as contaminated;

16.3. in growing sites, which are located in the contaminated area, at least three years after detection of contamination:

16.3.1. to collect, store and move seed potatoes separately from potatoes intended for other purposes (food, processing, animal feed); and

16.3.2. to perform cleaning, as well as where appropriate disinfection, of any agricultural machinery, storehouses and covered areas related to potato growing.

17. In order to determine the potential spread of the organism and the initial place of origin of contamination, the Service shall examine:

17.1. the potato tubers and plants thereof, which are related to the batch of contaminated potatoes; and

17.2. farms, which are located near the farm where contaminated potatoes were detected.

18. If the Service detects the organism in the initial selection material, the Service shall perform examinations of the initial selection material, breeder seeds, basic seeds or highest reproduction seeds of potatoes if the origin thereof is related to the batch of contaminated seed potatoes, and shall send a sample for laboratory analyses.

19. The Service shall:

- 19.1. indicate in the decision the deadline for the performance of phytosanitary measures;
- 19.2. supervise and control the performance of phytosanitary measures;
- 19.3. perform examinations of potatoes after harvesting in the time specified in the decision in sites, which have been specified as contaminated or potentially contaminated ;
- 19.4. supervise farms, warehouses and undertakings related to the movement of potato tubers, as well as farms, which use agricultural machinery on the basis of a contract together with farms where contaminated potatoes were detected; and
- 19.5. append the map of the contaminated area to the decision.

20. The Service shall perform the examinations referred to in Paragraph 3 of these Regulations in the contaminated area for at least three subsequent vegetation periods after detection of contamination.

21. If a potato grower has not performed the phytosanitary measures specified by the Service and has planted contaminated or potentially contaminated, or uncertified potatoes in the contaminated or potentially contaminated areas, the Service may destroy plantations of potatoes under compulsion by chemical or mechanical means. Expenditure related to the destruction of the potatoes shall be covered by the potato grower.

### **III. Provision of Information to the European Commission and European Union Member States**

22. The Service shall inform the European Commission and European Union Member States without delay regarding each case when a positive testing result is obtained in a laboratory analysis. The notification shall specify the following information:

- 22.1. the registration number of the farm registered in the register of persons engaged in the circulation of plants and plant products subject to phytosanitary control if a growing site that has been specified as contaminated is located in the farm;
- 22.2. numbers of the phytosanitary certificates or plant passports appended to the contaminated cargo or batch of host plants;
- 22.3. names of the varieties of contaminated seed potatoes or potatoes intended for other purposes (food, processing, animal feed) and the category of the seed; and
- 22.4. the results of the tests referred to in Paragraph 10 of these Regulations regarding the extract, a slide prepared for the immunofluorescence test, material of contaminated eggplants (*Solanum melongena*) and purified culture of the organism.

23. The Service may apply such phytosanitary measures for the usage of the contaminated or potentially contaminated potatoes, which are not referred to in Paragraph 14 of these Regulations. Prior to application of the referred to measures the Service shall inform the European Commission and European Union Member States about it.

24. If there is a risk of contamination from potatoes that are brought in from another European Union Member State or brought out to another European Union Member State and potato ring rot is confirmed, the Service shall inform the respective Member State about it without delay, in submitting a notification together with a copy of the plant passport and delivery document. The notification shall indicate:

- 24.1. the name of the contaminated potato batch variety;

- 24.2. the given name, surname or the name and address of the consignor and the consignee;
- 24.3. the delivery date of the potatoes;
- 24.4. the size of the delivered potato batch; and
- 24.5. the registration number of the producer or the seller.

25. The Service shall inform the European Commission regarding each case of approval of the organism by submitting a notification. The notification shall provide:

25.1. the description of the specified contamination. The number of production sites, the number of potato batches and potato varieties shall be indicated in the description. The category of the seed potatoes shall be indicated;

25.2. the description of investigation. The date of confirmation of contamination, the size and traceability of the contaminated batch (sources of contamination and the potential spread of contamination) shall be indicated in the description; and

25.3. a description of the demarcated area. The number of production sites, which are not recognised as contaminated, but are included in the respective area, shall be indicated in the description.

#### **IV. Closing Provisions**

26. Cabinet Regulation No. 569 of 26 July 2005, *Procedures for the Combating and Limiting of the Spread of Potato Ring Rot (Latvijas Vēstnesis, 2005, No. 124)* is repealed.

27. Paragraph 5 of these Regulations shall come into force on 1 January 2009.

#### **Informative Reference to European Union Directives**

These Regulations contain legal norms arising from:

- 1) Council Directive 93/85/EEC of 4 October 1993 on the control of potato ring rot; and
- 2) Commission Directive 2006/56/EC of 12 June 2006 amending the Annexes to Council Directive 93/85/EEC of 4 October 1993 on the control of potato ring rot.

Prime Minister

A. Kalvītis

Minister for Agriculture

M. Roze

## Detection of the Organism in Potato Tubers and Plants

### 1. Sample preparation:

#### 1.1. Potato tubers:

1.1.1. the standard sample size shall be 200 tubers per test. More intense sampling shall require more tests on samples of this size. Larger number of tubers in the sample will lead to inhibition or difficult interpretation of the results. However, the procedure can be conveniently applied for samples with less than 200 tubers where fewer tubers are available. Validation of all detection methods described below shall be based on testing of samples of 200 tubers;

1.1.2. optional pre-treatment in advance to sample preparation. Wash the tubers. Use appropriate disinfectants (chlorine compounds when a PCR test is to be used in order to remove eventual pathogen DNA) and detergents between each sample. Air dry the tubers. This washing procedure is particularly useful (but not required) for samples with excess soil and if a PCR test or direct isolation procedure is to be performed;

1.1.3. remove with a clean and disinfected scalpel or vegetable knife the skin at the heel end of each tuber so that the vascular tissue becomes visible. Cut out a small core of vascular tissue at the heel end and keep the amount of non-vascular tissue to a minimum. Set aside any tubers with suspected organism symptoms and test separately. If during removal of the heel end core suspect symptoms of ring rot organism are observed, the tuber shall be visually inspected after cutting near the heel end. Any cut tuber with suspected organism symptoms shall be suberised at room temperature for two days and stored under quarantine (at 4 to 10°C) until all tests have been completed. All tubers in the sample, including those with suspicious symptoms, shall be kept;

1.1.4. collect the heel end cores in unused disposable containers, which can be closed or sealed (in case containers are reused they shall be thoroughly cleaned and disinfected using chlorine compounds). Preferably, the heel end cores shall be processed immediately. If this is not possible, store them in the container without addition of a buffer, refrigerated for not longer than 72 hours or for not longer than 24 hours at room temperature. Drying and suberisation of the cores, and growth of saprophytes during storage may hinder detection of the potato ring rot bacterium;

1.1.5. process the heel end cores by one of the following procedures:

1.1.5.1. cover the cores with sufficient volume (approximately 40 ml) of extraction buffer in accordance with Annex 3 to these Regulations and agitate on a rotary shaker (50 – 100 revolutions per minute) for four hours at below 24°C or for 16 to 24 hours refrigerated;

1.1.5.2. homogenise the cores with sufficient volume (approximately 40 ml) of extraction buffer (Annex 3), either in a blender (Waring or Ultra Thurax) or by crushing in a sealed disposable maceration bag (for example, Stomacher or Bioreba strong gauge polythene, 150 mm x 250 mm; radiation sterilised) using a rubber mallet or suitable grinding apparatus (for example, Homex). The risk of cross-contamination of samples is high when samples are homogenised using a blender. Take precautions to avoid aerosol generation or spillage during the extraction process. Ensure that freshly sterilised blender blades and vessels are used for each sample. If the PCR test is to be used, avoid carry-over of DNA on



containers or grinding apparatus. Crushing in disposable bags and use of disposable tubes is recommended where PCR is to be used;

1.1.6. decant the supernatant. If excessively cloudy, clarify either by slow speed centrifugation (at not more than 180 g for 10 minutes at a temperature between 4 to 10°C) or by vacuum filtration (40 to 100 µm), washing the filter with additional (10 ml) extraction buffer;

1.1.7. concentrate the bacterial fraction by centrifugation at 7 000 g for 15 minutes (or 10 000 g for 10 minutes) at a temperature between 4 to 10°C and discard the supernatant without disturbing the pellet;

1.1.8. resuspend the pellet in 1,5 ml pellet buffer. Use 500 µl to test for the organism, 500 µl for *Ralstonia solanacearum* and 500 µl for reference purposes. Add sterile glycerol to final concentration of 10 to 25% (v/v) to the 500 µl of the reference aliquot and to the remaining test aliquot, vortex and store at – 16 to – 24°C (weeks) or at – 68 to – 86°C (months). Preserve the test aliquots at 4 to 10°C during testing. Repeated freezing and thawing is not advisable. If transport of the extract is required, ensure delivery in a cool box within 24 to 48 hours;

1.1.9. all positive controls and samples of the organism shall be treated separately to avoid contamination. This shall apply to the IF test slides and to all tests.

## 1.2. potato plants:

1.2.1. for detection of latent populations of the organism it is advised to test composite samples. The procedure can be conveniently applied for composite samples of up to 200 stem parts (where surveys are performed they shall be based on a statistically representative sample of the plant population under investigation). With a clean and disinfected knife or pruning shears, remove 1 to 2 cm segment from the base of each stem, just above the soil level. Disinfect stem segments briefly with ethanol 70% and immediately blot dry on tissue paper;

1.2.2. collect stem segments in a closed sterile container according to the following sampling procedures:

1.2.2.1. process the stem segments by one of the following procedures:

1.2.2.1.1. cover the segments with sufficient volume (approximately 40 ml) of extraction buffer and agitate on a rotary shaker (50 – 100 revolutions per minute) for four hours at below 24°C or for 16 to 24 hours refrigerated;

1.2.2.1.2. process immediately. Homogenise the segments with an appropriate volume of extraction buffer by crushing in a sealed disposable maceration bag (for example, Stomacher or Bioreba) using a rubber mallet or suitable grinding apparatus (for example, Homex). If this is not possible, store the stem segments refrigerated for not longer than 72 hours or for not longer than 24 hours at room temperature;

1.2.2.2. decant the supernatant after settling for 15 minutes;

1.2.2.3. further clarification of the extract or concentration of the bacterial fraction is not usually required but may be achieved by filtration or centrifugation as described previously;

1.2.2.4. divide the neat or concentrated sample extract into two equal parts. Maintain one half at 4 to 10°C during testing and store the other half with 10 to 25% (v/v) sterile glycerol and store at – 16 to – 24°C (weeks) or at – 68 to – 86°C (months) in case further testing is required;

## 2. IF test:

2.1. the use of the IF test as the principal screening test is recommended;

2.2. when the IF test is used as the principal screening test and the IF reading is positive, the PCR or FISH test shall be performed as the second screening test. When the IF

test is used as the second screening test and the IF reading is positive, further testing according to the flow scheme is required to complete the analysis. Always use a polyclonal antibody, when the IF test is used as the principal screening test. In case of a positive IF reading with a polyclonal antibody further screening of the sample with a monoclonal antibody may provide more specificity but can be less sensitive. Antibodies to the referred strain of the organism shall be used. It is recommended that the titre is determined for each new batch of antibodies. The titre is defined as the highest dilution at which the optimum reaction occurs when testing a suspension containing  $10^5$  to  $10^6$  cells per millilitre of the homologous strain of the organism and using an appropriate fluorescein isothiocyanate (FITC) conjugate according to the manufacturer's recommendations. The crude polyclonal or monoclonal antibodies shall have an IF titre of at least 1:2 000. During testing, the antibodies shall be used at a working dilution (WD) close to or at the titre. Use validated antibodies. The test shall be performed on freshly-prepared sample extracts. If necessary, it can be successfully performed on extracts stored at  $-68$  to  $-86^\circ\text{C}$  under glycerol. Glycerol can be removed from the sample by addition of 1 ml pellet buffer, re-centrifugation for 15 minutes at 7 000 g and resuspension in an equal volume of pellet buffer solution. This is often not necessary, especially if slide samples are fixed to the slides by flaming. Prepare separate positive control slides of the homologous strain or any other reference strain of the organism, suspended in potato extract, as specified in Annex 9 to these Regulations, and optionally in buffer. Naturally infected tissue (maintained by lyophilisation or freezing at  $-16$  to  $-24^\circ\text{C}$ ) shall be used where possible as a similar control on the same slide. As negative controls, use aliquots of sample extract which previously tested negative. Use multiwell microscope slides with preferably 10 windows of at least 6 mm diameter. Test control material in an identical manner as the sample;

2.3. prepare the test slides by one of the following procedures:

2.3.1. for pellets with relatively little starch sediment. Pipette a measured standard volume (15  $\mu\text{l}$  is appropriate for a 6 mm window diameter – scale up volume for larger windows) of a 1/100 dilution of the resuspended potato pellet onto the first window. Subsequently pipette a similar volume of undiluted pellet (1/1) onto the remaining windows on the row. The second row can be used as a duplicate or for a second sample (Picture 1);

**Figure 1.**

**Preparation of the test slide for specification of the titre of antibodies**

Dilutions of the resuspended pellet						Dilution of the resuspended pellet			
	1/100	1/1	1/1	1/1	1/1	[ ]			
(T = titre)	T/2	T/4	T/2	T	2T	[ ]	Twofold	dilutions	of the
							antiserum/antibody		

Sample 1	● <sub>1</sub>	● <sub>2</sub>	● <sub>3</sub>	● <sub>4</sub>	● <sub>5</sub>
Duplicate of sample 1 or sample 2	● <sub>6</sub>	● <sub>7</sub>	● <sub>8</sub>	● <sub>9</sub>	● <sub>10</sub>

2.3.2. for other pellets. Prepare decimal dilutions (1/10 and 1/100) of the resuspended pellet in the pellet buffer. Pipette a measured standard volume (15  $\mu\text{l}$  is appropriate for 6 mm window diameter – scale up volume for larger windows) of the resuspended pellet and each dilution on a row of windows. The second row can be used as a duplicate or for a second sample (Figure 2);

**Figure 2.**

### Preparation of the test slide

Working dilution of the antiserum/antibody					Decimal dilution of the resuspended pellet
1/100	1/1	1/1	1/1	1/1	
[ ]					
Sample 1	● <sub>1</sub>	● <sub>2</sub>	● <sub>3</sub>	● <sub>4</sub>	● <sub>5</sub>
Duplicate of sample 1 or sample 2	● <sub>6</sub>	● <sub>7</sub>	● <sub>8</sub>	● <sub>9</sub>	● <sub>10</sub>

2.4. dry the droplets at ambient temperatures or by warming at a temperature of 40 to 45°C. Fix the bacterial cells to the slide either by heating (15 minutes at 60°C), flaming, with 95% ethanol or according to the specific instructions from the suppliers of the antibodies. If necessary, fixed slides may then be stored frozen in a sealed desiccated box for as little time as necessary (up to a maximum of 3 months) prior to further testing;

#### 2.5. IF procedure:

##### 2.5.1. test slides shall be prepared:

2.5.1.1. prepare a set of twofold dilutions of the antibody in the IF buffer. The first well shall have ½ of the titre (T/2), the other ¼ of the titre (T/4), ½ of the titre (T/2), the titre (T) and twice the titre (2T);

2.5.1.2. prepare test slides. Prepare the working dilution (WD) of the antibody in IF buffer. The working dilution affects the specificity;

2.5.2. arrange the slides on moist paper. Cover each test window completely with the antibody dilution. The volume of antibody applied on each window shall be at least the volume of extract applied. The following procedure shall be carried out in the absence of specific instructions from the suppliers of the antibodies:

2.5.2.1. incubate the slides on moist paper under a cover for 30 minutes at ambient temperature (18 – 25°C);

2.5.2.2. shake the droplets off each slide and rinse carefully with IF buffer. Wash by submerging for five minutes in IF buffer-Tween and subsequently for five minutes in IF buffer. Avoid causing aerosols or droplet transfer that could result in cross-contamination. Carefully remove excess moisture by blotting gently;

2.5.2.3. arrange the slides on moist paper. Cover the test windows with the dilution of FITC conjugate used to determine the titre. The volume of conjugate applied on the windows shall be identical to the volume of antibody applied;

2.5.2.4. incubate the slides on moist paper under a cover for 30 minutes at ambient temperature (18 – 25°C);

2.5.2.5. shake the droplets of conjugate off the slide. Rinse and wash as before. Carefully remove excess moisture;

2.5.2.6. pipette 5 to 10 µl of 0.1 M phosphate-buffered glycerol or a commercially antifading mountant on each window and apply a coverslip;

#### 2.6. reading the IF test:

2.6.1. examine test slides on an epifluorescence microscope with filters suitable for excitation of FITC, under oil or water immersion at a magnification of 500 to 1 000. Scan windows across two diameters at right angles and around the perimeter. For samples showing no or low number of cells observe at least 40 microscope fields. Check the positive control slide first. The cells must be bright fluorescent and completely stained at the

determined antibody titre or working dilution. The IF test shall be repeated if the staining is aberrant;

2.6.2. observe for bright fluorescing cells with the characteristic morphology of the organism in the test windows of the test slides. The fluorescence intensity shall be equivalent or better than the positive control strain at the same antibody dilution. Cells with incomplete staining or with weak fluorescence shall be disregarded. If any contamination is suspected the test shall be repeated. This may be the case when all slides in a batch show positive cells due to the contamination of the buffer or if positive cells are found (outside of the slide windows) on the slide coating;

2.6.3. there are several problems inherent to the specificity of the immunofluorescence test. Background populations of fluorescing cells with atypical morphology and cross reacting saprophytic bacteria with a size and morphology similar to the organism are likely to occur in potato heel end core and stem segment pellets;

2.6.4. consider only fluorescing cells with typical size and morphology at the titre or working dilution of the antibodies;

2.7. interpretation of the IF reading:

2.7.1. if bright fluorescing cells with characteristic morphology are found, estimate the average number of typical cells per microscope field and calculate the number of typical cells per millilitre of resuspended pellet. The IF reading is positive for samples with at least  $5 \times 10^3$  typical cells per millilitre of resuspended pellet. The sample is considered potentially contaminated, and further testing is required;

2.7.2. the IF reading is negative for samples with less than  $5 \times 10^3$  typical or atypical cells per millilitre resuspended pellet, and the sample is considered negative. Further testing is not required.

### 3. FISH test:

3.1. when the FISH test is used as the first screening test and found to be positive, the IF test shall be performed as a second compulsory screening test. When the FISH test is used as the second screening test and found to be positive, further testing according to the flow scheme is required to complete the diagnosis. Use validated *C. m. subsp. sepedonicus*-specific oligoprobes. Preliminary testing with this method shall permit reproducible detection of at least  $10^3$  to  $10^4$  cells of the organism per millilitre added to sample extracts which previously tested negative. The following procedure shall preferably be performed on freshly prepared sample extract but can also be successfully performed on sample extract that has been stored under glycerol at  $-16$  to  $-24^\circ\text{C}$  or  $-68$  to  $-86^\circ\text{C}$ . As negative controls, use aliquots of sample extract that previously tested negative for the organism. As positive controls prepare suspensions containing  $10^5$  to  $10^6$  cells per millilitre of the organism (for example, strain NCPPB 4053, or PD 406) in 0,01 M phosphate buffer (PB) from a three to five day culture. Prepare separate positive control slides of the homologous strain or any other reference strain of the organism, suspended in potato extract. The use of the FITC-labelled eubacterial oligoprobe offers a control for the hybridisation process, since it will stain all eubacteria that are present in the sample. Test control material in an identical manner as the sample;

3.2. potato extract fixation:

3.2.1. the following protocol is based upon Wullings et al. (1998);

3.2.2. prepare fixative solution;

3.2.3. pipette 100  $\mu\text{l}$  of each sample extract into an Eppendorf tube and centrifuge for eight minutes at 7 000 g;

3.2.4. remove the supernatant and dissolve the pellet in 500  $\mu\text{l}$  of fixative prepared at least 24 hours previously. Vortex and incubate overnight at  $4^\circ\text{C}$ . An alternative

fixative is 96% ethanol. To use this dissolve the intended pellet in 50 ġl 0,01 M PB and 50 ġl 96% ethanol. Vortex mix and incubate at 4<sup>0</sup>C for 30-60 minutes;

3.2.5. centrifuge for eight minutes at 7 000 g, remove the supernatant and resuspend the pellet in 75 ġl of 0,01 M PB;

3.2.6. spot 16 ġl of the fixed suspension onto a clean multitest slide (Picture 3);

**Figure 3.**

### Layout of FISH slide

Sample 1 ○ window 1	Blank ○ window 2	Blank ○ window 3	Blank ○ window 4	Sample 2 ○ window 5
Sample 1 ○ window 6	Blank ○ window 7	Blank ○ window 8	Blank ○ window 9	Sample 2 ○ window 10
Coverslip 1			Coverslip 2	

Apply two different undiluted samples per slide, and use 10 ġl to make a 1:100 dilution (in 0,01 MB). The remaining sample solution (49 ġl) can be stored at – 20<sup>0</sup>C after addition of 1 volume of 96% ethanol. In case the FISH assay requires repeating, remove the ethanol by centrifugation and add an equal volume of 0,01 M PB (mix by vortexing);

3.2.7. air-dry the slide (or on slide dryer at 37<sup>0</sup>C) and fix them by flaming. At this stage the procedure may be interrupted and the hybridisation continued the following day. Slides shall be stored dust-free and dry at room temperature;

3.3. pre-hybridisation and hybridisation:

3.3.1. prepare a lysozyme solution containing 10 mg lysozyme (Sigma L-6876) in 10 ml buffer (100 mM Tris-HCl, 59 mM ADTA, pH 8,0). This solution can be stored but it shall only be freeze-thawed once. Cover each sample well with approximately 50 ġl of lysozyme solution and incubate for 10 minutes at room temperature. Then dip the slides in demineralised water, once only and dry them with filter paper. Alternatively, instead of lysozyme add 50 ġl of 40 to 400 ġg ml<sup>-1</sup> proteinase K in buffer (20 mM Tris-HCl, 2 mM CaCl<sub>2</sub>, pH 7,4) and incubate at 37<sup>0</sup>C for 30 minutes;

3.3.2. dehydrate the cells in a graded ethanol series of 50%, 80% and 96% ethanol. Air dry the slides in a slide-holder;

3.3.3. prepare a moist incubation chamber by covering the bottom of an air-tight box with tissue or filter paper soaked in 1 x hybmix. Pre-incubate the box in the hybridisation oven at 55<sup>0</sup>C for at least 10 minutes;

3.3.4. prepare the hybridisation solution allowing 45 ġl per slide, and preincubate for five minutes at 55<sup>0</sup>C;

3.3.5. place slides on a hot plate at 45<sup>0</sup>C and apply 10 ġl of hybridisation solution to each of the four wells on the slide;

3.3.6. apply two coverslips (24 x 24 mm) to each slide without trapping air. Place the slides in the pre-warmed moist chamber and hybridise overnight in the oven at 55<sup>0</sup>C in the dark;

3.3.7. prepare three beakers containing 1 l of ultra pure water, 1 l of 1 x hybmix (334 ml 3x hybmix and 666 ml ultra pure water) and 1 l of 1/2x hybmix (167 ml 3x hybmix and 833 ml ultra pure water). Pre-incubate each in a waterbath at 55<sup>0</sup>C;

3.3.8. remove the coverslips from the slides and place the slides in a slide holder;

3.3.9. wash away excess probe by incubation for 15 minutes in the beaker with 1x hybmix at 55<sup>0</sup>C;

3.3.10. transfer the slide holder to ½ hybmix washing solution and incubate for a further 15 minutes;

3.3.11. dip the slides briefly in the ultra pure water and place them on filter paper. Remove excess moisture by covering the surface gently with filter paper. Pipette 5 to 10 µl of anti-fading mountant solution (for example, Vectashield, Vecta Laboratories, Canada, USA or equivalent) on each window and apply a large coverslip (24 x 60 mm) over the whole slide;

#### 3.4. reading the FISH test:

3.4.1. observe the slides immediately with a microscope fixed for epifluorescence microscopy at 630 or 1 000 magnifications under immersion oil. With a filter suitable for fluorescein isothiocyanate (FITC) eubacterial cells (including most gram negative cells) in the sample are stained fluorescent green. Using a filter for tetramethylrhodamine-5-isothiocyanate, Cy3-stained cells of the organism appear fluorescent red. Compare cell morphology with that of the positive controls. Cells must be brightly fluorescent and completely stained. The FISH test shall be repeated if the staining is aberrant. Scan windows across two diameters at right angles and around the perimeter. For samples showing no or low number of cells observe at least 40 microscope fields;

3.4.2. observe for bright fluorescing cells with characteristic morphology of the organism in the test windows of the test slides. The fluorescence intensity must be equivalent or better than that of the positive control strain. Cells with incomplete staining or with weak fluorescence shall be disregarded;

3.4.3. if any contamination is suspected the test shall be repeated. This may be the case when all slides in a batch show positive cells due to the contamination of buffer or if positive cells are found (outside of the slide windows) on the slide coating;

3.4.4. there are several problems inherent to the specificity of the FISH test. Background populations of fluorescing cells with atypical morphology and cross reacting saprophytic bacteria with size and morphology similar to the organism may occur, although much less frequent than in the IF test, in potato heel end core and stern segment pellets;

3.4.5. consider only fluorescing cells with typical size and morphology.

#### 3.4.6. Interpretation of the FISH test results:

3.4.6.1. valid FISH test results are obtained if bright green fluorescent cells of size and morphology typical of the organism are observed using the FITC filter and if bright red fluorescent cells using the rhodamine filter in all positive controls and not in any of the negative controls. If bright fluorescing cells with characteristic morphology are found, estimate the average number of typical cells per microscope field and calculate the number of typical cells per millilitre of resuspended pellet (Annex 4). Samples with at least  $5 \times 10^3$  typical cells per ml of resuspended pellet are considered potentially contaminated. Further testing is required. Samples with less than  $5 \times 10^3$  typical cells per millilitre of resuspended pellet shall be considered negative;

3.4.6.2. the FISH test is negative if bright red fluorescent cells with size and morphology typical of the organism are not observed using the rhodamine filter, provided that typical bright red fluorescent cells are observed in the positive control preparations when using the rhodamine filter;

#### 4. PCR test:

4.1. when the PCR test is used as the principal screening test and found to be positive, the IF test shall be performed as a second compulsory screening test. When the PCR test is used as the second screening test and found to be positive, further testing according to the flow scheme is required to complete the diagnosis. Full exploitation of this method as principal screening test is only recommended when specialised expertise has been acquired. Preliminary testing with this method should permit reproducible detection of  $10^3$  to  $10^4$  cells of the organism per millilitre added to sample extracts which previously tested negative. Optimisation experiments may be required to achieve maximum levels of sensitivity and specificity in all laboratories. Use validated PCR reagents and protocols. Preferably select a method with an internal control. Use appropriate precautions to avoid contamination of sample with target DNA. The PCR test shall be performed by experienced technicians, in dedicated molecular biology laboratories, in order to minimise the possibility of contamination with target DNA. Negative controls (for DNA extraction and PCR procedures) shall always be handled as final samples in the procedure, to make evident whether any carry over of DNA has occurred;

4.2. the following negative controls shall be included in the PCR test:

4.2.1. sample extract that previously tested negative for the organism;

4.2.2. buffer controls used for extracting the bacterium and the DNA from the sample;

4.2.3. PCR-reaction mix;

4.3. the following positive controls shall be included:

4.3.1. aliquots or resuspended pellets, to which the organism has been added;

4.3.2. a suspension of  $10^6$  cells per millilitre of the organism in water from a virulent isolate (for example, NCPPB 2140 or NCPPB 4053);

4.3.3. if possible also use DNA extracted from positive control samples in the PCR test. To avoid potential contamination positive controls shall be prepared in a separate environment from samples to be tested. Sample extracts shall be as free as possible from soil. It could therefore in certain cases be advisable to prepare extractions from washed potatoes if PCR protocols are to be used;

4.4. DNA purification methods:

4.4.1. use positive and negative control samples as described above. Prepare control material in an identical number as the sample;

4.4.2. a variety of methods are available for purification of target DNA from complex sample substrates, thus removing inhibitors of PCR and other enzymatic reactions and concentrating target DNA in the sample extract;

4.4.3. the following method has been optimised for use with the validated PCR method:

4.4.3.1. method according to Pastrik (2000):

4.4.3.1.1. pipette 220  $\mu$ l of lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8,0], 1 mM EDTA [pH 8,0]) into a 1,5 ml Eppendorf tube;

4.4.3.1.2. add 100  $\mu$ l sample extract and place in a heating block or waterbath at 95°C for 10 minutes;

4.4.3.1.3. put tube on ice for 5 minutes;

4.4.3.1.4. add 80  $\mu$ l Lysozyme stock solution (50 mg lysozyme per ml in 10 mM Tris-HCl, pH 8,0) and incubate at 37°C for 30 minutes;

4.4.3.1.5. add 220  $\mu$ l of Easy DNA® solution A (Invitrogen), mix well by vortexing and incubate at 65°C for 30 minutes;

4.4.3.1.6. add 100  $\mu$ l of Easy DNA® solution B (Invitrogen), vortex vigorously until the precipitate runs freely in the tube and the sample is uniformly viscous;

4.4.3.1.7. add 500  $\mu$ l of chloroform and vortex until the viscosity decreases and the mixture is homogenous;

4.4.3.1.8. centrifuge at 15 000 g for 20 minutes at 4°C to separate phases and form the interphase;

4.4.3.1.9. transfer the upper phase into a fresh Eppendorf tube;

4.4.3.1.10. add 1 ml of 100% ethanol (– 20°C), vortex briefly and incubate on ice for 10 minutes;

4.4.3.1.11. centrifuge at 15 000 g for 20 minutes at 4°C and remove ethanol from pellet;

4.4.3.1.12. add 500  $\mu$ l of 80% ethanol (– 20°C) and mix by inverting the tube;

4.4.3.1.13. centrifuge at 15 000 g for 10 minutes at 4°C, save the pellet and remove ethanol;

4.4.3.1.14. allow the pellet to dry in air or in a DNA speed vac;

4.4.3.1.15. resuspend the pellet in 100  $\mu$ l sterile ultra pure water and leave at room temperature for at least 20 minutes;

4.4.3.1.16. store at – 20°C until required for PCR;

4.4.3.1.17. spin down any white precipitate by centrifugation and use 5  $\mu$ l of the supernatant containing DNA for the PCR;

4.4.3.2. other DNA extraction methods (for example, Qiagen DNeasy Plant Kit) could be applied providing that they are proven to be equally as effective in purifying DNA from control samples containing  $10^3$  to  $10^4$  pathogen cells per millilitre.

#### 4.4.4. PCR:

4.4.4.1. prepare test and control templates for PCR according to the validated protocol. Prepare one decimal dilution of sample DNA extract (1:10 ultra pure water);

4.4.4.2. prepare the appropriate PCR reaction mix in a contamination-free environment according to the published protocol. The validated PCR protocol is a multiplex reaction that also incorporates an internal PCR control;

4.4.4.3. add 5  $\mu$ l of DNA extract per 25  $\mu$ l PCR reaction in sterile PCR tubes;

4.4.4.4. incorporate a negative control sample containing only PCR reaction mix and add the same source of ultra pure water as used in the PCR mix in place of sample;

4.4.4.5. place tubes in the same thermal cycler which was used in preliminary testing and run the appropriately optimised PCR programme in accordance with Annex 5 to these Regulations;

#### 4.4.5. analysis of the PCR product:

4.4.5.1. resolve PCR amplicons by agarose gel electrophoresis. Run at least 12  $\mu$ l of amplified DNA reaction mixture from each sample mixed with 3  $\mu$ l loading buffer (Annex 5) in 2,0% (w/v) agarose gels in tris-acetate-EDTA (TAE) buffer at 5 to 8 V/cm. Use an appropriate DNS marker, for example, 100 bp ladder;

4.4.5.2. reveal DNA bands by staining in ethidium bromide (0,5 mg/L) for 30 to 45 min taking appropriate precautions for handling this mutagen;

4.4.5.3. observe stained gel under short wave UV transillumination (for example,  $\lambda = 302$  nm) for amplified PCR products of the expected size and document;



4.4.5.4. for all new findings/cases verify authenticity of the PCR amplicon by performing restriction enzyme analysis on a sample of the remaining amplified DNA by incubating at the optimum temperature and time with an appropriate enzyme and buffer. Resolve the digested fragments by agarose gel electrophoresis as before and observe characteristic restriction fragment pattern under UV transillumination after ethidium bromide staining. Compare with the undigested and digested positive control;

4.4.6. interpretation of the PCR test result:

4.4.6.1. the PCR test is negative if the organism-specific PCR amplicon of expected size is not detected for the sample in question but is detected for all positive control samples (in case of multiplex PCR with plant specific internal control primers: a second PCR-product of expected size must be amplified with the sample in question);

4.4.6.2. the PCR test is positive if the organism-specific PCR amplicon of expected size and restriction pattern (when required) is detected, providing that it is not amplified from any of the negative control samples. Reliable confirmation of a positive result can also be obtained by repeating the test with a second set of PCR primers. Inhibition of the PCR may be suspected if the expected amplicon is obtained from the positive control sample containing *C. m. subsp. sepedonicus* in water but negative results are obtained from positive controls with *C. m. subsp. sepedonicus* in potato extract. In multiplex PCR protocols with internal PCR controls, inhibition of the reaction is indicated when neither of the two amplicons are obtained. Contamination may be suspected if the expected amplicon is obtained from one or more of the negative controls.

5. Bioassay test:

5.1. preliminary testing with this method shall permit reproducible detection of  $10^3$  to  $10^4$  colony-forming units of the organism per millilitre added to sample extracts that previously tested negative. Highest sensitivity of detection can be expected when using freshly prepared sample extract and optimal growth conditions. However, the method can be successfully applied to extracts that have been stored under glycerol at  $-68$  to  $-86^\circ\text{C}$ . Some varieties of eggplant provide an excellent selective enrichment medium for the growth of the organism even in the absence of symptoms and also provide an excellent confirmatory host test. Growth conditions shall be optimal to reduce the risk of false negative test results in accordance with the cultural details indicated in Annex 7 to these Regulations;

5.2. distribute the whole of the remaining test aliquot of the resuspended pellet between eggplants by one of the methods given below. Use only plants at leaf stage two to three up to full expansion of the third true leaf. In order to ensure complete utilisation of the resuspended pellet as well as effective inoculation, the procedures outlined below will require 15 to 25 eggplants per sample;

5.3. do not water eggplants for one to two days prior to inoculation to reduce turgor pressure;

5.4. slit inoculation:

5.4.1. holding the plant between two fingers, pipette a drop (approximately 5 to 10  $\mu\text{l}$ ) of the suspended pellet on the stem between the cotyledons and the first leaf;

5.4.2. using a sterile scalpel, make a diagonal slit, about 1,0 cm long and approximately 2/3 of the stem thickness deep, starting the cut from the pellet drop;

5.4.3. seal the cut with sterile vaseline from a syringe;

5.5. syringe inoculation:

5.5.1. inoculate the eggplant stems just above the cotyledons using a syringe fitted with a hypodermic needle (not less than 23G). Distribute the sample between the eggplants;

5.5.2. as the positive controls, inoculate 5 plants with an aqueous suspension of  $10^5$  to  $10^6$  cells per millilitre of a known culture of the organism and, where possible, with naturally infected tuber tissue by the same inoculation method;

5.5.3. as the negative control, inoculate 5 plants with sterile pellet buffer by the same inoculation method;

5.5.4. incubate plants in quarantine facilities for up to four weeks at 18 to 24°C. Incubate plants with sufficient light and high humidity (70 to 80%) and water to prevent water logging or wilting through water deficiency. Cells of the organism are killed at temperatures above 30°C and the optimum temperature is 21°C. To avoid contamination incubate positive control and negative control plants on clearly separated benches in a glasshouse or growth chambers or, in case space is limited, ensure strict separation between treatments. If plants for different samples must be incubated close together, separate them with appropriate screens. When fertilising, watering, inspecting and any other manipulations take great care to avoid cross-contamination. It is essential to keep glasshouses and growth chambers free of all insect pests since they may transmit the bacterium from sample to sample;

5.5.5. examine regularly for symptoms starting after a week. Count the number of plants showing symptoms. Organisms cause leaf wilting in eggplants, which may commence as a marginal or interveinal flaccidity. Wilted tissue may initially appear dark green or mottled but turns paler before becoming necrotic. Interveinal wilts often have a greasy water-soaked appearance. Necrotic tissue often has a bright yellow margin. Plants are not necessarily killed; the longer the period before symptoms develop, the greater the chance of survival. Plants may outgrow the infection. Young eggplants are much more susceptible to low populations of the organism than are older plants, hence the necessity to use plants at or just before leaf stage 3. Wilts may also be induced by populations of other bacteria or fungi present in the tuber tissue pellet. These include *Ralstonia solanacearum*, *Erwinia carotovora*, subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*, *Erwinia chrysanthemi*, *Phoma exigua* var. *Foveata*, as well as large populations of saprophytic bacteria. In particular *Erwinia chrysanthemi* can cause leaf symptoms and wilt that is very similar to symptoms of *C. m. sepedonicus*. The only difference is blackening of the stems in case in *Erwinia chrysanthemi* infections. Other wilts can be distinguished from those caused by the organism since whole leaves or whole plants wilt rapidly. Also a Gram stain can be prepared: this test will differentiate the organism from *Erwinia* spp.;

5.5.6. as soon as symptoms in eggplants are observed reisolation shall be performed, using sections of wilted leaf tissue or stem tissue from plants. Surface disinfect the eggplant leaves and stems by wiping with 70% ethanol. Perform an IF or PCR on the eggplant sap and isolate on to suitable (selective) media. A Gram stain may also be prepared. Identify purified cultures of the presumptive organism and confirm pathogenicity;

5.5.7. under certain circumstances, in particular where growing conditions are not optimal, it may be possible for the organism to exist as a latent infection within eggplants even after incubation periods up to 4 weeks. If no symptoms are observed after 4 weeks, perform IF or PCR on a composite sample of 1 cm stem sections of each test plant taken above the inoculation site. If the test is positive, reisolation on suitable (selective) media shall be performed. Identify purified cultures of the presumptive organism and confirm pathogenicity as referred to before;

5.6. interpretation of the bioassay test results:

5.6.1. valid bioassay test results are obtained when plants of the positive control show typical symptoms, the bacteria can be reisolated from these plants and no symptoms are found on the negative controls;

5.6.2. the bioassay test is negative if test plants are not infected by the organism, and provided that the organism is detected in positive controls;

5.6.3. the bioassay test is positive if the test plants are infected by the organism.

## 6. Isolation of the organism:

6.1. diagnosis is only completed if the organism is isolated, subsequently identified and confirmed by a pathogenicity test. Although the organism is a fastidious organism, it can be isolated from symptomatic tissue. However, it may be outgrown by rapidly growing saprophytic bacteria and, therefore, isolations directly from the tuber or stem tissue pellet is difficult. With selective medium and appropriate dilution of the resuspended pellet from the heel end cores or stems of potatoes direct isolation of the organism may be possible. Isolations shall be made from all symptomatic potato tubers or stem segments and from eggplants where no symptoms are observed but IF or PCR test from the composite sample was positive. As positive controls, prepare decimal dilutions from a suspension of  $10^6$  colony-forming units per millilitre of the organism (for example, NCPPB 4053 or PD 406). To avoid any possibility of contamination, prepare positive controls totally separated from samples to be tested. For each newly prepared batch of a selective medium suitability thereof for growth of the pathogen shall be tested before it is used to test routine samples. Test control material in an identical manner as the sample;

### 6.2. selective plating:

6.2.1. from a 100 g/l aliquot from a resuspended potato pellet sample or eggplant sap make 10-fold dilutions in pellet buffer (Annex 3);

6.2.2. isolation from undiluted potato pellet usually fails due to the fastidious growth habit of *Cms* and competition by saprophytes. Since the bacterium is usually present in high populations in infected tissues, the saprophytes can usually be diluted out, whilst the pathogen remains. It is therefore recommended to spread 100 g/l from each of the sample, 1/100 up to 1/10 000 dilutions onto MTNA medium or NCP-88 medium (if using 90 mm diameter Petri dishes – adjust volume for alternative dish sizes), using spreaders (hockey sticks) and the spread plate technique. An alternative strategy is to spread out the initial 100 g/l potato pellet aliquot onto a first agar plate with a spreader and then remove the spreader to a second agar plate, streaking out any residue left on the spreader; finally repeat this with a third plate, thus giving a dilution plating effect via the spreader;

6.2.3. incubate plates in the dark at 21 to 23°C;

6.2.4. initial examinations of the plates including, by reference to the control plates, counts of any organism like colonies are made after 3 days, with further counts after 5, 7 and eventually 10 days;

### 6.3. purification of suspicious colonies:

6.3.1. subculturing of the organism-like colonies shall be carried out onto YGM media for eggplant inoculation and/or subsequent identification; this shall be done before the plates become too overgrown, after three to five days;

6.3.2. streak the organism-like colonies on to one of the following media:

6.3.2.1. nutrient dextrose agar (for use in subculturing only);

6.3.2.2. yeast peptone glucose agar;

6.3.2.3. yeast extract mineral salts agar;

6.3.2.4. incubate at 21 – 24°C for up to 10 days. The organism is slow-growing, usually producing pin-point, cream, domed colonies within 10 days;

6.4. re-streak to establish purity. Growth rates are improved with subculture. Typical colonies are creamy-white or ivory, occasionally yellow, rounded, smooth, raised, convex-

domed, mucoid-fluidal, with entire edges and usually 1 to 3 mm in diameter. A simple Gram stain may help to select colonies for further testing;

6.5. Identify presumptive cultures and perform a pathogenicity test.

## 7. Identification:

7.1. identify pure cultures of the presumptive organism isolates using at least two of the following tests based on different biological principles. Include known reference strains where appropriate for each test performed;

7.2. nutritional and enzymatic identification tests:

7.2.1. determine the following phenotypic properties, which are universally present or absent in the organism according to the methods of Lelliot un Stead (1987), Klement et.al. (1990), *Schaad* (2001), Anonymous (1987);

7.2.2. all media shall be incubated at 21<sup>0</sup>C and examined after six days. If no growth has occurred, incubate for up to 20 days;

7.2.3. All tests shall include a known organism control. Nutritional and physiological tests shall be made using inocula from nutrient agar subcultures. Morphological comparisons shall be made from nutrient dextrose agar cultures:

<i>Tests</i>	<i>Expected result</i>
Oxidation/Fermentation (O/F) test	Inert or weakly oxidative
Oxidase activity	
Growth at 37 <sup>0</sup> C	
Urease activity	
Aesculin hydrolysis	+
Starch hydrolysis	- or weak
Tolerance of 7% NaCl	
Indole production	
Catalase activity	+
H <sub>2</sub> S production	-
Citrate utilisation	
Gelatin liquefaction	-
Acid glycerol	
Acid from lactose	- or weak
Acid from rhamnose	
Acid from salicin	
Gram stain	+

## 7.3. IF test:

7.3.1. prepare a suspension of approximately 10<sup>6</sup> cells per millilitre in IF buffer;

7.3.2. prepare a 2-fold dilution series of an appropriate antiserum;

7.3.3. apply the IF procedure;

7.3.4. a positive IF test is achieved if the IF titre of the culture is equivalent to that of the positive control;

#### 7.4. PCR test:

7.4.1. prepare a suspension of approximately  $10^6$  cells per millilitre in ultra pure water;

7.4.2. heat 100  $\mu$ l of the cell suspension in closed tubes in a heating block or boiling waterbath at  $100^{\circ}\text{C}$  for four minutes. If required, addition of freshly-prepared NaOH to a final concentration of 0,05M may assist cell lysis. The samples may then be stored at  $-16$  to  $-24^{\circ}\text{C}$  until required;

7.4.3. apply appropriate PCR procedures to amplify the organism specific amplicons;

7.4.4. a positive identification of the organism is achieved if the PCR amplicons are the same size and have the same restriction fragment length polymorphisms as for the positive control strain;

#### 7.5. FISH test:

7.5.1. prepare a suspension of approximately  $10^6$  cells per millilitre in ultra pure water;

7.5.2. apply the FISH procedure;

7.5.3. a positive FISH test is achieved if the same reactions are achieved from the culture and the positive control;

#### 7.6. fatty acid profiling (FAP):

7.6.1. grow the culture on trypticase soy agar (Oxoid) for 72 hours at  $21^{\circ}\text{C}$  ( $\pm 1^{\circ}\text{C}$ );

7.6.2. apply an appropriate FAP procedure (Janse, 1991; Stead; 1992);

7.6.3. a positive FAP test is achieved if the profile of the presumptive culture is identical to that of the positive control. The presence of characteristic fatty acids 15:1 Anteiso A, 15:0 Iso, 15:0 Anteiso, 16:0 Iso, 16:0 and 17:0 Anteiso are highly indicative of *C. m. sepedonicus*. Other genera such as *Curtobacterium*, *Arthrobacter* and *Micrococcus* also have some of these acids but 15:1 Anteiso A is a rare acid in these bacteria but occurs in all *Clavibacter* spp. at between 1 to 5%. In *C. m. sepedonicus* the value is usually around 5%;

#### 7.7. BOX-PCR:

7.7.1. prepare a suspension of approximately  $10^6$  cells per millilitre in ultra pure water;

7.7.2. apply the test according to the procedure (Smith et al., 2001).

#### 8. Confirmation test:

8.1. the pathogenicity test shall be performed as final confirmation of a diagnosis of the organism and for assessment of virulence of cultures identified as the organism;

8.2. prepare an inoculum of approximately  $10^6$  cells per ml from three day cultures of the isolate to be tested and an appropriate positive control strain of the organism;

8.3. inoculate 5 to 10 eggplant stems of young seedlings at leaf stage 3;

8.4. incubate at  $18$  to  $24^{\circ}\text{C}$  with sufficient light and high relative humidity with appropriate watering to avoid water-logging or drought stress. With pure cultures, typical wilting shall be obtained within 2 weeks but plants not showing symptoms after this time shall be incubated up to 3 weeks at temperatures conducive to eggplant growth but not exceeding  $25^{\circ}\text{C}$ . If after 3 weeks symptoms are not present, the culture cannot be confirmed as being a pathogenic from of the organism;

8.5. isolate from symptomatic plants by removing a section of stem 2 cm above the inoculation point. Commminute and suspend in a small volume of sterile distilled water or 50 mM phosphate buffer. Isolate from the suspension by dilution spreading or streaking onto MTNA and YPGA, incubate for 3 to 5 days at  $21$  to  $23^{\circ}\text{C}$  and observe the formation of colonies typical of the organism.

Minister for Agriculture

M. Roze

**Media for Isolation and Culture of the Organism**

## 1. General growth media

Nutrient agar (NA)

Nutrient agar (Difco) 23,0 g

Distilled water 1,0 l

1.1. dissolve ingredients and sterilise by autoclaving at 121<sup>0</sup>C for 15 minutes;

1.2. nutrient dextrose agar (NDA);

1.3. Difco bacto nutrient agar containing 1% D(+) glucose (monohydrate). Sterilise by autoclaving at 115<sup>0</sup>C for 20 minutes;

1.4. yeast peptone glucose agar (YPGA);

Yeast Extract (Difco)	5,0 g
Bacto-Peptone (Difco)	5,0 g
D(+) Glucose (monohydrate)	10,0 g
Bacto-Agar (Difco)	15,0 g
Distilled water	1,0 l

1.5. dissolve ingredients and sterilise by autoclaving at 121<sup>0</sup>C for 15 minutes;

1.6. yeast extract mineral salts medium (YGM);

Bacto-Yeast-Extract (Difco) 2,0 g

D(+) Glucose (monohydrate) 2,5 g

K <sub>2</sub> HPO <sub>4</sub>	0,25 g
KH <sub>2</sub> PO <sub>4</sub>	0,25 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0,1 g
MnSO <sub>4</sub> . H <sub>2</sub> O	0,015 g
NaCl	0,05 g
FeSO <sub>4</sub> .7H <sub>2</sub> O	0,005 g
Bacto-Agar (Difco)	18 g
Distilled water	1,0 l

1.7. dissolve ingredients and sterilise 0,5 l volumes of medium by autoclaving at 115<sup>0</sup>C for 20 minutes.

## 2. Validated selective growth media

### MTNA medium

Unless otherwise stated all media components are from BDH:

Yeast extract (Difco) 2,0 g

Mannitol 2,5 g

K<sub>2</sub>HPO<sub>4</sub> 0,25 g

H <sub>2</sub> PO <sub>4</sub>	0,25 g
NaCl	0,05 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0,1 g
MnSO <sub>4</sub> ·H <sub>2</sub> O	0,015 g
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0,005 g
Agar (Oxoid no. 1)	16,0 g
Distilled water	1,0 l

2.1. dissolve ingredients, adjust pH to 7,2. After autoclaving (at 121<sup>0</sup>C for 15 minutes) and cooling down to 50<sup>0</sup>C, add the antibiotics: trimethoprim 0,06 g, nalidixic acid 0,002 g, amphotericin B 0,01 g;

2.2. stock antibiotic solutions: trimethoprim (Sigma) and nalidixic acid (Sigma) (both at 5 mg/ml) in 96% methanol, amphotericin B (Sigma) (1 mg/ml) in dimethyl sulfoxide. Stock solutions are filter-sterilised;

Note:

Durability of basal medium is 3 months. After antibiotics are added durability is 1 month when stored refrigerated.

### NCP-88 medium

Nutrient agar (NA)	23 g
Yeast extract (Difco)	2 g
D-mannitol	5 g
K <sub>2</sub> HPO <sub>4</sub>	2 g
KH <sub>2</sub> PO <sub>4</sub>	0,5 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0,25 g
Distilled water	1,0 l

2.3. dissolve ingredients, adjust pH to 7,2. After autoclaving and cooling down to 50<sup>0</sup>C, add the following antibiotics: Polymyxin B sulphate (Sigma) 0,003 g, nalidixic acid (Sigma) 0,008 g, Cycloheximide (Sigma) 0,2 g;

2.4. dissolve antibiotics in stock solutions as follows: nalidixic acid in 0,01 M NaOH, cycloheximide in 50% ethanol, Polymyxin B sulphate in distilled water. Stock solutions are filter-sterilised.

Note:

Durability of basal medium is 3 months. After antibiotics are added durability is 1 month when stored refrigerated.

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**Buffers for Test Procedures**

1. Unopened sterilised buffers may be stored for up to one year;

1.1. Buffers for the extraction procedure. Extraction buffer (50 mM phosphate buffer, pH 7,0). This buffer is used for extraction of the bacterium from plant tissues by homogenisation or shaking:

$\text{Na}_2\text{HPO}_4$  (anhydrous) 4,26 g

$\text{KH}_2\text{PO}_4$  2,72 g

Distilled water 1,00 l

1.2. dissolve the ingredients, check the pH and sterilise by autoclaving at 121°C for 15 minutes. Additional components may be used as follows:

	Purpose	Quantity (per L)
Lubrol flakes	Deflocculant (*)	0,5 g
DC silicone antifoam	Anti-foam agent (*)	1,0 ml
Tetra-sodium pyrophosphate	Anti-oxidant	1,0 g
Polyvinylpyrrolidone-40 000 (PVP-40)	Binding of PCR inhibitors	50 g

1.3. Sediment buffer (10 mM phosphate buffer, pH 7,2).

This buffer solution is used for resuspension and dilution of potato tuber heel-end core extracts following concentration to a pellet by configuration.

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  2,7 g

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  0,4 g

Distilled water 1,00 l

Dissolve the ingredients, check the pH and sterilise by autoclaving at 121°C for 15 minutes.

2. Buffers for the IF test:

2.1. IF-buffer (10 mM phosphate buffered saline, (PBS) pH 7,2). This buffer is used for the dilution of antibodies:

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  2,7 g

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  0,4 g

$\text{NaCl}$  8,0 g

Distilled water 1,0 l

Dissolve the ingredients, check the pH and sterilise by autoclaving at 121°C for 15 minutes.

2.2. IF-buffer-Tween. This buffer is used to wash slides. Add 0,1% Tween 20 to the IF buffer.

(\*) For use with homogenisation extraction method.



2.3. Phosphate buffered glycerol, pH 7,6. This buffer is used as a mountant fluid on the windows of the IF slides to enhance fluorescence.

Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O	3,2 g
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	0,15 g
Glycerol	50 ml
Distilled water	100 ml

Anti-fading mountant solutions are commercially available, for example, *Vectashield*® (*Vector Laboratories*) or *Citifluor*® (*Leica*).

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### Determination of Contamination Level in IF and FISH Tests

1. Count the mean number of typical fluorescent cells per field of view (c).
2. Calculate the number of typical fluorescent cells per microscope slide window (C).  
 $C = c \times S/s$ , where  
S = surface area of the window of the multispot slide, and  
s = surface area of the objective field.  
 $s = \frac{\pi^2}{4G^2K^2}$ , where  
i = field coefficient (varies from 8 to 24 depending upon ocular type),  
K = tube coefficient (1 or 1,25),  
G = magnification of objective (100x, 40x etc.).
3. Calculate the number of typical fluorescent cells per millilitre for a resuspended pellet (N).  
 $N = C \times 1\,000/y^x \times F$ , where  
y = volume of the resuspended pellet on each window, and  
F = dilution factor of the resuspended pellet.

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**Validated PCR Protocol and Reagents**

1. Preliminary testing shall permit reproducible detection of at least  $10^3$  to  $10^4$  cells of *C. m. sepedonicus* per millilitre of sample extract. Preliminary testing shall also show no false positive results with a panel of selected bacterial strains.

2. Multiplex PCR protocol with internal PCR control (Pastrik, 2000)

2.1. Oligonucleotides

Forward oligonucleotide PSA-1 5'- ctc ctt gtg ggg tgg gaa aa -3'

Reverse oligonucleotide PSA-R 5'- tac tga gat gtt ctt ccc c -3'

Forward oligonucleotide PNS-7-F 5'- gag gca ata aca ggt ctg tga tgc -3'

Reverse oligonucleotide PNS-8-R 5'- ttc gca ggt tca cct acg ga -3'

Expected amplicon size from *C. m.* subsp. *sepedonicus* template DNA = 502 bp (PSA-oligonucleotide set).

Expected amplicon size from the 18S rRNA internal PCR control = 377 bp (PSA-oligonucleotide set).

2.2. PCR reaction mix

Reagent	Quantity per reaction	Final concentration
Sterile ultra pure water	15,725 ġl	
10 x PCR buffer <sup>(1)</sup> (15 mM MgCl <sub>2</sub> )	2,5 ġl	11 x (1,5 mM MgCl <sub>2</sub> )
BSA (fraction V) (10%)	0.25 ġl	0,1 %
dNTP mix (20 mM)	0,125 ġl	0,1 mM
Oligonucleotide PSA-1 (10 ġM)	0,5 ġl	0,2 ġM
Oligonucleotide PSA-R (10 ġM)	0,5 ġl	0,2 ġM
Oligonucleotide NS-7-F (10 ġM) <sup>(2)</sup>	0,1 ġl	0,04 ġM
Oligonucleotide NS-8-R (10 ġM) <sup>(2)</sup>	0,1 ġl	0,04 ġM
Taq polymerase (5 U/ġl) <sup>(1)</sup>	0,2 ġl	1,0 U
Sample volume	5,0 ġl	
Total volume:	25,0 ġl	

<sup>(1)</sup> Methods were validated using *Taq* polymerase from Perkin Elmer (AmpliTaq or Gold) and Gibco BRL.

<sup>(2)</sup> Concentration of oligonucleotides NS-7 F and NS-8-R were optimised for potato heel end core extraction using the homogenisation method and DNA purification according to Pastrik (2000). Re-optimisation of reagent concentrations will be required if extraction by shaking or other DNA isolation methods are used.

### 2.3. PCR reaction conditions

Run the following programme:

<b>1 cycle of</b>	3 minutes at 95 <sup>0</sup> C (denaturation of template DNA)
<b>10 cycles of</b>	1 minute at 95 <sup>0</sup> C (denaturation of template DNA)
	1 minute at 64 <sup>0</sup> C (annealing of oligonucleotides)
	1 minute at 72 <sup>0</sup> C (extension of copy)
<b>25 cycles of</b>	30 seconds at 95 <sup>0</sup> C (denaturation of template DNA)
	30 minutes at 62 <sup>0</sup> C (annealing of oligonucleotides)
	1 minute at 72 <sup>0</sup> C (extension of copy)
<b>1 cycle of</b>	5 minutes at 72 <sup>0</sup> C (final extension)
	hold at 4 <sup>0</sup> C

Note:

This programme is optimised for use with an MJ Research PTC 200 thermal cycler.

### 2.4. Restriction enzyme analysis of amplicon

PCR products amplified from *C. m. subsp. sepedonicus* DNA produce a distinctive restriction fragment length polymorphism with enzyme Bgl II after incubation at 37<sup>0</sup>C for 30 minutes. The restriction fragments obtained from *C. m. subsp. sepedonicus*-specific fragment are 282 bp and 220 bp in size.

### 3. Preparation of the loading buffer:

#### 3.1. Bromphenol blue (10%-stock solution)

Bromphenol blue 5 g

Distilled water (bidest) 50 ml

#### 3.2. Loading buffer

Glycerol (86%) 3,5 ml

Bromphenol blue (5.1.) 300 ġl

Distilled water (bidest) 6,2 ml

### 4. 10 x tris-acetate-EDTA (TAE) buffer, pH 8,0

Tris buffer 48,4 g

Glacial acetic acid 11,42 ml

EDTA (disodium salt) 3,72 g

Distilled water 1,00 l

Dilute to 1x before use.

Also commercially available (for example, Invitrogen or equivalent).

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### Validated Reagents for FISH Test

1. Oligo-probes:

1.1. Cms-specific probe CMS-CY3-01: 5'- ttg cgg ggc gca cat ctc tgc acg -3';  
Non-specific eubacterial probe EUB-338-FITC: 5'- gct gcc tcc cgt agg agt -3';

2. Fixative solution:

2.1. the fixative contains paraformaldehyde, which is toxic;  
2.2. heat 9 ml molecular grade water (for example, ultra pure water (UPW)) to about 60°C and add 0,4 g paraformaldehyde. Paraformaldehyde dissolves after adding 5 drops of 1 N NaOH and stirring with a magnetic stirrer;  
2.3. adjust pH to 7,0 by addition of 0,1 M phosphate buffer (PB, pH 7,0) and 5 drops of 1 N HCl. Check pH with indicator strips and adjust it if necessary with HCl or NaOH;  
2.3. filter the solution through a 0,22 µm membrane filter and keep dust-free at 4°C until further use. Alternative fixative solution: 96% ethanol;

3. 3x hybmix:

NaCl 2,7 M  
Tris-HCl 60 mM (pH 7,4)  
EDTA (filter sterilised and autoclaved) 15 mM  
Dilute to 1x as required.

4. Hybridisation solution:

1 x hybmix  
Sodium dodecyl sulphate (SDS) 0,01 %  
Probe EUB 338 5 ng/µl  
Probe CMSCY301 5 ng/µl

Prepare quantities of hybridisation solution according to the calculations in Table 1. For each slide (containing 2 different samples in duplicate) 90 µl hybridisation solution is required.

**Table 1****Suggested Quantities for Preparation of Hybridisation Mix**

		2 slides	8 slides
Sterile ultra pure water		50,1	200,4
3x hybmix		30,0	120,0
1 % SDS		0,9	3,6
Probe EUB 338 (100 ng/ġl)		4,5	18,0
Probe CMSCY301 (100 ng/ġl)		4,5	18,0
	Total volume (ġl)	90,0	360,0

All solutions containing light sensitive oligo-probes shall be stored in the dark at  $-20^{\circ}\text{C}$ .  
Protect from direct sunlight or electric light during use.

5. 0,1 M phosphate buffer, pH 7,0

$\text{Na}_2\text{HPO}_4$	8,52 g
$\text{KH}_2\text{PO}_4$	5,44 g
Distilled water	1,00 l

Dissolve ingredients, check pH and sterilise by autoclaving at  $121^{\circ}\text{C}$  for 15 minutes.

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**Eggplant Culture**

1. Sow seeds of eggplant (*Solanum melongena*) in pasteurised seed compost. Transplant seedlings with fully expanded cotyledons (10 to 14 days) into pasteurised potting compost;
2. Eggplants shall be grown in a glasshouse with the following environmental conditions:

Day length		14 hours or natural day length if greater
Temperature	day	21 to 24 <sup>0</sup> C
	night	15 <sup>0</sup> C
Susceptible varieties	of eggplant:	Black Beauty
		Long Tom
		Rima
		Balsas

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**Preparation of Positive and Negative Controls for the  
Core Screening Tests PCR or IF or FISH**

1. Produce a 72 hour culture of a virulent strain of the organism [NCPPB 4053 or PD 406] on a MTNA basal medium and suspend in a 10 mM phosphate buffer to obtain a cell density of approximately  $1 \text{ to } 2 \times 10^8$  colony-forming units per ml. This is usually a faintly turbid suspension equivalent to an optical density of 0,20 at 600 nm.
2. Remove the heel end cores of 200 tubers taken from a white skin variety production known to be free from the organism.
3. Process the heel ends as usual and resuspend the pellet in 10 ml.
4. Prepare 10 sterile 1,5 ml microvials with 900  $\mu$ l of the resuspended pellet.
5. Transfer 100  $\mu$ l of the suspension of the organism to the first microvial. Vortex. Establish decimal levels of contamination by further diluting in the next five microvials. The six contaminated microvials will be used as positive controls. The four non-contaminated microvials will be used as negative controls. Label the microvials accordingly.
6. Prepare aliquots of 100  $\mu$ l in sterile 1,5 ml microvials thus obtaining nine replicates of each control sample. Store at  $-16$  to  $-24^\circ\text{C}$  until use.
7. The presence and quantification of the organism in the control samples shall be first confirmed by IF.
8. For the PCR test perform DNA extraction from the positive and negative control samples with each series of test samples.
9. For the IF and FISH tests perform assays on the positive and negative control samples with each series of test samples.
10. For the IF, FISH and PCR assays the organism shall be detected in at least the  $10^6$  and  $10^4$  cells/ml of the positive controls and not in any of the negative controls.

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