Cms IFA

Immunofluorescence assay for *Clavibacter michiganensis* subsp. *sepedonicus*
Catalog number: IFA 70001

List of Contents

Lot number	Item	125 tests	250 tests	500 tests	1000 tests
_____	Antibody concentrate, mouse anti-*Cms* clone 9A1	0.1 ml	0.2 ml	0.4 ml	0.8 ml
_____	FITC conjugate, anti-mouse for *Cms*	0.1 ml	0.2 ml	0.4 ml	0.8 ml

The above items should be stored at 4° C. All Agdia reagents should be stored undiluted for maximum sensitivity.

Additional materials required

Item	Catalog Number	Source
Microscope slides (Toxoplasmosis slides)	5638-01940	Bellco Glass, Inc. 340 Edrudo Rd P.O. Box B
Cover slips (No. 1 thickness, 24 x 50 mm)	1916-24050	Vineland, NJ 08360 USA (856) 691-1075 1-800-257-7043 www.bellcoglass.com
potassium phosphate, dibasic (anhydrous) potassium phosphate, monobasic (anhydrous) sodium chloride glycerol p-phenylenediamine	P-3786 P-5379 S-9625 G-7893 P-6001	Sigma Chemical Co. P.O. Box 14508 St. Louis, MO 63178 USA (800) 325-3010 (314) 771-5750 www.sigma-aldrich.com
acetone (Histological or Certified ACS grade) sterile pipette tips	A16 or A18	Fisher Scientific 50 Fadem Road Springfield, NJ 07081 USA (800) 766-7000 www.fishersci.com

Acknowledgment

This product has been produced by Agdia, Inc. under an agreement with Agriculture Canada and the Province of Alberta. The pioneering research and test development was performed at the Agriculture Canada Research Station, Vancouver, B.C. The cooperation and assistance of both Agriculture Canada and the Province of Alberta is gratefully acknowledged.

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Intended use

This Agdia test is an immunofluorescence assay to detect *Clavibacter michiganensis* subsp. *sepedonicus* (synonym=*Corynebacterium sepedonicum*), the causal agent of bacterial ringrot of potatoes. This test is intended for use by persons trained in microscopic recognition of *Cms* and in immunohistochemistry.

The test requires about 3-4 hours to complete. Slides can be viewed by fluorescence microscopy immediately or stored for later observations. Please read instructions carefully before performing this test.

Test principle

This test is based on a monoclonal antibody of the IgM class, originally designated MAb 5 (De Boer and Wieczorek, 1984). This antibody was developed at the Agriculture Canada Research Station, Vancouver, B.C., against a cell wall antigen of *Cms.*

Precautions and limitations

This test has been optimized to work best with bacterial concentrations below 10^8 cfu/ml. Antigen excess may occur at or above this concentration.

Use sterile pipettes to dispense samples and Agdia reagents.

Dilute only the amount of antibody needed directly before using.

All incubations should be performed in a moisture-saturated box to prevent evaporation of liquids in test wells.

Thoroughly wash, sanitize, and dry all buffer containers after using or discarding buffers.

Preparing for the test

Prepare buffers

Prepare PBS buffer and mounting fluid according to the instructions on the back page

Prepare samples and controls

Tissue samples

We recommend testing tuber vascular tissue. Stem tissue from plants more than 90 days old may also be tested. Leaf samples, or stem tissue from plants less than 90 days old, may not contain *Cms* cells even if the plant is infected. To sample tuber vascular tissue, cut around the stem to remove a shallow cone of tissue, 10-20 mm in diameter and 3 mm thick, with the stem at the center.

Grind samples and add 1 ml PBS buffer for each 0.5 g of sample. Previously frozen tissue may be used, or the sample extract may be frozen after preparation. Store frozen sample extracts at -20° C.

Pure culture

To test a pure culture, wash and resuspend cells three times in PBS buffer. Prepare a suspension with an optical density of 0.1 at 660 nm. This preparation will contain about 10^8 to 10^9 colony-forming units per milliliter. You must wash cells thoroughly to remove loosely bound polysaccharides, which can prevent the cells from adhering to the slide.

Controls

Positive and negative controls must be included to ensure accurate results. Positive control slides, using a culture solution of 10^8 cfu/ml in 10-fold dilutions, should be prepared in batches, then fixed and stored. This will avoid the possibility of producing aerosols of the bacterium in the laboratory, which may contaminate other samples being tested.

If using Agdia lyophilized controls, add the recommended amount of PBS buffer to each vial and shake to mix.

Test procedure

1. Add sample and controls

Prepare three 10-fold dilutions of sample extract or sample bacterial cell suspension.

Apply 20 µl of each sample dilution and control to separate wells of a multi-well microscope slide and air dry. Slides can be dried on a slide warmer or in an incubator at a temperature of 50° C. Rapid drying at 50° C will result in a more uniform distribution of cells on the slide.

After the slides have dried, fix in cold acetone for 10 minutes and air dry. Because bacterial antigens can be denatured by intense heat, we do not recommend fixing slides by flaming.

2. Add anti-Cms antibody

Mix concentrated anti-*Cms* antibody thoroughly by vortexing or shaking. Dilute according to the label in PBS buffer.

Add 20 µl of anti-*Cms* antibody preparation to each well. Incubate slide for 1 hour at 37° C in a humid box. Placing the slide in a humid box prevents the preparation from drying on the slide.

3. Wash

Using a wash bottle of distilled water, wash the slide with a gentle, steady flow for about 30 seconds. Do not spray water directly into the wells. Then air dry the slide.

4. Add FITC

Dilute FITC anti-mouse conjugate in PBS buffer according to the dilution given on the label.

Add 20 µl of FITC preparation to each well. Incubate slide for 1 hour at 37° C in the dark, in humid box. The fluorescein conjugate will fade if exposed to direct light.

5. Wash

Wash slide with distilled water and air dry.

6. Add slip cover

Place a drop of mounting fluid into each well, so that when the coverslip is added, all wells are in contact with mounting fluid and the slide is sealed from air. Then add the cover slip.

7. Observe slide

Observe the slide preparation with a microscope set up for fluorescence: with a high intensity light source (mercury vapor or xenon) set up for epi-illumination and with a filter set for fluorescein fluorescence.

Bacterial cells may accumulate at the edge of the wells, even if the slides are dried at 50° C. Therefore, it is very useful to focus first on this plane.

Focus on the edge of a well (Teflon coating) using high-dry objective lens and the regular tungsten light source. Then switch to oil immersion, turn off the tungsten light, and open the shutter for the UV light source. If bacterial cells have clumped near the edge of the well, they should be readily apparent.

Cms cells, which are shaped like kidney beans, will fluoresce a bright green color at their edges. Only a few, or up to several hundred, bacterial cells can be seen per field of view using a 100X oil immersion lens. The fluorescent cell edges result from a thicker layer of the surface-staining antibodies near the edges of the cells.

Observe the positive control slide first, then the negative control. It is very important to observe the negative control, as non-specific binding of the reagent to plant cells, and to the polysaccharide layer of bacterial cells that are not properly washed, may occur.

Then observe the samples. For each sample, start by observing the 1:10 dilution. If there are too many *Cms* cells present (antigen excess), the field of view will appear black. Observe the other two dilutions. If you are unsure how to recognize *Cms* cells or how to determine if antigen excess is present, please contact us.

Stained slides can be stored in the dark at room temperature for 72 hours or several days in the dark at -10° C or below.

Results

This monoclonal antibody, now called MAb 9A1, is highly specific for *Cms*. MAb 9A1 reacted with all 19 strains of *Cms* tested from 8 different geographic areas. It did not react with any of the 10 other plant pathogenic *Corynebacterium* species, or with any of 13 unidentified nonpathogenic bacteria isolated from potato tissues.

MAb 9A1 reacts with *Cms* cells in potato extracts and in pure cultures. No fluorescent cells are usually detected in decayed potatoes that are free of ringrot. However, the possibility of detecting some cross-reacting bacteria cannot be entirely eliminated.

Positive reaction, bright fluorescence

Species	Strains
Clavibacter michiganensis subsp. *sepedonicus*	CS3, CS5, CS12, CS13, CS14, CS15, CS16, CS17, CS20, CS106, CS118, BRR7, P45, R1, R2, R3, R4, R5, R6

Negative reaction, bright fluorescence

Species	Strains
C. betae	CB101, CB103
C. fascians	CF17
C. flaccumfaciens	CF3
C. insidiosum	CI16
C. michiganensis pv. *iranicum*	CI147
C. michiganensis pv. *michiganensis*	CM1
C. michiganensis pv. *tritici*	CT102
C. oortii	CO101
C. poinsettiae	CP2
C. rathayi	CR1

Technical Service

If you have any questions about using this kit, please contact Agdia, Inc. Monday - Friday by phone at 1-574-264-2014 or by e-mail at info@agdia.com.

Reference

De Boer, S. H., and Wieczorek, A. 1984. Production of monoclonal antibodies to *Corynebacterium sepedonicus. Phytopathology* 74: 1431-1434.

Buffer formulations

PBS Buffer Stock Solution

Prepare solutions A and B.

Solution A: Dissolve 17.42 g of potassium phosphate, dibasic (K_2HPO_4) in 200 ml distilled water. Adjust volume to 250 ml.

Solution B: Dissolve 5.44 g of potassium phosphate, monobasic (KH_2PO_4) in 90 ml distilled water. Adjust volume to 100 ml.

Add solution B to solution A. Continuously monitor the pH of solution A while adding solution B until the pH is 7.2 +/- 0.05.

Store phosphate buffer stock solution at 4° C for a maximum of 2 weeks.

1X PBS Buffer (0.01M)

Dissolve 8.5 g of sodium chloride in 960 ml distilled water. Add 25 ml of phosphate buffer stock solution. Adjust volume to 1000 ml..

Store 1X PBS buffer at 4° C for a maximum of 2 weeks.

Mounting Fluid

Mix the following in a dark bottle and store at -20° C. Allow 2 to 3 days for p-phenylenediamine to dissolve.

Glycerol	90.0 ml
1X PBS buffer	10.0 ml
p-phenylenediamine	0.1 g
Store at -20° C.	

Warning: p-phenylenediamine is a carcinogen. Prepare mounting fluid in a chemical fume hood. Observe all safety precautions listed on the p-phenylenediamine material safety data sheet

Date _____ Test _____

Test performed by _____

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