Immunofluorescence Assay (IFA) for *Clavibacter michiganensis* subsp. *sepedonicus* (Cms)

Content List

Lot Number	Item	50 wells	100 wells	250 wells	500 wells	1000 wells
	Monoclonal 9A1, 20x concentrate	0.05 ml	0.10 ml	0.25 ml	0.50ml	1.00ml
	Anti-mouse-FITC conjugate, 20 concentrate	0.05 ml	0.10 ml	0.25 ml	0.50ml	1.00ml
	Instruction	1	1	1	1	1

All about items should be stored at 4 °C for maximum reactivity.

Safety and Storage

Always wash hands thoroughly after using this product. Prevent direct skin and eye contact with, or ingestion of, product components. Obtain medical attention in case of accidental ingestion of reagent components.

All reagent components should be stored at the recommended temperature to assure their full shelf life. Do not store prepared working solution from day to day.

Please contact ACD, Inc. if you have any questions about safety and storage of this product.

Preparing For The Test

Check all the components in the package of ELISA Reagents.

Prepare all of buffer solutions according to the attached buffer formulations.

Make sure all laboratory equipments and facilities (such as fluorescence microscope, slides and cover slips) required for the test are ready.

Prepare a humid box for incubation steps.

Preparing Samples

Tissue Samples: Tuber vascular tissue and stem tissue from plant more than 90 days are recommended to be tested. Leaf tissues, or stem tissues from plant less than 90 days old may not contain Cms bacterial cells even the plant is infected.

Cut the tube vascular tissue around the stem to remove a shallow cone tissues with the stem at the center (about 15 mm in diameter and 3 mm in thick). Grind the samples with PBS, at a ratio of 1ml PBS for each 0.5 of tissue.

Bacterial Culture: To test a pure Cms culture, wash cell suspension three times in PBS buffer and prepare a cell suspension with an optical density of 0.1 at 660 nm. This preparation will contain about 10^8 to 10^9 colony-forming units (CFU) per milliliter. The bacterial cells must be washed thoroughly to remove loosely bound polysaccharides, which can prevent the cells from adhering to the slide .

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

ACD's lyophilized Cms control is recommended to be used. Other positive control include Cms strains CS3, CS5, CS12, CS13, CS14, CS15, CS16, CS17, CS20, CS106, CS118, BRR7, P45, R1, R2, R3, R4, R5, R6.

Various bacterial strain can be used as negative controls in this test, which include*Clavibacter betae*, *C. fascians*, *C. flaccumfaciens*, *C. insidiosum*, *C. michiganensis* pv. *Iranicum*, *C. michiganensis* pv. *Michiganensis*, *C. michiganensis* pv. *Tritici*, *C. oortii*, *C. poinsettiae*, and *C. rathayi*.

If you have any questions about sample and control preparations, please contact ACD, Inc.

Sample and Control Mounting/Fixing

Three 10-fold dilutions of sample extract or sample bacterial cell suspension are prepared. Add 20 µl of each sample dilution and control to separate wells of a multi-well microscope slide.

Slides can be air-dried, or dried on a slide warmer or in an incubator at a temperature of 37-50°C. Rapid drying at 37-50°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. We do not recommend fixing slides by flaming because bacterial antigens can be denatured by intense heat.

Incubating with Cms-Specific Antibody

Mix concentrated monoclonal antibody thoroughly by vortexing, and dilute with PBS at 1:20 and mix well.

Add 20 µl of anti-*Cms* antibody preparation to each well, and incubate slide for 1 hour at 37°C or 2-3 hours at room temperature (21-24 °C) in a humid box. Incubating the slide in a humid box prevents the preparation from drying on the slide.

Washing Slide

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

Preparing FITC-Conjugate

Mix concentrated anti-mouse-FITC conjugate by gently vortexing, and dilute with PBS at 1:20 and mix well.

Add 20 µl of conjugate preparation to each well, and incubate slide for 1 hour at 37°C or 2-3 hours at room temperature (21-24 °C) in a humid box. Avoid to expose the slide to direct light.

Washing Slide

Wash the slide with distilled water as above.

Placing cover slip

Place a drop of mounting fluid into each well and put cover slip on. All well surfaces are in contact with mounting fluid and the slide is sealed from air when the cover slip is on top.

Examining Test Slide

A microscope set up for fluorescence with a high intensity light source (mercury vapor or xenon) for epi-illumination and with a filter set for fluorescein fluorescence is needed to examine the slide preparation.

Examine the slide by focus on the edge of a well because bacterial cells may accumulate near the edge of the well and are readily apparent. Using high-dry objective lens and the regular tungsten light source first, and then switch to oil immersion, turn off the tungsten light, and open the shutter for the UV light source.

The antibody reacted slides can be stored in the dark at room temperature for 3-4 days, or 5-10 days in the dark at -10° C or below.

Evaluating Results

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

Sample examinations start by observing the 1:10 dilution. If there are too many *Cms* cells present (antigen excess), the field of view will appear black. Then examine 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 at ACD, Inc.

The anti-Cms monoclonal antibody used in this test is very specific for *Cms* and 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.

This IFA 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.

Buffer Formulations

PBS Buffer, 1x

Sodium phosphate, dibasic (anhydrous)	1.15 g
Potassium phosphate, monobasic (anhydrous)	0.2 g
Sodium chloride	8.0 g
Potassium chloride	0.2 g

Dissolve in distilled water and make to 1000 ml.
Adjust pH to 7.3.
Store at 4°C.

Mounting Fluid

Glycerol	90.0 g
1x PBS buffer	10.0 g
P-phenylenediamine	0.1 g

Prepare P-phenylenediamine under a fume hood.
Store at –20°C.