



# Microbiology Focus

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## New Fast and Specific Detection Method of Pathogenic Fungi in Tissue



*Fluorescence Detection of fungi in tissue*

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# Fast and Highly Specific Histological Detection of Pathogenic Fungi in Human Tissue

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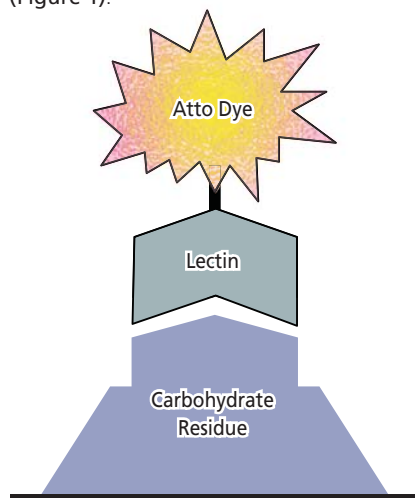
Jakob Zbaeren, Thrombose Laboratory, Inselspital Bern

*Lectins are ubiquitous proteins or glycoproteins that can be isolated from plant and animal sources and can bind to specific carbohydrate moieties.*

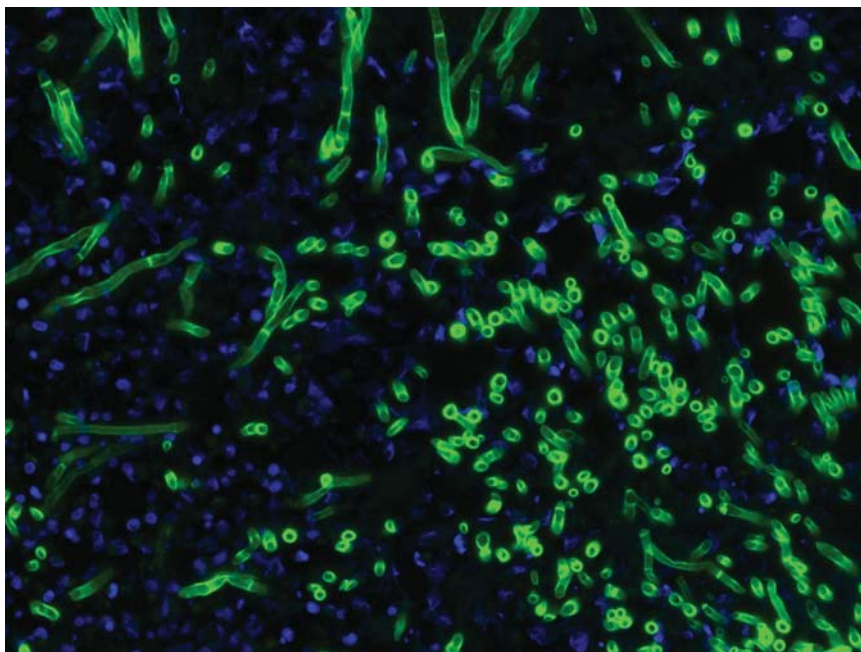
Due to their high affinity to sugar residues, lectins have become important tools for sensitive detection of cellular carbohydrates, revealing subtle alteration in glycosylation between otherwise indistinguishable cells. This allows identification of cellular surface structures, e.g. cell surface, cytoplasm, and nuclear structures. Furthermore, lectin affinity binding allows for the detection of pathogenic degeneration of tissue as well as pathogenic infestations such as fungi.

Histochemical studies are of importance in the histological and pathological investigation of tissue in clinical research. Lectin histochemistry can be performed on living cells in suspension, on cell smears, tissue imprints, fixed tissue sections or fresh cryostat sections.

The recently developed Atto-dye labeled lectins have many applications, including carbohydrate, mitogenic and histochemical studies. Atto-dyes have very bright fluorescent signals and high photo stability, which enable a direct one step tissue-binding protocol. Time-consuming multistage amplification procedures are not required for Atto-dye lectin conjugates. Here, we demonstrate a highly specific identification of pathogenic fungi on human tissue via direct fluorescence detection using fluorescently labeled lectin (Figure 1).



**Figure 1:** Direct one step binding of fluorescently labeled lectins.



**Figure 2:** Fluorescence microscopy of human skin tissue section (paraffin fixation) with fungal infection. The target carbohydrate subunit chitotriose [(GlcNAc)<sub>3</sub>] of the pathogenic fungi are specifically bound to lectin from *Phytolacca americana* Atto 488 conjugate (green). The nuclei are counterstained with DAPI (blue). Image by J. Zbaeren, Inselspital Bern, Switzerland.

Lectin histology was performed on both polymer and paraffin embedded human skin tissue. The lectin conjugate used was *Phytolacca americana*- Atto 488 (Cat. No. 39905). The conjugate was diluted 100 times in PBS buffer (pH 7.4) before incubating with each specimen for 30 min. After washing to remove any unbound lectin and counterstaining the nuclei with DAPI (Cat. No. 32670), the samples were examined using a microscope equipped for epifluorescence with a 450–490 nm excitation bandpass filter and a 520–560 nm barrier (emission) filter.

The images obtained show a very specific labeling of pathogenic fungi infecting human tissue (see Figure 2). The image demonstrates the fine filaments of the fungi containing typical *mycelium*, and individual fungi cells are clearly visible. A slightly higher fluorescence

is observed in the separating cross-walls between two cells (septa), which are due to a higher concentration of target carbohydrates. Very low background is observed.

Fungal cell walls contain chitin, a polymer of  $\beta$ -(1→4) linked *N*-acetyl-D-glucosamine, while animal and plant cells do not synthesize chitin. The lectin *Phytolacca americana* targets the fungal carbohydrate fragment chitotriose [( $\beta$ -*N*-Acetyl-D-glucosamine)<sub>3</sub>, (GlcNAc)<sub>3</sub>] shown in green ( $\lambda_{ex}$  485 nm). Due to the lack of the target carbohydrate chitotriose in the skin tissue, no specific interaction between the lectin *Phytolacca americana* and the tissue is observed. The bright and stable fluorescence properties of the Atto 488 dye provide a strong fluorescent signal without requiring additional amplification steps. Further experiments with staining different fungal in-

fectured tissues were carried out. Similar results confirm this approach to be a successful and reliable way to detect fungi. This application may encourage scientists to investigate further histological phenomena by using lectin interactions.

The Atto-dye lectin conjugates below are now available from Sigma Life Science. Additional lectins and lectin conjugates from Sigma Life Science may be found on at [sigma-aldrich.com/enzymeexplorer](http://sigma-aldrich.com/enzymeexplorer).

Description	Iex/Iem (nm)	Carbohydrate Specificity	Cat. No.	Package Size
Concanavalin A - Atto 565-conjugate	563 / 592 in PBS	α-Mannose, α-Glucose	69535	1 mg
Lectin from Artocarpus integrifolia -Atto 594 conjugate	601/ 632 in PBS	O-Methyl-α-Galactose	76158	1 mg
Lectin from Ulex europaeus - Atto 488 conjugate	501 / 523 in PBS	α-L-Fucose	19337	0.5 mg
Lectin from Ulex europaeus - Atto 550 conjugate	554 / 576 in PBS	α-L-Fucose	94165	0.5 mg
Lectin from Ulex europaeus - Atto 594 conjugate	601/ 632 in PBS	α-L-Fucose	73873	0.5 mg
Lectin from Phaseolus vulgaris - Atto 488 conjugate (Leucoagglutinin)	501 / 523 in PBS	GlcNAc - Man	75319	1 mg
Lectin from Phaseolus vulgaris - Atto 550 conjugate (Leucoagglutinin)	554 / 576 in PBS	GlcNAc - Man	90852	1 mg
Lectin from Phaseolus vulgaris - Atto 647N conjugate (Leucoagglutinin)	644 / 669 in PBS	GlcNAc - Man	77363	1 mg
Lectin from Phytolacca Americana - Atto 488 conjugate	501 / 523 in PBS	(GlcNAc) <sub>3</sub>	39905	1 mg
Lectin from Phytolacca Americana - Atto 550 conjugate	554 / 576 in PBS	(GlcNAc) <sub>3</sub>	94816	1 mg
Lectin from Phytolacca Americana - Atto 647N conjugate	644 / 669 in PBS	(GlcNAc) <sub>3</sub>	03065	1 mg
Lectin from Triticum vulgare - Atto 488 conjugate	501 / 523 in PBS	((GlcNAc) <sub>2</sub> , α-N-acetylneuraminic acid	16441	1 mg
Lectin from Triticum vulgare - Atto 532 conjugate	532 / 558 in PBS	(GlcNAc) <sub>2</sub> , α-N-acetylneuraminic acid	68917	1 mg

**Table 1:** Fluorescence labeled lectins

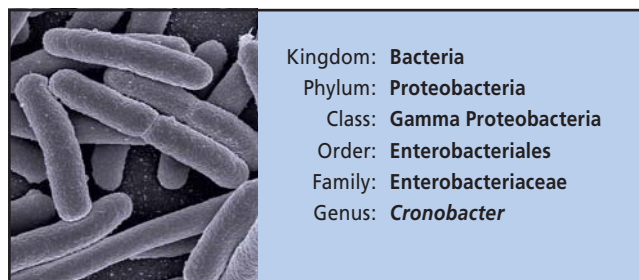
**Reference:** Lectin Methods and Protocols; J. M. Rhodes; J. D. Milton; Humana Press, Totowa, New Jersey, 1997

## Chronobacter spp. Classic and New Detection Methods

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*Enterobacter sakazakii*, now reclassified as a novel genus called *Cronobacter*, is known to be related to neonatal infections.

*Cronobacter* is a rod-shaped, motile and facultatively-anaerobic bacteria of the family Enterobacteriaceae. Originally *Cronobacter* spp. were listed as yellow-pigmented *Enterobacter cloacae* (see **Table 1**). The bacteria was then later on called *Enterobacter sakazakii*. Taxonomic studies have determined that *E. sakazakii* comprises a high genetic heterogeneity and should be reclassified as a novel genus, "Cronobacter" [1].



*Cronobacter* spp. are ubiquitous and frequently found on vegetable, meat, fermented bread, dairy products and especially in baby food. Consumption of contaminated powdered infant formula milk (IFM) can result in sepsis, infant meningitis and necrotising enterocolitis. In most cases the victims are pre-term infants, low birth weight infants or immunocompromised infants in the first weeks. Mechanisms are not fully understood now but it is assumed that endotoxins are produced. The bacterium can adhere to and penetrate into various types of cells (e.g. endothelial cells). It also survives in macrophages.

As Enterobacteriaceae are susceptible to heat they do not survive most production processes and it is most likely that contamination happens after the process. The post process contamination can come from



**Figure 1:** Drinking baby

the addition of heat sensitive additives such as vitamins or other micronutrients or incorrect handling while reconstitution or storage. It has been shown, that stationary phase *Cronobacter spp.* are remarkably resistant to osmotic and dry stress compared with other species of the Enterobacteriaceae group. It is difficult to isolate *Cronobacter spp.* from samples as they are most likely stressed, unevenly distributed throughout the batch and also numbers will probably be fairly low, often lower than 1 CFU per g.

For the classical microbiological tests a pre-enrichment is used to recover the stressed cells followed by a selective enrichment step.

The FDA method recommends Enterobacteriaceae enrichment (EE or Mossel) broth which is then streaked onto VRBG agar and suspect colonies are subcultured onto TSA agar where the yellow pigmented colonies are confirmed by oxidase test and other biochemical tests. See also **Table 1** with all kind of biochemical reaction from *Cronobacter spp.* species (not only from FDA).

ISO/TS 22964:2008 methods recommends buffered peptone water (BPW) as pre-enrichment medium and modified lauryl sulphate broth with vancomycin (mLST) incubated at 44 °C for the secondary selective enrichment step. The next step is then a chromogenic agar for isolation and identification (see **Table 2**).

There are diverse chromogenic agars available which help to save work and time to result and are more reliable than traditional media. The detection principle is based on the alpha-glucosidase possessed by *Cronobacter spp.* (not by most other Enterobacteriaceae) which cleaves the 5-bromo-4-chloro-3-indolyl-a-D-glucopyranoside or similar substrates. The result is a plate with e.g. blue colonies in case of *Cronobacter spp.* (see **Figure 2** and **3**) but biochemical confirmation is still required.



**Figure 2:** HiCrome™ *Cronobacter spp.* Agar (Fluka 92324); *Cronobacter spp.* (blue), *E. aerogenes* (green) *K. pneumoniae* (yellow)



**Figure 3:** HiCrome™ *Cronobacter spp.* Agar, Modified (Fluka 14703), with *Cronobacter spp.* colonies

Today there are also studies that not all *Cronobacter spp.* giving yellow pigmented colonies on tryptic soy agar and it was showed that some type of strains did not grow at 44-45 °C. Also some selective media may be too selective to recover all the *Cronobacter* species. There is still some work to do to improve the official methods.



## Did you know...

why this novel genus is called *Cronobacter*?

Cronos, was one of the Titans of the greek mythology who swallowed each of his children as soon as they were born. As *Cronobacter* species are harmful to neonates, the name was found to be adequate.

**20% Test Discount**  
for *Cronobacter spp.*  
media

(see table 2; Promo Code T62.  
Valid until 30.09.2009)

Biochemical Test	Reaction of <i>Cronobacter</i>
Gram	-
oxidase	-
catalase	+
H <sub>2</sub> S production	-
nitrate reduction	+
citrate utilization	+
esculin hydrolyzation	+
arginine hydolysation	+
Lysine	-
L-ornithine decarboxylation	+
Urease	-
Indole	-
ONPG	+
D-adonitol	-
L-arabinose	+
D-arabitol	-
D-cellobiose	+
Dulcitol	-/+
D-fructose	+
D-glucose	+
D-galactose	+
galacturonate	+
Inositol	+/-
Inulin	+
Lactose	+
Malonate	+/-
D-maltose	+
D-mannitol	+
D-mannose	+
D-melibiose	+
x-methyl-D-glucoside	+
D-raffinose	+
L-rhamnose	+
Salicin	+
Sorbitol	-
D-sucrose	+
D-trehalose	+
Xylose	+
Acetoin production (VP test)	+
methyl red test	-
tryptic soy agar at 25 °C	yellow pigmented

**Table 1:** Biochemical reactions from *Cronobacter spp.*

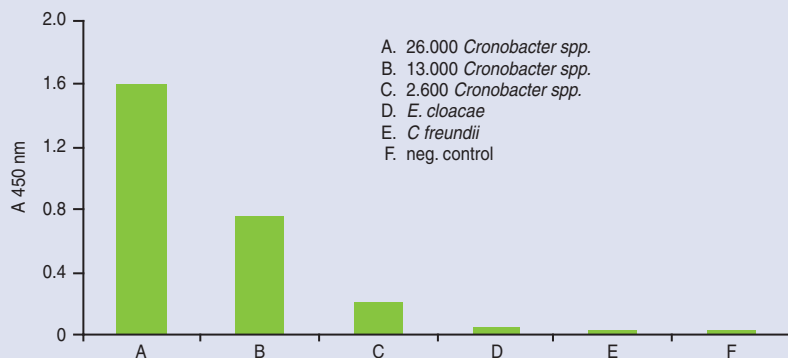
Brand	Cat. No.	Medium	Description
Fluka	92324	HiCrome™ <i>Cronobacter</i> spp. Agar	chromogenic media
Fluka	14703	HiCrome™ <i>Cronobacter</i> spp. Agar, Modified	chromogenic media according ISO
Fluka	69965	Mossel Broth (E.E. broth)	for pre-enrichment
Fluka	77187	Peptone Water, phosphate-buffered	for pre-enrichment
Fluka	22091	Tryptic Soy Agar	for isolation and differentiation
Fluka	79872	Tryptic Soy Agar	for isolation and differentiation

**Table 2:** Media for detection of *Cronobacter* spp.

## A New Molecular Biology Method

Rapid detection and identification of *Cronobacter* species is required, since even low cell numbers have been reported to cause a disease. HybriScan®*D Cronobacter* spp. is a new rapid molecular test system for detection of bacteria of the genus *Cronobacter* in food, especially in dried infant formula milk and its production environment. It is based on the detection of rRNA by sandwich hybridisation and so **no PCR** is needed. It is a 96 well microplate format and the workflow is very similar to an ELISA test.

Figure 4 shows the specificity of HybriScan®- *Cronobacter* spp. Different cell amounts and related *Enterobacteriaceae* were tested within a validation study. No signals were observed using  $2,3 \times 10^8$  *Enterobacter cloacae* cells or  $7 \times 10^8$  *Citrobacter freundii* cells per assay, whereas clear specific signals were detectable using  $2,6 \times 10^3$ ,  $1,3 \times 10^4$ , and  $2,6 \times 10^4$  cells of *Cronobacter* species, respectively. These results demonstrate that the HybriScan® system is highly specific for *Cronobacter* spp.



**Figure 4:** Specificity of HybriScan®- *Cronobacter* spp. Different cell numbers of *Cronobacter* spp. and related *Enterobacteriaceae* like *E. cloacae* and *Citrobacter freundii* were tested. Measurement data for HybriScan® analyses represent absorption at 450 nm.

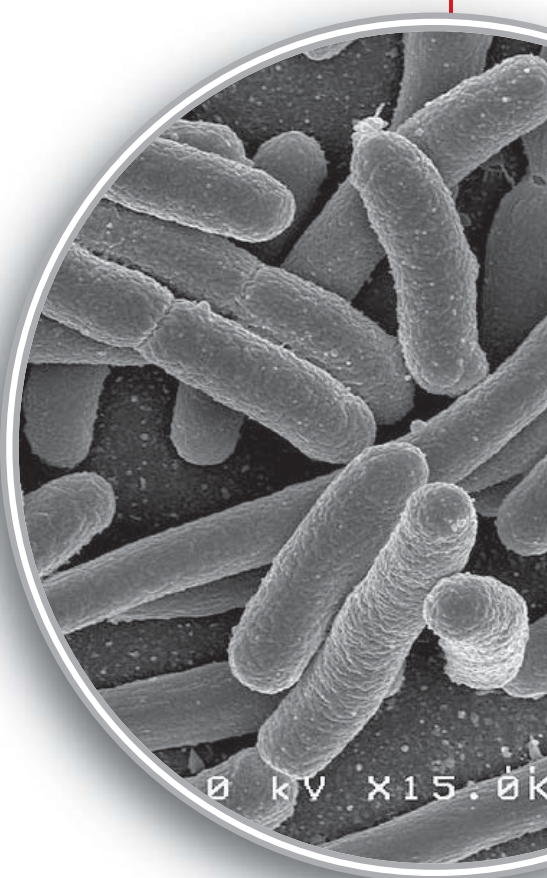
A validation study of HybriScan®- *Cronobacter* spp. was performed using two different enrichment procedures: (1) single step enrichment for 24 – 26 hours at 37 °C in ESSB broth (*Enterobacter sakazakii* selective broth) and (2) two step enrichment starting with a pre-enrichment for 18 – 20 hours at 37 °C in buffered peptone water and followed by a selective enrichment for 24 – 26 hours at 45 °C in mLST selective broth. The results of the above mentioned validation study are presented in Figure 4.

Brand	Cat. No.	Name	Assays
Fluka	12838	HybriScan® <i>D Cronobacter</i> spp.	96

**Table 2:** Ordering Information

### References:

1. Cawthorn, D.M.; Botha, S.; Witthuhn, R.S. Evaluation of different methods for the detection and identification of *Enterobacter sakazakii* isolated from South African infant formula milks and the processing environment. *International Journal of Food Microbiology*, 127:129–138 (2008)
2. Isolation and Enumeration of *Enterobacter sakazakii* from Dehydrated Powdered Infant Formula, U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition (2002)
3. Color Atlas and Textbook of Diagnostic Microbiology, 5th edition, Lippincott Williams &Wilkins (1997)
4. Iversen et al., The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov., *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies 1, *BMC Evolutionary Biology*, 7:64 (2007)
5. Bergey's manual of determinative bacteriology, D.H. Bergey, J.G. Holt, 9th Edition, Lippincott Williams & Wilkins (1994)
6. M.B. Kleiman et al., Meningoencephalitis and Compartmentalization of the Cerebral Ventricles Caused by *Enterobacter sakazakii*, *Journal of Clinical Microbiology*, p. 352-354 (1981)
7. ISO/TS 22964:2006 Milk and milk products detection of *Enterobacter Sakazakii*
8. O. Guillaume-Gentil, V. Sonnard, M.C. Kandahai, J.D. Mauragg, H. Jootsen, A simple and Rapid Cultural Method for Detection of *Enterobacter Sakazakii* in environmental samples. *Journal of Food. Protection*, 68 (1), 2005, pp. 64-69
9. K. Riedel, A. Lehner, Identification of proteins involved in osmotic stress response in *Enterobacter sakazakii* by proteomics. *Proteomics* 7, 1217-1231 (2007)
10. F.J. Pagotto, M. Nazarowec, S. Bidawid, J.M.Farber, *Enterobacter sakazakii*: Infectivity and enterotoxin production in vitro and in vivo; *J. of Food Protection*, Vol. 66, 3, p. 370-375 (2003)





# FLUKA-Microbiology Photography Competition!

## General Information

This competition for photography is sponsored by Sigma-Aldrich with the aim of encouraging microbiologists to show something about their work and science.

The four best photographic entries will be presented in the Microbiology Focus and the *Best of Show* will get a place on a cover! All 4 winners will be awarded a trendy USB stick.



## The competition will be judged by:

Prof. Mohammad Manafi  
Medical University of Vienna,  
Head of Department for Food Hygiene

Dr. Antje Breitenstein  
Scanbec GmbH, CEO

Prof. Dr. Corinne Gantenbein  
ZHSW, Head of Microbiology Department

Jvo Siegrist  
Sigma-Aldrich, Product Manager Microbiology

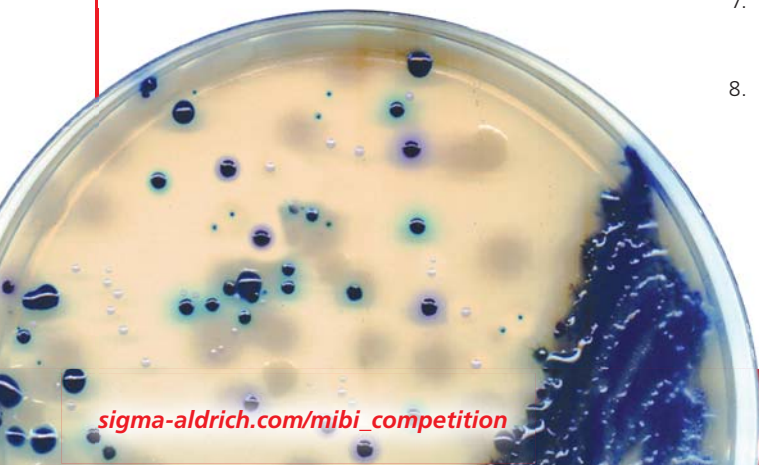
## Method of Entry

There is no entry fee. An individual may enter a maximum of 3 times and an entry form must be completed for each entry. To submit a photo, go to the following website:

[sigma-aldrich.com/mibi\\_competition](http://sigma-aldrich.com/mibi_competition)

## Rules of the Competition and Conditions of Entry

1. The competition is open to all residents worldwide.
2. Entries should illustrate any microorganisms (living or dead) or a microbiologist in action at work
3. Picture size should be at least 400 dpi and 90 x 120 mm (max 3 MB). The file format must be in jpg, tiff or pdf.
4. The entries will be judged on:
  - clarity of presentation
  - composition
  - illumination and contrast
  - congruency of subject matter and title of photograph
  - scientific interest and relevance
  - originality
5. Winning entries will be retained by Sigma-Aldrich, who will have sole rights of publication, reproduction and display.
6. Closing date will be 30th Sept. 2009.
7. Entries after the closing date will not be considered. Entries received incomplete, illegible, mutilated, altered or not complying exactly with the instructions and theme may be disqualified.
8. Decisions of the judges in all matters affecting the competition will be final and legally binding.



[sigma-aldrich.com/mibi\\_competition](http://sigma-aldrich.com/mibi_competition)

# Identifying Microorganisms Using Silver Staining Techniques

Mark Frei Technical Marketing Specialist.... [mark.frei@sial.com](mailto:mark.frei@sial.com)

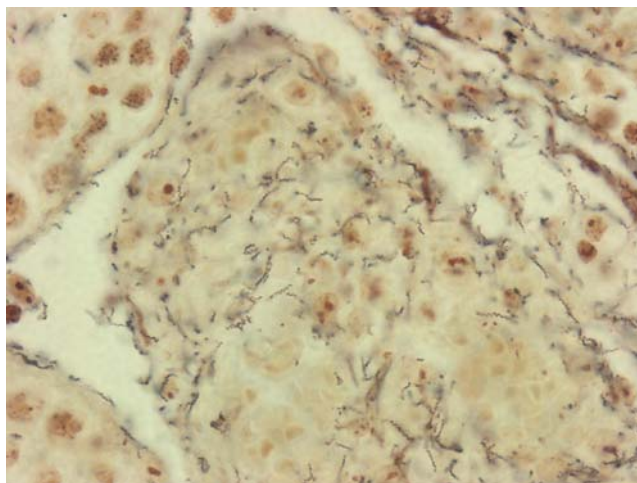
Silver staining techniques rely on basic chemical reactions for the microscopic examination and identification of microorganisms. These stains aid pathologists in the evaluation of disease states and help guide physicians in patient treatment.

Silver staining methods can be 10 – 100 times more sensitive than other staining techniques that rely on dyes that must penetrate the cell, however silver stains have a tendency to be capricious. Non-specific silver deposition and over-staining can produce a loss of detail. Clean glassware, proper technique and high quality reagents are necessary to obtain satisfactory results

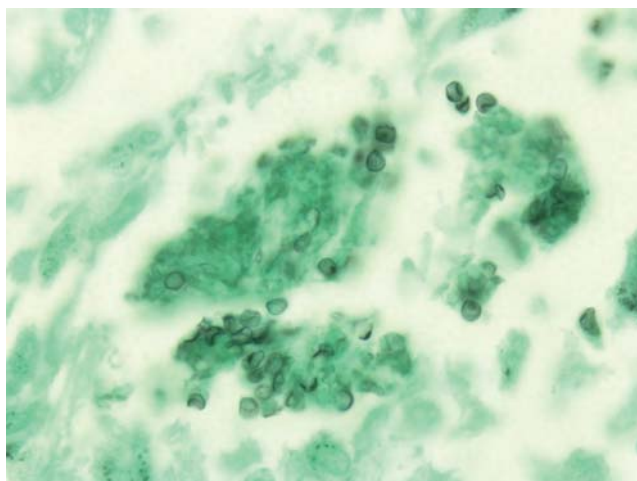
Sigma-Aldrich supplies two modified silver staining kits that allow more reliable and consistent staining compared to the traditional method.

The Silver Stain Kit (Modified Steiner-Steiner) is intended for the demonstration of spirochetes and non-filamentous bacteria in sections of paraffin-embedded tissue. The method provides the option to utilise a microwave oven to accelerate and accentuate the silver stain in tissue sections. The heat produced in the microwave facilitates the impregnation of silver nitrate into the tissues, resulting in a much cleaner background than the traditional method. The organisms stain black and the background is yellow-brown (see **Figure 1**). Try the Modified Steiner-Steiner Kit (HT101-A) for consistent and reproducible staining results.

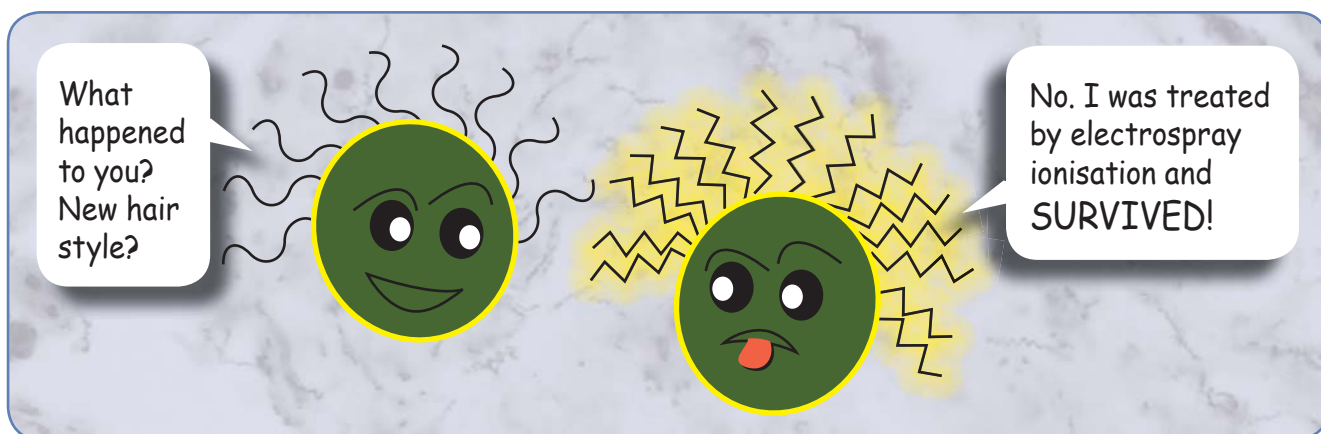
The Silver Stain Kit (modified GMS) is intended for use in histological visualisation of fungi, basement membrane and some opportunistic organisms. The organisms are stained black whilst other tissue elements are stained green (see **Figure 2**). Gomori Methenamine Silver (GMS) traditionally requires elaborate solution preparation and results can vary considerably due to the capricious nature of metal impregnation and photographic development. The Modified GMS Kit (HT100-A) incorporates a stable working silver methenamine salt along with buffer, toning reagent and developer, avoiding these issues. The use of a microwave oven allows for more rapid staining. Silver stains are of great importance in biomedical research applications and diagnostic pathology. Due to their low cost and ease of interpretation, silver stains will remain an important complement to emerging molecular biology technologies.



**Figure 1:** Steiner-Steiner silver stain of spirochetes



**Figure 2:** GMS silver stain of Pneumocystis



What bacteria think about new methods

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