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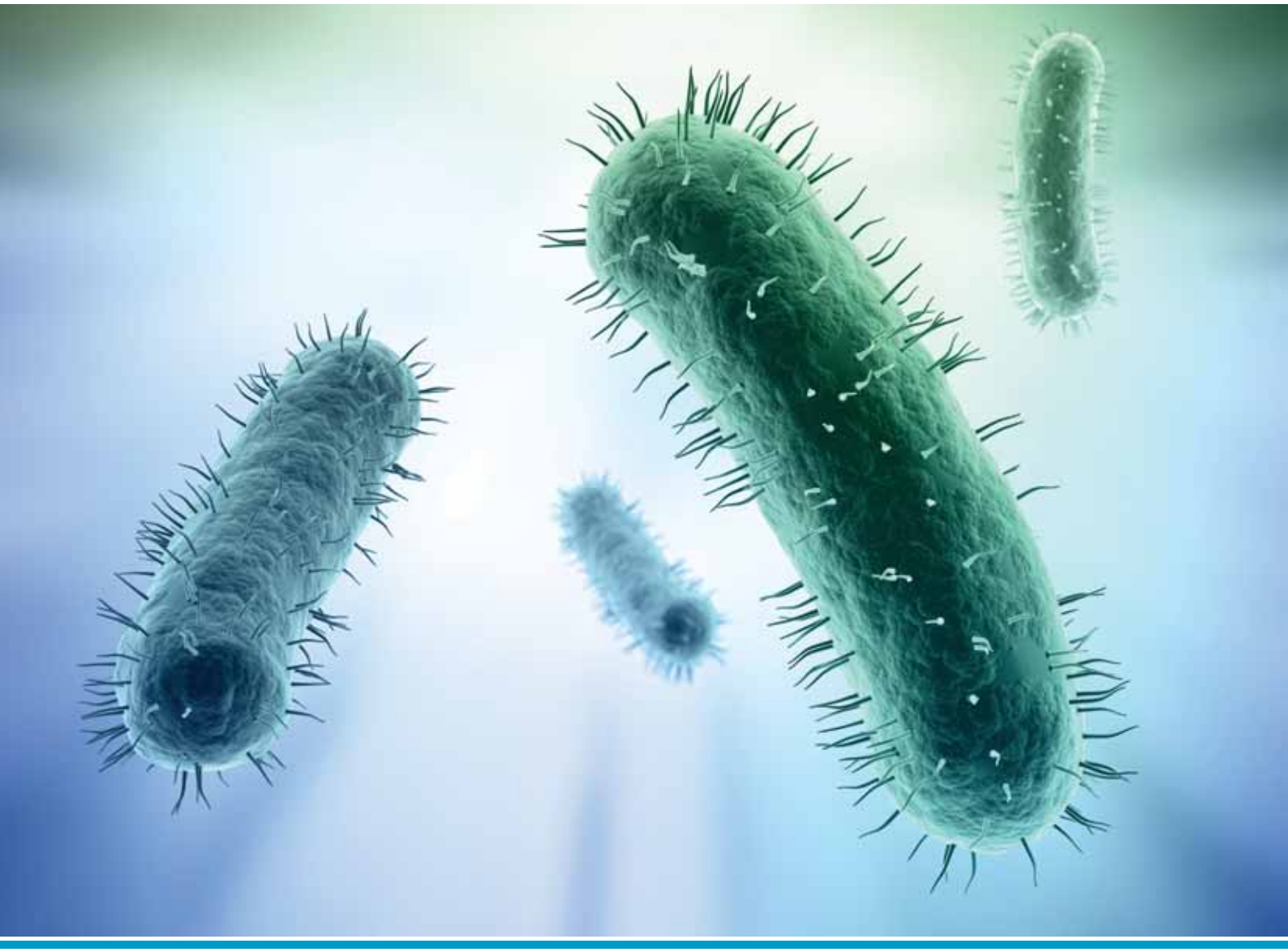
Prevention, Detection and Treatment of

Mycoplasma Contamination



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Mycoplasma Detection

Mycoplasma is a prokaryotic microorganism of the class Mollicutes that lack a true cell wall, and many of which are considered pathogenic.

Mycoplasma contamination is often detected in cell cultures, and consequently, virus cultures, vaccines and other biological materials produced in cells become contaminated as well.

Mycoplasma contamination in cell lines used for research poses a serious problem. In most cases, visual detection of such contaminations or detection with the aid of a microscope is impossible. Although mycoplasma does not cause visible damage to cells, it undeniably affects cell metabolism, cell growth in culture, protein synthesis, cytokine secretion, and even causes damage to DNA and RNA. Hence, results obtained from experiments are liable to be biased when mycoplasma is present. Various studies show that the percentage of contaminated cultures in cell banks is 10%-80%. Mycoplasma contamination can originate from bovine serum, laboratory employees, other contaminated cultures, or the animals from which the cells have been harvested.

The most prevalent species of mycoplasma detected in contaminated cell cultures include M. fermentans, M. hyorhinis, M. arginini, M. orale, M. salivarium, M. hominis, M. pulmonis and M. pirum.

Testing Methods

Several methods for the detection of mycoplasma have been published:

- Cultures on agar, liquid media, or semi-solid media.
- DAPI Staining – staining DNA with fluorescent dyes (4', 6-diamine-2-phenylindole dihydrochloride).
- DNA hybridization.
- Antibodies for specific mycoplasma species.
- Electronic microscope.
- PCR: specific primers.
- Biochemical – detection of mycoplasmal enzymes by colorimetric or luminescence assay.

Using PCR for the Detection

The testing required by the regulatory authorities is seeding in culture (agar and liquid media). This test is complicated, time consuming (about 5 weeks), and some mycoplasma species are difficult to detect with this method. In recent years, the disadvantages of these methods have been acknowledged (such as sensitivity, specificity and long and complex procedures), and use of PCR for the detection of contaminations in cell cultures has become increasingly widespread.

PCR has been shown to be a highly sensitive, specific and rapid method for the detection of mycoplasma contamination in cell cultures. Specific primers have been designed from DNA that

is coded to the ribosomal RNA (16SrRNA). The gene sequences for RNA are considered conserved sequences and are similar in the various mycoplasma species, which have not undergone significant mutation. Consequently, primers can be designed for these areas, which are specific to the mycoplasma and will not detect bacterial or animal DNA sequences.

The literature describes several PCR methods for the detection of mycoplasma, such as using a number of primers to obtain detection of specific mycoplasma species, and nested PCR (two consecutive PCR cycles using different primers) for amplifying sensitivity and specificity.

PCR testing techniques are based on amplification of a DNA fragment using primers prepared in advance, and fragment identification is usually carried out with electrophoresis.

In conjunction with Prof. Shlomo Rottem of the Mycoplasma Laboratory at the Hebrew University-Hadassah Medical School, Jerusalem, Biological Industries has developed the EZ-PCR Mycoplasma Test Kit (Cat. # 20-700-20; 20-700-10), a PCR-based mycoplasma test kit that simplifies testing and detection of mycoplasma contamination in cell cultures. The kit includes a unique reaction mix that contains all the ingredients required for

PCR: nucleotides, primers, Taq Polymerase and magnesium. No prior preparations are required for PCR, other than the sample to be tested (centrifugation and suspension in the buffer supplied with the kit). After performing agarose gel electrophoresis, positive samples will yield a 270bp fragment. The test takes approximately five hours to complete.

The primers have been designed to detect the mycoplasma species responsible for most contaminations in cell cultures (including Acholeplasma). The primers were tested and found to be specific to mycoplasmatic DNA, and do not react with animal or bacterial DNA.

In sensitivity tests for the detection of defined mycoplasmas, the EZ-PCR Mycoplasma Test Kit was found to be very sensitive in comparison to other test kits currently available on the market (Table 1). The ability to routinely conduct rapid and simple tests to detect mycoplasma contamination in cell cultures facilitates the eradication or treatment of contaminated cells.

Table 1: Minimal concentration of mycoplasma detected with EZ-PCR Mycoplasma Test Kit

	Without Sample Preparation	After Sample Preparation (conc. 1/20)*
M. fermentans	240 CFU/ml	12.00 CFU/ml
M. capricolum	110 CFU/ml	5.50 CFU/ml
M. penetrans	200 CFU/ml	10.00 CFU/ml
M. hyorhinis	210 CFU/ml	10.50 CFU/ml

* According to EZ-PCR Mycoplasma Test Kit instructions for use

Ready-to-use PCR Mix for the detection of mycoplasma in cell culture

EZ-PCR Mycoplasma Test Kit

Product Name	Cat. No.	Size	Temp.
EZ-PCR Mycoplasma Test Kit	20-700-20	20 Assays	-20°C

EZ-PCR Mycoplasma Test Kit is designed to detect the presence of mycoplasma contaminating biological materials, such as cultured cells.

Mycoplasma detection by the direct culture procedure is time-consuming and some mycoplasma species are difficult to cultivate. With PCR testing, results are obtained within a few hours, since the presence of contaminant mycoplasma can be easily detected simply by verifying the bands of

amplified DNA fragments in electrophoresis. There is no need to prepare probes labeled with radioisotopes, or to calculate enzyme, dNTPs or buffer concentrations. Instead, a ready-to-use, optimized PCR mix is supplied. The reaction mix contains a precipitant for direct loading of PCR products onto agarose gel.

The primer set allows detection of various mycoplasma species, with high sensitivity and specificity such as:

M. fermentans	M. arthritidis
M. arginini	M. bovis
M. hyorhinis	M. pneumoniae
M. orale	M. pirum
M. hominis	M. capricolum
M. salivarium	Acholeplasma
M. pulmonis	Spiroplasma

Kit Component:

	20-700-20
Reaction Mix	200µl
Buffer Solution	1ml
Positive Template Control	20µl

Reagents not supplied in the kit

1. Mineral oil
2. Agarose gel
3. Sterile distilled water

Equipment required

1. Authorized thermal cycler for PCR
2. Microcentrifuge tubes
3. Agarose gel electrophoresis apparatus
4. Microcentrifuge
5. Micropipettes and pipette tips (autoclaved)

Storage -20ºC

Avoid repeated changes in the Reaction Mix temperature.
When in use, always keep the Reaction Mix on ice!

Principle

rRNA gene sequences of prokaryotes, including mycoplasmas, are well conserved, whereas the lengths and sequences of the spacer region in the rRNA operon (for example the region between the 16S and 23S genes) differ from species to species. The detection procedure utilizing the PCR process with this primer set consists of:

1. Amplification of a conserved and mycoplasma-specific 16S rRNA gene region using two primers.
2. Detection of the amplified fragment by agarose gel electrophoresis.

This system does not allow the amplification of DNA originating from other sources, such as tissue samples or bacteria, which affect the detection result. Amplification of the gene sequence with PCR using this primer set enhances not only the sensitivity, but also the specificity of detection.
Amplified products are then detected by agarose gel electrophoresis.

Instructions for use

1. Test sample preparation

Transfer 0.5-1.0ml cell culture supernatant into a 2ml centrifuge tube.
To pellet cellular debris, briefly centrifuge the sample at 250 x g. Transfer the supernatant into a fresh sterile tube and centrifuge at 15,000-20,000 x g for 10 minutes to sediment mycoplasma. Carefully decant the supernatant and keep the pellet (the pellet will not always be visible). Re-suspend the pellet with 50µl of the Buffer Solution and mix thoroughly with a micropipette. Heat to 95ºC for 3 minutes.
At this stage the test sample can be stored at -20ºC for later use.

2. PCR amplification

a. Prepare the reaction mixture in a PCR tube by combining the reagents shown below:

Reagents	Volume
H_2O	35µl
Reaction Mix	10µl
Test sample	5µl

b. Overlay mineral oil (approximately 40µl) to avoid evaporation of the reaction mixture, if required.
c. Place all tubes in DNA thermal cycler. Set the parameters for the following conditions and perform PCR.

94°C	30 seconds	
94°C	30 seconds	35 cycles
60°C	120 seconds	
72°C	60 seconds	
94°C	30 seconds	
60°C	120 seconds	
72°C	5 minutes	

3. Analysis of amplified products by gel electrophoresis

a. Apply 20µl of the PCR product to the gel electrophoresis. Do not add loading buffer to the samples. Use 2% agarose gel.
b. Perform agarose gel electrophoresis with the PCR amplified samples to verify the amplified product and its size. The size of DNA fragments amplified using the specific primers in this kit is 270bp.

4. Control Template

By using 1µl Positive Template Control as a test sample, PCR efficiency can be checked. The size of the PCR product obtained using the positive template with primer pairs is 270bp.

Reference

Rottem, S., Barile, F.M. (1993), TIBTECH, 11:143-150

Treatment of Mycoplasma-Infected Cells with Antibiotics

Using Antibiotics to Disinfect Cell Cultures

The increasingly widespread use of more sophisticated and sensitive methods for the detection of mycoplasma contamination in cell cultures has resulted in contamination being detected in numerous cultures. This raises the issue of how to eliminate mycoplasma contamination. Naturally, the ideal solution is to discard the contaminated cells. However, if the cells that are stored in liquid nitrogen are also contaminated, a solution is required for eliminating the mycoplasma and preparing a new cell bank, particularly if the cells are unique and the result of extensive work.

A number of effective metho ds for the elimination of mycoplasma contamination in cell cultures have been published, such as:

- Treatment with specific hyperimmune serum (antibodies).
- Passage of contaminated cells in thymus-deficient mice.
- Exposure to analogs of nucleic acids that prevent reproduction of mycoplasma.
- Treatment with antibiotics.
- Exposure of contaminated cells to mouse macrophages.
- A technique that combines growing cells on soft agar and treatment with antibiotics.

The preferred method in terms of simplicity is treatment with antibiotics, which do not damage or alter cells.

Antibiotics such as penicillin, which attacks bacterial cell walls, are ineffective in this instance, since mycoplasma lacks a true cell wall. Several antibiotics eliminate mycoplasma effectively, such as Tylosin, Neomycin, Tetracycline and Gentamycin. However, the efficacy of these antibiotics is restricted to specific mycoplasma species and frequently only reduce the concentration of mycoplasmas rather than disinfect the cell culture.

Consequently, as soon as treatment is concluded, contamination will recur.

Two methods are recommended for treating contaminated cells with antibiotics. The first is based on alternating treatment with two types of antibiotics (Tiamutin and Minocycline), and the second on treatment with one type of antibiotic (Ciprofloxacine).

Summary

Heightened awareness regarding mycoplasma contamination, and increased use of sensitive and effective methods for the detection and treatment of mycoplasma contaminations, will lead to a reduction in the percentage of contaminated cultures. In addition to isolating contaminated cultures, and discarding or treating them, meticulous work procedures should be followed, and only mycoplasma-free raw materials should be used.

The contamination of cells with mycoplasma is a very common problem, although it often goes unnoticed since no cloudiness appears in the cell culture. Nevertheless, the contamination often causes biochemical changes as well as changes in the immunological properties of the cells.

Since mycoplasma-infected cells cannot always be discarded, many complex methods have been suggested for the elimination of mycoplasma.

Biological Industries is now offering a combination of antibiotics, which have been shown to be effective in the elimination of mycoplasma species that account for 90% of the contamination found in cell cultures.

When used according to the following instructions, no cytotoxic effects will occur. Store the BIOMYC solutions at -20.

BIOMYC-1 & BIOMYC-2

BIOMYC-1 is based on the antibiotic Tiamutin, which is produced by the fungus pleurotus mutilus. BIOMYC-2 is based on minocycline, which is a tetracycline derivative. These two antibiotic solutions are generally used sequentially in combination.

Instructions for Use

1. Do not use the two solutions together, but sequentially.
2. Add 1ml BIOMYC-1 to 100ml medium, and maintain the contaminated cells in this mixture for 4 days. Any fresh medium added should also contain BIOMYC-1.
3. After 4 days, add 1ml BIOMYC-2 to 100ml fresh medium, and maintain the cells in this second mixture for 3 days.
4. The above, together, are considered as one treatment cycle. It may be necessary to repeat this cycle 2-3 times.
5. During the process, the cells can be tested for mycoplasma contamination, and results can then be used to shorten the process when possible.

Prevention of Contamination

BIOMYC-3

BIOMYC-3 is based on the ciprofloxacin antibiotic, which is a member of the fluoroquinolone group. Many mycoplasma species have been found to be sensitive to BIOMYC-3, including A.laidlawii, M.orale, M.hyorhinis, M.fermentans and M.arginini. These species are responsible for most of the contamination in cell culture. At the recommended concentrations, no cytotoxic effects have been found, and the treatment is quite easy to perform.

Instructions for Use

1. Add 1ml BIOMYC-3 to 100ml medium.
2. Continue the treatment for a total of 14 days, changing the medium (containing BIOMYC-3) every 2-3 days.
3. Retain the cells in the growth medium for an additional 14 days before re-testing for mycoplasma.

Product Name	Cat. No.	Size	Temp.
BIOMYC-1	03-036-1D	10ml	-20°C
100X Conc.	03-036-1C	20ml	-20°C
	03-036-1B	100ml	-20°C
BIOMYC-2	03-037-1D	10ml	-20°C
100X Conc.	03-037-1C	20ml	-20°C
	03-037-1B	100ml	-20°C
BIOMYC-3	03-038-1D	10ml	-20°C
100X Conc.	03-038-1C	20ml	-20°C
	03-038-1B	100ml	-20°C

Reference

Schmitt, k. et al., J. Immunol. Methods, 109: 17-25 (1988)

Fast, reliable and highly efficient disinfection of incubators and lab surfaces

Pharmacidal

The problem of contamination in incubators and/or sterile workbenches is often serious, leading to extensive damage. Pharmacidal solution prevents contamination and growth of a broad range of fungi (and spores), bacteria (and spores) mycoplasma and viruses (including HIV and Hepatitis B).

Pharmacidal is safe and easy to use. The solution is available as ready-to-use sprays and it is non-irritant and non-corrosive. The active ingredients are quaternary benzylammonium compounds, and the solution does not contain mercury, formaldehyde, phenol or alcohol. Furthermore, Pharmacidal is non-toxic and biodegradable.

Pharmacidal has been found to be fully compatible with all common work surfaces, and it is especially effective against disagreeable smells.

Instructions for Use

Spray incubators once every 2 weeks.
Spray sterile benches once a day, or preferably before each laboratory worker begins using the work area.
The surfaces to be disinfected should be completely saturate by the spraying.

Allow to dry, no rinsing necessary.

Product Name	Cat. No.	Size	Temp.
Pharmacidal	IC-110100-B	100ml	AMB
	IC-110100-L	250ml	
	IC-110100-1	1 liter	
	IC-110100-G	5 liter	

Related Products for Disinfection

Aquaguard-1

100ml of 100X concentrated solution for disinfecting CO_2 water baths.

(Use 50ml per 5 liters of water in bath).

The water required for humidity is a source of contamination that disperses in the incubator. In order to disinfect the water we recommend Aquaguard-1 Solution, which contains a disinfectant that does not cause damage to the stainless steel tray, is non-toxic, non-volatile, and extremely effective.

The water should be replaced with sterile water every two to four weeks, adding 50ml of Aquaguard-1 per 5 liters of water.

Preventive treatment as described above will prevent damage that can be caused as a result of contamination to the tissue culture. In addition, it will also prevent the necessity of dealing with contamination that has dispersed in the incubator and causes repeated contamination of the tissue culture.

Aquaguard-2

50ml of 500X concentrated solution for prevention of microbial growth in water baths. (Use 2ml per 1 liter of water) Aquaguard-2 is intended for disinfecting various kinds of water baths from bacteria and fungi. It is recommended to use 2ml of Aquaguard-2 for each liter of water in the bath, and to repeat the procedure every 1-2 weeks. After 4-6 weeks, the bath should be emptied and refilled with water containing Aquaguard-2.

Product Name	Description	Cat. No.	Size	Temp.
AQUAGUARD-1 Solution	For disinfecting water baths of CO_2 incubators. Use 10ml per liter	01-867-1B	100ml	-20°C
AQUAGUARD-2 Solution	For disinfecting ordinary water baths. Use 2ml per liter	01-916-1E	50ml	-20°C

Biological Industries (BI) has been committed for over 30 years to provide optimal and innovative solutions for cell culture practice.
BI manufactures and supplies life science products to biopharmaceutical, academic and government research facilities, as well as to biopharma companies.

Our diverse portfolio of products and services includes all of the following:

- Liquid and powdered cell culture media
- Sterile sera (Foetal Bovine Serum, Newborn Calf serum, Donor horse, etc.)
- Novel serum-free and animal component-free media and supplements
- Products for stem cell culture
- Products for cytogenetics
- Products for mycoplasma detection and treatment
- Disinfectants
- ECM-coated plastic ware
- Products for molecular biology
- Contract manufacturing and custom formulations

BI's production and filling processes are according to GMP Quality System guidelines in a cGMP compliant facility. The products are manufactured in a controlled environment (clean rooms), according to ISO 14644:2001 and EU\GMP annex 1.
The clean rooms are monitored on a regular basis for particulate and microbial levels to ensure that the air handling system, cleaning protocol and personnel maintain standards control.
The production and the preparation area are class 100,000 (ISO 8) environments.
Sterile bottles and equipment are stored in class 10,000 (ISO 7) environment.
The filling room is a class 1,000 (ISO 6) environment with class 100 (ISO 5) laminar air flow sterile bench.
Only properly trained personnel are authorized to perform the product preparation, filtration and filling. The personnel entering the production and filling rooms are gowned as per the gowning procedure of BI Ltd.
In addition, BI Ltd. holds ISO 9001:2008, ISO 13485:2003 and also comply with the legal requirements set in the European IVD Directive 98/79/EC.

BI also exports its products to more than 40 countries worldwide, via a network of exclusive distributors. Over the years the company has established a reputation for fast delivery, and excellent technical support.
From the outset, the policy of BI has been based on the need to maintain an active Research and Development program in all facets of company activities. The company has its own in-house R&D department, and in addition, maintains active contact with science-based companies and research institutions in Israel and abroad, including know-how agreements with several such institutions. These ongoing efforts have led to the introduction of a series of serum-free medium products, and a novel product line for stem cell research, as well as many other products for cell culture and molecular biology.

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