

Understanding and Managing Cell Culture Contamination

Technical Bulletin



Life
Sciences

*John Ryan, Ph.D.
Corning Incorporated
Life Sciences
Acton, MA 01720*

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Introduction

No cell culture problem is as universal as that of culture loss due to contamination. All cell culture laboratories and cell culture workers have experienced it. Culture contaminants may be biological or chemical, seen or unseen, destructive or seemingly benign, but in all cases they adversely affect both the use of your cell cultures and the quality of your research. Contamination problems can be divided into three classes:

- ▶ *Minor annoyances* — when up to several plates or flasks are occasionally lost to contamination;
- ▶ *Serious problems* — when contamination frequency increases or entire experiments or cell cultures are lost;
- ▶ *Major catastrophes* — contaminants are discovered that call into doubt the validity of your past or current work.

Table 1. Some Consequences of Contamination

- ▶ Loss of time, money, and effort
- ▶ Adverse effects on the cultures
- ▶ Inaccurate or erroneous experimental results
- ▶ Loss of valuable products
- ▶ Personal embarrassment

The most obvious consequence of cell culture contamination is the loss of your time, money (for cells, culture vessels, media and sera) and effort spent developing cultures and setting up experiments. However, the less obvious consequences are often more serious (Table 1). First there are the adverse effects on cultures suffering from undetected chemical or biological contaminants. These hidden (cryptic) contaminants can achieve high densities altering the growth and characteristics of the cultures. Worse yet are the potentially inaccurate or erroneous results obtained by unknowingly working with these cryptically contaminated cultures. Products, such as vaccines, drugs or monoclonal antibodies, manufactured by these cultures will probably be useless. For some researchers the most serious consequence of contamination is suffering the embarrassment and damage to their reputation that results when they notify collaborators or journals that their experimental results are faulty and must be retracted due to contaminants in their cultures.

Preventing all cell culture contamination has long been the dream of many researchers, but it is an impractical, if not impossible, dream. *Contamination cannot be totally eliminated, but it can be managed to reduce both its frequency of occurrence and the seriousness of its consequences.* The goal of this bulletin is to review the nature of cell culture contamination and the problems it causes, and then to explore some of the key concepts and practical strategies for managing contamination to prevent the loss of valuable cultures and experiments.

What Are the Major Cell Culture Contaminants?

A cell culture contaminant can be defined as some element in the culture system that is undesirable because of its possible adverse effects on either the system or its use. These elements can be divided into two main categories: chemical contaminants and biological contaminants.

Chemical Contamination

Chemical contamination is best described as the presence of any nonliving substance that results in undesirable effects on the culture system. To define further is difficult; even essential nutrients become toxic at high enough concentrations. Nor is toxicity the only concern since hormones and other growth factors found in serum can cause changes that, while not necessarily harmful to cultures, may be unwanted by researchers using the system. (Reviewed in references 1-3.)

Media

The majority of chemical contaminants are found in cell culture media and come either from the reagents and water used to make them, or the additives, such as sera, used to supplement them. Reagents should always be of the highest quality and purity and must be properly stored to prevent deterioration. Ideally, they should be either certified for cell culture use by their manufacturer or evaluated by the researcher before use. Mistakes in media preparation protocols, reading reagent bottle labels, or weighing reagents are other common sources of chemical contamination.

Sera

Sera used in media have long been a source of both biological and chemical contaminants. Due to cell culture-based screening programs currently used by good sera manufacturers, it is unusual to find a lot of fetal bovine sera that is toxic to a majority of cell cultures. However, it is common to find substantial variations in the growth promoting abilities of different lots of sera for particular cell

culture systems, especially for cultures that have specialized or differentiated characteristics. Uncontrollable lot-to-lot variation in hormone and growth factor concentrations makes this problem inevitable; careful testing of sera before purchase, or switching to serum-free media can avoid these problems.

Table 2. Types and Sources of Potential Chemical Contaminants

- ▶ Metal ions, endotoxins, and other impurities in media, sera, and water
- ▶ Plasticizers in plastic tubing and storage bottles
- ▶ Free radicals generated in media by the photoactivation of tryptophan, riboflavin or HEPES exposed to fluorescent light
- ▶ Deposits on glassware, pipettes, instruments etc., left by disinfectants or detergents, antiscaling compounds in autoclave water, residues from aluminum foil or paper
- ▶ Residues from germicides or pesticides used to disinfect incubators, equipment, and labs
- ▶ Impurities in gases used in CO₂ incubators

Remember also that serum proteins have the ability to bind substantial quantities of chemical contaminants, especially heavy metals, that may have entered the culture system from other sources, rendering them less toxic. As a result, switching from serum-containing medium to a serum-free system can unmask these toxic chemical contaminants, exposing the cells to their adverse effects.

Water

The water used for making media and washing glassware is a frequent source of chemical contamination and requires special care to ensure its quality. Traditionally, double or triple glass distillation was considered to be the best source of high quality water for cell culture media and solutions. Newer purification systems combining reverse osmosis, ion exchange and ultrafiltration are capable of removing trace metals, dissolved organic compounds and endotoxins and are increasingly popular. However, these systems must be properly maintained and serviced to ensure continued water quality. Because of its

aggressive solvent characteristics, highly purified water can leach potentially toxic metal ions from glassware or metal pipes, and plasticizers from plastic storage vessels or tubing. These contaminants can then end up in media or deposited on storage vessels and pipettes during washing and rinsing. Water used to generate steam in autoclaves may contain additives to reduce scale buildup in pipes; these potentially toxic additives can also end up on glassware.

Endotoxins

Endotoxins, the lipopolysaccharide-containing by-products of gram negative bacteria, are another source of chemical contaminants in cell culture systems. Endotoxins are commonly found in water, sera and some culture additives (especially those manufactured using microbial fermentation) and can be readily quantified using the **Limulus Amebocyte Lysate** assay (LAL).

These highly biologically reactive molecules have major influences *in vivo* on humoral and cellular systems. Studies of endotoxins using *in vitro* systems have shown that they may affect the growth or performance of cultures and are a significant source of experimental variability (Reviewed in references 6 and 39). Furthermore, since the use of cell culture produced therapeutics, such as hybridomas and vaccines, are compromised by high endotoxin levels, efforts must be made to keep endotoxin levels in culture systems as low as possible.

In the past, sera have been a major source of endotoxins in cell cultures. As improved endotoxin assays (LAL) led to an increased awareness of the potential cell culture problems associated with endotoxins, most manufacturers have significantly reduced levels in sera by handling the raw products under aseptic conditions. Poorly maintained water systems, especially systems using ion exchange resins, can harbor significant levels of endotoxin-producing bacteria and may need to be tested if endotoxin problems are suspected or discovered in the cultures.

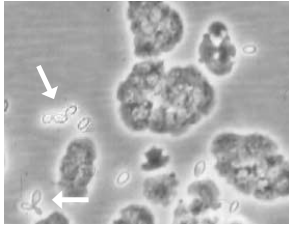


Figure 1. Photomicrograph of a low level yeast infection in a liver cell line (PLHC-1, ATCC # CRL-2406). Budding yeast cells can be seen in several areas (arrows). At this low level of contamination, no medium turbidity would be seen; however, in the absence of antibiotics, the culture medium will probably become turbid within a day.

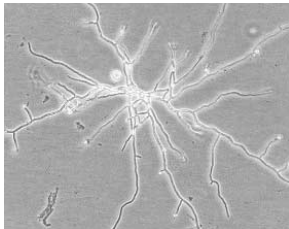


Figure 2. Photomicrograph of a small fungal colony growing in a cell culture. At this point, this colony would still be invisible to direct visual observation. If this culture was subcultured at this point, all of the cultures or experiments set up from it would soon be lost to fungal contamination.

Storage Vessels

Media stored in glass or plastic bottles that have previously contained solutions of heavy metals or organic compounds, such as electron microscopy stains, solvents and pesticides, can be another source of contamination. The contaminants can be adsorbed onto the surface of the bottle or its cap (or absorbed into the bottle if plastic) during storage of the original solution. If during the washing process they are only partially removed, then once in contact with culture media they may slowly leach back into solution. Residues from chemicals used to disinfect glassware, detergents used in washing, or some aluminum foils and wrapping papers for autoclaving or dry heat sterilization can also leave potentially toxic deposits on pipettes, storage bottles and instruments.

Fluorescent Lights

An important but often overlooked source of chemical contamination results from the exposure of media containing HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) — an organic buffer commonly used to supplement bicarbonate-based buffers), riboflavin or tryptophan to normal fluorescent lighting. These media components can be photoactivated producing hydrogen peroxide and free radicals that are toxic to cells; the longer the exposure the greater the toxicity (4,5). Short term exposure of media to room or hood lighting when feeding cultures is usually not a significant problem; but leaving media on lab benches for extended periods, storing media in walk-in cold rooms with the lights on, or using refrigerators with glass doors where fluorescent light exposure is more extensive, will lead to a gradual deterioration in the quality of the media.

Incubators

The incubator, often considered a major source of biological contamination, can also be a source of chemical contamination. The gas mixtures (usually containing carbon dioxide to help regulate media pH) perfused through some incubators may contain toxic impurities, especially oils or other gases such as carbon monoxide, that may have been previously used in the

same storage cylinder or tank. This problem is very rare in medical grade gases, but more common in the less expensive industrial grade gas mixtures. Care must also be taken when installing new cylinders to make sure the correct gas cylinder is used. Other potential chemical contaminants are the toxic, volatile residues left behind after cleaning and disinfecting incubators. Disinfectant odors should not be detectable in a freshly cleaned incubator when it is placed back into use.

Keep in mind that chemical contaminants tend to be additive in cell culture; small amounts contributed from several different sources that are individually nontoxic, when combined together in medium, may end up overloading the detoxification capabilities of the cell culture resulting in toxicity-induced stress effects or even culture loss.

Biological Contamination

Biological contaminants can be subdivided into two groups based on the difficulty of detecting them in cultures:

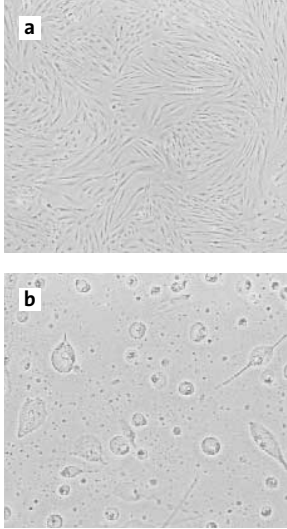
- those that are usually easy to detect — bacteria, molds and yeast;
- those that are more difficult to detect, and as a result potentially more serious culture problems, — viruses, protozoa, insects, mycoplasmas and other cell lines.

For a comprehensive review, see references 7 and 8.

Ultimately, it is the length of time that a culture contaminant escapes detection that will determine the extent of damage it creates in a laboratory or research project.

Bacteria, Molds, and Yeasts

Bacteria, molds and yeasts are found virtually everywhere and are able to quickly colonize and flourish in the rich and relatively undefended environment provided by cell cultures. Because of their size and fast growth rates, these microbes are the most commonly encountered cell culture contaminants. In the absence of antibiotics, microbes can usually be readily detected in a culture within a few days of becoming contaminated, either by direct microscopic observation. (See Figures 1 and 2.) or by the effects they have on the



Figures 3a and 3b. Photomicrographs of a winter flounder (*Pseudopleuronectes americanus*) fibroblast-like cell culture. Figure 3a shows an apparently healthy early passage culture; Figure 3b shows the same culture approximately 24 hours later. Electron microscopy showed virus-like particles in these cells. Multiple attempts to establish cell lines from this species were unsuccessful and showed cytopathic effects that appeared to be caused by an unknown virus.

culture (pH shifts, turbidity, and cell destruction). However, when antibiotics are routinely used in culture, resistant organisms may develop into slow growing, low level infections that are very difficult to detect by direct visual observation. Similar detection problems can occur with naturally slow growing organisms or very small or intracellular bacteria that are difficult to see during routine microscopic culture observation. These cryptic contaminants may persist indefinitely in cultures causing subtle but significant alterations in their behavior. By the time these cryptic contaminants are discovered, many experiments and cultures may have been compromised.

Viruses

Due to their extremely small size, viruses are the most difficult cell culture contaminants to detect in culture, requiring methods that are impractical for most research laboratories. Their small size also makes them very difficult to remove from media, sera, and other solutions of biological origin. However, most viruses have stringent requirements for their original host species' cellular machinery (may also be tissue specific) which greatly limits their ability to infect cell cultures from other species. Thus, although viruses may be more common in cell cultures than many researchers realize, they are usually not a serious problem unless they have cytopathic or other adverse effects on the cultures. (Reviewed in Ref. 7, 40.) Since cytopathic viruses usually destroy the cultures they infect, they tend to be self-limiting. Thus, when cultures self-destruct for no apparent reason and no evidence of common biological contaminants can be found, cryptic viruses are often blamed. (See Figures 3a and 3b.) They are perfect culprits, unseen and undetectable; guilty without direct evidence. This is unfortunate, since the real cause of this culture destruction may be something else, possibly mycoplasma or a chemical contaminant, and as a result will go undetected to become a more serious problem.

A major concern of using virally infected cell cultures is not their effects on the cultures but rather the potential health hazards they pose for laboratory personnel. Special safety pre-

cautions should always be used when working with tissues or cells from humans or other primates to avoid possible transmission of viral infection (HIV, hepatitis B, Epstein-Barr, simian herpes B virus, among others) from the cell cultures to laboratory personnel (9). Contact your safety office for additional assistance if in doubt as to appropriate procedures for working with potentially hazardous tissues, cultures or viruses.

Protozoa

Both parasitic and free-living, single-celled protozoa, such as amoebas, have occasionally been identified as cell culture contaminants. Usually of soil origin, amoebas can form spores and are readily isolated from the air, occasionally from tissues, as well as throat and nose swabs of laboratory personnel. They can cause cytopathic effects resembling viral damage and completely destroy a culture within ten days. Because of their slow growth and morphological similarities to cultured cells, amoebas are somewhat difficult to detect in culture, unless already suspected as contaminants (7). Fortunately, reported cases of this class of contaminants are rare, but it is important to be alert to the possibility of their occurrence.

Invertebrates

Insects and arachnids commonly found in laboratory areas, especially flies, ants, cockroaches and mites, can both be culture contaminants as well as important sources of microbial contamination. Warm rooms are common sites of infestation. By wandering in and out of culture vessels and sterile supplies as they search for food or shelter, they can randomly spread a variety of microbial contaminants. Occasionally they are detected by the trail of "foot prints" (microbial colonies) they leave behind on agar plates, but usually they don't leave any visible signs of their visit other than random microbial contamination. Mites can be a serious problem in plant cell culture facilities, especially those doing large scale plant propagation. Although bacteria, molds and yeast may sometimes appear to 'jump' from culture to culture, these multilegged contaminants really can. While not nearly as common as other culture contaminants, it is important to be alert to the presence of these invertebrates in culture areas.

Mycoplasmas

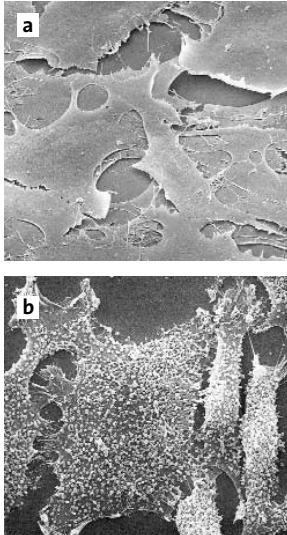
Mycoplasmas were first detected in cell cultures by Robinson and coworkers in 1956. They were attempting to study the effects of PPLO (**p**leu**p**neumonia**l**ike **o**rganisms — the original name for mycoplasma) on HeLa cells when they discovered that the control HeLa cultures were already contaminated by PPLO (10). In addition, they discovered that the other cell lines currently in use in their laboratory were also infected with mycoplasma, a common characteristic of mycoplasma contamination. *Based on mycoplasma testing done by the FDA, ATCC, and two major cell culture testing companies, at least 11 to 15% of the cell cultures in the United States are currently infected by mycoplasmas* (Table 3). Since many of these cultures were from laboratories that test routinely for mycoplasma, the actual rates are probably higher in the many laboratories that do not test at all (11-13). In Europe, mycoplasma contamination levels were found to be even higher: over 25% of 1949 cell cultures from the Netherlands and 37% of 327 cultures from former Czechoslovakia were positive (14). The Czechoslovakia study had an interesting, but typical finding: *100% of the cultures from labs without mycoplasma testing programs were contaminated, but only 2% of the cultures from labs that tested regularly.* Other countries may be worse: 65% of the cultures in Argentina and 80% in Japan were reported to be contaminated by mycoplasma in other studies (11).

Unfortunately, mycoplasmas are not relatively benign culture contaminants but have the ability to alter their host culture's cell function, growth, metabolism, morphology, attachment, membranes, virus propagation and yield, interferon

induction and yield, cause chromosomal aberrations and damage, and cytopathic effects including plaque formation (12). Thus, the validity of any research done using these unknowingly infected cultures is questionable at best. (See references 11, 12, and 15-18 for good overviews of this very serious mycoplasma contamination problem.)

What gives mycoplasmas this ability to readily infect so many cultures? Three basic characteristics: a) these simple, bacteria-like microbes are the smallest self-replicating organism known (0.3 to 0.8 μm in diameter), b) they lack a cell wall, and c) they are fastidious in their growth requirements. Their small size and lack of a cell wall allow mycoplasmas to grow to very high densities in cell culture (10^7 to 10^9 colony forming units/mL are common) often without any visible signs of contamination — no turbidity, pH changes or even cytopathic effects. (See Figures 4a and 4b.) Even careful microscopic observation of live cell cultures cannot detect their presence. These same two characteristics also make mycoplasmas, like viruses, very difficult to completely remove from sera by membrane filtration. In addition, their fastidious growth requirements (unfortunately, easily provided for by cell cultures) make them very difficult to grow and detect using standard microbiological cultivation methods. Thus, these three simple characteristics, combined with their ability to alter virtually every cellular function and parameter, make mycoplasmas the most serious, widespread, and devastating culture contaminants.

Mycoplasmas have been described as the “crabgrass” of cell cultures, but this is too



Figures 4a and 4b. These scanning electron micrographs show 3T6 cells (ATCC # CCL-96) with (4b) and without (4a) mycoplasma infections. The level of contamination of these cells by the mycoplasma shown here is typical of contaminated cells. Examination of this contaminated culture by phase contrast microscopy did not show any evidence of contamination; nor did the medium show any turbidity.

Table 3. Mycoplasma Contamination of Cell Cultures

Number of Cultures Tested	Number Positive
Food and Drug Administration (FDA) (1970's to 1990's) (11) 20,000 cultures tested	over 3000 (15%)
Bionique Testing Laboratories (several years prior to 1993) (41) 11,000 cultures tested	1218 (11.1%)
Microbiological Associates (1985 to 1993) (13) 2,863 cultures tested	370 (12.9%)
American Type Culture Collection (ATCC) (1989 to 1994) (42) 5362	752 (14%)

benign a description for what are the most significant and widespread cell culture contaminants in the world. Unfortunately, even with the advances in detection methods (discussed in detail later) mycoplasma infection rates (Table 3) have not changed noticeably since they were first detected in cell cultures. Aggressive management against mycoplasma contamination must be the central focus for any cell culture laboratory contamination or quality control program (16).

Cross-Contamination by Other Cell Cultures

With the advent of improved karyotyping methods in the late 1950's, it soon became apparent that some cell lines were cross-contaminated by cells of other species (7). In 1967, isoenzyme analysis was used to show that 20 commonly used human cell lines were intraspecies contaminated by HeLa cells (19,20). Contaminated is actually a misnomer since in fact 100% of the original cells had been replaced by the HeLa contaminant. Unfortunately, the scientific community was slow to respond to this very serious problem. Tests done at one research center on 246 cell lines over an 18 month period prior to 1976 showed that nearly 30% were incorrectly designated: 14% were the wrong species and 25% of the human cell lines were HeLa cells (21). A 1981 survey of cultures showed over 60 cell lines that were actually HeLa cells, 16 other human cell lines contaminated by non-HeLa human cell lines, and 12 cases of interspecies contamination (See Table 4). Nor is the problem limited to contamination by HeLa cells. The advent of DNA analysis

has shown that cells from a variety of sources have contaminated many other cell lines (42).

The seriousness of cross-contamination, while not as common as microbial contamination, cannot be overstated. The validity of experimental results from cultures having inter- or intraspecies contamination is, at the very least, questionable. Furthermore, their use can lead to the embarrassment of having to retract published results. Whenever the invading cell is better adapted to the culture conditions and thus faster growing than the original cells, it will almost always completely replace them. Because of the outward physical similarities of different cell lines and the wide morphological variations that can be caused by the culture environment, it is impossible to rely only on microscopic observation to screen for cross-contamination of cultures. Simple accidents are one of the most common means by which other cell lines gain entry into cultures and will be discussed separately in the next section.

Remember, the seriousness of any culture contaminant is usually directly proportional to the difficulty of detecting it; those that go undetected the longest have the most serious consequences. Cultures containing nonlethal (but not harmless), cryptic chemical or biological contaminants are sometimes used in research for months or even years before being uncovered; during this time the quality and validity of all research done with those cultures is compromised, as is the reputation of the researchers using them.

Table 4. Some HeLa Contaminated Cell Lines

Detroit 6 (CCL-3)	Conjunctiva (CCL-20.2)*
Minnesota-EE (CCL-4)	AV3 (CCL-21)*
L132 (CCL 5)*	HEp-2 (CCL-23)*
Intestine 407 (CCL-6)*	J-111 (CCL-24)
Chang Liver (CCL-13)	WISH (CCL-25)*
KB (CCL-17)*	Giardia Heart (CCL-27)
Detroit 98 (CCL-18)	Wilm's Tumor (CCL-31)
NCTC 2544 (CCL-19)	FL (CCL- 62)*

CCL# is the ATCC catalog designation. All except CCL-20.2, CCL-31 and CCL-62 were shown to be HeLa by Gartler in 1968 (20). Those marked with an asterisk can be found in the Cell Biology Collection on the ATCC web site (www.atcc.org) where they are clearly marked as HeLa contaminants.

Table 5. How Do Biological Contaminants Enter Cultures?

- ▶ Contact with nonsterile supplies, media, or solutions
- ▶ Particulate or aerosol fallout during culture manipulation, transportation, or incubation
- ▶ Swimming, crawling, or growing into culture vessels
- ▶ Accidents and mistakes

What Are the Sources of Biological Contaminants?

To reduce the frequency of biological contamination, it is important to know not only the nature and identity of the contaminants but also where they come from and how they gain entry into cultures. This section will detail some of the most common sources of biological contaminants (3).

Nonsterile Supplies, Media and Solutions

Unintentional use of nonsterile supplies, media or solutions during routine cell culture procedures is a major source of biological contaminants. These products may be contaminated as a result of improper sterilization or storage, or may become contaminated during use.

Glassware, including storage bottles and pipettes, is usually sterilized by autoclaving or dry heat sterilization. Serious contamination outbreaks are frequently traced to improper maintenance or operation of sterilization autoclaves and ovens. Packing too much into an autoclave or dry heat oven will cause uneven heating, resulting in pockets of nonsterile supplies. Using too short a sterilization cycle, especially for autoclaving volumes of liquids greater than 500 mL per vessel or solutions containing solids or viscous materials, such as agar or starches, is a common mistake. The size, mass, nature and volume of the materials to be sterilized must always be considered and the cycle time appropriately adjusted to achieve sterility (23). Then, once achieved, sterility must be maintained by properly storing the supplies and solutions in a dust- and insect-free area to prevent recontamination. Care must also be taken to avoid condensation on bottles of solutions stored in refrigerators and cold rooms. Of course,

good aseptic technique is also required to maintain the sterility of properly sterilized supplies and solutions once they are in use.

Plastic disposable cell culture vessels, pipettes, centrifuge tubes, etc. are usually sterilized by their manufacturer using a high intensity gamma or electron beam radiation source after they are sealed in their packaging. This is a very reliable process, however care must be taken when opening and resealing the packaging to avoid contaminating the products within.

Most media, sera and other animal-derived biologicals are not heat sterilizable and require membrane filtration (Sometimes radiation is also used.) to remove biological contaminants. Products filter sterilized in your laboratory should always be tested for sterility before use (discussed in detail later); commercially produced sterile products are tested by the manufacturer before being sold. While filtration through 0.2 μm membranes is very effective in removing most biological contaminants, it cannot guarantee the complete removal of viruses and mycoplasmas, especially in sera (16,18,24). In an excellent review of the rates and sources of mycoplasma contamination (25), Barile and coworkers reported that 104 out of 395 lots (26%) of commercial fetal bovine sera tested were contaminated by mycoplasma. They concluded in the early 1970's that animal sera were among the major sources of cell culture contamination by mycoplasma. Many sera manufacturers responded to this problem over the next decade by improving both filtration and testing procedures; they currently use serial filtration through at least three filter membranes rated at 0.1 μm or smaller to remove mycoplasmas. This approach has been very successful at reducing the problem of mycoplasma in sera and other animal-

derived products (16). While these products are no longer a major source of mycoplasma contamination, they must still be considered as potential sources to be evaluated whenever mycoplasmas are detected in cultures.

Airborne Particles and Aerosols

In most laboratories, the greatest sources of microbial contamination are airborne particles and aerosols generated during culture manipulations. The microbial laden particles are relatively large (generally 4 to 28 μm in diameter) and settle at a rate of approximately one foot per minute in still air. As a result, the air in a sealed, draft-free room or laboratory (no people, open windows or doors, air handling units, air conditioners, etc.) is virtually free of biological contaminants. However as soon as people enter the room, particles that have settled out will be easily resuspended. In addition certain equipment and activities can generate large amounts of microbial laden particulates and aerosols: pipetting devices, vacuum pumps and aspirators, centrifuges, blenders, sonicators, and heat sources such as radiators, ovens, refrigerators and freezers. Animal care facilities and the animals they house are especially serious particle and aerosol generators, and should always be kept as far from the culture area as possible.

McGarrity used a cell culture that was intentionally infected with mycoplasma as a model to study how mycoplasmas are spread in a laminar flow hood during routine subculturing procedures (26). (This reference is especially recommended for a better understanding of how mycoplasma can be spread in a lab.) Following trypsinization of the infected culture in a laminar flow hood, live mycoplasma were isolated from the technician, the outside of the flask, a hemocytometer, the pipettor, and the outside of the pipette discard pan. Live mycoplasma could even be successfully recovered from the surface of the laminar flow hood *four to six days later!* A clean culture, that was subcultured once a week in the same hood following the work with the contaminated cells, tested positive for mycoplasma after *only 6 weeks*.

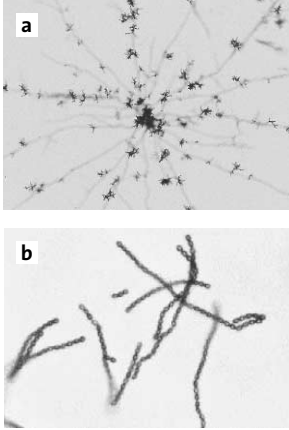
It is easy to understand from this study how the entry of a single mycoplasma infected culture into a laboratory can quickly lead to the infection of all the other cultures in the laboratory. This explains the frequent finding that if one culture in a laboratory is mycoplasma contaminated then usually most if not all of the other cultures will be as well. *Currently, the major source of mycoplasma contamination is infected cultures acquired from other research laboratories or commercial suppliers.*

Another major source of particulates and aerosols are laboratory personnel. Street clothes and dirty lab coats are dust magnets. Placing a dust-laden sleeve into a laminar flow hood generates a cloud of dust particles that can easily fall into and contaminate cultures during routine processing. Talking and sneezing can generate significant amounts of aerosols that have been shown to contain mycoplasma (26). Mouth pipetting is both a source of mycoplasma contamination and a hazard to personnel and must not be permitted under any circumstances. Dry, flaky skin is another source of contamination laden particles; this common condition is aggravated by the frequent hand washing required in the laboratory; even the lotions designed to moisten dry skin have occasionally been found to be contaminated. Some laboratory personnel shed yeast-containing particles for several days following bread making or beer brewing at home. Attempts by these individuals at cell culturing during this period have routinely ended in failure due to yeast contamination.

Incubators, especially those maintained at high humidity levels, can be a significant source of biological contamination in the laboratory. Dirty water reservoirs, and shelves or culture vessels soiled by spilled media, allow the growth of spore-generating fungi. The fans used in many incubators to circulate the air and prevent temperature stratification can then spread these spores and other particulates. Some incubators humidify incoming gases by bubbling them through the water reservoirs at the bottom of the incubator; the

aerosols generated by this will quickly spread any contaminants in the water.

While laminar hoods and incubators are the major sites where biological contamination occurs, transporting cultures between these two sites also provides opportunities for contamination. Most cell culture laboratories try very hard to keep their incubators and laminar flow areas clean, but sometimes they overlook the potential sources of contamination found in less clean laboratory areas traversed going from one location to the other. Rooms containing open windows, air conditioners, microbiology and molecular biology work areas, and the other major particle generators discussed above, add to the potential hazards of moving cultures around the laboratory. This problem increases both with the distance traveled and when the culture vessels are unsealed.



Figures 5a and 5b. Photomicrographs of contaminants growing on the outside surfaces of culture vessels. Eventually, these organisms may grow into the culture.

Swimming, Growing, and Crawling into Cultures

Unsealed culture plates and dishes, as well as flasks with loose caps to allow gas exchange, provide another common way for contaminants to enter cultures. It is very easy for the space between the top and bottom sidewalls of a dish, or a flask and its cap to become wet by capillary action with medium or condensation. This thin film of liquid then provides a liquid bridge or highway for microorganisms to either swim or grow into the culture vessel.

Even without any detectable film, fungi, as well as other microorganisms, can grow on the outside of culture vessels (Figures 5a and 5b); eventually their hyphae grow right up the side wall of the dish or past the cap into the neck of the flask. This is more often observed in long term cultures (a month or more) maintained in the same unsealed culture vessel. Small insects and other invertebrates can also make temporary visits into unsealed cultures, especially dishes and plates, leaving behind (unless they fall in and drown) only the contaminants carried on their feet.

Accidents

Accidents are often overlooked as a significant source of cell culture problems. An accident is defined as “an undesirable or unfortunate happening, unintentionally caused and usually resulting in harm, injury, damage or loss” (Webster’s Encyclopedic Unabridged Dictionary, 1989). Cell culture-related accidents are one of the leading causes of cross-contamination by other cell cultures. The following actual cases demonstrate how relatively simple accidents can result in serious cross-contamination problems:

- ▶ A technician retrieved a vial labeled WI-38 from a liquid nitrogen freezer thinking it contained the widely used diploid human cell line. Once in culture, it was immediately discovered to be a plant cell line derived from a common strain of tobacco called Wisconsin 38, also designated WI-38.
- ▶ Two separate research laboratories, both attempting to develop cell lines from primary cultures, shared a walk-in incubator. One lab used the acronyms HL-1, HL-2, etc. to identify the primary cultures they derived from human lung. The other lab worked with cultures derived from human liver, but they too (unknowingly) used the identical coding system. It wasn’t long before a culture mix up occurred between the two laboratories.

Fortunately, both of the above accidental cross-contamination cases, although serious, were caught before they caused catastrophic problems. But how many times have similar accidents occurred and not been caught? Based on continuing reports in the literature (7,8,19-22) many researchers have not been lucky enough to identify their mistakes.

The information presented above is designed to provide you with an increased awareness and understanding of the nature of biological and chemical contamination, and its serious consequences. The remaining sections will cover some basic ideas, techniques and strategies for actively detecting and combating cell culture contamination in your own laboratory.

How Can Cell Culture Contamination Be Controlled?

Cell cultures can be managed to reduce both the frequency and seriousness of culture-related problems, especially contamination. Lack of basic culture management procedures, especially in larger laboratories, frequently leads to long term problems, making contamination more likely for everyone. One solution is to actively manage your cultures to reduce problems and if necessary set up a program for use in your laboratory (27,28). This program should be designed to meet the needs of your specific working conditions and be based on the nature of your past cell culture problems; it can be very simple and informal, or more structured if required.

The first step in managing cultures is to determine the extent and nature of the culture losses in your lab. Everyone in the laboratory should keep an accurate record for a month or more of all problems, no matter how minor or insignificant, that result in the loss of any cultures. These problems may not only be contamination related but can also be from other causes such as incubator or equipment failures. Next, review the problems as a group to determine their nature, seriousness and frequency. The group's findings may be surprising: what were thought to be individual and minor random occurrences of contamination often turn out to have a pattern and be more extensive than any individual realized. This problem sharing is often a painful process, but remember the goal is not to place blame but to appreciate the extent and nature of the problems confronting the laboratory. A critical part of this process is understanding the seriousness and actual costs of culture loss; placing a dollar value on these losses is often required before the full extent of the losses can be appreciated. It is very important for everyone in the laboratory to know the answers to the following questions:

1. How much time, money and effort have been invested in your cultures and experiments?
2. What are the consequences of their loss?

3. How expensive or difficult will it be to replace them?

Once the nature and consequences of the problems in the laboratory are better understood, the need for a management system, if necessary, can be determined. Basic problem solving tools (2) can be used to help identify the source of problems; changes to minimize or prevent the problems from reoccurring can then be implemented.

The following suggestions, concepts and strategies, combined with basic management techniques, can be used to reduce and control contamination (Table 6). These may require modification to fit your own needs and situation.

Use Good Aseptic Techniques

Aseptic technique is designed to provide a barrier between microorganisms in the environment, and your cultures and sterile supplies, yet permit you to work with them. There are many successful techniques for achieving and maintaining aseptic cell cultures; ultimately, your technique is "good" if it routinely protects both you and your cultures from contamination. Teaching aseptic technique is beyond the scope of this guide; the goal here is to review some of its basic tenets and present some suggestions for improving it. The reader is referred to Freshney (3) for a basic introduction to this very important area.

Table 6. Steps for Reducing Contamination Problems

- Use good aseptic techniques
- Reduce accidents
- Keep the laboratory clean
- Routinely monitor for contamination
- Use frozen cell repository strategically
- Use antibiotics sparingly if at all

The first step in developing sound, rational aseptic techniques is a solid understanding of both the nature and potential sources of biological contamination. This is reviewed in the beginning of this bulletin and covered in many of the references.

The second step, based on the nature of your work, is to determine the level of risk or danger to yourself and other laboratory personnel and then design your culture techniques accordingly. This is especially true when working with cultures that are virally contaminated or derived from human and other primate sources. Ensure that all laboratory personnel have been trained in the safe handling and disposal of any potentially hazardous cultures and materials; refer to your facility's safety office for any necessary assistance or guidance (9).

Next, based on the potential costs and consequences if the cultures are lost, determine how rigorous your technique must be, and what degree of redundancy if any, is required. Very valuable or irreplaceable cultures can be carried by two or more workers using media from different sources and separate incubators to reduce the chance of their simultaneous loss (27,28). Evaluate whether workers need to be gloved, gowned and masked to reduce the potential for contamination.

The nature of your working environment and any problems it may present must also be considered in choosing appropriate aseptic techniques. Certified laminar flow hoods and safety cabinets are recommended for use whenever possible. Some of the aseptic techniques taught in introductory microbiology classes for use on the open bench, such as flaming, while popular, are not appropriate or necessary in laminar flow hoods (16). Hood manufacturers recommend against the use of Bunsen burners and other sources of flames in hoods; they disrupt the moving curtain of filtered air and the resulting turbulence can increase the probability of contamination by microbial laden aerosols and particles generated during routine culture manipulation.

The following suggestions are recommended to reduce the probability of contamination:

- ▶ Make it more difficult for microorganisms to gain entry by using sealed culture vessels whenever possible, especially for long term cultures. The multiple well plates can be sealed with labeling tape or placed in sealable bags,

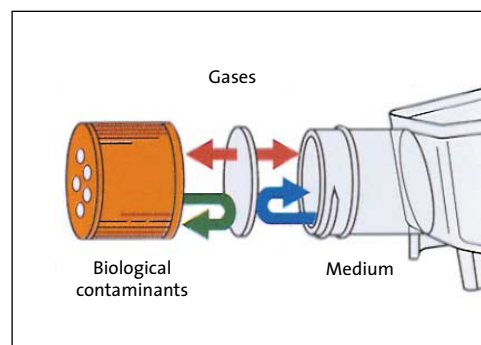


Figure 6. Vented cap flasks greatly reduce the opportunities for contamination in culture systems requiring gas exchange.

35 and 60 mm dishes can be placed inside 150 or 245 mm dishes. Use vented cap flasks (See Figure 6) whenever possible. These have hydrophobic filter membranes that allow sterile gas exchange but prevent the passage of microorganisms or liquids.

- ▶ Avoid pouring media from cell culture flasks or sterile bottles by using 50 or 100 mL pipettes to transfer larger volumes. Using a disposable aspirator tube and vacuum pump is an economical way to quickly and safely remove medium from cultures. A drop of medium remaining on the vessel's threads after pouring can form a liquid bridge when the cap is replaced providing a means of entry for bacteria, yeasts and molds. If pouring cannot be avoided, carefully remove any traces of media from the neck of the vessel with a sterile gauze or alcohol pad.
- ▶ Always carry unsealed cultures in trays or boxes to minimize contact with airborne contaminants. Square 245 mm dishes are excellent carriers for 384 and 96 well plates as well as for 35mm and 60 mm dishes.
- ▶ Do not use the hood as a storage area. Storing unnecessary boxes, bottles, cans etc. in the hood, besides adding to the bioburden, disrupts the airflow patterns.
- ▶ Never mouth pipette. Besides the risk of injury to laboratory personnel, mouth pipetting has been implicated as the likely source of human mycoplasma species (*M. orale* and *M. salivarium*) often found in cell cultures (15).

- ▶ Use clean lab coats or other protective clothing to protect against shedding contaminants from skin or clothes. Their use should be restricted to the cell culture area to avoid exposure to dirt and dust from other areas.
- ▶ Work with only one cell line at a time in the hood, and always use separate bottles of media, solutions, etc. for each cell line to avoid possible cross-contamination. Use disinfectant to wipe down the hood's work surfaces between cell lines.
- ▶ Use antibiotic-free media for all routine culture work; this is a very important concept and will be discussed in detail below.
- ▶ Whenever possible, package sterile solutions, such as trypsin, L-glutamine and antibiotics, in small volumes (i.e., stored in 15 mL tubes) to reduce the number of times each tube must be entered and thus reduce the probability of contamination.
- ▶ Leave laminar flow hoods running 24 hours a day. Only turn them off when they will not be used for extended periods.

Reduce Opportunities for Accidents

Accidents usually involve people, and reducing them must take into consideration both human nature and stress. Based on personal experience, accidents are far more likely on: a) Friday afternoons, b) the day before a vacation begins, c) with new employees, or d) when people are stressed, overworked or rushed. The following suggestions can help reduce the confusion and misunderstanding that cause many accidents to happen in the laboratory.

- ▶ Be very careful when labeling solutions, cultures, etc. Always clearly indicate if solutions or other supplies have been sterilized. Reduce misunderstandings in crowded or busy labs by using a color coding system: assign each worker their own color for labeling tape and marking pen inks.
- ▶ Be very careful with the use and choice of acronyms. Everyone in the laboratory should understand and agree to their meaning.

- ▶ Whenever possible use standardized recordkeeping forms; this simplifies their use and makes it more likely that good records will be kept.
- ▶ Use written protocols and formulation sheets when preparing media and solutions, listing the reagents used, lot numbers, weights, volumes, pH and any special treatments that were done. These will both reduce the potential for errors as well as provide a valuable aid in tracking down the cause of problems.

Clean Up the Work Area and Surrounding Environment

Reducing the amount of airborne particulates and aerosols in the laboratory, especially around the incubator and the laminar flow hood, will reduce the amount of contamination. Routinely wipe floors and work surfaces to keep down dust. Incubators, especially those that maintain high humidity levels, require periodic cleaning and disinfecting. Often overlooked but important sources of contaminants are the water baths used to thaw sera and warm media. Dirty water baths not only coat bottles with a layer of heavily contaminated water right before they are placed under the hood, but the water dripping from bottles generates heavily contaminated aerosols which can end up on lab coats and hands. Water baths should be emptied and cleaned on a regular basis, well before odor or visible turbidity develops. Pipette disposal trays and buckets, and other waste containers provide a source of potentially heavily contaminated materials in close proximity to the laminar flow hood and are a potential mycoplasma source (26). Waste containers should be emptied daily and the wastes disposed of safely. Autoclaving of any wastes that have been in contact with cells is recommended.

The cooling coils on refrigerators and freezers are a major source of microbial laden airborne particulates that are often overlooked in otherwise very clean laboratories. These should be vacuumed at least yearly; besides removing a significant source of contamination, regular vacuuming will extend the life of the cooling units and allow them to run more efficiently.

Some laboratories may also need to consider a pest management program to reduce the presence of mice, ants, cockroaches and other multilegged creatures that can be sources of contamination. Potted plants, although attractive, can provide a home for these creatures and should not be kept in the culture vicinity. Care must be taken when using pesticides as part of a pest management program to prevent accidentally chemically contaminating the cultures in the laboratory.

Sterility Testing

The best strategy for reducing contamination is to be proactive by routinely monitoring supplies, media and solutions, work areas and, most importantly, cell cultures for contaminants before they are used in critical applications and experiments. The key to developing a realistic contamination monitoring program is to keep it as simple as possible so that people use it, yet ensure that it can get the job done. Unfortunately there are no easy solutions: no single microbiological medium can detect all types of biological contaminants, and practical testing methods often miss low levels of contaminants. The process of detection is made even more difficult by the presence of antibiotics. The techniques and concepts presented below offer some practical approaches for monitoring contamination that can be readily adapted to meet the needs of most cell culture laboratories.

All autoclaves and dry heat ovens used to sterilize glassware, solutions and other supplies must be regularly maintained, and personnel properly trained in their loading and operation. Thermometers and chart recorders should be tested and calibrated periodically to ensure their accuracy. Inexpensive (when compared to the cost of a single autoclave failure) autoclave thermometers, spore test strips and capsules, or other testing devices can be placed inside autoclaves or into bottles of solutions or other packaged supplies during every run, or as necessary, to ensure proper loading and operation.

Samples of all in-house filter-sterilized solutions should be tested for sterility each time they are prepared and the solu-

tions not used until testing is complete. Standard microbiological testing methods for bacteria, yeasts and fungi usually require placing samples for testing into several different broths (trypticase soy, thioglycolate and Sabouraud broths, for example) and semisolid media (brain-heart infusion, blood agar), and then incubating them at both 30° and 37°C for at least two weeks (29).

Cell culture media, especially unopened bottles of media that are outdated or no longer used in the lab (as long as they do not contain any antibiotics) can provide a very rich, readily available and useful substitute for standard microbiological media. A small amount of serum (3 to 5% — again outdated or unwanted sera can be used) should be added to promote growth. The medium can be dispensed in 10 mL amounts into sterile 16 mm by 125 mm glass or plastic screw cap culture tubes or clear 15 mL plastic centrifuge tubes and be stored at 4°C until needed. The sterility of either filtered solutions or cultures and products suspected of being contaminated can be routinely and easily checked by placing a small sample into each of two tubes and incubating one at 30° and the other at 37°C for at least two weeks.

This sterility test media substitute is also very useful for evaluating the amount or source of particulate contamination in an area, near a piece of equipment or by a technique. Hoods, and especially incubators, are frequently blamed by laboratory personnel as the source of their contamination problems as in: “my cultures keep getting contaminated because something is wrong with the hood” (or incubator). Until these areas are screened and eliminated as the source of the problem, the real problem, often simply aseptic technique, can not be dealt with effectively. These suspected problem areas can be screened by dispensing the test medium into 96 well culture plates or 100 mm culture dishes (use agar-gelled media for the dishes). The vessels are then opened (with unopened vessels as controls) for 30 to 60 minutes at several locations within the test site prior to being sealed and incubated. Cultures can be initially

checked for contamination after two to three days although slow growing contaminants may take two weeks or longer to appear. The rate of contamination (number of colonies or contaminated wells/vessel or unit area/unit time) can then be calculated and analyzed. Besides giving an accurate level of the bioburden in that area, microscopic observation of the contaminants in the liquid test media also allows their morphological comparison with the microorganisms found causing problems in the cell cultures. Past experience with this approach has shown it is a very useful tool when teaching aseptic technique as it clearly demonstrates that the air in a room, or even inside a humidified incubator is usually not a major source of contamination in a well maintained laboratory. It is also a useful tool in tracking down mysterious contamination outbreaks.

Detecting Mycoplasma in Cultures

No monitoring program is complete unless it can effectively detect contaminated cultures, especially those infected by mycoplasma. Unfortunately mycoplasma detection is not simple, and because of this, and a lack of awareness, many cell culture users simply don't bother to test. (As many as 50%, see survey results presented in Table 7.) As a result, it is estimated that at least 15% of all cell cultures in the United States are contaminated with mycoplasma. Because of these outrageously high levels of contamination and the proven ease with which mycoplasmas can be spread from contaminated cultures (26), it is very important to quarantine all cultures coming into the laboratory until they have been tested for mycoplasma. This is especially true of gifts of cell lines from other labs; often these "gifts" end up infecting your cultures.

There are two basic testing methods for mycoplasma: direct culture in media, or indirect tests that measure specific characteristics of mycoplasma (16). Direct culture is the most effective and sensitive method for detecting mycoplasma, but it is also the most difficult and time consuming. It requires several carefully tested liquid and semisolid media and controlled environmental conditions (See reference 30

for detailed protocols), and must be run with live mycoplasma controls. Additionally, although direct culture is the most sensitive method, it is the slowest (requiring up to 28 days) and it may not reliably detect some fastidious strains of mycoplasma, making it less than 100% effective. Budget permitting, direct culture testing is best contracted to an outside testing facility for two reasons: first, given the ease with which mycoplasma can spread in the laboratory, bringing live mycoplasma into a cell culture facility for the required controls is not recommended; second to do it well, direct testing requires a serious effort and commitment of resources better spent in doing cell culture. These tests are commercially available at a reasonable cost from several cell culture testing companies. (Visit www.atcc.org or www.bionique.com for additional information on mycoplasma testing services.)

There are a wide variety of indirect test methods available for mycoplasma detection, including PCR-based kits, DNA fluorochrome staining, autoradiography, ELISA, immunofluorescence and specific biochemical assays. These tests are faster than direct culture, all are commercially available in kit form, and they can detect the fastidious, difficult to cultivate strains that are occasionally missed by direct culture. However they lack the sensitivity of direct culture, requiring much higher levels of contamination for detection. As a result, they have more frequent false negatives than direct culture methods, potentially leaving researchers who rely solely on a single indirect test with a false sense of security. (Reviewed in references 11, 12, and 18.)

The most widely used and recommended indirect test is DNA fluorochrome staining. (See reference 31 or the Corning web site for detailed protocols.) This easy and relatively fast procedure stains DNA using a fluorescent dye. When stained and fixed cells are examined under a UV microscope equipped with the proper filter package, DNA fluoresces brightly. (See Figure 7a and 7b.) Not only will this test detect mycoplasma but as an added benefit it will also detect any other

microbial contaminants. This staining method can be combined with an indicator cell line to increase its sensitivity. Interpreting results is not always easy, especially with hybridoma cultures; suitable positive and negative control slides should always be used to help interpret staining results. These positive and negative mycoplasma control slides are commercially available; since they have already been fixed, they are safe to use in the laboratory.

The best overall testing approach is a combination of both methods: direct culture can provide very high sensitivity while DNA fluorochrome staining can detect any fastidious mycoplasma that the direct culture misses. Both the FDA and USDA requires this approach for cell culture derived products, such as monoclonal antibodies, vaccines and drugs, and the cells required to produce them. If resources do not permit the combined approach, then the DNA fluorochrome staining procedure using an indicator cell line, combined with one other indirect test method should provide a minimum level of security.

Detecting Other Biological Contaminants in Cultures

The traditional microbiological media described earlier for testing the sterility of solutions can be adapted for testing cultures for bacteria, yeasts and fungi (29). However, the direct culture tests and the indirect DNA fluorochrome test for mycoplasma, although not designed for this purpose, will also detect most bacteria, yeasts and fungi, including intracellular forms, reducing the need for the traditional tests. Special culture procedures are also available for detecting suspected protozoan contaminants in culture. (Details can be found in reference 32.)

There are several other important quality control tests that should be used to both identify and characterize the cell cultures used in your research. Besides the serious and widespread problem of cross-contamination by other cell lines described earlier, cells are also continually evolving in culture: important characteristics can be lost, mutations can occur, or chromo-

some can undergo rearrangements or changes in number. Monitoring these changes is important because altered cell cultures can have a significant impact on the reproducibility of your research. (Reviewed in reference 33.) The following characterization methods are recommended for monitoring cell cultures; refer to the cited references for details. Most laboratories should incorporate at least one of these methods as part of their monitoring program:

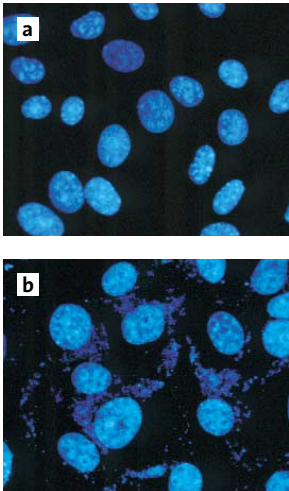
- Karyotyping, a relatively simple method used to determine the modal chromosome number and presence of any unique marker chromosomes (34).
- Electrophoresis and isoenzyme analysis to generate a protein ‘fingerprint’ that can be used to determine species or for future comparisons (33).
- Immunological or biochemical techniques to detect markers that are unique to the tissue, cell line or the species from which it is derived (33).
- DNA fingerprinting, a relatively new technique but one that is becoming increasingly useful, can be used to detect both intra- and interspecies contamination (35).

The results from these characterization tests can serve as an important baseline against which any future changes can be compared.

Recommendations for a Testing Program

The cell culture testing program you choose should be the best you can afford, as it is the cornerstone of your research. An inadequate program (or worse, no program at all) provides a false sense of security and can eventually lead to failure compromising the validity of your research. The following steps are recommended for setting up a sound, yet practical culture monitoring program:

Test all current in-house cell lines using the methods described above to ensure they are free from mycoplasma and other microbial contaminants, and to check their identity. Then incorporate these tested cultures into your cell repository and rely only on them for all future experiments.



Figures 7a and 7b. Photomicrographs (1000x) of VERO cells stained with Hoechst 33258 dye. DNA-containing nuclei and mycoplasma stain brightly under ultraviolet light allowing the clean culture (7a) to be easily distinguished from the infected culture (7b).

(Photomicrographs courtesy of Bionique Testing Laboratories, Inc.)

Quarantine and then test all incoming cell lines and any cultures currently stored in your cell repository that were not tested when they were frozen.

Test all cell lines that are in continuous use at least every three to four months and any time they behave suspiciously. Better yet, save time, money and effort by periodically discarding these cultures and replacing them with cultures from your tested cell repository. (This strategy will be discussed in detail later in the section on using a cell repository.)

New lots of sera should be evaluated for any critical applications before widespread use. The simplest test method is to use the new serum in an indicator cell culture for several weeks and then test the culture for mycoplasma contamination using DNA staining or other suitable test.

Detecting Chemical Contaminants

Determining that a chemical contaminant is the cause of a cell culture problem is usually much more difficult than with biological contaminants because it is so hard to detect. Often the first signs that something is wrong are widespread alterations in the growth, behavior or morphology of the cultures in the laboratory; however, it may take weeks before these changes are noticed. Once noticed, the cause is frequently misconstrued to be of biological origin; only after extensive and unsuccessful testing for the usual microbial suspects does attention focus on the possibility it might be a chemical contaminant.

Begin the problem solving process by identifying all changes that have occurred in the lab in the weeks prior to the problem being noticed, especially in equipment, solutions, media and supplies, that may be related to the problem. Good record keeping is essential for this process to be successful. Bring together laboratory personnel to brainstorm for all of the possible causes and then select the best possibilities for evaluation. Simple comparison experiments can then be done to eliminate each possibility as the source of the problem; media, solutions, sera and other products to use as controls in the

testing can be obtained from other labs or sources. The best way to avoid chemical contamination is to test all new lots of reagents, media and especially sera, and test the water purity at least yearly using the most sensitive culture assay available.

Strategic Use of a Frozen Cell Repository

A cryogenic cell repository is commonly used in laboratories to reduce the need to carry large numbers of cultures and to provide replacements for cultures lost to contamination or accidents. Freezing cultures also stops biological time for them, preventing them from acquiring the altered characteristics that can normally occur in actively growing cells as a result of environmental or age related changes. However, a cell repository is only a reliable resource if the cultures it contains have been properly tested, labeled and stored. (Reviewed in reference 36.)

Equally important, a cell repository can also be used strategically to convert continuously carried cultures into a series of short-term cultures, thereby greatly reducing both the amount of quality control testing required and potential problems from cryptic contaminants. When cultures are continuously carried for long periods in the laboratory they should be tested for contaminants at least every three to four months (more often for critical applications). If they are not tested regularly, then when a cryptic contaminant, such as a mycoplasma or another cell line, is finally uncovered, it is impossible to determine how long it has been in the culture and how much research has been invalidated by its presence. In addition, if the contaminant is mycoplasma, it is likely to have spread by then to other cultures. However, regular testing, although very important to ensure the integrity of your cultures, can require considerable effort, especially in laboratories using multiple cell lines. Rather than test cultures several times a year, it is easier to simply discard them every three months replacing them from the repository with cultures from the same lot or batch that have been previously tested to ensure their integrity.

Tested stocks should be set up in the cell repository for each culture that is routinely used in your laboratory. The cultures should be grown for at least two weeks in antibiotic-free media, then thoroughly tested to check their viability, ensure they are free of contamination, and confirm their identity and presence of any important characteristics. Testing should be done both immediately before and after freezing; however, if you don't mind assuming some added risk, testing can be left until after freezing. The freezer stock should always be prepared from pooled cultures and contain enough vials, assuming a consumption rate of five vials per year (or higher based on your experience), to last the planned lifetime of any research projects involving them. A better alternative may be to first develop a seed or master stock (10 to 20 vials is usually sufficient, depending on your envisioned needs), and then from that develop a working stock (approximately 20 vials). When the original working stock is depleted, it is replaced by using a vial from the seed stock to develop a new working stock. Assuming a consumption rate of five vials per year, each working stock will be good for four years, with the seed stock lasting for 40 to 80 years. Hopefully, this will be long enough to finish a research project! This approach reduces the amount of routine testing to practical levels since only newly introduced cultures will require testing. Equally important, discarding cultures after growing them for three months also destroys any undiscovered biological contaminants that may have gained access to the cultures, limiting both their damage to the integrity of the research and their spread to other cultures.

Strategic Use of Antibiotics

When used intelligently, antibiotics are a useful tool in cell culture, but they can be very dangerous when overused or used incorrectly. Experienced cell culture users have recommended for many years that antibiotics never be used routinely in culture media (3,7,12,17,18,26,27). In a major study, Barile found that 72% of cultures grown continuously in antibiotics were contaminated by mycoplasma, but

only 7% grown without antibiotics were contaminated, a ten-fold difference (37). Similar results are common: workers who routinely and continuously use antibiotics in their media tend to have higher contamination problems, including mycoplasma, than workers who don't. Over reliance on antibiotics leads to poor aseptic technique. It also leads to increased antibiotic resistance among common culture contaminants. In an ongoing study (41) of the antibiotic sensitivity of culture-derived mycoplasmas, 80% were resistant to gentamycin, 98% to erythromycin, and 73% to kanamycin, all commonly used antibiotics widely claimed to be effective against mycoplasmas. Mycoplasmas also showed resistance to the antibiotics recommended and sold specifically for cleaning up mycoplasma infected cultures: 15% were resistant to ciprofloxacin, 28% to lincomycin, and 21% to tylosin.

Why does the routine use of antibiotics lead to higher rates of mycoplasma contamination? Everyone generates and sheds a relatively constant flow of particles, consisting of fibers, aerosols and droplets, as they work in the laboratory. These particles can have a mixture of bacteria, yeast, fungi, and even mycoplasmas bound to them. If one of these contamination-laden particles enters an antibiotic-free culture, the chances are that at least one of the contaminants will produce a highly visible infection within 24 to 48 hours. As a result the contaminant is quickly detected and the culture discarded. It is very unlikely that particles shed by laboratory personnel would ever consist of just difficult to detect contaminants, such as mycoplasmas, that could enter cultures and not cause visible signs of contamination. However, if the culture contains antibiotics, there is a chance that the antibiotics will prevent the growth of the usually more easily detected contaminants but allow mycoplasma or other cryptic contaminants to grow undetected. As a result, instead of being discarded, the cryptically infected culture remains in use, is utilized in experiments, and becomes a potential source of serious contamination for the other cultures in the laboratory.

Antibiotics should never be used as a substitute for good aseptic technique, however they can be used strategically to reduce the loss of critical experiments and cultures. The key is to use them only for short term applications: for the first week or two of primary cultures, during the initial production stages of hybridomas, for experiments in general where the cultures will be terminated in the end. Whatever their use, the antibiotics ultimately chosen should be proven effective, noncytotoxic and stable (37).

Curing Contaminated Cultures

Autoclaving is the preferred method for dealing with contaminated cultures — it always works and is guaranteed to keep the infection from spreading to other cultures. However, occasionally contamination will be found in a valuable culture that cannot be replaced and attempts will be made to save it. This is a task that should not be undertaken lightly as it usually entails considerable effort and frequently turns out to be unsuccessful. In addition, cultures can lose important characteristics as a result of the clean up procedure. If the contaminant is a fungus or yeast, success is unlikely since antifungal agents, such as amphotericin B (Fungizone) and Nystatin, will not kill these organisms, but only prevent their growth. Many bacterial culture contaminants come from human or animal sources and are likely to have developed resistance to most commonly used cell culture antibiotics.

Most clean up attempts, however, are usually made against mycoplasma infected cultures. Treating with antibiotics is the most widely used approach, but as discussed earlier, cell culture mycoplasma strains are often resistant to some of the antibiotics specifically recommended for cleaning up mycoplasma infected cultures. Furthermore, the more attempts made at cleaning up contaminated cultures with these antibiotics the more likely resistant mycoplasma strains will develop. Other approaches, usually combining the use of antibiotics with specific antisera or other chemical treatments, can be used as well. (Reviewed in references 11, 16, and 37.) However, none of these

methods are 100% successful and clean up should only be tried as a last resort. A word of caution: often these treatments reduce the level of contamination below that which can be detected by indirect methods such as DNA staining or PCR. As a result, clean up attempts often appear successful for the first month or more following treatment because the low level of surviving mycoplasmas can escape detection. But eventually the few remaining undetected mycoplasmas recover leading to more serious problems. Budget permitting, there is at least one commercially available mycoplasma clean up service for contaminated cultures, it is relatively expensive but usually successful (17).

Table 7. Contamination Survey Results^a

- A. Do you consider microbial contamination (bacteria, yeast, fungi, mycoplasmas) of your cultures to currently be a problem?
- 50%** Yes, minor
 - 8%** Yes, serious
 - 33%** No
 - 9%** Not sure
- B. How often is it a problem?
- 67%** 1-5 times/year
 - 20%** 6-10 times/year
 - 12%** More than 10 times/year
- C. Have you ever encountered mycoplasma contamination in any of your cultures?
- 9%** Yes, once
 - 14%** Yes, several times
 - 33%** Never
 - 44%** Maybe, not sure
- D. Do you currently test your cultures for mycoplasma?
- 50%** No
 - 32%** Yes, occasionally
 - 18%** Yes, an average of 4 times/year
- E. Do you use antibiotics in your culture medium?
- 65%** Yes, usually
 - 7%** Yes, short term only
 - 17%** Occasionally
 - 11%** Never

^aCombined summary of three surveys (130 respondents) conducted at Corning seminars in Baltimore, Boston and St. Louis in 1990.

A Final Warning

In the United States alone, losses due to cell culture contamination, especially by mycoplasma, cost cell culture users millions of dollars annually; this is money that could otherwise be used for additional research. Unfortunately this serious problem does not appear to be getting any better. As shown by the survey results in Table 7 (page 19), contamination is a problem for most cell culture workers. At least 23% of respondents have experienced mycoplasma contamination of their cultures, but an additional 44% suspected mycoplasma contamination but were not sure. The reason for their uncertainty is clarified by the response to the next question: 50% of all respondents do not test for mycoplasma, as a result they are unaware of the status of their cultures. The answer to the last question points out one important reason for widespread contamination problems – the over use of antibiotics. With 65% of respondents using antibiotics on a regular basis, the continued frequent occurrence of cryptic contaminants, especially mycoplasmas, is likely.

Because of the very serious nature of mycoplasma contamination and its widespread distribution, it is important to summarize the major sources of mycoplasma contamination and review the basic steps for preventing it from happening in your laboratory. Currently, the

number one source of mycoplasma contamination is other infected cell lines; it is essential to quarantine all cultures brought into the laboratory until they have been screened for mycoplasma contamination, and to use only tested cultures in research. The second common source is the cell culturist; good aseptic technique combined with the strategic use of a tested cell repository and limited use of antibiotics will greatly reduce the opportunities for contamination via this route. The last important source of mycoplasma is sera and other biologicals that are sterilized by filtration; buy only from sources that have a good reputation and that use currently acceptable filtration (0.1 µm or smaller) and testing procedures.

Cell culture contamination will never be totally eliminated, but through a better understanding of the nature of contamination and the implementation of some basic concepts it can be better controlled and its damage greatly reduced. The information in this bulletin has been compiled to provide you with the foundation (Figure 8) upon which you can build a contamination management program designed to fit your own needs. For additional assistance in these areas, please visit www.corning.com/lifesciences, or call Corning Incorporated Technical Information Center at **1.800.492.1110**. International customers please call **978.635.2200**.



Figure 8. Key building blocks for successfully managing cell culture contamination

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Cell Culture Protocols and Technical Articles

For additional cell culture protocols and technical articles, please visit the Corning Technical web site at www.corning.com/lifesciences. The following are some of the technical articles that can be found there:

Introduction to Animal Cell Culture

This 10-page Corning Technical Guide is designed to serve as a basic introduction to animal cell culture. It is appropriate for both laboratory workers who are using this tool for the first time or for those who interact with cell culture researchers and who want a better understanding of the key concepts and terminology in this interesting and rapidly growing field. Topics covered include:

- ▶ What is animal cell and tissue culture?
- ▶ How are cell cultures obtained?
- ▶ What are cultured cells like?
- ▶ What are the problems faced by cultured cells?
- ▶ How to decide if cultured cells are “happy”?
- ▶ What is cell culture used for?

Identifying and Correcting Common Cell Growth and Attachment Problems

This Corning Technical Guide reviews some of the common and not so common cell growth and attachment problems that are often very difficult to identify and eliminate. Topics covered include:

- ▶ Identifying the causes of unusual growth patterns due to technique or incubator problems.
- ▶ Problems with culture media, including HEPES and fluorescent light-induced problems.
- ▶ Applying cell culture problem solving strategies.

General Guide for Cryogenically Storing Animal Cell Cultures

This 8-page Corning Technical Guide examines both the theoretical and practical aspects of cryogenic preservation and reviews key strategies for managing a cell repository. Special attention will be given to:

- ▶ Understanding and controlling the freezing process
- ▶ Selecting cryogenic storage systems
- ▶ Record keeping
- ▶ Quality control procedures to minimize culture loss

Endotoxins and Cell Culture

This 8-page Corning Technical Guide discusses endotoxins and their effects on cell cultures. Special attention will be given to:

- ▶ What are endotoxins?
- ▶ Sources of endotoxins in cell culture
- ▶ Endotoxin effect on in vitro cell growth and function

Helpful Hints to Manage Edge Effects of Cultured Cells for High Throughput Screening

This technical note is a compendium of techniques, collected from Corning Cell Culture facilities and customers, to reduce the occurrence of irregular patterns of cell adhesion or “edge effect” in microplates.

Analysis of CHO Cell Requirements for Assay Miniaturization in High Throughput Screening

This technical note analyzes the culture conditions for optimal growth of Chinese hamster ovary (CHO) cells for assay miniaturization in high-throughput screening.

Mycoplasma Detection Using DNA Staining

This 3-page protocol provides a detailed procedure for using Hoechst stain #33258 to stain the DNA in cell monolayers for detecting mycoplasmas or other prokaryotic organisms.

Roller Bottles Selection and Use Guide

This 9-page guide describes the characteristics of all the glass and plastic Corning roller bottles and offers tips on solving cell growth and attachment problems that can occur in roller bottles.

Transwell® Permeable Supports Selection and Use Guide

Selecting the right Transwell inserts for your research application does not have to be difficult. This 11-page guide describes all of the importance physical and performance characteristics of the Corning Transwell permeable support products and offers tips on using them.

Transwell and Snapwell™ Bibliography

This bibliography lists several hundred references, organized by research application, citing the use of Corning Transwell permeable supports in cell culture research.

Corning Incorporated
Life Sciences

45 Nagog Park
Acton, MA 01720
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