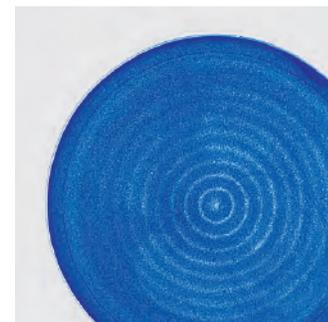


Corning Guide for Identifying and Correcting Common Cell Growth Problems

Technical Bulletin



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Introduction

Although very popular among life science researchers, cell culture can be a very difficult tool to work with in the laboratory. Unlike other common laboratory tools such as electrophoresis or chromatography, cell culture makes dynamic, ongoing use of living organisms. These living cultures often respond to our mistakes not only by the erratic behavior common to other laboratory tools, but by dying - the total, irreplaceable destruction of the tool itself.

As one of the major suppliers of vessels used in cell culture, Corning Life Sciences often receives calls from anxious customers who are experiencing growth or attachment problems with their cell lines. Usually the customer, searching for a cause (and solution) to their problems, suspects that a change or mistake in the manufacturing process is responsible, or that perhaps the special surface treatment process used on most cell culture vessels was not properly done. Culture media and sera producers also receive similar calls from customers trying to determine who is responsible for the erratic behavior or loss of their cultures.

Because of the complex nature of cell culture, identifying the underlying causes of culture behavior problems is often a difficult, time-consuming task. Erratic culture behavior can take many forms; unusual growth patterns or inconsistent, spotty, and uneven cell attachment are the most com-

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The Corning Life Sciences Technical Information Center (Call 800.492.1110 toll free in the USA; 978.442.2200 outside the USA) is available 8:30 a.m. - 5:00 p.m. EST Monday through Friday to work with customers on their culturing problems. Or, use the Corning Life Sciences web site at www.corning.com/lifesciences.

mon problems. Gradual or abrupt changes in growth rate or unexplainable experimental results are also experienced occasionally. In cell culture, any sudden change is suspect and a potential problem and, therefore, to be avoided.

Corning Life Sciences has spent many years helping customers deal with these problems. By using some of the information gained from these customer contacts, Corning has produced this guide to help identify and solve some of the common and uncommon causes of cell culture problems. The focus will be limited to three common problem areas: technique, incubators and media. In addition to the information provided in this guide, it is strongly suggested that you refer to the articles listed in the references or on the Corning Life Sciences web site (www.corning.com/lifesciences) for additional help and recommendations.

Surface Treatment Process

The initial and most common suspects for cell culture problems are usually either the culture vessels or the medium being used. Problems associated with culture medium will be dealt with in a later section. Much of the suspicion surrounding plastic cell culture vessels is due to the mystery or lack of understanding concerning the special treatment process used to modify the surface of the plastic. Virgin polystyrene, the resin used to manufacture most cell culture vessels, is hydrophobic in its untreated state. Protein attachment factors do not bind well to this natural surface resulting in poor cell adhesion and growth. Because of this, either a corona discharge or plasma treatment is used under carefully controlled conditions during the manufacturing process to insert oxygen atoms (in the form of carboxyl groups) into the backbone of the polystyrene chain (Ramsey et al, 1984; Amstein and Hartman, 1975; Hudis, 1974). This alteration of the plastic polymer (not a peelable coating) results in a hydrophilic surface with a net negative charge that creates a surface suitable for cell attachment and growth. The culture vessels are then sterilized and thoroughly evaluated by quality control tests to insure they received the proper degree of treatment.

Since this modified surface is not visibly different from the untreated surface, there is no easy way, short of growing cells, for customers to check the adequacy of the treatment process. As a result, many customers assume cell attachment and growth problems are caused by mistakes made during manufacturing. It is very important that the plastic culture vessel's status as the suspected cause of the problem be resolved as quickly as possible so the real cause can be identified and eliminated. Usually, the first step is comparing the performance of the suspected vessel against the same product from a different production lot, or against similar products from another manufacturer. If a difference is found or the results are not clear, then it is time to contact the product manufacturer for assistance. Once the vessel is eliminated as the problem's cause, the customer can then continue to look elsewhere for a solution. The following examples will help identify some common problems and causes often incorrectly associated with poor surface treatment of plastic vessels and, where possible, will offer some solutions that can be used to eliminate them.

Problems Related to Technique

The greatest opportunity for cell culture problems occurs during the day-to-day activities of feeding and maintaining the cultures. Culture loss due to contamination is one of the most frequent and more serious of these problems. However, many other problems, while less serious and not as noticeable as contamination, still adversely affect the cultures and experiments. Often, the first sign that something is wrong occurs when the cells are microscopically examined and uneven or unusual patterns of cell attachment or growth are observed. Identifying the problem's cause is the first and usually most difficult step, followed by finding the right solution. Sometimes transient growth problems occur and then disappear without ever identifying a cause. Please note that many of these growth problems are not readily observed during routine microscopic observation of live cultures. The occurrence and extent of these problems are best observed when sample cultures are first fixed (2.5% glutaraldehyde or 70% ethanol) and stained (1% crystal violet stain) prior to observation.



Figure 1. Examples of spotting in flasks caused by bubbles in the medium. The flask on the bottom right shows a normal bubble-free cell monolayer. These flasks were fixed and stained to show the effects of bubbles on cell attachment and subsequent growth.

For information on preventing culture loss from contamination, refer to the Corning Life Sciences Technical Bulletin *Understanding and Managing Cell Culture Contamination*. This is available at www.corning.com/lifesciences. (Click on Technical Information, then on Cell Culture.)

Growth Problems in Flasks, Dishes, and Plates

Spotting

Clear areas, often resembling single or small clusters of viral plaques, occur along the sides or in the middle of the vessels (Figure 1). This usually results when the initial cell inoculum contains foam or bubbles. Although they appear to float on top of the medium, bubbles also push below the surface, displacing the cell-containing medium and reducing the cells available for attaching. Should the bottom of the bubble contact the bottom of the vessel, it will prevent cells from attaching in that area. Bubbles only need to stay intact for a short period to have this effect. Bubbles that occur during refeeding of cultures, but after cells have attached, may cut off the cells from the fresh medium. This will result in cell necrosis under those areas, if the bubbles last long enough. Bubbles can usually be avoided by careful attention to mixing and pipetting techniques.

Uneven Growth

Inadequate mixing of cell inoculum with medium during addition to vessels, especially dishes, can result in uneven distribution, attachment and growth of cells (Figure 2). Experience or simple experimentation will generally result in effective ways of mixing medium thoroughly without creating bubbles or foam.

Uneven growth can also occur as a result of the shear forces generated by medium sweeping across cell monolayers during medium changes or while moving cultures between the laminar flow hood and the incubator (Tchao, 1996). This effect is often more pronounced in serum-free cultures.

Static Electricity

Static electrical charges that build up on plastic vessels can also adversely affect cell attachment. This problem occurs more frequently when the relative humidity is very low during the winter (or year round in some laboratory locations). Wiping the outside of vessels with a clean damp towel, increasing the room humidity, or using commercially available antistatic devices may eliminate or reduce this problem. Extra care should be used to avoid rubbing the vessels against the packaging when opening them (especially roller bottles) as this can increase the static charge.



Figure 2. The cell inoculum was not adequately mixed when added to this flask. Consequently, part of the flask surface was not covered by medium when it was placed in the incubator.

Meniscus Rings

When too small a volume of cell inoculum is used, a pattern of heavier growth will appear along the sides of flasks or as a ring or halo in dishes. (This halo effect is often more pronounced in multiple well plates.) This pattern occurs because the meniscus along the sides of the vessel is deeper and contains more cell inoculum and medium per unit surface area than does the thinner film of inoculum toward the center of the vessel. A similar effect is observed when too little medium is used in refeeding cultures. As a rule of thumb, 0.2 to 0.3 milliliters of medium should be used for every square centimeter of growth surface (Table 1).

Mycoplasma

Although the problem of cell culture contamination is beyond the scope of this guide, it is important to draw attention to another potential and widespread source of mysterious cell attachment and growth problems. For more detailed information on the problem of cell culture contamination, refer to Lincoln and Gabridge, 1998; Rottem and Barile, 1993; McGarrity, 1982; McGarrity, 1976. Due to the very high densities they can achieve in cell culture (up to 108/mL), mycoplasmas (unlike other contaminants such as bacteria and fungi) cause serious adverse effects on cell cultures without clouding the medium or being observed under the microscope. Mycoplasmas often grow attached to the cell membrane; as a result, a single cell may have several hundred mycoplasma on its membrane which greatly affects the ability to attach and grow. An ongoing mycoplasma screening program is an essential requirement for all cell culture labs working with cell lines (Lincoln and Gabridge, 1998; Masover and Buck, 1983; McGarrity et al, 1985; McGarrity, 1982). Without such a program, mycoplasma contamination, along with the associated problems, is likely to occur at some point.

Table 1. Recommended Medium Volumes for Corning® Dishes and Flasks*

Corning Plastic Culture Flasks (area)	Recommended Medium Volumes
25 cm ²	5 to 7.5 mL
75 cm ²	15 to 22.5 mL
150 cm ²	30 to 45 mL
162 cm ²	32 to 48 mL
175 cm ²	35 to 52.5 mL
225 cm ²	45 to 67.5 mL

Corning Plastic Culture Dishes (diameter)	Recommended Medium Volumes
35 mm	1.6 to 2.4 mL
60 mm	4.2 to 6.3 mL
100 mm	11 to 16.5 mL
150 mm	30 to 45 mL
245 mm (square)	100 to 150 mL

*Based on using 0.2 to 0.3 mL medium per cm² of growth area.

For additional information on detecting and preventing mycoplasma contamination, refer to the Corning Life Sciences protocol *Mycoplasma Detection Using DNA Staining or Technical Bulletin Understanding and Managing Cell Culture Contamination*. These are available at www.corning.com/lifesciences. (Click on Technical Information, then on Cell Culture.)

Growth Problems in Roller Bottles

The constant movement of the medium across the surface of the bottle, as slow as it appears, can make it more difficult for cells to attach and grow in roller bottles compared to stationary vessels such as flasks and dishes. The constant motion of the medium can also lead to a more stressful cell environment than is found in stationary culture systems. Consequently, any technique-related issues that reduce the attachment ability of cells is magnified and clearly stands out (Freshney, 1994). Please note that many of these growth problems are not readily observed during routine microscopic observation of live cultures. The occurrence and extent of these problems are best observed when sample cultures are fixed and stained prior to analysis.

Uneven Cell Attachment and Clumping

One of the most frequently encountered problems using roller bottles is difficulty getting the cells to attach and form an even monolayer in the bottle. Rotating bottles at inappropriate speeds is a common cause of attachment problems. If the bottles are rotated too quickly for cells to easily attach, areas of heavy cell growth often appear as circular bands towards both ends of the bottles (Figure 3). This is because the medium flow is slightly slower at the ends than in the middle of the bottles. Rotating bottles too fast may also result in large clumps of cells. This results from the tendency of cells to form clumps since they find it easier to adhere to each other than to the surface of the roller bottle. Eventually these clumps become large enough to attach to the bottle surface. A recommended starting speed for



Figure 3. The roller bottle on the left was rotated too fast resulting in uneven distribution of cells to the ends of the bottle. The bottle on the right was rotated at the correct speed.

initiating roller bottle cultures is 0.5 to 1.0 revolutions per minute (rpm) to start. However, if cells have difficulty attaching, slower speeds (0.1 to 0.4 rpm) should be used until the cells are attached.

Cell damage during subculturing, or incomplete inactivation or removal of dissociating enzymes can also make it more difficult for cells to attach and may result in banding or clumping. The protein-based cell receptors used to initiate cell attachment become damaged by the dissociating procedures and must be replaced before the cells can reattach. Poorly regulated incubator temperatures (temperatures that are too high or too low) will also make it more difficult for cells to evenly attach to roller bottles.

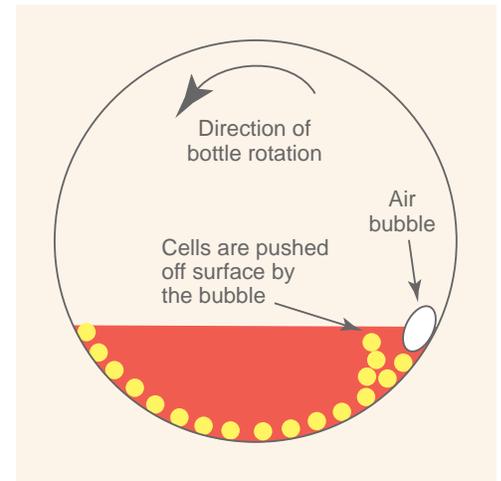


Figure 4. Most of the cells have attached to the bottom end of this bottle as a result of the roller apparatus not being level. This also can occur when bottles are stood on end for too long immediately after seeding it with cells.

If the bottles are initially rotated too slowly, or if they slip or stop turning even for a short time during the initial cell attachment period, uneven longitudinal bands of cell growth may appear. Cleaning the rollers on the roller apparatus should alleviate slipping bottles. If necessary, rubber bands can be placed around the ends of the bottles to improve traction.

Bands of heavy growth at just one end of the bottle are often the result of the roller apparatus not being level, causing an increased amount of medium and cells at the end of the bottle that is lower (Figure 4). Furthermore, the longer it takes the cells to attach, the more time there is for them to gradually roll down the side of the bottle to the lower end before attaching. Standing a bottle on end for too long after initially seeding it with cells can have a similar effect.



Figure 5. These clear bands were caused by debris in the medium scraping away cells as the bottle was rotated.

Clear Bands

Occasionally, clear circular bands will occur on roller bottles where the cells appear to have been swept away (Figure 5). While small pieces of rolling debris or large cell clumps may cause this to occur, one of the most common causes is the short-term presence of bubbles in the initial cell inoculum. These bubbles, when in contact with the sides of the slowly rotating bottle, can act as miniature plows, scraping off the cells as they begin to attach. Avoid bubble formation by carefully pouring medium down the sides of the bottles, or by pipetting it directly into the bottom of the bottles. Cell suspensions used for inoculating roller bottles should be carefully prepared to ensure they are bubble-free.

Streaking

Condensation (essentially pure water) falling onto exposed cells can cause some unusual patterns and events. This problem usually occurs in roller bottles that have been removed from an incubator and are standing upright at cooler room temperatures awaiting processing. Due to temperature differences, water vapor will condense on the inside of the cap. The resulting droplets may then coalesce and run down the sides of the bottle across the cells that are now only covered by a very thin film of medium. These cells will then undergo a strong osmotic shock. If they have formed a confluent monolayer, they may tear or pull apart from each other along the path the water takes, creating a visible dagger-like streak (Figure 6). Cells that have not reached confluency may round up and float off into the medium, leaving behind a long clear streak devoid of cells.



Figure 6. A clear streak free of cells resulting from cap condensation running down the cell sheet while the bottle was temporarily stored upright awaiting processing.

Peeling

Heavily confluent cell monolayers (especially fibroblasts) will occasionally start to peel away from the surface of the roller bottle. This also occurs in flasks, dishes and microplates. This results, not from surface treatment failure, but from the formation of a flexible sheet of tightly interconnected cells and cell-manufactured extracellular matrix. Over time, mechanical stresses can develop in the cell sheet from cellular movements and contractions that may



Figure 7. A cell monolayer damaged by scraping with a pipette (Left). Cells peeling away from the surface of the bottle (Right).

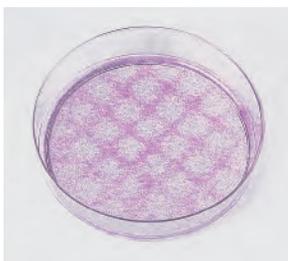


Figure 8. Growth pattern showing differential attachment and growth of WI-38 human fibroblasts. This pattern matches the pattern of holes on the shelf on which the dish was incubated.

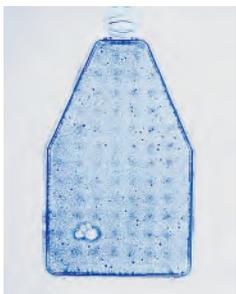


Figure 9. Growth pattern showing differential attachment and growth of cells in a flask. Here the growth was heavier over the holes. Note also the clear area caused by bubbles.

then cause the cell sheet to tear or pull away from the roller bottle (Figure 7). Physical damage from pipetting directly onto the cell sheet, tearing it with the end of the pipette, or other manipulations to the cell sheet may also initiate cell sheet peeling.

Problems Related to Incubators

Cells spend nearly all of their existence in incubators, yet these units may not always provide the stable, consistent environment cells require. Besides the obvious function of maintaining temperature, incubators used in open culture systems also control humidity, the gaseous environment around the cells, and indirectly, the pH of the culture medium. Ideally these parameters should be constant and not a source of experimental variation. Unfortunately, variation does occur and can be a major problem if not recognized and eliminated (Freshney, 1987, Chapters 2 and 3).

Temperature

Temperature differences within the incubator, even though small, can create problems even when the differences are only a few tenths of a degree. Constant opening and closing of poorly insulated incubator doors can result in significant temperature reductions, usually localized toward the front of the incubator. Often this effect is first noticed when heavy condensation forms on vessels located near the front of the incubator. As a result of slightly cooler temperatures, these vessels may have considerably slower growth rates than their neighbors to the rear as well as being more prone to fungal contaminants from the condensate. One solution is to set aside a separate incubator, with reduced traffic in and out, for all critical experiments, thus minimizing temperature fluctuations. Where this is not practical, those areas within the incubator that have the least temperature fluctuation (usually towards the rear) should be utilized for critical work.

Usually temperature effects are difficult to visualize and are less likely to be recognized. Figure 8 shows a distinctive growth pattern for WI-38 cells that was caused by minor temperature fluctuations in a dish. The dish was lightly inoculated with cells, placed in an incubator on a perforated metal shelf for 48 hours and then fixed and stained. The relatively clear areas on the plate perfectly match the perforations on the metal shelf showing the cells preference for the warmer areas directly over the metal portion of the shelf. These conditions usually occur when incubators are frequently opened, especially during the first few hours after freshly inoculated cultures are placed inside.

A similar pattern in a flask is shown in Figure 9. Here the cell growth and attachment is better over the holes in the shelving. This indicates the temperature in the incubator is a bit too high for the cells. These patterns indicate that cells are sensitive to very small temperature changes (less than half a degree).

Stacking vessels together can also result in vessel-to-vessel differences in temperature and growth rate. The vessel on the bottom of the stack, which is in contact with the metal shelf, warms up fastest when initially placed in the incubator. The vessel on the top is likely to cool faster, while a vessel in the middle is more insulated from any temperature fluctuations. It is very important to consider these positional effects when designing experiments where growth rates will be evaluated. Using spacers or empty “dummy” vessels to avoid direct contact with perforated shelves, and not stacking vessels, although more difficult, may help avoid these problems.

Evaporation

Evaporation induced changes in the osmolality of the culture medium can affect both the cell growth rate and, occasionally, the patterns of growth. Evaporation effects are easily observed in 96 well plates where the outer peripheral wells (especially the four corner wells) often show a marked reduction in media volume over time. While tighter fitting lids can reduce this effect, it cannot be eliminated. Visible evaporation effects in other types of culture vessels, although present, are much harder to detect.

Evaporation losses depend on the type of culture vessel used (flasks, dishes, multiple well plates, etc.), their location in the incubator, and frequency of entry into the incubator. The humidification system, positioning of circulating fans, amount of insulation, and general airflow patterns will all help determine local evaporation levels. Evaporation can be minimized by keeping water reservoirs full and humidifying incoming gases (usually carbon dioxide) using a gas washing cylinder (Corning Cat. No. 31770-500EC for example) filled with purified water in-line with any gases being fed into the incubator.

The following method can be used to create a ‘map’ for an incubator showing both the expected evaporation levels and those areas to be avoided for experiments requiring long term incubation.

Procedure for determining evaporation levels in different sections of an incubator

1. Determine the total number of dishes that will be needed. How many shelves and dishes per shelf will be tested? At least 3 shelves (top, middle, and bottom) with 9 to 16 dishes per shelf (in a 3 × 3 or 4 × 4 matrix) are recommended for each test.
2. Consecutively number the bottoms of all dishes to be used. Then accurately pipette the appropriate amount of medium (4 to 5 mL for a 60mm dish) into each dish, and record the weight (without the cover in place). Water can be substituted for medium without affecting accuracy, thus eliminating some expense and the problem of contamination. Cover the dish and place in the appropriate position in the incubator. Make sure that the position of each dish is recorded so that the map can later be accurately constructed.
3. Incubate for the desired time period. This is usually the length of your average experiment. It is preferable during this period to continue normal use of the incubator to better reflect actual expected evaporation levels. If not, normal use should be simulated by periodically opening the incubator.
4. Remove the dishes in small groups from the incubator and quickly weigh each without the covers in place. Any cover condensation represents water lost from the medium and should never be counted in the weighing process. The difference between the initial dish plus medium or water weight (in grams) and final weight, after evaporation losses, for each dish divided by the volume of water in milliliters initially added (× 100) will very closely approximate the total evaporation loss for the dish expressed as a percent.

$$\frac{\text{Initial weight (in grams)} - \text{Final weight}}{\text{Volume of water added (in mL)}} \times 100 = \% \text{ Evaporation Loss}$$

5. Construct the map showing evaporation levels on the different incubator shelves. Any “hot spots” will clearly stand out; their cause can then be determined and corrected or the area can be avoided in the future. Evaporation levels higher than 10 to 15% may have adverse effects on cultures. Wide differences in evaporation levels in different locations within the incubator will cause considerable variation in experimental results as well.



Figure 10. Vibration-induced concentric ring pattern in a 100mm dish containing chick embryo fibroblasts that was stained 1 hours after plating.

Vibration

Incubator vibration is responsible for some of the most bizarre growth patterns that occur in culture vessels. Its effect primarily occurs on cells when they are trying to initially attach to the surface following inoculation of the vessel. In dishes, vibration will push cells to the edges or middle of the vessel or will sometimes form concentric rings of cells. Figure 10 shows this type of pattern in a chick embryo fibroblast culture. The entire batch of 40 dishes placed in the incubator showed similar patterns. Although the vibration that caused this problem was always present in the laboratory, this pattern never occurred until the CO₂ tank supplying the incubator emptied at the same time the cultures were incubated. This loss of CO₂ raised the pH of the medium and made it more difficult for the cells to attach, allowing time for the vibration to have its effect.

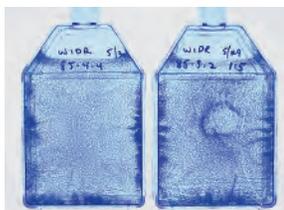


Figure 11. Flasks containing WiDr (human tumor) cells from the same incubator show varied vibration effects depending on their location in the incubator and the amount of damage the cells received during the subculturing process.

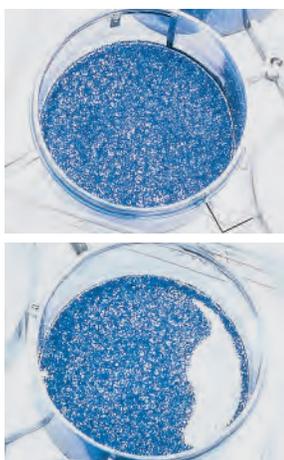


Figure 12. Wells from two 6 well plates. The well on the top shows an evenly stained cell monolayer from a plate on a level shelf; the well on the bottom shows the effect of a tilted shelf on cell growth.



Figure 13. Vibration has caused the cells in this flask to attach in parallel rows. Moreover, the heavier cell density on the left side of this flask indicates the incubator shelf was not level.

Figure 11 shows typical patterns that occur in flasks because of vibration. Patterns in flasks tend to be more varied due to the irregular shape of the vessel. Changes in the frequency and amplitude of the vibration or vessel location on the incubator shelf will also affect the cell patterns. Sharpness or intensity of the resulting patterns weaken over time, especially with more motile cell lines, and are most noticeable when vessels are stained after 24 to 48 hours of incubation.

Finding the source of the vibration is difficult. It may be as simple as a loose fan motor within the incubator but is far more likely to be caused by a more remote source. Heavy foot traffic, air handling units, compressors, laminar flow hoods, centrifuges, refrigerators and other motorized appliances should be kept as far from incubators as possible.

Incubators should be placed on heavy, sturdy tables or benches that are not shared with any vibrating equipment. Positioning them on a floor directly over major structural supports will reduce the effects of natural building vibration. Spaces along well-supported outside walls, if located away from heavy traffic areas both inside and out, will often have less vibration than the central spaces. If the incubators are not fully supported and carefully leveled, then vibration effects can be enhanced. Setting up experiments at the end of the day may help eliminate some of the vibration caused by people-associated activities.

Levelness

Unusual patterns also occur when the vessels in the incubator are not level. Improper stacking of vessels or using shelves that have not been leveled prior to use often causes this. Figure 12 shows stained wells from two 6 well plates, one of which was not on a level incubator shelf. The ‘crescent moon’ effect from the lack of medium caused by the tilted shelf is clear. Shelves should be checked with a spirit or bubble level and adjustments made following the incubator manufacturer’s recommendations. It is recommended that shelves be checked periodically to make sure they are level, especially after their removal for cleaning.

Sometimes an unusual growth pattern indicates that more than a single problem is causing the pattern. Figure 13 shows a stained flask that has parallel rows of cells caused by an incubator vibration problem. However, there is also heavier cell growth on the left side of the flask indicating either that the incubator shelf was not level or that the flask was improperly stacked on other flasks.

Gases

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.

Problems Related to Culture Media

As mentioned earlier, both culture medium and culture vessels are prime suspects whenever mysterious cell growth or attachment problems occur without obvious causes. Unless heavily contaminated, good culture medium is not visibly different in appearance from defective culture medium. The only good way to determine medium quality is to attempt to grow cells with it; this is the basic quality control procedure used by most media manufacturers and the only good method for homemade media as well.

Cell cultures respond to deficient or toxic media in different ways depending upon both the nature and the degree of the problem. These responses can range from minor changes in growth rate or cell attachment to the total destruction of the culture. Determining if the

medium is responsible for a problem is relatively easy; simply test the suspected batch against a sample proven effective. Determining why the medium is defective is extremely difficult due to the numerous reagents and complex steps involved. Therefore, time and energy are much better spent preventing media problems than trying to find and fix them later; management by prevention is the key to successful media production. The following sections will discuss some of the common and uncommon problem areas for making and using culture media (Jakoby and Pastan, 1979, Chapter 5).

Formulation Errors

The first step is deciding which medium is best for your applications. Usually recommendations from scientific literature or colleagues are good starting points. Unfortunately, there is much confusion in this area from the many formulations using the same or similar acronyms. The current catalog of one major media manufacturer offers 17 different formulations of 1X liquid media collectively called **MEM** for Eagle's **M**inimal **E**ssential **M**edium. This does not include six formulations for α -MEM. The same catalog lists 22 additional liquid media formulations for **D**ulbecco's **M**odified **E**agle **M**edium (DMEM) that have very different formulations from Eagle's MEM. It should be no surprise that confusion and mistakes are frequently made when selecting or ordering media.

Formulations printed in scientific journals also contain occasional errors or intentional changes that add to the confusion (Burke and Croxall, 1983). Special care and effort must be taken to ensure that the medium you make or buy is what was recommended and that you know its contents.

When making media from scratch, the formulations used should be carefully checked with at least two reputable sources. The most obvious media problems arise from mistakes made during media preparation. A master formulation sheet and preparation protocol, including any special instructions or precautions, should be prepared for all media and solutions, and then used by everyone in the lab. Preparation log sheets listing all reagents used, their lot numbers, weight or volumes (both desired and delivered), pH, date, preparers and storage conditions, are essential to insure consistency of product, reduce the potential for errors and track down mistakes if they occur.

Reagent Quality

The next major source of problems is the medium ingredients including the water, reagents and any special supplements such as sera and antibiotics. The highest quality water available should always be used. Special care must be taken to remove all trace metals, dissolved organics and endotoxins. Endotoxins can have a variety of effects on cultured cells and are often found in water purified by systems that use ion-exchange resins. Purified water should not be stored for long periods before use. Water quality should be periodically checked using sensitive growth assays at clonal densities against known standards (Freshney, 1994, Chapter 7).

It is wise to purchase chemicals of the highest purity available to avoid problems associated with trace contaminants. This is especially important for serum-free media where the effects of trace contaminants are not masked by sera. Once purchased, the optimum storage conditions for the chemicals must be carefully maintained to prevent any breakdown in quality. The same lot of chemicals should be used each time medium is made; when a lot is replaced, the new medium should be immediately compared against a previous batch to insure the replacement medium is satisfactory.

Buffers

After mixing, the medium is then buffered by the addition of sodium bicarbonate and adjusted to the proper pH. It is also helpful to check the osmolality at this point; mistakes in adding reagents can be uncovered as a result of finding unexpected deviations in osmolality measurements.

For information on the effect of endotoxins on cell culture, refer to the Corning Life Sciences Technical Bulletin *Endotoxins and Cell Culture*. This is available at www.corning.com/lifesciences. (Click on Technical Information, then on Cell Culture.)

Many growth problems result when customers do not supply the CO₂ levels required by the bicarbonate-based buffering system of the medium they are using. This results in poor pH control and will adversely affect cell attachment and growth. Usually the higher the level of sodium bicarbonate, the higher the level of CO₂ required for optimum buffering capacity. (Table 2.)

The most common system is a medium buffered with Earle's Balanced Salts containing 2.2 g/L of sodium bicarbonate. This system is designed for use in open culture vessels (dishes, microplates, or flasks with loose caps) that allow gas exchange with a humidified and enriched CO₂ environment (usually 5%). The second system is a medium buffered with Hanks' Balanced Salts containing only 0.35 g/L of sodium bicarbonate. This buffering system is designed for use in a sealed or gas-tight system and is not suitable for dishes and microplates.

Often, the above bicarbonate-based buffer systems are supplemented with the addition of HEPES, a widely used organic buffer. The use of this organic buffer can lead to additional problems upon exposure of the medium to fluorescent light. (For more information, see the section on Fluorescent Light-induced Toxicity below.)

Table 2. Some commonly used cell culture media with the amounts of sodium bicarbonate used for buffering. Higher levels of sodium bicarbonate usually require higher levels of CO₂ added to the incubator.

Cell culture media	Sodium bicarbonate levels (g/L)	Extra CO ₂ needed
Leibovitz's L-15 Medium	None	No
Eagle's MEM with Hanks' salts	0.35	No
Medium 199 with Hanks' salts	0.35	No
Ham's F12	1.176	Yes
DMEM/F12	1.2 to 2.438	Yes
RPMI 1640	2.0	Yes
Eagle's Minimal Essential Medium (MEM) with Earle's salts	2.2	Yes
McCoy's 5A	2.2	Yes
Medium 199 with Earle's salts	2.2	Yes
MEM Medium with Earle's salts	2.2	Yes
CMRL 1066 Medium with Earle's salts	2.2	Yes
Iscove's Modified Dulbecco's medium	3.024	Yes
Dulbecco's Modified Eagle's Medium (DMEM)	3.7	Yes

Filtration

Sterile filtration is usually the last step prior to the addition of any sterile supplements. There are two potential problems that may occur: first, interfering substances may be washed into the medium from the membrane or, second, valuable medium components may be lost by binding to the filter membrane. While most membranes contain usually harmless trace levels of leachable (extractable) substances, some membranes, especially some cellulose-based membranes, contain wetting agents that, at higher concentrations, may affect cell growth. These agents can be easily and safely removed from filters prior to use by running a small amount of warm high purity water through the filter unit. Some membrane materials, such as cellulose nitrate or nylon, may bind specific medium components, especially peptides and proteins used as growth or attachment factors (Brock, 1983). Testing should be performed to ensure these important supplements or factors are not being lost due to filtration.

For information on selecting the best filters for cell culture, refer to the Corning Life Sciences *Filter Selection and Use Guide*. This is available at www.corning.com/lifesciences. (Click on Technical Information, then on Filters.)

Sera

The sera used as media additives have long been a source of problems in cell culture. These problems include the high cost of serum, its variable quality and performance, and its potential as a source of mycoplasmas, endotoxins and other contaminants. While many advances have been made in the use of serum-free and reduced serum media, many cell culturists still use sera in their media. When practical, it is still a good idea to pretest several lots of serum before choosing one for purchase. The most sensitive assay possible that reflects the expected use of the serum should be used.

Fluorescent Light-induced Toxicity

The deleterious effect of fluorescent light on culture media may be the single most overlooked source of chemically induced cytotoxicity. It is very important to store media and cells growing in culture vessels in the dark away from sources of fluorescent light that will interact with light sensitive media components (riboflavin, tryptophan and HEPES). These interactions result in the production of hydrogen peroxide and free radicals that are directly toxic to cells. This well-documented problem is often ignored when there are cell growth issues (Wang, 1976, Wang and Nixon, 1978). Since the toxic effects of improperly stored media slowly increase with time, this problem is particularly difficult to identify. Besides direct cytotoxicity, other light-induced damaging effects include genetic damage (increase in mutation rates and chromosomal aberrations).

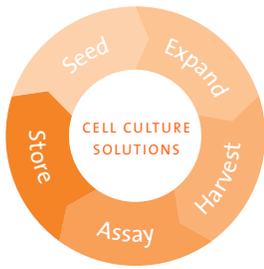
Problem Solving Suggestions

This guide has attempted to identify and solve some of the basic causes of cell culture problems. Many other problems can and will occur. Below are some recommended steps that can be used to help identify cell culture problems and find their causes:

1. Clearly identify and define the problem. It may require additional testing to repeat or duplicate the problem. It helps to make this a team effort, utilizing everyone in the laboratory whose knowledge or experience might contribute any helpful information.
2. It often helps to break up complex problems into smaller pieces that can be handled and understood more easily.
3. Organize all known facts surrounding the problem. Be specific, look for cause and effect relationships and then discard all facts that clearly do not apply and work with the rest. Don't overlook the obvious. A problem well stated is a problem half solved.
4. Once there is a clear understanding of the problem, begin looking for the cause. Try to avoid the urge to fix problems by changing everything; it may worsen the situation or mask the original problem.
5. Brainstorm and search for all the possible causes. Identify all changes that have occurred in the lab, in the cultures, in media, solutions, etc. that may relate to the problem. Good record keeping is essential for this step. Then select the best possibilities and begin to evaluate if they are actually contributing to the problem. Be creative! This may require some testing and experimentation.
6. Determine and implement the best long-term solution, not only to fix the problem but also to minimize or prevent any chance of a recurrence of the problem. This will take very careful planning.

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