

# Tissue Culture Trouble-Shooting

Many laboratories refer to tissue culture as “black art” or voodoo, due to the numerous variables that make it difficult to determine solutions when problems arise. A systematic approach that examines the symptoms and meticulously retraces each step in the culture process will help narrow a problem down to one material or critical procedure that has created the issue.

Almost every problem encountered in cell culture can be identified as one of the following:

1. The cells are growing poorly or not at all.
2. The cells have an abnormal morphology.

Each of these problems can usually be traced to one of the four following items:

1. **Materials:** They were poor quality, inappropriate, compromised, or contaminated.
2. **Equipment:** It may need to be calibrated or the laboratory has gotten away from suggested manufacturer usage.
3. **Environment:** The cultures were exposed to the wrong type of environment.
4. **Cells:** They were exposed to toxicity, contamination, or nutritional deficiency.
5. **Technique:** The procedures or protocols were not correct for the cell type.

Retracing steps will be easier, if the laboratory has maintained accurate and complete records of all materials, procedures, and culture work performed. This not only helps the laboratory find critical mistakes, it also saves time when dealing with manufacturers. A technician may be able to pinpoint the problem on paper, illuminating the need for long trials. Also keeping records of lot numbers, seeding densities, protocols, etc. will limit your time on the phone with manufacturers, when trying to identify if the problems lie outside the laboratory.

## Materials

### Plastics

If the plastics are the reason for growth problems, it will generally fall into one of two issues: manufacturers quality of plastics or cell line. While most plastic manufacturers make quality plastics not every plastic will work with every cell line. How each manufacturer treats their plastics is different (and proprietary) and some cell lines may adhere better to one plastic versus another. While most cell lines will grow well with most plastics, there are a few plastics that offer better performance.

When identifying the plastics as a problem manufacturing is more likely the problem versus cells adhering to the plastic. Generally they fall into these two areas:

1. Quality manufacturing practices.
2. Handling issues.

In manufacturing there are several practices that affect the quality of the end product, such as; the molding, the environment, quality control, and packaging. Most TC plastics are injection molded. Problems can arise from molds wearing down and not being replaced often enough or to overall poor designs. A mold problem will most often show up in as an uneven growth surface. If you experience “spotting” or “clumping” of cells (this is when cells grow better in the middle or in spots) this can be an uneven growth surface. Since TPP, Falcon, Nunc, Corning, and Sarstedt are all ISO9001 certified this should not be a problem. What is more likely the problem is poor design.

Again, since 5 of the 6 major TC plastic manufacturers are ISO9001 certified, the clean-room environment should never be a problem. What is a consideration is the certification that a manufacturer will give to their products. Here is a chart that shows who will certify their products non-pyrogenic and/or RNase and DNase free:

<i>Manufacturer</i>	<i>NON-Pyrogenic Certification</i>	<i>RNase / DNase Certification</i>
<b>TPP</b>	Yes	Yes
<b>Corning</b>	Yes	No
<b>Falcon</b>	Yes	No
<b>Greiner</b>	Yes	Yes
<b>Nunc</b>	No	No
<b>Sarstedt</b>	No	No

If contamination, uneven growing surfaces, or variability in cell growth are problems, utilizing a manufacturer that will certify their products may be worth investigating. Most manufacturers or distributors of these products are very helpful with sampling.

Another issue that can cause cross-contamination and/or variability in cell culture is how a plastic is handled in the laboratory. Since no one can guarantee perfect performance with cell culture, look for a manufacturer that provides benefits for handling. TPP plates and dishes with gripping edges for easier handling, there are pipettes that will leak less and cause less arm fatigue, and there are even vacuum filters that operate faster with less chance of tipping. TPP also offers black alpha-numerics on their plates for easier reading

and well identification under a microscope or hood. Overall improvements like these can mean more success for cell growth, less wasted media, serum, and plastics through less dropping, cross contamination, spilling, and all around easier handling of materials.

If plastics are identified as the main problem, it is worthwhile to do a side-by-side comparison of all the manufacturers to see if results vary and ease of operation can improve. While many laboratories do not want to take risks with their cells, the return of improved plastic performance will pay off immediately and in the long term.

## Media

Picking the “right” culture medium is vital to the cells' growth and vitality. The original supplier of a cell line can provide the best recommendation to use. A media supplier will provide information on storage, preparation, and disposal.

**Storage:** Incorrect media storage or expired media can make even the best medium choice null and void. Media should always be stored as directed: This always means in the dark and usually refrigerated at 2° to 8°C. Media components can break down when exposed to light and to temperature extremes. In some situations toxic by-products will form due to poor storage practices. Disposal requirements of expired media or media that appears cloudy or contains precipitates should always be followed. Freezing of media may cause irreversible precipitation of critical components.

**Preparation of Dry Powder Media:** Media incorrectly prepared in the lab from dry powder also can be a source of problems. Common mistakes include not letting the powder fully dissolve, incorrect pH adjustment, or using water above 30° C. Sterile filtering should be done with a final 0.2 µm cellulose acetate or other low-protein binding filter. Filters coated with wetting agents should not be used to avoid the inadvertent addition of cytotoxic detergents to the media. Filters made of nylon or glass may remove necessary lipids and fatty acids, while nitrocellulose filters can strip proteins from the medium. Filtering sterile medium after supplementing with serum or other growth factors also is not recommended. Although there are exceptions, most media contain heat labile components and should not be autoclaved. Serum-free medium should not be filtered through membrane porosities of less than 0.2 µm as this may remove critical components.

To reduce the chances of microbial contamination or cross-contamination of cell lines, use separate media containers for each cell line and do not share the same bottle of material with co-workers. Aseptically dividing material into a separate vessel for each person and for each cell line is good laboratory practice. Never work with more than one cell line at a time.

## Supplements

The two most common cell culture media supplements are serum and L-glutamine. The formation of precipitates or contamination can happen by incorrect handling of serum. When using a new serum (or even a new lot) expect a slight lag in growth. One of the top errors made in cell culture is using media that is deficient in L-glutamine. The half-life for L-Glutamine in liquid form, at refrigerated temperatures, is an estimated 6 to 8 weeks. To achieve optimum results, periodically replenish L-Glutamine levels in media.

## Glassware

Always clean cell culture glassware with phosphate-free detergents. Thoroughly rinsing glassware will remove residue from cleaning detergents that can be toxic to cells. Also a film of surfactants and cell debris can develop in spinner flasks over time. Stripping and recoating the vessels with a silicon reagent will reduce shear and friction. Repeated use of glassware may be a source of endotoxin. To eliminate glassware as a problem, schedule periodic washing in sodium hydroxide (NaOH) or dry oven exposure at 250 C for three hours.

## Equipment

Some laboratories will look to their equipment as the last place to check, but often a contamination problem can be as simple as cleaning the incubator. All manufacturers of CO<sub>2</sub> incubators and Bio-Safety cabinets supply helpful trouble-shooting in their manuals and some even do it on their websites. Here are a few common errors that can cause big problems:

<i>Common Equipment Areas to Check to Limit Contamination</i>
<b>CO<sub>2</sub> Incubator Calibration:</b> All manufacturers recommend a regular calibration (at least once a year) using a FYRITE CO <sub>2</sub> Gas Analyzer.
<b>Monitor CO<sub>2</sub> Tank Levels:</b> Letting a CO <sub>2</sub> tank run empty will compromise your cell lines and may kill them. Many auto tank switches are not also alarms.
<b>General Maintenance of Incubators and Bio-Safety Cabinets.</b> As the manufacturer recommends units need to be cleaned and decontaminated, CO <sub>2</sub> supply lines checked for leaks, replace filters, etc., on a regular basis.
<b>General Operation Requirements:</b> All general operation requirements from manufacturers are designed for safety and proper operation of the unit(s). For example, disconnecting the window alarm on a bio-safety cabinet is a problem waiting to happen. Also, on a bio-safety cabinet, if the window is not used at its appropriate height airflow will not operate properly and a safe clean environment is drastically compromised.

## *Environment*

**Carbon Dioxide (CO<sub>2</sub>):** Mammalian cell culture media typically contain buffering systems that require a CO<sub>2</sub> atmosphere. Severe pH shifts can result if adequate CO<sub>2</sub> levels are not maintained. 5% CO<sub>2</sub> is usually sufficient in most culture systems; however, this level may need to be adjusted to accommodate or enhance the growth of certain cell lines. It is also important to leave the flask caps of these cultures open slightly to allow adequate gas exchange. Insect cell culture media, as well as a few mammalian media such as L-15, contain buffering systems that are not dependent on CO<sub>2</sub>. These cultures should be exposed to atmospheric conditions. As a general rule, media and buffers containing Earle's Balanced Salts require CO<sub>2</sub> for optimum performance, while those with Hanks' Balanced Salts are for use under atmospheric conditions.

**Humidity:** For culture vessels that must have the caps loose for gas exchange, humidity levels are also important. Low humidity can lead to the evaporation of water from the medium resulting in concentrated salt levels and conditions that cause cell lysis. A pan of water placed in the bottom of the incubator usually is sufficient to supply the proper humidity level. You may want to add clean water and change the water daily.

**pH:** When conditions are too acidic or basic, poor cell growth and permanent cell damage can occur. For the best results keep mammalian cells at pH range of  $7.2 \pm 0.2$  and with insect cells approximately  $6.2 \pm 0.1$ . Monitor cultures periodically and perform media changes as needed, because the pH of a medium will change as cells take up nutrients and as the levels of respiratory by-products increase.

**Temperature:** Fluctuation of temperature out of normal ranges can lead to permanent damage of cells. For example, when incubating optimum growth of mammalian cells is at  $37 \pm 1$  C, while insect cultures must be kept at  $25 \pm 2$  C.

## *Cells*

**Toxicity:** Cell toxicity, or cytotoxicity, is the inhibition of cell growth or deterioration of cells due to excessive concentrations of a particular agent or ingredient. Abnormal morphology is the main indicator of cytotoxicity and can include one or more of the following: giant cells, multi-nucleated cells, a granular or "bumpy" appearance, vacuoles in the cytoplasm or in the nucleus, and/or ragged cell edges.

To identify which material (medium, serum, supplements, flasks, etc.) is causing the cytotoxicity, substitute them one at a time with control reagents, and screen for the signs of toxicity listed above. Serial dilutions of a suspected material can be used to determine the level at which that component becomes cytotoxic to the cultures.

**Contamination:** Microbial contamination comes in many forms including gram positive and gram negative bacteria, mycoplasma, virus, mold, and yeast. Indicators of contamination include turbid culture media, changed growth rates, abnormally high pH, poor attachment, multi-nucleated cells, grainy cellular appearance, vacuolization, inclusion bodies, and cell lysis.

One initial method to confirm suspicion of contamination is to transfer fluids from the suspected culture to a "clean" culture, incubate, and observe daily for cell deterioration. Specific identification assays are available for determining the group or type of contamination present. Positively identifying the contaminant is crucial to determining its source and in planning corrective action.

Contaminated cultures should be autoclaved and discarded immediately to reduce the risk of spreading the contamination to other cultures or other areas of the lab.

Antibiotics such as gentamicin, streptomycin, and penicillin often are used in cell culture media to control low levels of contamination. However, some researchers feel that it is wise to avoid using antibiotics altogether. It is better to detect a contamination right away and handle it immediately than to mask it with antibiotics. Antibiotics also can be cytotoxic when used at elevated concentrations, and low serum or serum-free cultures are especially susceptible to this problem.

**Nutritional Deficiencies:** Because different cell types and cell lines have different nutritional requirements, it is difficult to have one medium system that will satisfy all of your cultures. Unsatisfactory cell performance (poor growth, poor attachment, and/or monolayer sloughing) often occurs if the cell's nutritional requirements are not being met. Other indicators are giant cells, vacuoles, and ragged cell edges. Deficiencies can arise from a certain component or components being absent altogether or from levels being exhausted too quickly.

Supplementing medium with additional amino acids (particularly L-glutamine), vitamins, glucose, or higher levels of serum and observing the cell's response can help to identify the component(s) not present at sufficient concentrations. Depending on the degree of the deficiency, the cultures may be too thoroughly damaged to completely recover. It is often necessary to start new cultures once this problem has been identified.

## *Technique*

<i>Common Problems Due To Technique</i>	
<i>Symptom</i>	<i>Common Problem</i>
“Spotting”	Check for bubbles in medium that have attached and displaced cells. If this is not the problem it could be an uneven growth surface due to the plastics or the incubator
Streaking	Condensation buildup, which may not only be a technique problem, but also an incubator problem. Some plastic manufacturer’s, like TPP, have features that help compensate for this problem.
“Clumping” of Cells	Cells were not totally re-suspended prior to seeding, cells were damaged during sub-culturing, uneven growth surface due to an un-level incubator or poor plastic manufacturing
Cells that are Stringy	This mostly likely is due to an overexposure to trypsin, but it could be that your pipettes have poor vacuums and do not remove all the trypsin.
“Halo” Effect	Not enough media was used or poor distribution of media over cells. Poor distribution can sometimes be attributed to poor design of flasks, dishes, and/or pipettes or the incubator not being level.
Peeling or Sloughing	Prolonged incubation or over seeding can cause overgrowth.
Circular Holes	Can be caused by excessive foaming or uneven growth surfaces in your flasks, dishes, or plates.
Circular Banding in Roller Bottles	Rollers turning too quickly.
Banding on One Side of Roller Bottles	Rollers turning too slowly.

**Trypsinization:** The misuse of trypsin can lead to immediate and long-term problems with a set of cultures. Trypsin is a popular detachment factor because it digests the proteins that keep the cell attached to a surface. Prolonged exposure to trypsin can result in too much of the cell being digested, which can cause cell damage or destruction. A few keys to correctly using trypsin:

- Use the correct concentration for the cultures being used. Some cells are easy to detach and others are much more difficult to dissociate from the substrate. Different concentrations of trypsin are available with or without EDTA. EDTA, also called Versene, increases a trypsin solution’s effect by taking up calcium and magnesium ions.
- Watch the clock. Only leave the trypsin on the cultures as long as is absolutely necessary. Check the monolayer often to see if it is beginning to come off and at that point, a gentle tap against the side of the vessel may help to dislodge the cells. Be aware that forcing cells to detach by harsh mechanical treatment such as smacking the flask can cause the cells to “tear” off the surface and cause permanent damage.
- Neutralize the trypsin after the cells have detached by adding serum or gently centrifuging the cells into a pellet and re-suspending in fresh media. Because serum contains trypsin inhibitors, it is important to completely remove serum prior to trypsinization. This can be done by first rinsing the monolayer with serum-free medium or a calcium- and magnesium-free balanced salt solution such as DPBS.
- Control the trypsin by controlling the temperature. Cells that are sensitive to trypsin should be trypsinized at room temperature or refrigerated temperatures, while trypsinizing in a 37° C incubator can speed up the process for more stubborn cells and reduce the total exposure time.

**Seeding:** Using an inappropriate seeding density can lead to everything from overgrowth and cell sloughing to sparse cultures that may never become fully confluent. The correct density is dependent on the cell type, the medium and amount of serum used, the size of the vessel, and when the cultures are to be used or sub-cultured again.

If you are unsure about how many cells to add to each vessel, try several different concentrations and determine which grows to the appropriate cell density in the time period that is applicable for your transfection, assay, or cell stocks. Remember that high cell densities will use nutrients quickly and may require additional supplementation to avoid deficiencies.

**Adapting From Serum to Serum-Free:** Adapting cells to serum-free conditions can be a smooth transition, however, if it is done incorrectly, many problems can arise. This transition should typically be done over several passages to gradually select cells that can grow under serum-free conditions.

**Other Important Factors to Consider:**

- Use actively growing cells that are in their log phase of growth. Use cells that are 80 – 90% viable.
- Keep exposure to trypsin at a minimum. Cells in the absence of serum are very susceptible to trypsin, so use cold trypsinization procedures or dilute trypsin with calcium- and magnesium-free DPBS.
- Handle the cells gently. Do not centrifuge at high speeds or roughly re-suspend the cells.
- Use a serum-free media that is designed for your type of application.
- Feeding and sub-culturing the cells at more frequent intervals than used with serum-containing conditions may be necessary.