Promises and Pitfalls of Cell Line Adaptation

Some Basic Protocols

by Terrell Johnson

Optimal cell growth is routinely achieved using a defined basal medium supplemented with relatively high levels of serum or proteins. To manufacture highly purified biopharmaceuticals of consistent quality requires a well-defined production process. Thus it is desirable to eliminate serum or other animal-derived proteins. Chemically defined serum-free or protein-free cell culture media offer lot-to-lot consistency and reduced production costs. Such media also facilitate downstream processing, regulatory approval, and improved product biosafety. An increasing demand for biopharmaceuticals produced using mammalian cell culture, coupled with capacity limitations, necessitates improving the productivity and robustness of manufacturing processes. Therefore, it is frequently necessary to replace a medium in use with a more defined formulation.

Changes in culture conditions can alter the physiological stimuli cells receive, which initiates the adaptation process. This process involves metabolic changes associated with maintaining or growing cells in a new environment. It may be accompanied by induction or repression of protein synthesis in response to extracellular stimuli such as alternative nutrients. In extreme circumstances, adaptation can involve selecting a small subpopulation of cells from the much larger heterogeneous starting population. Given physiological shifts involved in this process, it is not surprising to encounter changes in cell properties (e.g., growth rates and productivity).

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The ease of cell adaptation to a new medium is a function of intrinsic factors associated with a given cell line as well as the degree of similarity or difference in medium compositions and handling protocols used during the adaptation process. Two general strategies are used to adapt cells to serum-free media. The simplest is *direct adaptation*, in which cells are switched directly from serum-supplemented medium into a serum-free alternative.

Sequential adaptation or *weaning* is the alternate approach that switches cells from serum-supplemented to a serum-free medium through several sequential steps. Weaning tends to be less harsh on cells than direct adaptation is, so it can be beneficial for adaptation of less robust cell lines. This can involve reducing serum levels (e.g., from 10% to 5% to 2.5% to 1%, then to 0%), increasing the ratio of new medium to the current medium (0:100, 25:75, 50:50, 75:25, 100:0), or altering both serum and media at sequential passages until cells are acclimated to serum-free conditions.

No single adaptation technique is applicable in all circumstances. But to maximize the chance for success, a number of points should be considered before beginning adaptation (See Adaptation Considerations Box).

Serum-free media will support the growth and attachment of adherent cells; however, most such formulas are designed for use in a suspension environment. The protocols listed here generally focus on adapting cells in suspension culture to growth in serum-free or protein-free media. Attached cells can be adapted to suspension growth, and other protocols are available for doing so. Alternatively, these protocols can be easily altered for adapting attached cells to new media while retaining the existing properties of attached cell growth. My protocols are generally quite conservative and can be shortened in many cases. However, because of differences in cell line properties, some modifications may be required for a given cell line. Initial cell inocula, cell yields, and culture periods given here are intended as starting points for subsequent optimization.

From Serum Supplemented to Serum Free

Attached Cell Lines: Some cells can be directly adapted from serum-

containing to serum-free media (SFM). Direct adaptation of attached cell lines is achieved by seeding monolayer cultures at a minimum of twice the normal inoculation density. Dilute cells from a parent culture (serum-containing) by adding prewarmed SFM. The use of 75-cm^2 and 162-cm^2 disposable sterile T-flasks is recommend so that parent cells can be diluted in a total working volume of 20–30 mL for T-75 flasks and 40–50 mL for T-162 flasks. Subculture cells every three to five days at a 1:5 dilution.

Suspension Cell Lines: For direct adaptation of cells in suspension culture, an inoculum should be at least twice the normal density. Monitor cells daily and remove culture medium when it becomes acidic, then add an equivalent replacement volume of fresh SFM. Maintain cell densities between 2×10^5 cells/mL and 1.4×10^6 cells/mL. If cell density shifts below the lower limit of that recommended range, adjust the medium volume to a level that will reestablish a mid–log-phase cell density.

Cells are fully adapted to serum-free medium when growth rates and densities return to normal parameters for a minimum of three subcultures in 100% serum-free medium. Stock cultures adapted to serum-free medium should be subcultured in serum-free medium every three to five days when their cell densities are $1–3 \times 10^6$ with 90% viability.

Weaning or sequential adaptation is gentler on cells than direct adaptation. This alternative method for less robust cell lines involves a sequential medium and/or serum reduction. Some different protocols that have been used are summarized below.

The most traditional approach for sequentially adapting cells from serum-supplemented medium to serum-free medium is the *serum reduction method*. Grow cells in a basal medium supplemented with a normal level of fetal bovine serum (FBS), usually 5–15%, until they approach confluence in an attached culture — or near the peak of the linear log phase in a suspension culture. Subculture cells at

Adaptation Considerations

Not all cell lines are sufficiently robust to undergo direct adaptation. Sequential adaptation (weaning) may be needed to avoid the danger of population selection.

Some cells may adapt readily to new media, whereas others may have more difficulty adjusting to changes. Medium change should be performed before any additional changes in a culture system.

Before adaptation begins, it is critical that cells be in their midlogarithmic phase of growth, with viability >90% .

Duration of the growth lag at low density depends on the split density used. In general, the lower the split density used (higher split ratio), the longer the lag phase. Above a certain density threshold with each cell line, there will be little density dependence. Below a threshold density, there will be a prohibitively long lag, so routine exposure to such stress should be avoided.

Cells that can recover with a short (<12 hours) lag phase at low density (~25 cells/mL) are well suited for adaptation. This is a cell line characteristic, however, and a little investigation is necessary to find the density tolerance for a particular line. Some lines can be split to 54 cells/mL and recover easily; others won't recover well if split below 55 cells/mL. Due to the inherent stress associated with the adaptation process, cultures should be seeded in higher initial cell inocula than during regular subculturing.

The maximum density to which cells will grow on reaching their stationary phase depends on both the cell line and medium used. Cells should not be allowed to reach this phase during adaptation. After you split them into fresh medium, those remaining can be monitored for information purposes after reaching their stationary phase. Typically, you should avoid culturing cells above 80% of their characteristic maximum density, which frequently varies from 2×10^6 to over 12×10^6 cells/mL depending on the cell line and culture conditions. Cells should not be allowed to overgrow during adaptation because it increases your chances of selecting a subpopulation.

Most serum-free media contain much less protein than serum-supplemented media, so they are less able to protect cells from stress in culture. The following factors involved in cell stress should be considered during the adaptation process:

- Reduce (at least 10-fold) or eliminate antibiotics, if possible.
- Minimize extreme changes in pH, temperature, and osmolality because cells can be very sensitive during adaptation.
- Minimize mechanical forces and enzyme treatments. Do not centrifugate.
- When switching to SFM, it is advisable to decrease trypsin (EDTA) concentration if cell adherence is not strong. A more gentle dissociation solution such as versene is preferred for weakly adherent cells. Because SFM generally have little or no protein, a trypsin inhibitor must be used to quench trypsin and protect the cells.

Adaptations require routine monitoring of cell density and viability, as well as pH of the culture medium.

Controlled, consistent cryopreservation and recovery techniques are essential.

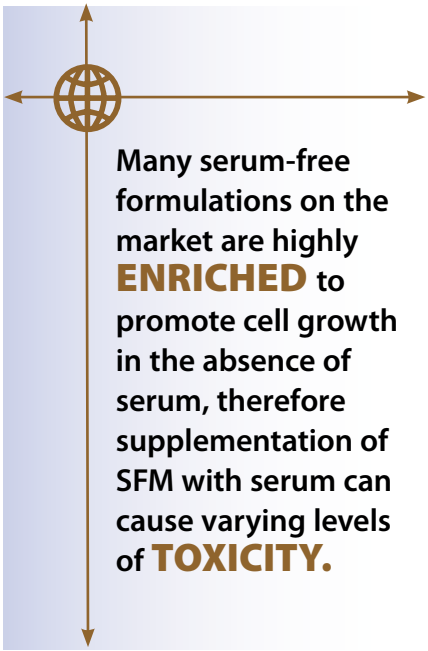
To ensure against loss of adapted cell cultures, keep a backup culture in the previous mixture during each step of this process until cells have adapted to a new combination of medium and/or serum. Several passages may be needed at a given step in the process before it is possible to proceed to the next.

Cryopreserve cultures periodically during adaptation to provide additional protection against loss.

Cell viability may drop to very low levels during adaptation. This is expected as part of the process. For example, a switch from serum-supplemented to SFM may cause most cells in a culture to die, followed by outgrowth of a surviving subpopulation. Although this phenomenon has been referred to in literature as *adaptation* or *weaning*, it is more likely a selective process by which surviving cells differ phenotypically from their parental population.

While cells are being adapted, production can be monitored. But until they are placed in an aerated production system, such results will not be indicative of their true performance in the new serum-free conditions.

As with any new process, commitment, careful observation, and patience are essential to cell line adaptation.



twice the normal split ratio into a serum-free medium containing 5% (v/v) FBS. When cells approach saturation density, subculture (as above) in serum-free medium containing 1% FBS. At each subsequent subculture, reduce FBS by 50% until the FBS concentration is below 0.06%. At that point, cells can be maintained in a serum-free medium.

If cell growth declines at any point during adaptation, return serum concentration to the previous level that promoted cell growth. Allow cell growth to stabilize at that concentration before proceeding with serum reduction. After cells have been passaged for three subcultures in 100% serum-free medium, they are adapted.

One caution should be noted for supplementing SFM with serum. Because many serum-free formulations on the market are highly enriched to promote cell growth in the absence of serum, supplementation of SFM with serum (even at low levels) can cause varying levels of toxicity. In such instances, use of an alternative method such as subculture is suggested.

The *subculture or split method* may be especially beneficial in circumstances where a selected SFM does not allow serum supplementation. Grow cells to 90% saturation density — usually about 10^6 cells/mL in suspension culture — in their normal medium (basal medium containing 5–10% serum). In a T-25 flask, add 2.5 mL of cell seed stock and 2.5 mL of fresh serum-free medium at a 1:1 split ratio. After 24 hours split the cells 1:1 (2.5 mL cells and 2.5 mL fresh serum-free medium) into a new flask, feeding the original flask with 3 mL of fresh serum-free medium.

At some point, cell doubling will decrease as the time interval between cell cultures increases. Continue to subculture the cells 1:1 as necessary until they must again be split daily. At that point, cells are fully adapted and a normal subculturing program can be adopted. Assuming doubling times have normalized, subculture within the linear log portion of the growth curve twice a week.

Another approach is sometimes needed for adapting cells **from serum-supplemented medium to protein-free medium** when the above protocols are unsuccessful. Using one of those methods, first adapt the cell line to a low-protein, serum-free medium. After cells have adapted to serum-free conditions, use one of the methods below to adapt the cell line to growth in the desired protein-free medium.

Adaptation from one SFM to another or to a protein-free medium, in some circumstances, may be more challenging than the original adaptation from serum-supplemented to serum-free conditions. This can be a greater or lesser issue depending on the ability of cells to adapt to the altered physiological stimuli of their new medium. It also may depend on to what degree heterogeneity in the cell line is reduced as a result of selection during adaptation from the original serum-supplemented medium. So the techniques for adaptation from one serum-free formulation to another are similar to but less aggressive than those described above.

Direct Adaptation from Serum-Free to Protein-Free

Direct adaptation of cells in serum-free culture to another serum-free or protein-free medium can be performed by seeding them at two to three times the normal seeding density into the new medium using either stationary or spinner cultures. Subculture cells every three to five days at a 1:5 dilution. Alternatively, they can be allowed to reach midlog phase and then be maintained at that density by daily medium addition.

When medium addition is about 50% daily, cells can be split using standard subculturing techniques. Either way they will be adapted after they have been successfully subcultured in their new medium for three passages with a normal growth rate. The time interval needed to achieve normal cell growth varies according to cell line.

When adaptation is undertaken in **stationary culture**, many suspension cell lines can grow very loosely attached or in suspension as clusters of two to 10 cells without agitation. Therefore, trituration at each subculture may be required to achieve a single cell suspension.

Alternatively, cells can be adapted by **weaning**. In this process, cells are split into a ratio of 25% in the new medium to be adapted to and 75% in the old medium (current culture medium) at a density at least two times that for normal seeding. Those cells are expanded to the mid-log phase and their growth parameters monitored. Cells continue splitting at this density in the same medium combination until their growth parameters are within an acceptable range. That may take as little as a single passage if there is no significant change in growth parameters.

Once those cultures stabilize, split them at the normal seeding density and monitor their subsequent growth. Continue splitting at this new density in the same medium combination until the growth parameters are again within an acceptable range. This too may take as little as a single split. However, the cells may begin to slow their doubling time. If that happens, give them more time to adapt. Again, keep the cell density high to aid in the adaptation. When cells return to their normal behavior, proceed to the next stage. If they fail to achieve normal behavior, the new medium may simply be inappropriate. If growth parameters reach a new

equilibrium (different from what has been normal but still acceptable), then you can proceed with the next stage.

Next, increase the proportion of the new medium to 50%. Repeat the adaptation step, initially seeding cells at two times their normal seeding density. As previously, subculture while monitoring growth parameters. Continue until cells can be split at normal seeding density in this medium combination and their growth parameters are within an acceptable range.

Now, increase the proportion of the new medium to 75% and repeat the adaptation process described above. Finally, increase the proportion of the new medium to 100% and repeat again. Stock cultures of SFM-adapted cells should achieve a cell density of 1–3 × 10^6 cells/mL, with 90% viability, when subcultured in SFM every three to five days. Once a culture has been successfully passaged at least three times in 100% of the new medium, it is adapted to the new medium.

Final Steps

Significant advantages can be gained in a downstream manufacturing process by transferring from a serum-containing to a serum-free production system. Many cell lines of commercial interest can be moved directly from serum-containing into serum-free or protein-free media without adaptation procedures. Other lines require more gradual, sequential adaptation (weaning) to successfully adapt to serum-free or protein-free conditions. Specific cell growth characteristics and production rates following adaptation depend on both the cell line and the culture medium. With care and monitoring of cell adaptation, you can adapt most cell lines to serum-free or protein-free conditions while simultaneously maintaining high cell viabilities and productivities. In some cases, productivity and longevity may be increased.

Please note that the adaptation process can lead to altered cellular characteristics, including changes in growth rates and productivities, due to modifications of cellular metabolism or adaptive selection of a subpopulation from the original cell line. Therefore, it is important to characterize all growth parameters after adaptation to determine whether they have shifted substantially as a result of the process. Similarly, it is also important to reexamine productivity and product characteristics such as glycosylation and/or phosphorylation to ensure that no significant shifts have occurred.

For Further Reading

Freshney RI. *Culture of Animal Cells: A Manual of Basic Technique* (Fourth Edition). Wiley-Liss: New York, NY, 2000.

Mather JP, Roberts PE. *Introduction to Cell and Tissue Culture: Theory and Technique.* Plenum Press: New York, NY, and London, UK, 1998.

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