Growing More Cells: A Simple Guide to Small Volume Cell Culture Scale-Up

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Table of Contents

Introduction

We need more cells… Life science researchers are constantly being challenged to produce more cells for cell-based experiments and assays, or for producing recombinant proteins, antibodies and viral vectors. Researchers must choose from a sometimes confusing variety of cell culture vessels, systems and methods to meet their needs for larger quantities of cells. This Corning guide is designed to help researchers select the vessels and methods that can best meet all of their needs for growing more cells or producing more cell products. It will focus on basic systems suitable for producing at least 1 x 10^9 cells (approximately one gram). This guide is not designed for researchers who need to produce much larger quantities of cells or for clinical or industrial level production, although much of the information presented here may also be applicable to these situations.

One goal of this guide is to keep the scale-up process as simple as possible to increase the likelihood of success. A second goal is to keep costs as low as possible to make the scale-up process more affordable to research laboratories faced with tight budgets. As a result, hollow fiber perfusion systems, fixed- and fluidized-bed reactors, airlift and stirred tank bioreactors, as well as other systems requiring extensive investment in equipment or automation are not covered here. The reference section provides a useful starting point for additional information on these more expensive or complicated scale-up systems and strategies.

CORNING

System Choices

The first step in choosing a system for scale-up is to review the systems that Corning recommends for use in research laboratories (Figure 2). Each system should be evaluated in light of your own unique needs and situation. Corning offers six systems for scaling-up attachment-dependent cells:

- Large surface area flasks
- 245 mm square dishes
- Roller bottles
- Multilayer CellSTACK® Culture Chambers
- HYPER*Flask*™ Vessels
- E-Cube™ bioreactors

For scaling-up suspension cultures Corning offers:

- Disposable plastic and reusable glass spinner flasks
- Disposable plastic and reusable glass Erlenmeyer style shaker flasks

Figure 1. Life science researchers having been using Corning® products for cell culture for over 80 years

Flasks

The first cell culture flasks were developed by Alexis Carrel in 1923. These round flat bottom flasks were manufactured from PYREX® glass and had either a canted or straight neck. They were called "D" flasks where the "D" referred to their diameter: thus a D-3.5 flask was 3.5 cm in diameter. William Earle introduced glass "T" flasks in 1947 that were hexagonal or rectangular. The "T" referred to the total surface area of the flask that was available for cell growth: thus a T-25 flask had a 25cm^2 growth area. By the 1960s, straight neck T-flasks were available molded from polystyrene that was treated to enhance cell attachment. Corning developed the first canted neck polystyrene T-flasks in 1974 to offer researchers better pipette access to the cell monolayer. Corning offers these in five sizes ranging from 25 cm^2 to 225 cm^2 growth areas (Table 1). Most of these flasks are available with either the standard cell culture surface or the Corning® CellBIND® Surface for enhanced cell attachment. Corning flasks are available with several cap styles. To reduce contamination problems in CO_2 incubators the use of vented caps is highly recommended.

Advantages

- Traditional and easy to use
- Good pipette access and easy to feed or harvest cells
- Cell growth can be quickly determined with a microscope
- Reduce media spills and culture contamination
- No additional equipment required

Disadvantages

- Labor intensive for large numbers: requires 44 T-225 flasks to grow 1 x 10^9 cells (Figure 3)
- Use more incubator space

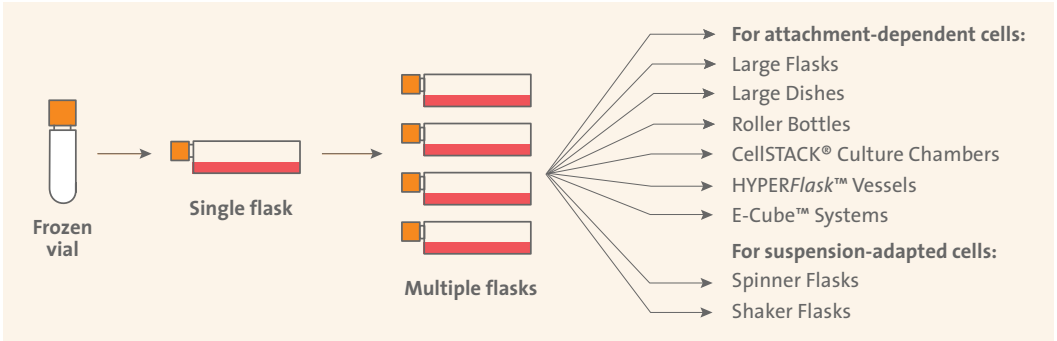

Figure 2. Corning offers systems for scaling up both attachment-dependent and suspension-adapted cell cultures.

Table 1. Expected Cell Yields and Recommended Medium Volumes for Corning® Flasks

Corning Flask	Average Cell Yield*	Medium Volume
25 cm^2	2.5 x 10^6	5 to 7.5 mL
75 cm^2	7.5 x 10^6	15 to 22.5 mL
150 cm^2	1.5 x 10^7	30 to 45 mL
175 cm^2	1.75 x 10^7	35 to 52.5 mL
225 cm^2	2.25 x 10^7	45 to 67.5 mL

* Assumes an average yield of 1x10^5 cells/cm^2 from a 100% confluent culture.

Figure 3. Large Corning® T-225 flasks provide a simple and practical approach for growing large amounts of cells.

Dishes

Round glass culture dishes were developed by Richard Petri in 1877 when he was an assistant in Robert Koch's microbiology lab. The simple glass Petri dish was widely used in the early days of cell culture as a sterile container for holding hanging drop cultures. Polystyrene Petri dishes that are surface treated for cell culture became available in the 1960s. Corning offers round cell culture dishes in four sizes with approximately 35 mm to 150 mm diameters (Table 2). The 150 mm dish has approximately 148 cm^2 growth area. However, for producing much larger amounts of cells Corning offers a 245 mm square dish with 500 cm^2 growth area (Figure 4).

Advantages

- Simple economical alternatives to flasks
- Direct access to the cell surface and easy to harvest (especially by scraping)
- Require less incubator space than flasks
- Cell growth can be quickly determined with a microscope
- No additional equipment required

Disadvantages

- Labor intensive to produce large numbers of cells: 20 500 cm^2 dishes to grow 1 x 10^9 cells
- More prone to accidental media spills and contamination due to open design

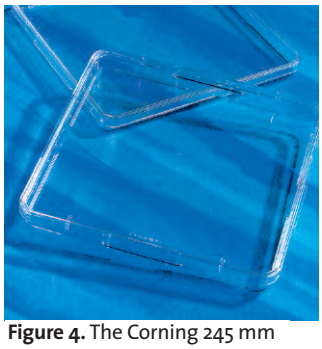

Figure 4. The Corning 245 mm square dish offers 500 cm^2 growth area.

Table 2. Expected Cell Yields and Recommended Working Volumes for Corning Dishes

Corning Dishes	Growth Area	Average cell yield*	Medium Volume
35 mm	8 cm^2	8.0 x 10^5	1.6 to 2.4 mL
60 mm	21 cm^2	2.1 x 10^6	4.2 to 6.3 mL
100 mm	55 cm^2	5.5 x 10^6	10 to 15 mL
150 mm	148 cm^2	1.48 x 10^7	30 to 45 mL
245 mm (square)	500 cm^2	5.0 x 10^7	100 to 150 mL

* Assumes an average yield of 1 x 10^5 cells/cm^2 from a 100% confluent culture.

Roller Bottles

The concept of growing cells as rotating cultures was developed by George Gey (1933) at Johns Hopkins University as a means of growing larger quantities of attachment-dependent cells. His work was primarily done in glass roller tubes. By the late 1950s, much larger glass roller bottles were in common use for growing large numbers of cells, especially for viral vaccine production (Whittle and Kruse; 1973; Mather; 1998b). Corning offers disposable polystyrene roller bottles in four sizes with 490 cm^2 through 1750 cm^2 growth areas (Figure 5 and Table 3). These roller bottles are available with either the standard cell culture surface or the Corning® CellBIND® Surface for enhanced cell attachment. Corning also offers reusable roller bottles manufactured from PYREX® glass for optical clarity and mechanical strength. Corning reusable PYREX glass roller bottles are designed to withstand repeated wet or dry sterilization and are available with approximate growth areas of 670 to 1,330 cm^2.

Figure 5. Corning plastic roller bottles are available with 490 cm² to 1750 cm² growth areas

Advantages

- Prevent gradients from forming within the medium that may adversely affect growth
- Provide superior gas exchange
- Very economical for cultivating large quantities of anchorage-dependent cells: same culture techniques as flasks but less labor
- Cell growth can still be determined using most inverted microscopes

Disadvantages

- Require more incubator space than flasks or dishes
- Require a moderate amount of labor to produce large numbers of cells: 12 850 cm² roller bottles to grow 1 x 10^9 cells.
- Expect to spend at least $1,500 for equipment to handle 12 bottles.
- May cause cell attachment problems due to the constant bottle rotation, which may be aggravated when low or serum-free media are used. The Corning® CellBIND® surface was developed to solve these problems.

Table 3. Expected Cell Yields and Recommended Working Volumes for Corning Polystyrene Roller Bottles

Corning Roller Bottles	Average Cell Yield*	Medium Volume
490 cm² roller bottle	4.9 x 10^7	100 to 150 mL
850 cm² roller bottle	8.5 x 10^7	170 to 255 mL
1700 cm² roller bottle	1.7 x 108	340 to 510 mL
1750 cm² roller bottle	1.75 x 10^8	350 to 525 mL

*Assumes an average yield of 1 x 10^5 cells/cm² from a 100% confluent culture.

Corning CellSTACK® Culture Chambers

Figure 6. CellSTACK-10 chambers have 6,360 cm² growth surface and save on space and labor

The Corning CellSTACK Culture Chambers are multilayered vessels that provide both a space and a labor-saving solution for the problem of growing large amounts of anchorage dependent cells in flask-like conditions (Figure 6). CellSTACK Chambers are available in four sizes that are suitable for use in a research laboratory setting. The single layer 1-STACK with 636 cm² cell growth area, 2-STACK with 1,272 cm² cell growth area, 5-STACK with 3,180 cm² cell growth area and the 10-STACK with 6,360 cm² cell growth area (Table 4). The fifth and largest CellSTACK-40 Chamber has 25,440 cm² cell growth area and can easily produce over 2.5 x 10^9 cells. However, due to its large size, it requires special handling equipment and is not recommended for research laboratory use. CellSTACK Chambers are available with either the standard cell culture surface or the Corning CellBIND surface for enhanced cell attachment. CellSTACK Chambers have two 26 mm diameter filling ports that allow direct access to the chamber bottom and offer greater flexibility for aseptically filling and removing medium and cells. Optional filling caps with 1/8" or 3/8" diameter tubing for direct aseptic transfer of media, solutions or cells with peristaltic pumping or by gravity feeding are recommended for larger CellSTACK Chambers for safe aseptic liquid transfer (Figure 7).

Larger CellSTACK Chambers require some practice to be able to handle efficiently. To help researchers unfamiliar with using CellSTACK Chambers, Corning has a technical video guide on www.corning.com/lifesciences that demonstrates using these products.

Advantages

- Less labor than dishes, flasks or roller bottles to produce cells: only 2 CellSTACK-10 chambers required to grow 1 x 10^9 cells
- Easy scale-up by adding more layers or more chambers
- Require less incubator space than flasks and roller bottles; can be stacked
- No additional equipment required

Disadvantages

- Larger CellSTACK chambers are heavy when filled, more difficult to manipulate, requires strong shelves

Figure 7. Optional filling caps allow medium to be aseptically transferred in and out of Corning CellSTACK chambers

- Larger CellSTACK® chambers are more difficult to use than flasks, dishes and roller bottles due to larger media volumes not easily supplied by pipetting
- Hard to check cell growth by microscopy in 10-layer CellSTACK Chambers because of their height. However, 1-layer companion cultures grown side-by-side under identical conditions can be used to monitor growth

Table 4. Recommended Media Volumes for Corning® CellSTACK Culture Chambers

Corning Vessel	Growth area	Average cell yield*	Media volume
1-Stack	636 cm^2	6.36 x 10^7	130 to 190 mL
2-Stack	1,272 cm^2	1.27 x 10^8	260 to 380 mL
5-Stack	3,180 cm^2	3.18 x 10^8	650 to 900 mL
10-Stack	6,360 cm^2	6.36 x 10^8	1,300 to 1,900 mL
40-Stack	25,440 cm^2	2.54 x 10^9	5,200 to 7,200 mL

* Assumes an average yield of 1 x 10^5 cells/cm^2 from a 100% confluent culture.

Corning HYPER*Flask*™ Vessels

Figure 8. 10-layer Corning HYPER*Flask* vessels have been successfully used for cell propagation (both adherent and suspension cells), protein and virus production, and transfections.

The multilayered Corning HYPER*Flask* vessels use a gas permeable polystyrene film to provide gas exchange between the cells and culture medium and the atmospheric environment surrounding it. This allows for a much greater cell growth surface area than in the traditional flasks (175 cm^2) of the same overall footprint space. The HYPER*Flask* vessel is designed to be filled entirely with medium and sealed with a solid cap (Figure 8). There is no need to crack the cap or use a vented cap due to the gas exchange through the ultra-thin film. Each HYPER*Flask* vessel has a total growth area of 1720 cm^2 or 10 times the growth area of a standard T175 flask.

Advantages

- Ten interconnected polystyrene film growth surfaces allow for gas exchange directly into the medium
- No additional equipment required
- 10-fold higher cell yields than 175 cm^2 flasks with the same footprint increases productivity while reducing processing time and incubator space
- Requires much less incubator space than roller bottles, flasks, dishes or CellSTACK chambers

Disadvantages

- High density HYPER*Flask* vessels are more difficult to use than flasks, dishes and roller bottles because each vessel requires about 565 mL of medium not easily supplied by pipetting
- Only 4 of the 10 layers in HYPER*Flask* vessels can be directly observed by microscopy

Corning E-Cube™ System with CellCube® Modules

The E-Cube system is a small (25.4 cm x 35.6 cm footprint) simple perfused bioreactor system that uses Corning's patented parallel-plate 10-Stack CellCube module with 8,500 cm^2 cell growth area. CellCube modules have a tissue culture treated polystyrene growth surface for cell attachment and are continually perfused with media for increased cell productivity. Corning developed the CellCube module as a parallel plate growth chamber that is integrated into a bioreactor system for the mass culture, growth, and process control of substrate attached cells. The system is ideal for producing large amounts of cells, or recombinant proteins, viral vaccines and vectors for gene therapy (Kotani, et al.; 1994). Unlike hollow fiber bioreactors, cells can be harvested from the E-Cube system.

The E-Cube system includes an oxygenator, medium reservoir, multiple access ports and all required tubing and fittings and uses a disposable 10-Stack CellCube module. It requires a peristaltic pump (Figure 9) and it fits in most CO_2 incubators. The recommended operating volume with a 10-Stack CellCube module is 1,700 to 2,550 mL medium.

Figure 9. The Corning E-Cube system is a simple to use perfused bioreactor with an 8,500 cm² growth area parallel plate culture chamber.

To help researchers unfamiliar with the E-Cube™ system, Corning provides an instruction manual on www.corning.com/ lifesciences that demonstrates the setup and use of this product.

Advantages

- Requires less labor than dishes, flasks or roller bottles to produce cells: a single 10-Stack CellCube® module can grow approximately 1 x 10^9 cells.
- Higher cell yields due to continuous perfusion of cells with medium
- Easily scaled up by a switching to a 25-Stack CellCube module with 21,250 cm^2 of growth area
- Requires less incubator space than flasks and roller bottles

Disadvantages

- No direct microscopic viewing of cells, requires more expertise to use than flasks, roller bottles and CellSTACK® chambers.
- Requires initial several thousand dollar investment
- Requires a user-supplied peristaltic pump

Spinner Flasks

Figure 10. The Corning® reusable glass spinner flasks are available in a wide range of sizes including the 250 mL and 1L shown here.

W. Cherry and R.N. Hull in 1956 used a suspended magnetic stirrer to grow cells in suspension in round bottom flasks. In 1957 W. F. McLimans and colleagues developed the modern glass spinner flask and successfully scaled them up to 20L fermenters by 1958. For those cell lines that can grow in suspension, spinner flasks offer a very economical and simple way to produce large numbers of cells. Many suspension-adapted cell lines can achieve densities of 1 to 2 x 10^6 cells/mL or higher in these spinner flasks (Mather; 1998b, Iyer et al.; 1999). Corning offers reusable glass spinner flasks in working volumes of 125 mL to 36L with either angled or vertical sidearms, and a variety of cap designs (Figure 10 and Table 5). Disposable polystyrene spinner flasks in 125 mL, 500 mL, 1L and 3L working volumes are also available (Figure 11). These disposable spinner flasks are ready to use without any labor or effort required to clean, assemble and sterilize.

Advantages

- Very economical and compact
- Simple to harvest
- Much less labor than using dishes, flasks, roller bottles or multilayered chambers for growing an equivalent number of cells
- Easier to scale up

Disadvantages

- Only for suspension adapted cells (unless microcarriers are used)
- Difficult to feed cultures
- Require a magnetic stirrer which also requires more incubator space
- Require significant labor to decontaminate, clean, reassemble and sterilize glass spinner flasks

Figure 11. Ready-to-use disposable Corning spinner vessels avoid the cleaning and sterilizing problems common with reusable glass spinner flasks.

Table 5. Expected Cell Yields and Recommended Working Volumes for Corning Spinner Flasks

Corning Spinner Flasks	Average Cell Yield*	Working Volume
125 mL Spinner Flask	1.25 x 10^8	100 to 125 mL
250 mL Spinner Flask†	2.5 x 10^8	125 to 250 mL
500 mL Spinner Flask	5.0 x 10^8	250 to 500 mL
1L Spinner Flask	1.0 x 10^9	0.5 to 1L
3L Spinner Flask	3.0 x 10^9	1.5 to 3L
6L Spinner Flask†	6.0 x 10^9	3.0 to 6.0L
15L Spinner Flask†	1.5 x 10^{10}	7.5 to 15L
36L Spinner Flask†	3.6 x 10^{10}	18 to 36L

*Assumes an average yield of 1 x 10^6 cells/mL from a culture used at maximum working volume.
†Only available in glass.

Figure 12. PYREX® glass Erlenmeyer and shaker flasks are available in a variety of sizes and closure styles.

Figure 13. Corning® 3L plastic Fernbach flasks have large vented caps for enhanced gas exchange and higher cell yields.

Erlenmeyer Style Shaker Flasks

W. R. Earle used Erlenmeyer flasks in 1954 to grow L929 cells in suspension. Today they are widely used on shaker apparatus to grow bacteria, fungi and plant and animal cells in suspension. They are especially useful for growing insect cell lines that have high oxygen requirements. Corning offers disposable plastic and reusable glass Erlenmeyer flasks in a variety of sizes (50 mL to 6L), styles and cap designs (Figures 12, 13, and Table 6). The plastic Erlenmeyer flasks are available with baffles to improve mixing and vented caps to increase gas exchange.

Advantages

- Very economical and compact
- Simple to sample and harvest
- Baffled Erlenmeyer flasks provide better gas exchange than spinner flasks
- Require much less labor than using dishes, flasks, roller bottles or multilayered chambers
- Easy to scale-up to 1L media volumes in a 3L flask

Disadvantages

- Only for suspension adapted cells
- Require shaker apparatus

Table 6. Expected Cell Yields and Recommended Working Volumes for Corning® Shaker Flasks

Corning Shaker Flasks	Average Cell Yield*	Working Volume
50 mL Erlenmeyer Flask†	2.0 x 10^7	15 to 20 mL
125 mL Erlenmeyer Flask	5.0 x 10^7	37.5 to 50 mL
250 mL Erlenmeyer Flask	1.0 x 10^8	75 to 100 mL
500 mL Erlenmeyer Flask	2.0 x 10^8	150 to 200 mL
1L Erlenmeyer Flask	4.0 x 10^8	300 to 400 mL
2L Erlenmeyer Flask	8.0 x 10^8	600 to 800 mL
3L Fernbach Flask	1.2 x 10^9	1.0 to 1.2L
4L Erlenmeyer Flask†	1.6 x 10^9	1.2 to 1.6L
6L Erlenmeyer Flask†	2.4 x 10^9	2.0 to 2.4L

*Assumes an average yield of 1 x 10^6 cells/mL from a culture used at maximum working volume.
†Only available in glass.

Selecting the Best System

The key to selecting the best of the above systems for your needs is to thoroughly understand and evaluate these five key areas:

- Attachment requirements
- Cell or product yields desired
- Equipment and space requirements
- Labor requirements and availability
- Investment in technology and expertise to operate (ease of use)

Once each of these five areas have been thoroughly evaluated and understood, selecting the system that best meets your needs will be much easier.

Matching the Culture System with the Attachment Requirements of the Cells

The first and most important step is to determine the attachment requirements of your cells. Some cell lines are attachment-dependent, that is, they can only grow when attached to a suitable substrate such as a flask or roller bottle. Many other cell lines, frequently designated as transformed cell lines, are able to grow either attached to a substrate or floating unattached in suspension; they are attachment-independent.

In general, if the cells allow it, it is always easier to grow large amounts of animal cells in suspension culture than in attached culture. Some cell lines used to produce genetically engineered proteins will also have higher yields when grown in suspension culture. If cells are attachment-dependent, it may be possible to adapt them to grow in suspension. However, even if the cells can be adapted to grow in suspension, they may not maintain the same characteristics or produce the same amount of products that they did when grown attached (Iyer et al.; 1999). It may also be necessary to screen the cultures after adaptation to select for high producers or the desired cell characteristics.

For short term projects, it may not be worth the effort to attempt to adapt attachment-dependent cells to grow in suspension. For longer term or ongoing projects, it is usually worth the effort to attempt to adapt cultures to grow in suspension.

Advantages of growing cells in suspension culture systems:

- Easier to sample but harder to feed
- Most economical for large amounts of cells
- Less labor intensive
- May provide better gas exchange than static monolayer systems
- Less space needed because cells grow in three dimensions versus two in monolayer culture

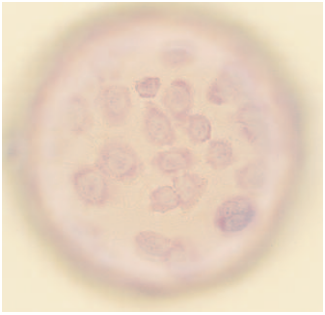

Figure 14. Attachment-dependent cells can also be grown in spinner flasks on microcarrier beads.

Another alternative for growing attachment-dependent cells in suspension is to use microcarriers in modified spinner flasks (Figure 14). Microcarriers are small (100 to 300 µm) sterile glass or polymeric beads to which the cells attach and grow while the beads are kept in suspension by the rotation of the paddle. Microcarriers do not work well with all cell types and have their own unique problems and requirements. They are beyond the scope of this guide; please refer to references (McLimans; 1979, Freshney; 2000) for information on using microcarriers.

The growth of cells attached to substrates, such as flasks and dishes, also offers some advantages, especially since this is the method used for growing most cell lines.

Advantages of growing cells in attached culture systems:

- Easier to feed but harder to subculture
- Easier to view cultures to check status
- Avoids physical stresses on cells caused by stirring or shaking which may lead to higher yields

Matching the Desired Cell or Product Yields with the Culture System

The next step is to calculate the amount of cells required for the project. In general, at least 1 x 10^5 cells/cm^2 can typically be produced when growing attached cells and 1 x 10^6 cells/mL or more can be produced in suspension (Table 8). Actual cell yields can be several times higher than this depending on the cell line and culture conditions. By optimizing all aspects of cell production, biopharmaceutical companies have achieved densities of 1 x 10^7 cells/mL

Table 8. Estimated Requirements* to Produce 1 x 10^9 (~1 gram) Mammalian Cells

Products (all disposable plastic)	Expertise Required	Vessels Required	Vessels Costs†	Equipment Costs	Labor Costs
225 cm^2 Flasks	Basic	44	$302	None	High
245 mm Dishes	Basic	20	$330	None	High
850 cm^2 Roller Bottles	Basic+	12	$87	$1500+	High
CellSTACK®-10 Chamber	Medium	2	$390	None	Medium
1720 cm^2 HYPER*Flask*™	Medium	6	$450	None	Medium
3L Shaker Flasks	Basic+	1	$48	$1,000+	Low
1L Spinner Flasks	Basic+	1	$124	$1,000+	Low
E-Cube™ System	High	1	$296	$3,000+	Low

* The above requirements are based on the assumption is that 1 x 10^5 cells/cm^2 can be produced when growing attached cells and 1 x 10^6 cells/mL when grown in suspension. The actual cell yields can be significantly higher than this depending on the cell line and culture conditions.
† Vessel costs are approximate and based on U.S. 2008 list prices.

or higher (Wurm; 2004). When producing cell-based products, running some preliminary tests will be required to determine product yields under the expected culture conditions. Protein yields of 10 mg/L are common but, as with cell yields, optimization can give much higher yields (Wurm; 2004). Based on these yields, it is then possible to calculate the total number of cells required. Larger amounts of cells can be produced by either increasing the number of culture vessels or by increasing the number of batches produced. For many research laboratories, producing multiple smaller batches may be easier and more practical in the long run. Taking time to optimize culture media and operating conditions can also significantly raise cell and product yields, especially for suspension cultures.

Evaluating Equipment and Space Requirements

Next determine how much incubator space and equipment is available in the laboratory. Some culture systems, such as spinner vessels, are far more compact than others. It may be possible to borrow incubator space from other laboratories especially if large walk-in warm rooms are available near your laboratory. Some culture systems also require additional equipment such as pumps, stirrers, shakers or roller apparatus. If this equipment is not available and cannot be borrowed, is there money in the budget to buy it?

Evaluating Labor Requirements and Availability

Next, determine who will be doing the culture work. Are people with the necessary cell culture skills available to do the required work and can they make the time commitment to see the project through to its end?

Evaluating the Required Investment in Technology and Expertise to Operate the System

How easy or user friendly is the system to use? This often depends on the experience and expertise of the people doing the work. Do the available people have the skills necessary to make the culture system work?

Making the Final Decision

By considering the key areas listed above with the advantages and disadvantages of each of the culture systems summarized in Tables 8 and 9 (page 10), you should now be able to choose the system or systems that will best meet your needs. Also keep in mind:

1. *Batch size*
   It may be easier and much more practical to produce the required number of cells in several smaller batches rather than one large batch.
2. *Practicality*
   The larger the capacity of individual vessels, the greater the loss from contamination or culture failure but larger vessels require less labor to set up, maintain and harvest.
3. *Simplicity*
   Keeping the process and cell culture system as simple as possible increases the likelihood of initial success (Griffiths; 1990).
4. *Monitoring*
   Frequently monitor culture status to ensure the cells are healthy and producing the expected yields. This is much easier to do in culture vessels where the cells can be directly examined using a microscope (dishes, flasks, roller bottles and small CellSTACK® Culture Chambers)
5. *Optimization*
   Time permitting, optimizing the basic areas – media, serum, seeding densities, feeding schedules – that impact the cultures the most will pay off in the long run.

Table 9. Comparison of Corning Systems for Scaling Up Cultures in a Research Laboratory Setting

Corning Vessel Type	Surface area (cm^2)	Recommended Working Volume (mL)	Advantages	Disadvantages
225 cm^2 Flasks	225	45-68	◗ Simple to use and harvest	◗ Very labor intensive ◗ Requires more space
245 mm Square Dishes	500	100-150	◗ Simple to use and harvest, easy scraping ◗ Saves on incubator space	◗ Very labor intensive ◗ Easy to spill from and contaminate (Spillguard system is available)
850 cm^2 Roller Bottles	850	170-255	◗ Simple to use and harvest ◗ Very economical	◗ Labor intensive ◗ Requires more space ◗ Requires roller apparatus
CellSTACK®-10 Chambers	6,360	1,300-1,900	◗ Reduces labor ◗ Easy to scale-up ◗ Saves on incubator space	◗ Difficult to see cells ◗ Liquid handling and harvesting are more difficult – requires expertise
HYPER*Flask*™ Vessels	1,720	560-565	◗ Very compact 10 layers ◗ Saves on incubator space ◗ 10-fold higher yields than 175 cm^2 flask but in same footprint	◗ Can only see 4 of the 10 layers with microscope ◗ Large medium volume makes handling more difficult
E-Cube™ System with 10-Stack CellCube® Modules	8,500	17,00-2,550	◗ Perfused bioreactor gives higher yields ◗ Reduces labor ◗ Easy to scale-up	◗ Requires more expertise to use – cannot view cells ◗ Requires pump
1L Spinner Flasks	NA	500-1,000	◗ Very economical and compact ◗ Simple to use, sample and harvest ◗ Reduces labor ◗ Easy to scale-up	◗ Only works with suspension-adapted cells ◗ Requires cleaning and autoclaving ◗ Requires magnetic stirrer and additional incubator space
3L Erlenmeyer Flasks	NA	500 - 1,000	◗ Simple to use, sample and harvest ◗ Very economical and compact ◗ Reduces labor	◗ Only works with cells that grow in suspension ◗ Requires shaker apparatus

Making It Work

This next section provides information to help optimize the culture process and increase your chances of success.

Media Issues

Use the Best Medium

While Eagle's original Minimal Essential Medium (E-MEM) is one of the most widely used media, it is often not the best for maximizing cell growth or production since it is a very simple "bare bones" formulation. For example it does not contain nonessential amino acids, such as proline which is required for growth of CHO cells. Richer media such as M199, F12, F12K, RPMI 1640, Dulbecco's Modified Eagle's Medium (D-MEM) or some of the fortified MEM formulations (i.e., Alpha MEM) will often work better and may require less serum supplementation. Simple side-by-side media and sera optimization experiments can be done with minimal effort and expense and may pay large benefits when it is time to scale-up (Mather; 1998a).

Adapting mammalian or insect cells to serum-free medium formulations may also be advantageous, especially if cell products need to be harvested from the medium and purified later. However, with some cell lines this conversion may require considerable time and effort. Several companies supply serum-free media customized to grow specific cell lines. Although more expensive than standard media, they can save a lot of time and effort when growing one of these cell lines. In addition, the savings obtained by replacing the fetal bovine serum should easily pay for the additional costs. Information on the availability and use of these media can be found on the Internet or by contacting media companies directly. The larger the scale-up effort the more sense it makes to optimize the media and culture conditions.

Control Culture pH

Many cell growth and attachment problems result when the culture pH is not properly maintained. These problems increase when using vessels with larger medium volumes because they take longer to reach pH equilibrium when initially set up or following medium changes. This problem is made worse when researchers do not supply the appropriate CO_2 levels required by the bicarbonate-based buffering system of the medium they are using, resulting in poor pH control. Media buffered with low levels of sodium bicarbonate, such as found in Eagle's Minimal Essential Media (MEM) buffered with Hanks' salts (0.35 g/L sodium bicarbonate), are designed for use in sealed (air-tight) culture vessels in incubators or walk-in warm rooms without elevated CO_2 levels. This buffer system is often used in large scale production using roller bottles or CellSTACK chambers since it does not require a CO_2 incubator.

However, most other bicarbonate-buffered media have higher levels of bicarbonate and require open culture vessels (dishes, plates or flasks with loose or vented caps) in a humidified incubator capable of maintaining 5% to 7% CO_2. Table 10 gives the bicarbonate levels found in some commonly used cell culture media. Usually the higher the level of sodium bicarbonate, the higher the level of CO_2 that must be supplied for optimum buffering capacity. There are also commercially available media formulations that do not use a bicarbonate-based buffering system to maintain pH levels; these may be useful for controlling pH when CO_2 incubators are not available.

Often, the above bicarbonate-based buffer systems are supplemented with the addition of HEPES (N-hydroxyethylpiperazine-N'-ethanesulfonic acid), a widely used organic buffer. The use of this buffer can lead to additional problems upon exposure of the medium to fluorescent light (see next section below).

Table 10. Bicarbonate Levels in Some Commonly Used Mammalian Cell Culture Media

Medium	Sodium bicarbonate levels (g/L)	Extra CO_2 needed
Eagle's Minimal Essential Medium (MEM) with Earle's salts	2.2	Yes
Eagle's MEM with Hanks' salts	0.35	No
Medium 199 with Earle's salts	2.2	Yes
Medium 199 with Hanks' salts	0.35	No
Alpha MEM Medium	2.2	Yes
Dulbecco's Modified Eagle's Medium (DMEM)	3.7	Yes
DMEM/F12	1.2 to 2.438	Yes
Ham's F12	1.176	Yes
MCDB 131 Medium	1.176	Yes
McCoy's 5A	2.2	Yes
RPMI 1640	2.0	Yes
CMRL 1066 Medium	2.2	Yes
Leibovitz's L-15 Medium	None	No

Avoid Media Exposure to Fluorescent Light

The deleterious effect of fluorescent light on culture media (with or without cells) is an often overlooked source of scale-up growth problems or complete culture failure. It is very important to store media and grow cells in the dark away from sources of fluorescent light (walk-in warm or cold rooms) 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).

Prewarm Medium

Always prewarm the medium when initially seeding cells into culture vessels, especially when using larger vessels, roller bottles or multilayer CellSTACK® chambers. The longer it takes for the medium to reach growth temperature, the longer it takes for cells to attach. Slow attachment can lead to uneven and poor growth problems especially in roller bottles. See *Help Cells Attach Quickly* (page 14) below for additional information on cell attachment problems.

Feed Cultures Appropriately

For optimum cell growth, change culture medium when necessary. Unfortunately, there is no single feeding regimen that works for all cell types. Since growing large amounts of cells requires large amounts of medium, feeding too often can be a waste of time, money and effort. However, saving money by scrimping on medium is usually counterproductive; it is better to over feed and have happy cells. It is worthwhile to run simple experiments in smaller culture vessels comparing different feeding strategies, for example, changing the entire medium every three days or changing half the medium every two days. Glucose monitoring kits and devices are available that can be used to check glucose levels and then change the medium when the glucose levels drop below a certain point. For cells that are being used to produce products, it is sometimes possible to feed with serum-free medium once the cells have reached confluency without affecting product yields.

Liquid Handling Issues

Figure 15. Corning aseptic transfer caps for spinner flasks offer a much safer alternative to pouring or pipetting when transferring large amounts of cells or medium.

Use Good Aseptic Technique

Culture contamination, especially by mycoplasma, is probably the single most frequent source of scale-up problems and failure (Lincoln and Gabridge; 1998). This problem is aggravated by the technical difficulty of aseptically transferring the larger volumes of sterile media and solutions required by many larger scale culture systems. The largest pipets only deliver 100 mL. As a result, pouring is often used but it can be messy and increases the likelihood of contamination. Aseptically pumping media through sterile tubing sets with aseptic connectors or filling bells is a better alternative to pouring. These tubing sets can be connected to media bags for quickly filling culture vessels with peristaltic pumps or by gravity. Corning makes a variety of liquid transfer accessories, including tubing sets for use in Corning® CellSTACK Culture Chambers and spinner flasks (Figure 15).

Although the problem of cell culture contamination is beyond the scope of this article, Corning offers a detailed technical brochure *Understanding and Managing Cell Culture Contamination* available on the Corning Life Sciences web site. This 23-page guide examines the problems and causes of culture loss due to contamination and explores some of the important strategies for preventing these losses by careful culture management.

Figure 16. Corning® 500 mL disposable centrifuge tubes are ideal for processing large amounts of cell suspensions.

Centrifuge Gently

Centrifugation is frequently used to separate cells from culture media or dissociating solutions. This process should always be done as quickly and gently as possible to form a soft cell pellet that is easy to resuspend without damaging the cells. Usually spinning cell suspensions for 5 minutes at 50 to 100 x g or RCF (Relative Centrifugal Force) is enough to sediment most cells without damaging them. In addition to the widely used 15 and 50 mL plastic centrifuge tubes, Corning also offers disposable plastic 250 mL and 500 mL V-bottom centrifuge bottles (Catalog Nos. 430776 and 431123) for centrifuging large media volumes (Figure 16).

Suspension Culture Issues

Reduce Shearing Damage

Because animal cells lack a protective cell wall, they can be easily damaged by the shear forces that develop if they are spun too fast or shaken too vigorously. Some cell lines, especially those grown in serum-free media, are very sensitive to shear forces and a difference of only 30 to 50 RPM in mixing speeds can lead to serious growth problems. For many cell

lines, shaking them is often gentler than stirring them and is a better approach when trying to suspension-adapt cells. For shaker flasks, Corning recommends starting with a shaking rate of 75 to 150 RPM on an orbital shaker with a medium volume of 30 to 40% of the nominal flask capacity (1L of medium in a 3L flask). For Corning® spinner flasks a starting speed of 50 to 150 RPM is suggested. The use of baffled spinner and shake flasks can reduce the required mixing rates considerably.

Some cell types, such as insect cells, require higher oxygen levels and may benefit from more vigorous mixing and the use of vented flasks or continuous gassing. It is strongly recommended to empirically determine the optimum stirring or mixing conditions. Start by choosing the lowest speed that appears to give an even cell distribution from the top to the bottom of the flask. However, to get adequate gassing, higher speeds may be necessary, Shearing damage from these higher speeds can be reduced by increasing the medium viscosity by adding carboxymethylcellulose (1 to 2%), BSA (100 µg/mL) or Pluronic® F-68 (0.1%) to the medium (Mather, 1998b). This is especially important when using reduced serum or serum-free medium.

Avoid Cell Clumping and Sticking

Some cell lines tend to form large clumps when grown in suspension. These larger clumps tend to settle to the bottom of the flask or may attach to the flask side walls and can result in lower cell viability and growth. Using a calcium-free medium (Joklik's MEM; S-MEM) will reduce cell clumping as these divalent cations are very important in cell to cell binding (McLimans; 1979). Coating the surface of glass suspension flasks with siliconizing solutions before sterilization will reduce cell clumps forming on the flask surface. Sigmacote®, AquaSil™, and Siliclad® are some commercially available siliconizing solutions suitable for cell culture applications. It is important to carefully follow the use and safety directions for these products to avoid culture toxicity.

Use Appropriate Seeding Densities

Using the correct initial seeding density is very important when growing cells in suspension. It is always better to add too many cells rather than too few. Start with a seeding density of 1×10^5 to 5×10^5 cells/mL; the higher concentration is better when cells are adapting to serum-free conditions. An alternative approach for spinner and shake flasks is to start with half the normal volume of medium. This reduces the number of cells required to reach the optimum seeding densities by 50%. After the cells are actively growing (after 24 to 48 hours), additional medium can be added to bring the vessel to its final operating volume.

Avoid Overheating Cultures

Flask cultures placed directly over magnetic stirrers or shaker motors may overheat as the result of excessive heat transfer from the motors to the flasks. In walk-in warm rooms this may affect only flasks placed directly over the motor but in small incubators it may cause the entire incubator to overheat. Check for this problem in advance by placing identical culture flasks filled with water in the incubator and monitoring the temperature for at least 48 hours. Sometimes heat transfer from the stirrer to the flask can be reduced by elevating the flask a few millimeters above the stirrer surface so that air can flow beneath it. Also make sure that any stirrers or shakers used in humidified CO_2 incubators are designed to withstand the corrosive atmosphere. Placing shakers in incubators will usually generate vibrations which may prevent cells from attaching to culture vessels in the same or adjoining incubator chambers.

Keep the Cells Happy

Check cell number and viability daily (Acton et al.; 1979). Keep suspension cell densities of mammalian cultures to 1×10^6 cells/mL or lower by dilution or harvesting until a growth curve has been done for the culture to determine the maximum density that can be supported by the system being used. Higher density cell populations (2×10^6 cells/mL and above) are often hard to maintain in good health and can crash suddenly. Cultures should be split or harvested while their viability is still high (above 90%). Adjust pH as necessary by adding

sterile bicarbonate solution to raise the pH if the medium is acidic (yellow) or by gassing with 5% carbon dioxide if the medium is alkaline (purple). Some insect cell lines routinely achieve densities of over 1 x 10^7 cells/mL in suspension culture especially if provided with adequate gas exchange.

The viability and growth rates of suspension cultures, especially at higher densities, can greatly benefit by periodic medium changes. Cultures can be fed by withdrawing 50% to 100% of the cell suspension, spinning down the cells in centrifuge bottles, discarding the old medium and resuspending the cell pellets in an equal volume of fresh medium which is then added back to the culture. Corning offers plastic disposable 250 mL and 500 mL centrifuge bottles (Corning Cat. Nos. 430776 and 431123) to make centrifuging large media volumes easier (Figure 15).

Attachment-Dependent Culture Issues

Use Appropriate Seeding Densities

Starting with the correct initial seeding density is also very important when growing attachment-dependent cells. It is always better to add too many cells than too few; seeding densities can always be lowered later. Start with a seeding density of 1 x 10^4 to 2 x 10^4 cells/cm^2; the higher concentration is better when using difficult to grow cells or cells are adapting to serum-free conditions (Behie et al.; 2004).

Figure 16. Corning CellBIND Surface can improve cell attachment especially under low serum or serum-free conditions.

Help Cells Attach Quickly

Cell attachment problems are often a serious problem, especially when growing cells in reduced- or serum-free medium. Prewarming the medium used for the initial cell seeding and pregassing larger culture vessels so the medium will reach its correct pH sooner will help cells attach more quickly. Pregassing larger vessels before seeding is highly recommended and should be done with filtered medical grade 5% CO_2/95% air mixtures.

For cells that have attachment problems on traditional cell culture vessel surfaces, Corning recommends trying the patented Corning® CellBIND® surface on flasks, roller bottles and CellSTACK® Culture Chambers (Figure 16). This surface is created by a novel microwave plasma process that improves cell attachment by incorporating significantly more oxygen into the cell culture surface than traditional plasma or corona discharge treatments, rendering it more hydrophilic (wettable) and increasing the stability of the surface.

Corning also offers a detailed technical brochure *Guide for Identifying and Correcting Common Cell Growth Problems* on the Corning Life Sciences web site. This 14-page guide reviews some of the common and not so common cell growth and attachment problems, such as incubator issues, that are often very difficult to identify and eliminate.

Figure 17. As these crystal violet stained roller bottles show, rotating bottles too quickly (left bottle) results in poor cell attachment and growth when compared to a bottle rotated at a slower speed (right).

Rotate Roller Bottles Slowly

The constant movement of the medium across the surface of the bottle, slow though 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 (Figure 17). A recommended starting speed for initiating roller bottle cultures is 0.5 to 1.0 revolutions per minute (rpm) to start. However, if cells have difficulty attaching (or staying attached), slower speeds (0.1 to 0.4 RPM) should be used until the cells are attached (Clark et al.; 1990).

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 are magnified and clearly stand out. Using prewarmed medium and pregassing the bottles with CO_2 so that pH shifts are minimized when inoculating cells will make it easier for the cells to quickly attach.

Corning also offers a detailed technical brochure *Roller Bottle Selection and Use Guide* on the Corning Life Sciences web site. 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.

Keep the Cells Happy

Maintaining optimal cell to medium ratios is important for obtaining good cell growth. As a starting point, use 0.2 to 0.3 mL medium for each square centimeter of culture vessel growth surface area (i.e., 5 to 7.5 mL for a 25 cm^2 flask). Using more medium may reduce the need for feeding (changing the medium) in the cultures but, due to the increased medium depth and the static nature of the environment, will also slow the diffusion of oxygen to the cells. (See also Feed cultures appropriately, page 11.) Sometimes gassing the cultures will increase cell yields and viability. This is not practical on dishes, flasks and roller bottles because of the large number of vessels involved but is useful with CellSTACK chambers.

It is also important to subculture cells before they reach confluency to keep them actively growing and healthy. In addition, epithelial-like cells will often form very strong cell-cell bonds at confluency making them much harder to remove from the substrate when sub-culturing them.

Harvest Cells Gently

Don't over dissociate cells when subculturing or harvesting. Too long exposure to harsh dissociating agents can reduce viability and make it very difficult for cells to reattach. This often occurs when attempting to harvest too many vessels simultaneously. It is better to harvest vessels a few at a time rather than attempt to harvest many at once. If using serum-free medium, make sure the dissociating agents are either inactivated or removed by gentle centrifugation. Keep harvested cells chilled until ready to reseed new vessels. This will maintain viability and reduce cell clumping. There are a variety of dissociating enzymes and agents available; experimenting with different combinations may improve both harvesting efficiency and cell viability (Freshney; 2000).

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