Insect Cell Lines

Version K
July 12, 2002
25-0127

Growth and Maintenance of Insect Cell Lines

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Important Information

Shipping and Storage

Shipping

- Cells are shipped on dry ice.

Storage/Passaging

- Store cells in liquid nitrogen. To initiate cell culture immediately upon receipt, see page 10.

Limited Use Label License No. 66 High Five™ Cells

The High Five™ cell line is patented by the Boyce Thompson Institute for Plant Research, Ithaca, New York, and is covered under U.S. patent no. 5,300,435. The High Five™ cell line is sold for research purposes only. Commercial use requires a license from Boyce Thompson. For more information, please contact: Joyce L. Frank, Tel: 607-254-1220; Fax: 607-254-1242; E-mail: jf51@cornell.edu.

Product Qualification

Sf9, Sf21, and High Five™ insect cells are qualified using the following criteria:

- The cells must be in logarithmic growth with 98% viability before they are frozen
- Cells must demonstrate that they can be recovered as healthy logarithmically growing cells within 2 to 3 days after thawing

Introduction

Cell Lines

Introduction

This manual is included with Sf9, Sf21, and High Five™ insect cells and provides general information about the growth and maintenance of insect cell cultures. The Sf9, Sf21, and High Five™ cell lines are suitable for use in expressing recombinant proteins with baculovirus and other insect expression systems (e.g. InsectSelect™ System).

Comparison of Cell Lines

The following table summarizes some general characteristics of the cell lines available from Invitrogen. For ordering information, see next page.

Cells	Doubling Time	Cell Appearance	Initial Medium to Use
Sf9	72 hours	Spherical with some granular appearance - regular in size. Firm attachment to surfaces	complete TNM-FH
Sf21	24 hours	Spherical with some granular appearance - different sizes. Firm attachment to surfaces	complete TNM-FH
High Five™	18 hours	Spherical with some granular appearance – different sizes. Loose attachment to surfaces	Express Five® SFM

Sf9 and Sf21 Cell Lines

Origin: Sf9 (Catalog no. B825-01) and Sf21 (Catalog no. B821-01) cell lines are the traditional cell lines used with baculovirus and originated at the USDA Insect Pathology Laboratory. The cell lines are also suitable for use in the InsectSelect™ System. These two cell lines originated from the IPLBSF-21 cell line, derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda* (O'Reilly *et al.*, 1992; Vaughn *et al.*, 1977).

Characteristics: Sf9 and Sf21 share the following characteristics:

- Grow well in monolayer and suspension culture
- Adaptable to serum-free medium

Size Differences: The Sf9 cell line is a clonal isolate of IPLBSF21-AE (Sf21) (O'Reilly *et al.*, 1992). The small, regular size makes them exceptional for the formation of monolayers and plaques. Sf21 cells are somewhat more disparate in size and form monolayers and plaques which are more irregular.

Uses: Both cell lines are suitable for transfection, plaque purification, generating high-titer stocks, plaque formation, and expression of recombinant proteins. If you are a first-time user of baculovirus, you may find it easier to use the Sf9 cells to isolate recombinant plaques. Sf21 cells may express more protein than Sf9 cells with some constructs (Hink *et al.*, 1991).

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Characteristics of High Five™ Cell Line

Origin: The High Five™ cell line (BTI-TN-5B1-4, Catalog no. B855-02) was developed by the Boyce Thompson Institute for Plant Research, Ithaca, NY and originated from the ovarian cells of the cabbage looper, *Trichoplusia ni* (Davis *et al.*, 1992; Granados *et al.*, 1994; Wickham *et al.*, 1992; Wickham and Nemerow, 1993).

Characteristics: This cell line has the following characteristics:

- Doubles in less than 24 hours
- Grows well in adherent cultures, but forms irregular monolayers, thus making plaques more difficult to identify
- Adaptable to suspension culture and serum-free medium
- Provides 5-10 fold (for selected proteins) higher secreted expression than Sf9 cells (Davis *et al.*, 1992)

Uses: High Five™ cells are excellent for expressing recombinant proteins. They can also be used for transfection and plaque purification; however, isolation of recombinant plaques may be difficult if recombinants are not blue.

Insect Expression Systems

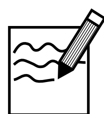
A number of insect expression systems are available from Invitrogen to facilitate expression of recombinant proteins in Sf9, Sf21, or High Five™ cells. The Bac-to-Bac® Baculovirus Expression System allows recombinant protein expression using baculovirus while the InsectSelect™ System allows protein expression using a non-lytic system. For more information about these expression systems, see our Web site (www.invitrogen.com) or call Technical Service (see page 32).

Which Cell Line is Best to Use?

We recommend Sf9 or Sf21 cells for transfection, purification, and amplification of recombinant virus. Sf9 cells are regular in size, easy to manipulate, and form good monolayers for plaque assays. Sf9 and Sf21 cells can also be used for expression of recombinant proteins, but the High Five™ cell line may produce higher levels.

We recommend the High Five™ cell line for expression of secreted recombinant proteins. They are grown in serum-free medium, adaptable to suspension culture, and produce high levels of recombinant protein (Davis *et al.*, 1992).

Note: Generally it is easier to use one cell line for procedures up to optimization of protein expression. Once you have confirmed expression of your recombinant protein, other cell lines can be tried for optimization of expression levels.



Note

When designing a scheme for purification of polyhistidine-tagged recombinant proteins, note that serum-free media cannot be applied directly to a metal-chelating resin (e.g. ProBond™ from Invitrogen) because media components will strip the nickel ions from the resin.

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Cell Lines, continued

Ordering Information for Cell Lines

See the table below for ordering information for frozen cell lines available from Invitrogen.

Cells	Frozen
Sf9	**Catalog no. B825-01** 1 x 10^7 cells in 1 ml 60% Grace's Media, 30% FBS, and 10% DMSO
Sf21	**Catalog no. B821-01** 1 x 10^7 cells in 1 ml 60% Grace's Media, 30% FBS, and 10% DMSO
High Five™	**Catalog no. B855-02** 3 x 10^6 in 1 ml 42.5% fresh Express Five® SFM, 42.5% conditioned Express Five® SFM, 5% FBS, and 10% DMSO

Insect Culture Media

Sf9, Sf21, and High Five™ cells can be grown in serum-required or serum-free media (see **Media Considerations**, page 8, for more information). We recommend the following media for each cell line (see table below).

Cells	Serum-Required Medium	Serum-Free Medium
Sf9	Grace's Insect Medium	Sf-900 II SFM
Sf21	Grace's Insect Medium	Sf-900 II SFM
High Five™	Grace's Insect Medium	Express Five® SFM

Ordering Information for Media

GIBCO® insect culture media are available from Invitrogen to culture Sf9, Sf21, and High Five™ insect cells. See the table below for ordering information.

Item	Amount	Catalog no.
Grace's Insect Medium, Unsupplemented	500 ml	11595-030
Grace's Insect Medium, Supplemented	500 ml	11605-094
Sf-900 II SFM	500 ml	10902-096
	1 L	10902-088
Express Five® SFM	500 ml	10486-017
	1 L	10486-025

Methods

Cell Handling Techniques

Introduction

The following terms and techniques are referred to throughout this manual. Take a moment to review them.

Sterile Technique

All handling of insect cell lines should be carried out under sterile conditions in a laminar flow hood.

Passaging/ Subculturing

Passaging/subculturing refers to diluting cells back to a density that maintains log phase growth and maximum viability.

Adherent Cultures: Adherent cultures should be passaged at confluency (see below for definition) and are typically diluted at a 1:5 dilution (volume of cells: final volume of medium) in order to maintain log phase growth.

Example: A 75 cm^2 flask containing 12 ml of medium is at confluency. Cells are dislodged into the 12 ml of medium. Two ml of the medium containing cells are transferred into another 75 cm^2 flask containing 8 ml of medium for a 1:5 dilution (2 ml of cells in 10 ml final volume).

Suspension Cultures: Suspension cultures should be passaged before they reach a density of 2.0 to 2.5 x 10^6 cells/ml and diluted back to 0.7 to 1.0 x 10^6 cells/ml.

Example: A 100 ml spinner containing 50 ml of cell culture is at a density of 2 x 10^6 cells/ml. Remove 25 ml of medium and cells, replace with 25 ml of fresh complete medium for a final density of 1 x 10^6 cells/ml.

Confluency

Confluency is a marker for when to subculture your cells.

Definition: A confluent monolayer is an adherent cell culture (dish, plate or flask) in which the cells have formed a single layer over the entire surface area available for growth. Once the cells have started to form clusters above the first layer or have started to lift up from the surface, the culture is past confluency.

Passaging past confluency: Cells that are repeatedly passaged at densities past confluency display decreased doubling times, decreased viabilities, and a decreased ability to attach. The culture is considered to be unhealthy.

Passaging before confluency: Cell cultures that have not reached confluency are more difficult to dislodge, and require more mechanical force to dislodge them from the monolayer. When repeatedly subcultured before confluency, cells display decreased doubling times and decreased viabilities (for more information, see **Troubleshooting Guide**, page 26. The culture is considered to be unhealthy.

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Floaters

Floaters are a normal occurrence and are often seen in older cultures and cultures which have overgrown.

Definition: Cells that are either loosely attached or suspended in the medium.

Note: If floaters constitute more than 5% of the culture, remove the old medium containing the floaters and replace with fresh medium before subculturing.

Viable floaters: Many floaters may still be viable. To check viability, remove a small aliquot of medium containing floaters and assay for trypan blue exclusion (see protocol on page 23). If the viability of floaters is high (>95%), the cells may be propagated by transferring the medium containing floaters to a new appropriately sized flask containing fresh medium.

Sloughing

This subculturing method is very gentle and results in high cell viabilities. We typically use this method to dislodge adherent cell cultures.

Definition: To dislodge cells from a surface by streaming medium over them. The protocol for this technique can be found in **Adherent Cell Culture**, page 13.

Doubling Time

Population doubling times for insect cells will vary depending on growth conditions.

Healthy Doubling Times:

- Sf9 cells double every 72 hours
- Sf21 cells double every 24 hours.
- High Five™ cells double every 18-24 hours.

Note: If cell doubling time exceeds 24-72 hours, there may be a problem with the cell seeding density, viability, medium, temperature, or oxygenation. For more information on what to do if doubling times exceed 24 hours, see **Troubleshooting Guide**, page 26.

Viability

Cell viability should be regularly assessed during cell counts in order to maintain optimal adherent and suspension cultures.

Definition: Cell viability refers to the percent of cells in a culture that are living. Cell viability is determined by treating the cells with trypan blue (see page 23). Trypan blue dye molecules are excluded from viable cell membranes, but readily enter non-viable cells. Cells that are blue are dead.

Minimum Requirements: Cell viability should be at least 95% for healthy log-phase cultures. Cells below 95% viability are not growing under optimal conditions and should not be used in experiments. See **Troubleshooting Guide**, page 26 if your cell viability is below 95%.

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Cell Log

It is very important to monitor the density and viability of your insect cell culture. Keeping a cell log will enable you to record the data necessary to make decisions about your cell culture. A cell log is also a very helpful troubleshooting tool (see page 25).

Definition: A cell log or cell notebook should include the following information:

- date of initiation of culture
- lot number of original shipment
- dates of passage
- passage number at each passage
- densities at passage
- viabilities at passage
- passage number at freeze down
- any notes or comments on cell appearance
- medium and medium lot number

Growth Temperature

Cells should be maintained at 27°C in a non-humidified environment. Fresh cell culture medium should be equilibrated to room temperature before use. Cells can be maintained at room temperature on the bench top or in a drawer, however, a 27°C controlled environment is recommended.

Below 27°C: Insect cells will grow more slowly below 27°C. They will recover and resume normal doubling times (see above) once the temperature is returned to 27°C.

Above 27°C: Insect cells will begin to show increased doubling times at temperatures between 27°C and 30°C. Above 30°C, cells may display decreased viabilities. Cells under prolonged exposure to temperatures above 30°C should not be used. They will not recover if the temperature is returned to 27°C.

CO_2

CO_2 exchange is not required for insect cell culture.

Concentrating Cells

Cells can be concentrated if their density is too low (less than 5 x 10^5 cells/ml) to support log phase growth. Concentrating cells to a higher density (1 x 10^6 cells/ml) will induce log phase growth (see page 23).

Cell Dispersion

Prior to performing transfections and plaque assays, cells need to be evenly distributed over the surface of a tissue culture plate.

Purpose: This ensures that:

a) cells do not distribute unevenly, leading to asymmetric monolayers.
b) maximum cell surface area is available for infection.

Procedure: To disperse cells, rock the flask or plate slowly by hand forward and backward, then side to side. Do this four times, watching carefully to be sure the liquid reaches all areas of the growth surface. Do not use a circular motion to disperse cells as this causes a concentration of cells around the edges of the plate rather than an even distribution.

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Cell Handling Techniques, continued

Important

All addition and removal of medium should be done in a laminar flow hood, using sterile conditions and equipment.

Addition and Removal of Medium - Flasks

When **removing** medium from a **flask** of cells, the flask should be tilted so that all the medium flows to one corner, away from the cell monolayer. Medium should be carefully removed with a pipet. Avoid touching the cell monolayer.

When **adding** medium to a **flask**, gently pipette the medium down the side of the flask away from the cell monolayer. Treat the cells gently as they are loosely adherent and some may dislodge.

Addition and Removal of Medium - Plates

When **removing** medium from a **plate** containing a cell monolayer, the plate should be tilted at a 45°C angle so that medium flows to one edge. Aspirate the medium completely and very carefully using a Pasteur pipette.

When **adding** medium to a **plate**, carefully and slowly add the medium against the side edge of the plate. Allow the medium to cover the entire surface of the plate. Be careful not to dislodge cells.

Addition and Removal of Medium - Spinners

When **removing** medium from a **spinner**, remove the screw cap from one spinner arm. Carefully insert pipette without touching the flask and remove medium from the culture. Carefully remove pipette from the arm of the spinner without touching the sides or dropping any medium. Change pipettes each time one is inserted into the spinner and then removed.

When **adding** medium to a **spinner**, remove the screw cap from one spinner arm. Carefully insert pipette without touching the flask and add medium. Carefully remove pipette from the arm of the spinner without touching the sides or dropping any medium. Change pipettes each time one is inserted into the spinner and then removed.

Media Considerations

Introduction

The following information will provide you with background on the media used in insect cell culture.

Working With Baculovirus

When working with recombinant or wild-type viral stocks (e.g. infecting cells), always maintain separate media bottles for cell culture and for virus work. Baculovirus particles can survive and be maintained in media at +4°C and will contaminate your cell cultures if added to plates or flask during passaging.

Grace's Insect Medium / TNM-FH

- **Grace's Insect Medium:** Grace's Insect Medium, Unsupplemented is available separately from Invitrogen (Catalog no. 11595-030). **Note:** This medium contains L-glutamine.
- **Grace's Insect Medium, Supplemented:** A supplemented form of Grace's Insect Medium is available separately from Invitrogen (Catalog no. 11605-094). The additional supplements are TC yeastolate and lactalbumin hydrolysate. This supplemented medium is referred to as **TNM-FH** (*Trichoplusia ni* Medium-Formulation Hink).
- **Complete TNM-FH:** TNM-FH is not considered to be a complete medium without the addition of 10% fetal bovine serum (FBS). Serum provides additional nutrients and also protects the cells from hydrodynamic stresses in spinner culture.
- **Serum Sensitivity:** Serum does not need to be heat-inactivated prior to use in insect cell culture. However, the quality of serum is important for optimal cell growth. Serum from different vendors or different lots from the same vendor can vary in their ability to support optimal cell growth. We strongly recommend that you test a small aliquot of a new lot before using it with all of your insect cell cultures. For more information on serum sensitivity, see **Troubleshooting Guide**, page 26.
- **pH:** TNM-FH and Grace's medium do not contain pH indicators. The normal pH for Sf9 cells in this medium is 6.2. Unlike mammalian cell cultures, the pH rises gradually as the cells grow, but usually does not exceed pH 6.4.
- **Stability:** Complete TNM-FH medium is stable for 1 month at +4°C.
- **Antibiotics:** Many antibiotics are suitable for use with insect cells. We routinely use 10 µg/ml gentamycin in the medium of our stock cultures. The following table summarizes some of the most commonly used antibiotics, their working concentrations, and their methods of action.

Antibiotic	Working Concentration	Method of Action
Gentamycin	10 µg/ml	Inhibits bacterial protein synthesis
Amphotericin B (Fungizone)	0.25 µg/ml	Binds sterols and interferes with membrane permeability
Penicillin-Streptomycin	100-200 U/ml 100 µg/ml	Inhibits bacterial cell wall synthesis Inhibits bacterial protein synthesis

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Media Considerations, continued

Preparation of Complete TNM-FH Medium

If you are using Sf9 or Sf21 cells, prepare complete TNM-FH medium. If possible, prepare the medium just prior to use. Complete TNM-FH medium may be stored at +4°C for up to 1 month if it does not become contaminated.

1. To 500 ml of **Grace's Insect Medium, Supplemented,** add 55 ml of FBS and 500 µl of a 10 mg/ml stock of gentamycin. Mix well by pipetting.
2. Filter this solution through a 0.2 µm filter into a sterile container.
3. Store complete TNM-FH medium at +4°C.
4. Equilibrate to room temperature before use.

Important

Grace's Medium will not support growth without the additional supplements and serum.

Serum-Free Media

We recommend the use of Sf-900 II SFM for Sf9 and Sf21 cell lines and Express Five® SFM for the High Five™ cell line.

- **Supplementation:** Serum-free media are complete without any supplementation or the addition of serum. However, serum can be added at 2% to decrease proteolysis during infections.
- **Surfactants:** Many serum-free media contain surfactants such as Pluronic® F-68 to decrease membrane shearing in suspension culture**. Note:** Both Sf-900 II SFM and Express Five® SFM contain surfactants. Additional surfactants do not need to be added.
- **Adaptation:** We recommend that cells growing in serum-containing medium be adapted to serum-free conditions, rather than simply transferred into a new medium without serum. This gradual adaptation protocol (see page 24) will ensure optimal cell growth.
- **Benefits:** There are a few important benefits to using serum free medium:

  1) Decreases cost as serum can be expensive.

  2) Simplifies purification of secreted recombinant proteins.

  3) Eliminates issues of serum sensitivity.

Preparation of Serum-Free Media

Follow manufacturer's instructions when using serum-free media. In general, there is no additional preparation required for serum-free media as they are supplied as complete media.

Note that Express Five ® SFM does not contain L-glutamine. To 500 ml Express Five ® SFM, add 45 ml of 200 mM L-glutamine (Catalog no. 25030-081) prior to use.

We recommend the addition of Gentamycin at 10 µg/ml. The addition of other antibiotics is optional (see Table, page 8).

Pluronic® is a registered trademark of BASF Corporation

Initiation of Cell Culture from Frozen Stock

Introduction

The following protocols will enable you to initiate a culture from a frozen stock of the cell line(s) you have chosen. Insect cells from Invitrogen (Sf9 and Sf21) are frozen down at a passage number less than 12.

Experimental Outline

The table below outlines the steps needed to initiate a culture of insect cells from frozen stock.

Step	Action
1	Remove cells from liquid nitrogen or dry ice (if just received)
2	Thaw cells into desired medium
3	Let cells attach for 30-45 minutes
4	Remove medium (with DMSO) and add fresh medium
5	Grow to confluency and passage
6	Subculture until cells are doubling every 18-24 hours and are 95% viable
7	Freeze down several vials of low passage cells as backup

Thawing the High Five™ Cell Line

We recommend that High Five™ cells be thawed into Express Five® SFM for the best results. However, there are many different formulations of serum-free insect media available. If you have a different serum-free medium or wish to culture the High Five™ cells in complete TNM-FH, you can thaw them directly into the new medium. It is generally safe to change over to a new formulation of medium by thawing directly into it. Note that the cell viability may drop and the growth rate will be slow until the cells have adapted to the new medium. Another alternative is to thaw and culture the High Five™ cells in Express Five® SFM, then adapt the cells to the new medium as described on page 24.

Initiating Culture with Frozen Cells

Thawed cells do not always appear round; some may be amorphous or have a wrinkled appearance. There may be a significant portion of the cells that have lysed or are floaters. The cellular debris and floaters will be eliminated through successive rounds of subculturing.

The freezing process can damage cells, resulting in a 20-25% mortality rate upon thawing. For example, if 1×10^7 cells are frozen, 7.5 to 8×10^6 cells will be recovered.

1. Remove vial of cells from dry ice or liquid nitrogen (if they were stored) and place in a 37°C water bath. Thaw rapidly with gentle agitation until cells are **almost** thawed. Remove cells from the waterbath. **Leaving cells at 37°C after they have thawed will result in cell death.**
2. Quickly decontaminate the outside of the vial by treating with 70% ethanol. Dry the vial, and place on ice.
3. Pre-wet a 25 cm^2 flask by coating the adherent surface with 4 ml complete TNM-FH.
4. Transfer the 1 ml cell suspension directly into the 4 ml of medium.
5. Transfer flask to a 27°C incubator and allow cells to attach for 30-45 minutes.

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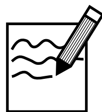
Initiation of Cell Culture from Frozen Stock, continued

Initiating Culture with Frozen Cells, continued

6. After the cells are attached, gently remove the medium. This must be done as soon as the cells have attached to remove the DMSO from the freezing medium. This step will also remove cellular debris and unhealthy cells that do not adhere.
7. Feed cells with 5 ml of fresh medium.
8. After 24 hours, change the medium. Viability of the cells should be greater than 70% when revived in this manner. Continue to incubate until cells have formed a confluent monolayer. At this point, they should be subcultured. Proceed to **Adherent Cell Culture**, page 13.

   If the viability of the cells does not appear to be 70% or greater, let cells sit again overnight at 27°C. If more cells continue to detach after another 24-48 hour incubation, call Technical Service (see page 32).



Note

Note that frozen High Five™ cells are supplied in 42.5% fresh Express Five® medium, 42.5% conditioned Express Five® medium, 10% DMSO, and 5% FBS. We have found that including serum helps the cells attach to the culture dish. After thawed cells have attached to the bottom of the culture dish (see previous page for protocol), remove the medium and replenish with Express Five® SFM.

Adherent vs. Suspension Culture

Introduction

The information in this section will help you to decide whether to maintain your insect cell culture as an adherent or suspension culture. The method you use for culturing cells will depend on your specific needs and laboratory setup. For protocols regarding maintenance of adherent cultures see page 13. For protocols regarding maintenance of suspension cultures, see page 15.

Adherent Cultures

The following table summarizes some important factors.

Pros	Cons
Easy and inexpensive to maintain.	Mechanical manipulations required for subculturing may decrease viability.
Allows easy visual inspection under inverted microscope to follow infection course.	Requires multiple flasks (e.g. several 150 cm^2 flasks) for large scale expressions and high-titer stocks.
All cell lines can be maintained with this method (Sf9, Sf21, High Five™).	Limited cell density/ml due to monolayer. This may limit protein yields/ml of cell culture.

Suspension Cultures

The following table summarizes some important factors.

Pros	Cons
Can generate from 250 ml to greater than 1 liter of high-titer stock or expressed protein expression per spinner flask. Facilitates scale up of protein expression.	Requires spinners, spin plates, incubator - can be expensive to start up.
Higher cell densities/ml (up to 3 x 10^6 cells/ml for Sf9 cells). This can increase protein yields per ml of cell culture.	Requires daily cell counts and viability determinations to follow growth patterns and infection courses.
Greater oxygenation and minimal manipulation may increase viability. Cell viabilities are typically 98% or greater.	Can be difficult to maintain sterility.
Sf9 and Sf21 cells require no adaptation and readily switch from adherent to suspension and vice versa.	High Five™ cells require adaptation to suspension culture.

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Adherent Cell Culture

Introduction

The following information will enable you to successfully subculture adherent insect cells. You must maintain the cell lines as adherent cultures prior to establishing a suspension culture. Frozen cells (Sf9, Sf21, and High Five™) from Invitrogen must be initiated into culture as adherent cells, then scaled up for suspension culture.

Maintaining Adherent Cultures

The following suggestions will help you to maintain healthy adherent cell cultures:

- **Check cells daily** until a confluent monolayer has formed. Once a confluent monolayer has formed, the cells can be subcultured.
- **Passage cells only at confluency** or slightly after when a few cells are starting to pull away from the bottom of the flask. Cells will be easier to dislodge. The less mechanical force used to dislodge the cells, the better the viability will be.
- **Do not overgrow cells.** Repeated subculturing of cells at densities past confluency will result in decreased doubling times and decreased viabilities.
- **Do not split cells back too far.** Densities lower than 20% confluency inhibit growth. The healthiest cells are those taken from log phase cultures. Log phase growth can be maintained by splitting cells at a 1:5 dilution.
- **Keep a record of the passage number.** After 30 passages or more (2-3 months in culture and increased doubling times of 28-32 hours), cells usually will start to lose their viability and infectivity. You will need to thaw new cells and start new cultures.
- **Keep a cell log.** Recording the density (% confluency), viability, passage number and general appearance of your cells in culture and at freeze down can be a very helpful tool in troubleshooting experiments. See page 25 for a sample cell log.

Recommended Culture Volumes

The table below summarizes the flask volumes that are recommended for the routine subculturing of insect cells.

Flask Size (cm^2)	Volume Range (ml)
25	5-10
75	15-20
150	40-50

Cell Densities at Confluency

The table below summarizes the cell densities that we typically obtain from confluent cultures of Sf9, Sf21, and High Five™ cells. Cell numbers may vary depending upon the culture conditions and the health of your cells (see suggestions above for maintaining healthy cultures). You may use these numbers as a general guide when setting up your experiments or when setting up flasks to seed a suspension culture (see **Suspension Cell Cultures**, pgs. 15-20), however, you should count the actual number of your cells before proceeding.

Flask Size (cm^2)	Sf9	Sf21	High Five™
25	4.0×10^6	3.8×10^6	3.0×10^6
75	1.2×10^7	1.1×10^7	9.0×10^6
150	2.4×10^7	2.3×10^7	1.8×10^7

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Adherent Cell Culture, continued

Methods for Subculturing Adherent Cells

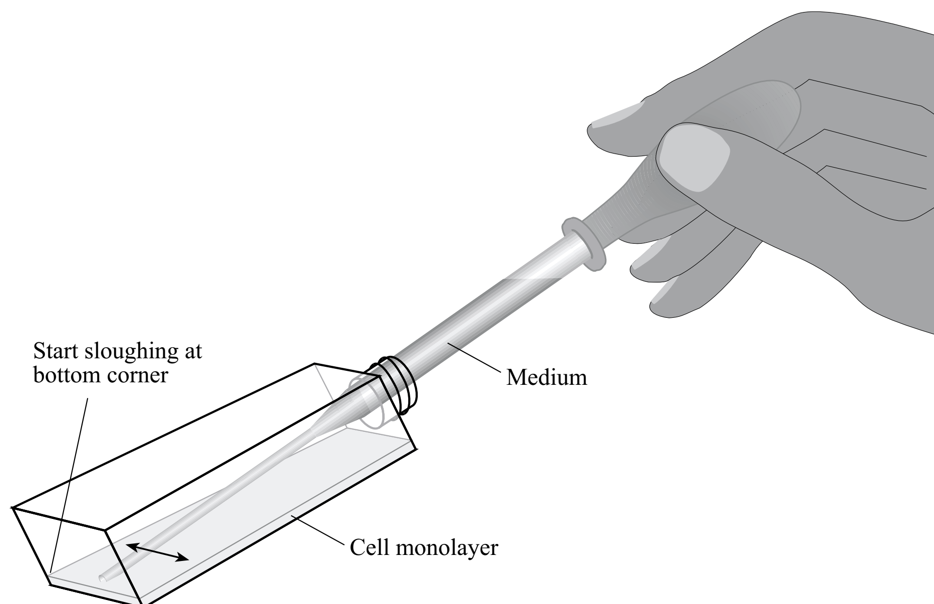
There are three methods that can be used to dislodge monolayers in adherent cell culture.

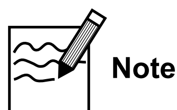
- **Sloughing** (this is the method we use at Invitrogen).
- Trypsinization.
- Tapping the flask until monolayer loosens.

Sloughing

We use sloughing exclusively as it dislodges the monolayer with the least manipulation and mechanical force, resulting in higher viabilities than the other two methods. Sloughing involves streaming medium over the monolayer using the following procedure.

1. Remove all but 5 ml of medium from your flask (independent of flask size).
2. Tilt flask on end so that the remaining medium flows to one corner, away from the cells.
3. Draw up some of the remaining medium into a sterile Pasteur pipette and starting at the bottom corner of the flask, stream medium across the cells. Use a side-to-side streaming motion as you move from the bottom corner up to the top opposite corner of the flask. Use a gentle stream to dislodge cells. See the graphic below.
4. Once the first cells are dislodged, cells above them will be easier to remove.





Note

If you find that High Five™ cells do not attach very well to the culture flask, add serum to 5%. Once the cells have attached and grown to 75-80% confluency, you may replace the serum-containing medium with Express Five® SFM.

Suspension Cell Cultures

Introduction

Information is provided below to enable you to establish and maintain suspension cultures.

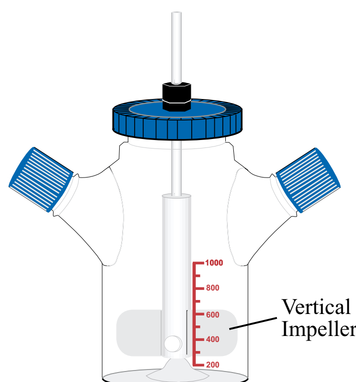
Important

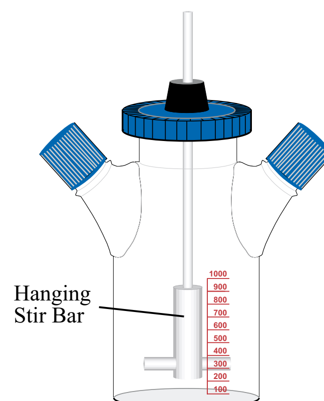
You must be able to maintain adherent cell lines prior to establishing a spinner culture. Frozen cells from Invitrogen must be initiated into culture as adherent cells. Healthy adherent cells can be scaled up for suspension culture.

Maintaining Suspension Cultures

The following information will help you to maintain healthy suspension cell cultures.

- **Monitor and Record Cell Growth:** Cells should be monitored daily through cell counts and viability. It is helpful if this data is maintained in a log or chart to help you make decisions on the health of your culture. See page 25 for a sample cell log.
- **Renew Cultures every month:** Cells can be kept in suspension for up to one month in log phase. At this point, a new culture should be initiated as cells begin to lose their infectivity and are no longer optimal for use in protocols.
- **Maintain Log Phase Cell Density:** When Sf9, Sf21 and High Five™ cells reach a density of 2 to 2.5 x 10^6 cells/ml, they should be diluted to no less than 7 x 10^5 cells/ml.
- **Use an Appropriate Flask:** Use a spinner flask with a vertical impeller rather than one with a hanging stir-bar assembly. The vertical impeller provides better aeration.





- **Maintain Volumes for Adequate Aeration:** The total culture volume in a spinner should not exceed 1/2 the indicated volume of the spinner for proper aeration (e.g. a 500 ml spinner should never contain more than 250 ml of culture).
- **Use a Surfactant to Decrease Shearing:** 0.1% Pluronic® F-68 is recommended for spinner cultures. Pluronic® F-68 is a surfactant that decreases cell membrane shearing due to impeller forces. **Note: Sf-900 II SFM and Express Five® SFM already contain surfactants**.
- **It is not necessary to change medium** when you are culturing cells in suspension. Regular subculturing requires the removal of cell suspension and the addition of medium sufficient to dilute the cell density to 1 x 10^6 cells/ml. This addition of medium is sufficient to replenish cell nutrients.
- **The impeller should be rotating smoothly** without any jerkiness or jumping motion. Smooth impeller motion is essential for adequate aeration and high cell viabilities.

continued on next page

Suspension Cell Cultures, continued

Maintaining Sterile Conditions in a Spinner Flask

To avoid contamination and maintain healthy, sterile spinner cultures:

- **Finger-tighten spinner caps** when incubating cell cultures. Loose caps are not necessary for aeration and can be a potential source of contamination.
- **Use a tissue culture hood** when opening spinner flasks to passage cells or remove medium.
- **Do not drip medium or touch the pipette** into or around the arm of the spinner when removing or adding medium to the flask.
- **Do not use soap** when cleaning spinners as residue always remains and will cause cell mortality. We recommend washing with 10% acetic acid or other commercially available spinner flask cleaners (see page 24).
- **Make sure all rings are tightened** around the spinner bar and the top of the flask prior to autoclaving. Moisture that can get into these spaces can be a cause of contamination. Spinner arm caps should be loose during autoclaving.
- **Autoclave spinner at least twice** when using them for infections with baculovirus constructs (e.g. high-titer stocks, protein expression). One wet cycle and one dry cycle is the minimum required to ensure proper sterility. Baculovirus particles can live through one cycle of autoclaving and cause contamination (see protocol for **Cleaning Spinner Flasks**, page 24).

Pluronic® F-68

We recommend adding Pluronic® F-68 (Catalog no. 24040-032) to your spinner culture medium at a final concentration of 0.1%. Pluronic® F-68 is a surfactant that helps reduce cell shearing due to the force of the impeller. Some serum-free media may already contain Pluronic® F-68 or other suitable surfactants. **Note: Both Sf-900 II SFM and Express Five® SFM contain surfactants. Additional surfactants do not need to be added.**

Minimum Requirements For Starting a Spinner Culture

To successfully initiate a spinner culture, you must meet the following minimum requirements:

- **Cell viability of 95%** or greater is required.
- **A minimum density of 1 x 10^6 cells per ml** is required.
- **The impeller must be submerged 1 cm** or more to ensure adequate aeration. A summary of minimum volumes for different spinner sizes follows:

Size of spinner (ml)	Minimum volume required (ml)
100	30
250	80
500	200

Note: We do not recommended initiating a spinner culture into a spinner flask larger than 500 ml. We suggest scaling up from smaller spinners that have already been established.

Suspension Cultures in Shake Flasks

You may wish to grow suspension cultures of insect cells in shake flasks as opposed to spinner flasks. Procedures to grow suspension cultures in shake flasks using either serum-containing or serum-free media are described in Richardson, 1995, chapters 3 and 4, respectively.

Suspension Cell Cultures: Sf9 and Sf21 Cell Lines

Introduction

A protocol is provided below to enable you to transfer adherent Sf9 and/or Sf21 cells from tissue culture flasks into spinner flasks. This procedure will allow you to initiate and maintain suspension cultures of Sf9 and Sf21 cells. For more information on suspension culture, see **Suspension Cell Cultures**, page 15.

Required Equipment and Materials

You will need the following:

- Adherent Sf9 or Sf21 insect cells
- Spinner flasks (Bellco #1965 Series)
- Magnetic stir plate
- 27°C constant temperature incubator, CO_2 not required

Suspension Culture of Sf9 or Sf21 Cells

When initiating any suspension culture, log phase cells of 95% viability or greater must be used to ensure success. To rapidly initiate a suspension culture of Sf9 or Sf21 cells:

1. Grow up enough log phase adherent cells to start a spinner of desired size with 1×10^6 cells/ml. Seeding at lower densities can cause decreased doubling times in the initial stages of culture. See **Cell Densities at Confluency** on page 13 to help you determine the number of flasks needed to obtain enough cells to seed a spinner culture.

   Note: We recommend starting off with a 100 ml or 250 ml spinner flask as these require fewer total cells than larger flasks to initiate culture.
2. Remove cells from flasks, count cells and make sure that viability is at least 95%.
3. Seed a clean, sterile spinner of the desired size with enough cells to reach a density of 1×10^6 cells/ml.

   Example: A 100 ml spinner with 50 ml of culture at 1×10^6 cells/ml would require a total of $(1 \times 10^6$ cells/ml$)(50$ ml$) = 5 \times 10^7$ viable cells.
4. Incubate the spinner(s) at 27°C with constant stirring at 80-90 rpm.
5. When the cells reach a density of about 2 to 2.5×10^6 cells/ml, add enough medium to dilute their density back to 1×10^6 cells/ml. This should occur in 24-72 hours. Cells should be checked daily for density and viability.

Maintenance of Suspension Sf9 and Sf21 Cells

Once you have reached the maximum volume allowed for a given spinner size (maximum volume allowed = 1/2 the assigned volume of the spinner), you can move up to a larger spinner or maintain the volume that you have.

To maintain volume: Subculture the cells when their density reaches about 2.0 to 2.5×10^6 cells/ml. Be sure to subculture the cells when the density is not greater than 4×10^6 cells/ml and keep the density above 1×10^6 cells/ml for log phase growth.

To increase spinner size: Transfer enough culture volume at 2×10^6 cells/ml to seed a larger spinner with at least 1×10^6 cells/ml. Be sure to maintain minimum volume requirements (see **Minimum Requirements**, page 16). Add enough fresh medium to bring the density of the culture to 1×10^6 cells/ml. Continue to maintain volume (as above) or repeat this process to increase spinner size until desired culture volume is reached.

Suspension Cell Cultures: High Five™ Cell Line

Introduction

In the following procedure, adherent High Five™ cells are transferred from tissue culture flasks into spinner flasks. Serum-free medium supplemented with heparin is used to reduce the aggregation of cells. The cells are grown for several passages until they are >95% viable and have a doubling time between 18 and 24 hours. At this point, the cells are weaned from heparin. If the cells continue to grow in suspension without forming large aggregates in the absence of heparin, they are fully adapted to growth in suspension culture.

Required Equipment and Materials

You will need the following:

- High Five™ insect cells (Invitrogen)
- Express Five® SFM (Invitrogen)
- Heparin, tissue culture grade (Sigma, Catalog no. H3149)
- Spinner flasks (Bellco #1965 Series)
- Magnetic stir plate
- Hemacytometer
- 27°C constant temperature incubator, CO_2 not required

Important

When growing the High Five™ cells, do not let the cell density exceed 2.5×10^6 cells/ml as they will begin to form large aggregates of cells.

If the cell viability drops below 60% during the initial growth phase (see next page), the cells are no longer healthy and the adaptation process must be started again with new High Five™ cells.

Use of Heparin

Heparin will help keep the High Five™ cells from aggregating. To maintain heparin in the culture, use serum-free medium supplemented with 10 units of heparin per ml of medium when splitting cells or increasing culture volume.

If at any time, suspension cells begin to develop large aggregates (> 10 cells per aggregate), add heparin to the medium at 10 units per ml of culture. Small aggregates of 5 to 10 cells, visible under a hemacytometer, are common to High Five™ suspension cells and will not effect expression.

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Initial Growth Phase

This initial phase will take 3 to 4 days.

1. Take four confluent 75 cm^2 flasks of adherent High Five™ cells, slough, and inoculate a 100 ml spinner flask. Total volume should be between 40-50 ml with a cell density of 0.7 to 1.0 x 10^6 High Five™ cells/ml. Use serum-free medium to adjust the volume or cell density and add 10 units of heparin per ml of culture. Do not exceed 50% of the spinner flask volume.
2. Incubate the spinner flask at 27°C at a constant stirring rate of 80-90 rpm. Grow the cells for 24 hours.
3. After 24 hours, count the cells and determine their viability and cell density. The cells should be ~90% viable. If the cell density is below 2.0 x 10^6 cells/ml, continue to grow the cells.
4. Once the cell density reaches 2.0 x 10^6 or greater, passage and expand the cells with heparin into a second 100 ml spinner flask to a cell density of 1.0 x 10^6 cells/ml. You will now have two 100 ml spinner flasks, each with 50 ml of culture. If cells must grow longer than 24 hours before they are passaged (i.e. over the weekend), the cell density may be reduced to 0.8 x 10^6 cells/ml.
5. Continue to grow the cells checking the cell density and viability daily. When cells reach 2 x 10^6 cell/ml and 95% viability, expand them into a 500 ml spinner flask.

Adaptation of Culture

Adaptation is defined as 98% viable and doubling every 18-24 hours in a 500 ml suspension culture. Transfer the cells to a 500 ml spinner flask as follows:

1. Pool the cells from the two 100 ml spinner flasks into one 500 ml spinner flask and add 150 ml of serum-free medium supplemented with heparin. Make sure that the cell density does not fall below 0.8 x 10^6 cells/ml.
2. Continue to grow and split the cells as described in Step 4, above, until they are 98% viable and doubling every 18-24 hours in the desired size spinner flask, typically 500 ml or 1 liter.
3. Once the cells are 98% viable and doubling every 18-24 hours, they can be slowly weaned from heparin. This is best accomplished by adding medium without heparin when splitting the cells.

Once the cells have been adapted, they should not aggregate in the absence of heparin.

Maintenance of Suspension High Five™ Cells

Once you have reached the maximum volume allowed for a given spinner size (maximum volume allowed = 1/2 the assigned volume of the spinner), you can move up to a larger spinner or maintain the volume that you have.

To maintain volume: Subculture the cells when their density reaches 2.0 to 2.5 x 10^6 cells/ml. Be sure to subculture the cells when the density is not greater than 2.5 x 10^6 cells/ml and keep the density above 1 x 10^6 cells/ml for log phase growth.

To increase spinner size: Transfer enough culture volume at 2 x 10^6 cells/ml to seed a larger spinner with at least 1 x 10^6 cells/ml. Be sure to maintain minimum volume requirements (see above). Add enough fresh medium to bring the density of the culture to 1 x 10^6 cells/ml. Continue to maintain volume (as above) or repeat this process to increase spinner size again until desired culture volume is reached.

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Long-Term Storage of Suspension High Five™ Cells

High Five™ cells that are adapted to suspension culture can be frozen down in freezing medium (42.5% conditioned medium, 42.5% fresh medium, 10% DMSO, and 5% FBS).

1. You will need 3 x 10^6 cells/ml per vial. One way to do this is to take 15 ml of a 2 x 10^6 cell/ml culture and centrifuge down the cells. Resuspend the cells in 10 ml freezing medium and aliquot 1 ml per vial.
2. Hold the cells at -80°C for 24 hours, then transfer to liquid nitrogen.

Thawing Suspension-Adapted High Five™ Cells

High Five™ cells that have been adapted to suspension culture and frozen down will first need to be grown as adherent cultures before adaptation to suspension cultures. They will need to be grown in the presence of heparin and weaned as described above. However, the adaptation process will not take as long as with High Five™ cells that have never been adapted to suspension culture.

Freezing Protocols

Introduction

Once cell lines are established and doubling regularly, they can be frozen. Cells should be 90% viable and 80-90% confluent. We recommend that you freeze down several vials as soon as your cell culture meets these requirements (e.g. at as low a passage number as possible). When you thaw cells, they will be optimal for use.

Procedure

Use the table below to make the freezing medium for each cell line. Note that Grace's Insect Medium does not contain supplements nor FBS.

Cell Line	Freezing Medium	Density (cells/ml)	Attachment Time, post-thaw (min)
Sf9	60% Grace's Insect Medium 30% FBS 10% DMSO	1 x 10^7	30-45
Sf21	60% Grace's Insect Medium 30% FBS 10% DMSO	1 x 10^7	30-45
High Five™	42.5% conditioned Express Five® SFM 42.5% fresh Express Five® SFM 10% DMSO 5% FBS	3 x 10^6	30-45

1. Count cells using a hemacytometer. You need enough cells to freeze down 2-4 cryovials at the densities shown in the above table. Cells can come from suspension or adherent culture.
2. Set up sterile cryovials on ice. Be sure to label.
3. Centrifuge the cells at 400-600 x g for 10 minutes at room temperature. Remove the supernatant. For High Five™ cells, save the conditioned medium in order to make the freezing medium.
4. Resuspend cells to the given density in the freezing medium indicated.
5. Transfer 1 ml of the cell suspension to sterile cryovials.
6. Place at -20°C for 1 hour, then transfer to -80°C for 24-48 hours.
7. Store in liquid nitrogen.

Appendix

Support Protocols

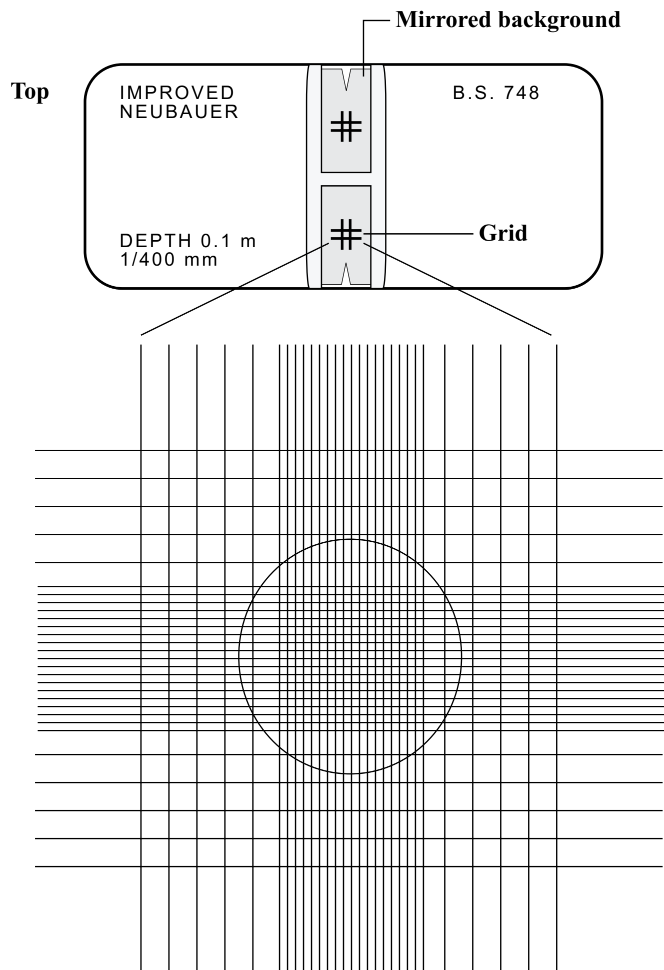
Introduction

Guidelines to maintain and optimize insect cell culture are provided in this section. Included are protocols for cleaning spinner flasks and for adaptation to serum-free medium.

Counting Cells in a Hemacytometer

Hemacytometers may be obtained from most major laboratory suppliers (e.g. Baxter Scientific). The procedure below provides some general directions on how to use the hemacytometer. For more details, refer to King and Possee, 1992.

1. Clean the chamber and cover slip with alcohol. Dry and fix the coverslip in position.
2. Harvest cells. Add 10 µl of the cells to the hemacytometer. Do not overfill.
3. Place chamber in the inverted microscope under the 10X objective. Use phase contrast to distinguish cells.
4. Count the cells in the large, central gridded square (1 mm^2). The gridded square is circled in the graphic below. Multiply by 10^4 to estimate the number of cells per ml. Prepare duplicate samples and average the count.



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Support Protocols, continued

Trypan Blue Exclusion

The following procedure will enable you to quickly and accurately determine cell viability. Cell viability is calculated as the number of viable cells divided by the total number of cells within the grids on the hemacytometer. If cells take up trypan blue, they are considered non-viable.

1. Determine the cell density of your insect cell line suspension using a hemacytometer.
2. Prepare a 0.4% solution of trypan blue in buffered isotonic salt solution, pH 7.2 to 7.3 (i. e. phosphate-buffered saline).
3. Add 0.1 ml of trypan blue stock to 1 ml of cells.
4. Load a hemacytometer and examine immediately under a microscope at low magnification.
5. Count the number of blue staining cells and the number of total cells. Cell viability should be at least 95% for healthy log-phase cultures.

   % viable cells = [1.00 - (Number of blue cells ÷ Number of total cells)] x 100

   To calculate the number of viable cells per ml of culture, use the formula below. Remember to correct for the dilution factor.

   Number of viable cells x 10^4 x 1.1 = cells/ml culture

Concentrating Cells

To concentrate cells from a suspension culture (or resuspended cells from monolayer culture):

1. Transfer cell suspension to a sterile centrifuge tube of appropriate size and centrifuge for 10 minutes at 800 x g.

   Note: Baculovirus cells are very sensitive to centrifugal force.
2. Carefully remove the supernatant without disturbing the pellet of cells.
3. Add the desired volume of fresh medium gently to the side of the tube and slowly pipette up and down 2 to 3 times to resuspend the cell pellet.
4. Transfer to the desired, sterile container.

continued next page

Support Protocols, continued

Adaptation to Serum-Free Medium

The following protocol will enable you to adapt your cell lines to the appropriate serum-free conditions. The procedure takes about 7 days and involves gradually decreasing the percentage of serum containing medium in the cell culture. Changing the medium over to serum-free without adaptation can lead to greatly decreased doubling times and cell mortality. This procedure may also be used to adapt High Five™ cells to a medium other than Express Five® SFM.

Note: This protocol can also be used to adapt cells to a new formula of medium.

1. Begin with adherent log phase cells that are 50% confluent (adherent cells).
2. Remove 25% of the serum-containing medium (TNM-FH) and add 25% of the serum-free medium of your choice (e.g. Sf-900 II SFM).
3. Grow cells to confluency.
4. Subculture to 50% confluency with 50% serum-containing medium and 50% serum-free medium.
5. Grow cells to confluency.
6. Subculture to 50% confluency with 25% serum-containing medium and 75% serum-free medium.
7. Grow cells to confluency.
8. Subculture to desired density/dilution using 100% serum-free medium.

Note: During the adaptation process it is normal for the growth rate of the cells to slow down. Keeping the cells at no lower than 50% confluency helps to keep them in log phase and minimizes time loss due to slow growth rates.

Cleaning Spinner Flasks

Proper spinner care is important for both cell health and to prevent cross contamination of your cultures with different viral stocks. Baculovirus particles can survive a cycle through the autoclave. We have developed a protocol that uses two cycles in the autoclave to eliminate virus contamination of future spinner cultures.

1. Wash with 7X cleaner (Bellco) or substitute 10% acetic acid. Wash while spinning, approximately 2 hours. The 7X glass cleaner is preferable as trace amounts are not detrimental to the cells. Acetic acid can be detrimental if not rinsed away completely. **Do not use detergents.**
2. Rinse 5 times with tap water.
3. Rinse 5 times with deionized water.
4. Tighten spinner cap and o-ring around impeller bar, spinner arm caps should be finger-tightened.
5. Autoclave once wet with deionized water for 45 minutes on liquid cycle.

   Note: You can repeat this step more than once if desired.
6. Autoclave once dry for 45 minutes on dry cycle.

Cell Log

Cell Line: **Cell Lot #/Date of Freeze down:**

Date of Initiation: **Medium:** **Medium Lot No.:**

Passage No.	Date of Passage	Cell Density	Cell Viability	Comments
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				
26				
27				
28				
29				
30				

Record any changes in medium and medium lot number in the "Comment" column as well as any notes about cell appearance.

Troubleshooting Guide

Introduction

The following section summarizes the most common problems associated with insect cell culture. Morphological changes in the cells or changes in the growth rate can indicate an underlying problem with the culture. If you have kept a record of cell viabilities and doubling times, this can be helpful in diagnosing a problem when it comes up. Use this table as a general guide for potential problems--what might cause them and what to do to solve and prevent them.

Adherent Cells: Morphology Changes - First Week in Culture

Problem	Potential Causes	Solutions
Cells are granular and/or floating post-thawing	Medium was not removed within 1 hour of thawing cells. DMSO in the freezing medium can be harmful to cells. **Note:** This applies to Sf9, Sf21 and High Five™ cells.	Remove medium, add fresh medium.
	High Five™ cells were not thawed into Express Five® SFM.	Cells may adapt over several days. Keep checking the cells. If possible, change medium to Express Five® SFM.
	Cells are exhibiting serum sensitivity.	Try a new type or lot of serum, thaw new cells (see page 10).
Cell lysis or debris	Aging cell culture with passage number greater than 30.	Thaw new cells of lower passage number.

continued on next page

Adherent Cells: Morphology Changes - More Than One Week in Culture

Problem	Potential Causes	Solutions
(Decreased viability and growth rate)	Contamination with wild-type or recombinant baculovirus. You will see lysis, debris and/or swelling of cells. Inclusions in the nucleus indicates wild-type infection.	**Discard the old culture and initiate a fresh culture.** • Thaw new cells. • Decontaminate hood and equipment. • Make up new medium.
	Too much mechanical manipulation during subculturing.	Thaw new cells. Switch to another method of subculturing (e.g. sloughing).
Floaters (greater than 5-10% of cells in culture)	Passaging too often before confluency.	• Remove medium/floaters. • Replace with fresh medium. • Passage only at confluency.
	Cells have overgrown past confluency.	• If this is the first time this has occurred, replace medium and split cells. • Thaw new cells if this has happened more than once.
	Sensitivity to a new lot or brand of serum (FBS).	Test your culture with another serum. At Invitrogen, we routinely use serum from GIBCO®.
Cells swollen, spots in the nucleus	Contamination with wild-type or recombinant baculovirus.	**Discard the old culture and initiate a fresh culture.** • Thaw new cells. • Make new medium. • Decontaminate equipment.

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Adherent Cells: Growth and/or Viability Decrease

Problem	Potential Causes	Solutions
Cell doubling time > 24 hours	Subculturing technique too rigorous.	Thaw new cells and try using a different subculturing technique (e.g. sloughing, page 14).
	Cells grown past confluency more than 1 time.	Thaw new cells and check your cell cultures daily to avoid overgrowth.
	Cells are at a high passage number (greater than 30 passages).	Thaw new cells and make sure that you are freezing down cells at a low passage number (less than 10) so that new cultures are optimal.
	Cells are repeatedly being passaged before confluency.	• Let cells grow to confluency for next passage. • If doubling time does not increase, thaw new cells.
	Cells are split back below 20% confluency repeatedly.	Concentrate cells to a confluency of 50% or greater and plate out (see page 23).
Cell viability is less than 90%	Subculturing technique too rigorous.	Thaw new cells and try using a different subculturing technique (e.g. sloughing, page 14).
	Cells are repeatedly being passaged before confluency, when they adhere more tightly, requiring more mechanical force to dislodge them.	• Let cells grow to confluency for next passage. • If viability does not increase, thaw new cells.
	Bacterial or fungal contamination.	**Discard the old culture and initiate a fresh culture.** • Decontaminate equipment. • Prepare fresh medium.
	Contamination with wild-type or recombinant baculovirus. You will see lysis, debris and/or swelling of cells. Inclusions in the nucleus indicates wild-type infection.	**Discard the old culture and initiate a fresh culture.** • Decontaminate equipment. • Prepare fresh medium. • Keep separate bottles of medium for cell culture and for virus work. • Do not work with cell culture at the same time that you are working with virus in the tissue culture hood.

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Spinner Culture: Morphology Changes

Problem	Potential Causes	Solutions
Clumping of cells	Spin rate is too slow.	Use 80-90 rpm.
Cell lysis or debris	Aging cell culture that has been maintained in suspension longer than two months.	Thaw new cells of low passage number and initiate a new spinner culture.
	Shearing due to impeller spin rate.	Add Pluronic® F-68 at 0.1% final concentration if needed.
	Contamination with wild-type or recombinant baculovirus. You will see lysis, debris and/or swelling of cells. Inclusions in the nucleus indicates wild-type infection.	**Discard the old culture and initiate a fresh culture.** • Thaw new cells and start a new spinner culture. • Decontaminate hood and equipment. See **Cleaning Spinner Flasks**, page 24. • Make up new medium. Keep separate bottles of medium for cell culture and viral work.
Cells swollen, spots in the nucleus	Contamination with wild-type or recombinant baculovirus.	• See above recommendations for baculovirus contamination.

Spinner Culture: Growth and/or Viability Decrease

Problem	Potential Causes	Solutions
Doubling time > 24 hours	Spinner seeded or split back to a cell density that is too low (less than 5 x 10^5 cell/ml).	Concentrate cells to 1 x 10^6 cell/ml (see page 23). This will boost them into log phase growth.
	Spinner overgrown to a cell density > 3 x 10^6 cells/ml.	1. Split cells to a density of 1.5 x 10^6 cells/ml. 2. Grow them overnight to a density of 2.5 to 3.0 x 10^6 cells/ml. 3. Split them back to a density of 1.0 x 10^6 cells/ml, continue normal maintenance (see page 15).

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Spinner Culture: Growth and/or Viability Decrease

Problem	Potential Causes	Solutions
Doubling time > 24 hours, continued	Inadequate aeration.	The volume of culture should not exceed 1/2 the designated capacity of the spinner.
		Add Pluronic® F-68 at 0.1% final concentration if needed.
		Volume of culture should meet minimum volume requirements (page 16).
		Impeller should spin with a smooth continuous motion. Jerky or jumping motion of the impeller does not provide good aeration.
Viability < 90%	Shearing due to impeller spin rate.	Add Pluronic® F-68 at 0.1% final concentration if needed.
	Inadequate aeration.	See above Recommendations for **Inadequate Aeration**.
	Bacterial or Fungal Contamination.	**Discard the old culture and initiate a fresh culture.** See Section on **Maintaining Sterile Conditions in a Spinner,** page 16. Try adding antibiotics to your medium (see page 8).
	Contamination wild-type or recombinant baculovirus. You will see lysis, debris and/or swelling of cells. Inclusions in the nucleus indicates wild-type infection.	**Discard the old culture and initiate a fresh culture.** Adequate sterilization of spinner flasks between uses where there is contact with baculovirus is required. See page 24.

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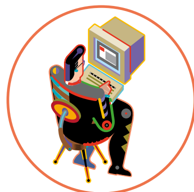
Troubleshooting Guide, continued

Problems with High Five™ Cells in Suspension

Problem	Potential Causes	Solutions
Clumping of cells	Cells were grown above a density of 2 to 2.5 x 10^6 cells/ml.	1. Let the spinner sit in the hood for 30-45 min. to settle large clumps. 2. Transfer top 1/3 of solution containing small clusters and single cells to a new spinner and continue to culture (see **Suspension Cell Culture**, page 15). **Note:** If clumps represent more than 50% of the culture, start a new adaptation.
	Spin rate is too slow.	Use 80-90 rpm.
	Adaptation did not work.	Try again, this is common on the first few attempts. If you do not have heparin in your spinner culture, add heparin (see **Adaptation of Culture**, page 19).
Cell lysis or debris	Aging culture that has been maintained in suspension longer than two months.	Thaw new cells of low passage number and initiate a new spinner culture.
	Jerky or jumping motion of impeller.	Adjust flask on spinner until impeller moves smoothly.
Cells swollen, spots in the nucleus, and/or lysis	Contamination with wild-type or recombinant baculovirus. You will see lysis, debris and/or swelling of cells. Inclusions in the nucleus indicates wild-type infection.	**Discard the old culture and initiate a fresh culture.** • Thaw new cells and start a new spinner culture. • Decontaminate hood and equipment. See **Cleaning Spinner Flasks**, page 24. • Make up new medium Keep separate bottles of medium for cell culture and viral work.
Viability < 90%	see **Spinner Culture: Growth and/or Viability Decrease**, page 30.	
Doubling time > 24 hours	see **Spinner Culture: Growth and/or Viability Decrease**, page 29.	

Technical Service

World Wide Web



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For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

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2. Follow instructions on the page and fill out all the required fields.
3. To request additional MSDSs, click the ‘Add Another’ button.
4. All requests will be faxed unless another method is selected.
5. When you are finished entering information, click the ‘Submit’ button. Your MSDS will be sent within 24 hours.

continued on next page

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