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Growth and maintenance of insect cell lines

USER GUIDE

Catalog Numbers B82101, B82501, and B85502

Publication Number MAN0000030

Revision B.0

ThermoFisher SCIENTIFIC

Manufacturer's address: Life Technologies Corporation | 5781 Van Allen Way | Carlsbad, CA 92008

Information in this document is subject to change without notice.

Revision history: Pub. No. MAN0000030

Revision	Date	Description
B.0	14 March 2017	Rebrand and update legal boilerplates
A.0	31 March 2015	Baseline for this revision

Contents

Product information 4

Introduction 4
Contents and storage 4
Cell lines 4

Methods 7

Cell handling techniques 7
Media guidelines 11
Initiate cell culture from frozen stock 13
Adherent vs. suspension culture 16
Adherent cell culture 17
Suspension cell cultures 19
Suspension cell cultures: Sf9 and Sf21 cell lines 23
Suspension cell cultures: High Five™ Cell Line 24
Freeze insect cell lines 26

Troubleshooting 28

Appendix A: Support protocols 33

Count and concentrate cells 33
Adapt cells to serum-free medium 34
Generate a growth curve 35
Clean glass culture vessels 36
Cell Log 37

Appendix B: Ordering information 38

Cells and media 38

Documentation and support 39

Customer and technical support 39
Limited product warranty 39

Safety 40

Chemical safety 41
Biological hazard safety 42

Product information

Introduction

This manual 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).

Contents and storage

Contents	Cat. No.	Amount	Storage[1]
Sf21 cells	B82101	1 mL	Liquid nitrogen
Sf9 cells	B82501	1 mL	
High Five™ cells	B85502	3 x 10^6 cells	

[1] Cells are shipped on dry ice.

Cell lines

Comparison of cell lines

The following table summarizes some general characteristics of the cell lines available from Thermo Fisher Scientific. For ordering information, see page 38.

Cells	Doubling time	Cell appearance	Initial medium
Sf9	24–30 hours	Spherical with some granular appearance (regular in size). Firm attachment to surfaces	complete TNM-FH
Sf21	24–30 hours	Spherical with some granular appearance (different sizes). Firm attachment to surfaces	complete TNM-FH
High Five™	18–24 hours	Spherical with some granular appearance (different sizes). Loose attachment to surfaces	Express Five™ SFM

Important

It has been confirmed by RT-PCR that High Five™ cells harbor an endogenous alphanodavirus. Under certain conditions, alphanodavirus particles are produced from High Five™ cells. Produced viral particles do not appear to adversely influence heterologous protein expression and or other research applications for which High Five™ cells are intended. Contact Technical Support for further information (page 39).

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Sf9 and Sf21 cell lines

Origin: Sf9 (Cat. No. B82501) and Sf21 (Cat. No. B82101) 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*.

Characteristics: Sf9 and Sf21 cells 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). 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.

High Five™ Cells

Origin: The High Five™ cell line (BTI-TN-5B1-4, Cat. No. B85502) was developed by the Boyce Thompson Institute for Plant Research, Ithaca, NY and originated from the ovarian cells of the cabbage looper, *Trichoplusia ni*.

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

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 Thermo Fisher Scientific 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, visit **thermofisher.com** or contact Technical Support (page 39).

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Cell lines, continued

Cell line selection

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.

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.

Design guidelines

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™ Nickel-Chelating Resin) because media components will strip the nickel ions from the resin.

Insect culture media

Sf9, Sf21, and High Five™ cells can be grown in serum-supplemented or serum-free media (see **Media guidelines**, page 11, for more information). We recommend the following media for each cell line (see page 38 for ordering information).

Cells	Serum-supplemented medium	Serum-free medium
Sf9 cells	Grace's Insect Medium, Supplemented	Sf-900™ II SFM, Sf-900™ III SFM
Sf21 cells	Grace's Insect Medium, Supplemented	Sf-900™ II SFM, Sf-900™ III SFM
High Five™ Cells	Grace's Insect Medium, Supplemented	Express Five™ SFM

Cell handling techniques

Sterile technique

Handle insect cell lines under sterile conditions in a laminar flow hood.

Passage/ subculture

Passaging/subculturing refers to diluting cells back to a density that maintains log phase growth and maximum viability. We recommend performing a cell count and viability every time the cells are passaged to determine the health of the cells and to ensure robust cell growth.

Note: The recommended seeding densities for suspension cultures throughout this manual allows for a 3–4 day subculture schedule. If the cells need to be passaged earlier than 3–4 days, seed them at a higher density. The important consideration is for the cells to be in the mid-log phase of growth when passaging at high densities.

Adherent cultures

Passage adherent cultures when the cells are in mid-log phase of growth. Generally, this is when the cells are ~90% confluent. Cells should be passaged at a seeding density of 2×10^4–5×10^4 viable cells/cm^2.

Example: Cell seeding density for a T-75 flask can range from 1.5×10^6 to 3.75×10^6 viable cells/flask depending on seeding density used. Volume of medium per flask should be ~12 mL.

Suspension cultures

Suspension cultures should be passaged when they reach mid-log phase of growth. Generally, mid-log phase of growth corresponds to a cell density of 2×10^6 to 4×10^6 cells/mL. Cells should be seeded between 3×10^5 to 5×10^5 viable cells/mL for suspension cultures.

Note: We recommend performing a growth curve to determine the accurate cell density range where mid-log phase of growth falls based on your culture conditions (i.e., incubator, equipment, technicians, etc.). Refer to **Generate a growth curve**, page 35, for more information.

Example: A 100-mL spinner containing 50 mL of cell culture is at a density of 2×10^6 cells/mL. Remove 12.5 mL of medium cell suspension and add to flask with 37.5 mL of fresh complete medium for a final density of 5×10^5 viable cells/mL.

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Cell handling techniques, Continued

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 increased 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 may be more difficult to dislodge, and require more mechanical force to dislodge them from the monolayer. When repeatedly subcultured before confluency, cells display increased doubling times and decreased viabilities (for more information, see **Troubleshooting**, page 28. The culture is considered to be unhealthy.

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.

Floaters are cells that are not tightly adherent and may detach from the culture vessel. These detached cells may still be viable.

To determine cell viability, take a small aliquot of floater cells and test their viability using trypan blue. If the cells are still viable, they can be removed and passaged at the recommended seeding density.

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 17.

Doubling time

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

Healthy doubling times:

- Sf9 cells double every 24–30 hours
- Sf21 cells double every 24–30 hours.
- High Five™ cells double every 18–24 hours.

Note: For more information on what to do if doubling times exceed the ranges provided above, see **Troubleshooting**, page 28.

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Cell handling techniques, Continued

Viability

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

Definition: Cell viability refers to the percent of cells in a culture that are living. Cell viability can be determined using trypan blue or other acceptable methods.

Minimum requirements: Cell viability should be greater than 90%. If viability is low, culture conditions may be the cause. See **Troubleshooting**, page 28, if your cell viability is below 90%.

Growth temperature

Maintain insect cells in a non-humidified incubator at 26–28°C. This temperature range is critical for maintaining robust cell growth as this is the temperature of insect blood.

Below 26°C: Culturing insect cells below 26°C will result in slower cell growth. Doubling times will be greater than 24–30 hours.

Above 28°C: Insect cells begin to show increased doubling times at temperatures between 28°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 may not recover if the temperature is returned to 26–28°C.

CO_2

CO_2 exchange is not required for insect cell culture.

Concentrate cells

If the cell density is too low and the cells have been in culture for 4–5 days, we recommend concentrating the cells by centrifuging them at 100 × *g* for 5 minutes and resuspending them in fresh medium. Cells should not be left in the same medium for more than 4–5 days as nutrients in the medium will have been used up by the cells in that period and the medium itself degraded due to prolonged exposure to warm temperatures.

Cells should also be centrifuged and concentrated if a lot of cell debris is observed in culture.

Disperse cells

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. Repeat this four times, watching carefully to ensure that the liquid reaches all areas of the growth surface. Do not use a circular motion to disperse the cells because 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

Cell log

It is very important to monitor the density and viability of your insect cell culture. Keeping a cell log enables you to record the data necessary to make decisions about your cell culture (see page 37 for a cell log template). A cell log is also a very helpful troubleshooting tool (see page 28).

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
- seeding densities at passage

Media guidelines

Work 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 stock cultures if introduced to culture plates or flasks during passaging.

Grace's Insect Medium/TNM-FH

- **Grace's Insect Medium:** Grace's Insect Medium, Unsupplemented (Cat. No. 11595030) is available separately.

  Note: This medium contains L-glutamine.
- **Grace's Insect Medium, Supplemented:** A supplemented form of Grace's Insect Medium (Cat. No. 11605094) is available separately. The additional supplements are 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), heat-inactivated. Serum provides additional nutrients and also protects the cells from hydrodynamic stresses in suspension culture conditions (i.e., spinner and shaker flasks). We recommend supplementing the medium with 0.1% Pluronic™ F-68 when culturing suspension cells in shaker or spinner flasks to reduce the shear stress on the cells.
- **Serum sensitivity:** 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 from a new lot before using it with all of your insect cell cultures. For more information on serum sensitivity, see **Troubleshooting**, page 28.
- **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 2–4 weeks at 2–8°C, protected from light.
- **Antibiotics:** Many antibiotics are suitable for use with insect cells. The following table summarizes some of the most commonly used antibiotics, their working concentrations, and their methods of action. See page 38 for ordering information.

Antibiotic	Working concentration	Method of action
Gentamicin	10 µg/mL	Inhibits bacterial protein synthesis
Amphotericin B (Fungizone™)	0.25 µg/mL	Binds sterols and interferes with membrane permeability
Penicillin-Streptomycin	50–100 U/mL 50–100 µg/mL	Inhibits bacterial cell wall synthesis Inhibits bacterial protein synthesis

Media guidelines, *Continued*

Prepare complete TNM-FH Medium

If 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 2–8°C for 2–4 weeks.

1. To 500 mL of Grace's Insect Medium, Supplemented**,** add 50 mL of FBS, heat-inactivated. Mix well.
2. *Optional*: Filter this solution through a low protein binding 0.2 µm filter into a sterile container, if you believe that its sterility has been compromised.
3. Store complete TNM-FH medium at 2–8°C, protected from light.
4. Equilibrate to room temperature before use. Protect the bottle from light exposure when warming.

Medium requirements

Complete THM-FH medium consisting of Grace's Medium, Supplemented containing yeastolate and lactalbumin hydrolysate and supplemented with 10% FBS, heat-inactivated, is required for the maintenance of robust cell growth.

Serum-free media

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

- **Supplementation:** Serum-free media are complete formulations and do not require any supplementation, including the addition of FBS, to maintain cell growth. 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: Sf-900™ II SFM, Sf-900™ III SFM, and Express Five™ SFM contain surfactants. You do not need to add additional surfactants.
- **Adaptation:** We recommend sequentially adapting the cells growing in serum-supplemented medium into serum-free medium. For more information, refer to **Adapt cells to serum-free medium** (page 34).
- **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.

Prepare serum-free media

Refer to the product manual for the specific serum-free media for usage instructions. Sf-900™ II SFM and Sf-900™ III SFM are complete media and do not require additional supplementation. Express Five™ SFM requires supplementation with 18 mM L-glutamine before use.

Initiate 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 (Sf9 and Sf21) are frozen down at a passage number less than 15.

Experimental outline

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

Step	Action
1	Remove cells from liquid nitrogen.
2	Quickly thaw cells in a 37°C water bath.
3	Perform a cell count to determine actual cell density and viability.
4	Seed cells at a density of 2×10^4–5×10^4 viable cells/cm^2 for adherent culture or 3×10^5–5×10^5 viable cells/mL for suspension culture.
5	Grow cells to ~90% confluency for adherent culture or a cell density of >2.0×10^6 cells/mL for suspension culture. Passage and expand cells when this confluency or density is reached.
6	Freeze down several vials of cells on the third passage as backup.

Thaw High Five™ cells

We recommend thawing High Five™ cells into Express Five™ SFM for best results. These cells have been adapted to Express Five™ SFM and cryopreserved in this formulation. If you wish to grow cells in a different medium, we recommend that you thaw and expand the cells in Express Five™ SFM first. Once the cells show robust growth in Express Five™ SFM, they can be adapted into other media formulations (i.e., complete TNM-FH, other serum-free media, etc.). Refer to 34**Adapt cells to serum-free medium** (page 34), if you wish to grow High Five™ cells in media other than Express Five™ SFM.

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Initiate cell culture from frozen stock, Continued

Initiate culture with frozen cells

Thawed cells do not always appear round; some may be amorphous or have a wrinkled appearance. You may observe cell debris and floaters in the culture upon thawing. This is normal and the cell debris will be eliminated through successive rounds of subculturing. Refer to **Troubleshooting**, page 28, for more information on cell debris.

Suspension culture conditions – shake flasks

This applies to Sf9 and Sf21 cells.

1. Warm growth medium to room temperature.
2. Remove a vial of cells from liquid nitrogen. Quickly thaw the cells in a 37°C water bath by agitating the cryovial until the cells are almost completely thawed with only a small ice crystal remaining (about 1–2 minutes). Do not submerge the entire cryovial in the water bath.
3. Decontaminate the outside of the vial by treating with 70% ethanol and place it in a laminar hood.
4. Remove the cells from the cryovial and transfer them into a sterile 15-mL conical tube.
5. Resuspend the cells by drop-wise adding medium that has been pre-warmed to room temperature and swirling the tube as the medium is being added. Resuspending the cells by this method reduces osmotic shock to cells and allows for increased cell recovery and viability.
6. Remove a small amount of the cell suspension and perform a cell count to determine cell density, viability, and health.
7. Transfer the cells into 125 mL shake flask with 25–30 mL of medium. The volume of medium to add is dependent on the cell density as determined above. Final cell density in the culture should be 3×10^5–5×10^5 viable cells/mL. The upper recommended cell seeding density is more ideal but a minimum of 3×10^5viable cells/mL is required for the initial thaw.
8. Place the flask on a shaker apparatus shaking between 125–150 rpm in a non-humidified incubator at 26–28°C. The recommended shaking speed is 130–135 rpm.
9. The cells should reach a density of $>2 \times 10^6$ cells/mL after 4–5 days in culture. If the cells do not reach this density in 4–5 days post-thaw, but the cell viability is good, centrifuge the cells at $100 \times g$ for 3–5 minutes, resuspend them in the same volume of fresh medium, and then place them back into the incubator.
10. After 1–3 days, determine the cell density and viability again.
11. Passage the cells when the cell density reaches 2×10^6 to 4×10^6 cells/mL.

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Initiate cell culture from frozen stock, Continued

Initiate culture with frozen cells, continued

Adherent culture conditions

1. Warm growth medium to room temperature.
2. Remove a vial of cells from liquid nitrogen. Quickly thaw the cells in a 37°C water bath by agitating the cryovial until the cells are almost completely thawed with only a small ice crystal remaining (about 1–2 minutes). Do not submerge the entire cryovial in the water bath.
3. Decontaminate the outside of the vial by treating with 70% ethanol and place it in a laminar hood.
4. Remove the cells from the cryovial and transfer them into a sterile 15-mL conical tube.
5. Resuspend the cells by drop-wise adding medium that has been pre-warmed to room temperature and swirling the tube as the medium is being added. Resuspending the cells by this method reduces osmotic shock to cells and allows for increased cell recovery and viability.
6. Remove a small amount of the cell suspension and perform a cell count to determine cell density, viability, and health.
7. Seed the cells into appropriately sized flask(s) at 2×10^4–5×10^4 viable cells/cm^2, based on the cell density and viability determined above.
8. Place the flasks with loosened caps into a 26–28°C, non-humidified, non-CO_2 incubator.
9. Passage the cells using the sloughing method when the cells are ~90% confluent. The protocol for the sloughing method can be found in **Adherent cell culture**, page 17.

Adherent vs. suspension culture

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™ cells).	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 shake flasks, spinner flasks, and expensive equipment such as shaker apparatus and incubators.
Higher cell densities/mL (up to 3×10^6 cells/mL for Sf9 cells). This can increase protein yields per mL of cell culture.	Requires routine cell counts to determine mid-log phase cell densities to obtain robust cell growth.
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.

Adherent cell culture

Introduction

The following information will enable successful subculturing of adherent insect cells. Frozen Sf9 and Sf21 cells from Thermo Fisher Scientific may be directly initiated into culture as adherent or suspension cells. High Five™ cells must be first established as an adherent culture, because the cell densities provided do not allow for direct initiation into suspension culture.

Maintain adherent cultures

- **Check cells daily** until monolayer cultures have reached ~90% confluency and subculture at this point. Cells should be passaged when in mid-log phase of growth and this is typically at ~90% confluency.
- **Do not overgrow or undergrow cells.** Subculturing overconfluent cells or cells that are less than 90% confluent (i.e., cells that are not in mid-log phase of growth) will result in increased doubling time. Routinely passaging cells that are not in the mid-log phase of growth may also lead to decreased viability.
- **Keep a record of the passage number.** In general, a fresh culture using frozen stocks should be initiated approximately once every 3 months. This is regardless of whether cells are passaged 2 or 3 times a week. Keeping a record of passage numbers ensures that the cells do not go through too many passages, which can affect cell health and experimental results.
- **Keep a cell log.** Recording the cell density, viability, passage number and general appearance of your stock cells provides useful information when troubleshooting cell growth issues. See page 36 for a sample cell log.

Recommended culture volumes

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

Flask size (cm^2)	Volume range (mL)
25	5–10
75	15–20
150	40–50

Cell densities at confluency

The cell densities that are typically obtained from confluent cultures of Sf9, Sf21, and High Five™ cells are listed below. Cell numbers may vary depending upon the culture conditions and cell health (see above for suggestions for maintaining healthy cultures). Use these numbers as a general guide when setting up experiments or setting up flasks to seed a suspension culture (see **Suspension cell cultures**, page 19).

Flask size (cm^2)	Sf9	Sf21	High Five™ Cells
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

Subculture adherent cells

These methods can be used to dislodge monolayers in adherent cell culture.

- **Sloughing** (this is the method we use).
- Tapping the flask until monolayer loosens.
- Scraping (this method should be used as a last resort, because it may damage the cells and result in decreased viability).

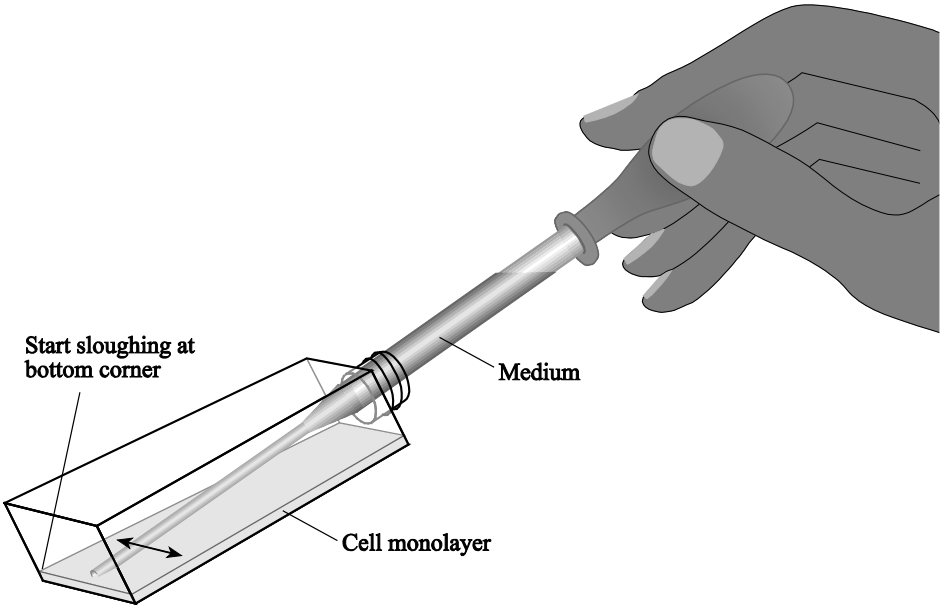
Note

Insect cells are very sensitive to physical force and agitation. Minimize harsh pipetting or agitation to maintain high viability.

Sloughing

The sloughing method dislodges the monolayer with the least manipulation and mechanical force, resulting in higher viabilities than the other 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 pipette and stream medium across the cells starting at the bottom corner of the flask. 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.



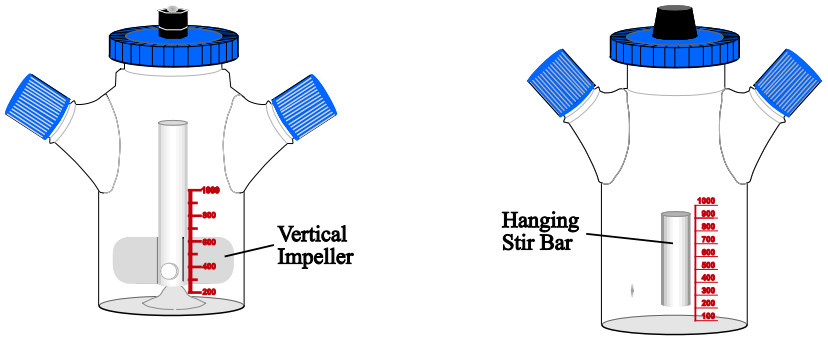
Suspension cell cultures

Guidelines

You must be able to maintain adherent cell lines prior to establishing spinner flask or shake flask cultures. Sf9 and Sf21 cells may be thawed directly into adherent or suspension cultures due to high cell densities provided. High Five™ cells must be initiated into culture as adherent cells, expanded, and adapted to suspension culture.

Maintain spinner flask suspension 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 troubleshoot potential issues that may arise later on. See page 37 for a sample cell log.
- **Renew cultures periodically.** As a general rule, a new culture should be initiated from frozen stocks once every 3 months as cells begin to lose their infectivity and are no longer optimal for use in protocols. A fresh vial of frozen stock may need to be thawed earlier than 3 months depending on the culture conditions and the number of passages per week.
- **Maintain mid-log phase cell density.** When Sf9, Sf21, and High Five™ cells reach a density of 2×10^6 to 4×10^6 cells/mL, they should be passaged at 3×10^5 to 5×10^5cells/mL.
- **Use an appropriate spinner flask.** Use a spinner flask with a vertical impeller rather than one with a hanging stir-bar assembly. The vertical impeller provides better aeration.



Note: Spinning speed needs to be optimized based on the impeller blade used. Start at the lowest spinning speed and incrementally increase the speed until it is optimized to achieve 24–30 hours doubling time, good cell viability, and low cell clumping.

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Suspension cell cultures, Continued

Maintain spinner flask suspension cultures, continued

- **Maintain volumes for adequate aeration.** The total culture volume in a spinner flask should not exceed one half of the indicated volume of the flask for proper aeration (e.g., a 500-mL spinner flask should not contain more than 250 mL of culture).
- **Use a surfactant to decrease shearing.** 0.1% Pluronic™ F-68 is recommended for spinner and shaker cultures. Pluronic™ F-68 is a surfactant that decreases cell membrane shearing due to impeller and shaking forces.

  Note: Sf-900™ II SFM, Sf-900™ III SFM, and Express Five™ SFM already contain surfactants.
- **It is not necessary to change medium** when culturing cells in suspension. Regular subculturing requires the removal of cell suspension and the addition of fresh medium based on the recommended seeding density of the cells. 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.

Maintain 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.
- **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 **Clean spinner flasks**, page 36).

Continued on next page

Pluronic™ F-68

We recommend adding Pluronic™ F-68 (Cat. No. 24040-032) to your spinner/shaker 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/shaking. Some serum-free media may already contain Pluronic™ F-68 or other suitable surfactants.

Note: Sf-900™ II SFM, Sf-900™ III SFM, and Express Five™ SFM already contain surfactants. You do not need to add additional surfactants**.**

Minimum requirements for 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 0.5×10^6 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	Minimum volume required
100 mL	30 mL
250 mL	80 mL
500 mL	200 mL

Note: Initiating a culture in spinner flasks larger than 500 mL is not recommended. We recommend starting from smaller spinner flasks and gradually adapting the culture to larger size flasks. Spinning speed may need to be optimized when moving to different size flask(s).

Continued on next page

Maintain shake flask suspension cultures

- **Monitor and record cell growth.** Cells should be routinely monitored for cell density and viability, and passaged when in log phase of growth. It is helpful if these data are maintained in a log or chart to help you make decisions on the health of your culture. See page 36 for a sample cell log.
- **Renew cultures periodically.** As a general rule, a new culture should be initiated from frozen stocks once every 3 months as cells begin to lose their infectivity and are no longer optimal for use in protocols. A fresh vial of frozen stock may need to be thawed earlier than 3 months depending on the culture conditions and the number of passages per week.
- **Maintain mid-log phase cell density.** Sf9 and Sf21 cells should be passage when densities reach 2×10^6 to 4×10^6 cells/mL. Generally, cells reach mid-log phase of growth in this density range. We recommend determining the cell density range that corresponds to the mid-log phase of cell growth in your specific culture by performing a growth curve. For more information on how to perform a growth curve, refer to **Generate a growth curve**, page 35.
- **Use an appropriate flask.** Use a shake flask ranging from 125 mL to greater than 1 L.
- **Maintain volumes for adequate aeration.** The recommended total culture volume of cells and media are provided in the table below.

Flask size	Total volume if cells and media
125 mL	30–50 mL
250 mL	75–100 mL
>500 mL	1/4 to 1/3 total flask volume

- **Maintain shaking speed to provide proper aeration.** Shaking speed for insect cells can range from 125 to 150 rpm. We recommend setting the shaking speed to 130–135 rpm.
- **Use a surfactant to decrease shearing.** Addition of 0.1% Pluronic™ F-68 is recommended for spinner and shake flask cultures. Pluronic™ F-68 is a surfactant that decreases cell membrane shearing due to impeller forces.
  Note: Sf-900™ II SFM, Sf-900™ III SFM, and Express Five™ SFM already contain surfactants.
- **It is not necessary to change medium** when culturing suspension cells. Regular subculturing requires the removal of cell suspension and the addition of fresh medium based on the recommended seeding density of the cells. This addition of medium is sufficient to replenish cell nutrients.
- **Maintain proper seeding density** for robust cell doubling time, growth, and viability. The recommended seeding density is 3×10^5–5×10^5 viable cells/mL. Subculturing cells using the recommended seeding density will provide cell densities of 2×10^6–4×10^6 cells/mL every 3–4 days, depending on the initial seeding density.

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 suspension culture in spinner or shake flasks. This procedure allows you to initiate and maintain suspension cultures of Sf9 and Sf21 cells. For more information on suspension culture, see **Suspension cell cultures**, page 19.

Required materials

- Adherent Sf9 or Sf21 insect cells
- Spinner flasks (Bellco #1965 Series) or shake flasks (Corning or Fisher)
- Magnetic stir plate or shaker apparatus
- 26–28°C incubator, CO_2 not required

Suspension culture 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 0.5×10^6 viable cells/mL. Seeding at lower densities can cause increased doubling times in the initial stages of culture. See **Cell densities at confluency**, page 17, for help in determining the number of flasks needed to obtain enough cells to seed a spinner or shake flask culture.

   Note: We recommend starting off with a 100-mL or 250-mL spinner flask or 125-mL shake flask, as these require fewer total cells than larger flasks to initiate suspension cultures.
2. Detach cells from flasks and count cells to determine cell density and viability. Viability needs to be >95% to ensure successful adaptation.
3. Seed a clean, sterile spinner or shake flask 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 0.5×10^6 viable cells/mL would require a total of $(0.5 \times 10^6 \text{ cells/mL}) \times (50 \text{ mL}) = 2.5 \times 10^7$ viable cells.
4. Incubate the spinner flask(s) at 26–28°C with constant stirring at 80–90 rpm or the shake flask(s) at 26–28°C at a constant shaking speed of 125–150 rpm (130–135 rpm is recommended).
5. When the cells reach a density of about 2×10^6 to 4×10^6 cells/mL, passage them at a seeding density of 3×10^5 to 5×10^5 viable cells/mL in appropriately sized flask(s).

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 of 18–24 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 materials

- High Five™ insect cells (Thermo Fisher Scientific)
- Express Five™ SFM (Thermo Fisher Scientific)
- Heparin, tissue culture grade (Sigma, Cat. no. H3149)
- Spinner flasks (Bellco #1965 Series) or shake flasks (Corning or Fisher)
- Magnetic stir plate or shaker apparatus
- 26–28°C incubator, CO_2 not required

Use heparin

Heparin helps 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 5–10 cells, visible under a hemacytometer, are common to High Five™ suspension cells and will not affect expression.

Adapt culture

1. Take four confluent 75-cm^2 flasks of adherent High Five™ cells, slough, and inoculate a 100-mL spinner or a 125-mL shake flask. Total volume should be between 40–50 mL for the spinner flask or 30–50 mL for the shake flask, and the cell density should be 2×10^5–4×10^5 viable cells/mL. Use serum-free medium to adjust the volume or cell density. Do not exceed 50% of the spinner flask volume.
2. Incubate the spinner flask at 26-28°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 2×10^6 cells/mL, continue to grow the cells.
4. When the cell density reaches $>2 \times 10^6$ cells/mL, passage the cells at a seeding density of 2×10^5–4×10^5 viable cells/mL into the desired flask and scale up accordingly.

   Note: The cell density range corresponding to the mid-log phase of growth should be determined based on culture conditions (i.e., technicians, incubator, equipment, etc.) by performing a growth curve (see **Generate a growth curve**, page 35).
5. Cells are considered adapted to suspension culture when robust cell growth with a doubling time of 18–24 hours and a viability of >90% is observed.

Continued on next page

Suspension cell cultures: High Five™ Cell Line, Continued

Maintain suspension High Five™ Cells

Maintain healthy cells by passaging them in mid-log phase of growth at the recommended seeding density.

Note: If the cells are passaged when the cell density is below or above the range corresponding to the determined mid-log phase of growth, doubling time will be longer. However, the doubling time will return to the normal range of 18–24 hours when these "distressed" cells are passaged in the mid-log phase of growth.

Storage High Five™ Cells suspension long-term

High Five™ cells that are adapted to suspension culture can be frozen down in freezing medium consisting of 45% conditioned medium, 45% fresh growth medium, and 10% DMSO.

Adherent cultures: Cryopreserve adherent cells at a minimum of 3×10^6 viable cells/vial. Cells frozen from adherent cultures can only be thawed into adherent culture conditions due to their lower cell density per vial.

Suspension cultures: Cryopreserve suspension cells at 1.0×10^7–1.5×10^7 viable cells/vial. Cells frozen at higher densities may be thawed directly into suspension or adherent conditions using the appropriate seeding density.

Freeze insect cell lines

Introduction

Once cell lines are established and are doubling regularly, they can be frozen. Cells should be >90% viable and in the mid-log phase of growth. For adherent cultures, this corresponds to ~90% confluency. For suspension cultures, the cell density range corresponding to mid-log phase of growth should be determined from the growth curve. We recommend that you freeze down several vials of healthy cells at as low a passage number as possible for optimal long-term use.

Freezing procedure

You should continue to maintain your stock cell culture until your frozen cells have been pre-qualified (see **Pre-qualification**, page 27).

1. Use the table below to prepare the freezing medium appropriate for the cell line and store at 2–8°C until use. Note that Grace's Insect Medium does not contain supplements or FBS.

Cell line	Freezing medium	Density (cells/vial)
Sf9	80% Grace's Insect Medium, Supplemented 10% FBS, heat-inactivated 10% DMSO	>1 × 10^7
Sf21	80% Grace's Insect Medium, Supplemented 10% FBS, heat-inactivated 10% DMSO	>1 × 10^7
High Five™ cells	45% conditioned Express Five™ SFM 45% fresh Express Five™ SFM 10% DMSO	>3 × 10^6

2. Prepare cryogenic storage vials (i.e., cryovials) by labeling each vial appropriately with the cell name, density, and date.
3. Transfer the stock cells to cryopreserve from the culture flask(s) into sterile 15-mL or 50-mL conical tube(s).

   Note: If maintaining cultures in multiple flasks, pool the cells from all flasks.
4. Take a small aliquot of the cells and determine the cell density and viability.
5. Centrifuge the cells at 100–200 × *g* for 5 minutes to pellet.
6. Remove the spent medium from tube(s). Flick the bottom of the tube with a finger a few times to dislodge the cell pellet and gently resuspend the cells in an appropriate volume of cold freezing medium to achieve the desired cell density to add to each cryovial (see table above).
7. Dispense aliquots of the cell suspension into labeled cryovials. As cells are dispensed into cryovials, frequently and gently mix the cell suspension to maintain a homogeneous solution.
8. Freeze the cells in a controlled rate freezing apparatus by decreasing the temperature approximately 1°C per minute. If equipment is not available, put cryovials into –5°C to –20°C for 30 minutes and then transfer them to –80°C for overnight storage.
9. The next day, transfer the cells left overnight at –80°C into the vapor phase of liquid nitrogen in the storage tank.

Continued on next page

Freeze insect cell lines, Continued

Pre-qualification

The day after transferring the frozen cells to liquid nitrogen storage, pre-qualify the frozen cells by thawing one vial and testing the thawed cells for bacterial, fungal, and mycoplasma contamination, normal cell morphology, and robust growth. Cell morphology and growth should be similar to the growing stock cells.

If thawed cells have become contaminated or do not look healthy, thaw a second vial from the same freeze. If the second vial shows the same results as the first, expand the growing stock cultures and freeze fresh aliquots of cells again.

We recommend pre-qualifying every freeze that has been transferred to liquid nitrogen storage. Cryopreservation does not guarantee quality cells unless the cells have been pre-qualified. Do not discontinue stock cultures until it has been shown that cell freeze has passed all pre-qualification tests.

Troubleshooting

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. Keeping a good record of cells (i.e., cell density, viability, and detailed notes on the health of the cells) will help in troubleshooting potential problems that may arise. Information below may be used as a guideline to help troubleshoot common problems observed in culturing insect cells.

Observation	Possible cause	Recommended action
Adherent cells: morphology changes—first week in culture		
Cells are granular and/or floating post-thawing	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 13).
	Aging cell culture with passage number greater than 30.	Thaw new cells of lower passage number.
Cell lysis or debris	Contamination with wild-type or recombinant baculovirus. You will see lysis, debris and/or swelling of cells. Inclusions in the nucleus indicate wild-type infection.	Discard the old culture and initiate a fresh culture. • Thaw new cells. • Decontaminate hood and equipment. • Make up new medium.
Decreased viability and growth rate	Too much mechanical manipulation during subculturing.	• Thaw new cells. • Switch to another method of subculturing (e.g., sloughing).
	Passaging too often before confluency.	• Remove medium/floaters. • Replace with fresh medium. • Passage only at confluency.
Floaters (>10% of cells in culture)	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.
Adherent cells: morphology changes—more than one week in culture		
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.

Troubleshooting, Continued

Observation	Possible cause	Recommended action
Adherent cells: growth and/or viability decrease		
Increased cell doubling times	Subculturing technique too rigorous.	Thaw new cells and try using a different subculturing technique (e.g., sloughing).
	Cells passaged above or below the mid-log phase of growth (i.e., passaged at >90% or <80% confluency).	Passage cells in mid-log phase of growth (~90% confluency) to obtain doubling times of 18–24 hours for High Five™ cells and 24–30 hours for Sf9 and Sf21 cells.
	Cells are at a high passage number (>30 passages).	Thaw new cells and make sure that you are freezing down cells at a low passage number (<10) so that new cultures are optimal.
Cell viability is less than 90%	Subculturing technique too rigorous.	Thaw new cells and try using a different subculturing technique (e.g., sloughing).
	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 indicate 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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Troubleshooting, Continued

Observation	Possible cause	Recommended action
Suspension culture: morphology changes		
Cell lysis or debris	Aging cell culture due to high passage numbers.	Thaw new cells of low passage number and initiate a new suspension culture.
	Shearing due to impeller spin rate or shaking speed.	• Reduce impeller spin rate or shaker speed. • Pluronic™ F-68 may also be added 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 **Clean glass culture vessels**, page 36 . • Make up new medium. Keep separate bottles of medium for cell culture and viral work.
	Stock cells are routinely allowed to overgrow, causing cell stress and reduce viability.	• Passage cells in mid-log phase of growth. • Generate a growth curve to determine the cell density range where the cells would be in mid-log phase of growth.
	Residual detergent from cleaning of the glass culture vessels stick to the glass, causing cell toxicity.	Clean glass culture vessels using an acid wash.
Cells swollen, spots in the nucleus	Contamination with wild-type or recombinant baculovirus.	See above recommendations for baculovirus contamination.

Continued on next page

Troubleshooting, Continued

Observation	Possible cause	Recommended action
Suspension culture: growth and/or viability decrease		
Increased cell doubling times	Cells passaged at a low seeding density.	Centrifuge the cells at 100 × *g* for 5 minutes. Remove the spent medium, flick the bottom of the tube to dislodge the cell pellet, and resuspend the cells in a smaller volume to achieve a cell density of 5×10^5 viable cells/mL.
	Cells not passaged when in mid-log phase of growth.	Passage cells in mid-log phase of growth. A general guideline is to passage the cells when they reach a density of about 2×10^6 viable cells/mL. Ideally, a growth curve should be created to determine the cell density range that indicates mid-log phase of growth based on your culture conditions.
Increased cell doubling times	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.
		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 high impeller spin rate or shaker speed	• Reduce impeller spinning spin rate or shaker speed. • Pluronic™ F-68 may also be added at 0.1% final concentration, if needed.
	Inadequate aeration.	See above or **Inadequate aeration**.
	Bacterial or Fungal Contamination.	Discard the old culture and initiate a fresh culture. • See **Maintain sterile conditions in a spinner flask,** page 20. • Add antibiotics to your medium.
	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.

Continued on next page

Troubleshooting, Continued

Observation	Possible cause	Recommended action
Problems with High Five™ Cells in suspension		
Clumping of cells	Cells were grown to densities too high.	1. Let the spinner sit in the hood for 30–45 minutes 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 cultures**, page 19). 3. Passage cells in mid-log phase of growth. Generate a growth curve to determine the cell density range where the cells would be in mid-log phase of growth. **Note:** If clumps represent more than 50% of the culture, start a new adaptation.
	Spinning rate or shaking speed too high.	Use 80–90 rpm for spinner flask and 125–150 rpm for shake flask.
	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 **Adapt culture**, page 24).
Cell lysis or debris	Aging culture due to high passage numbers.	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 **Clean glass culture vessels**, page 36. • Make up new medium Keep separate bottles of medium for cell culture and viral work.
Viability <90%	see **Suspension culture: growth and/or viability decrease**.	
Increased cell doubling times	see **Suspension culture: growth and/or viability decrease**.	

Appendix A: Support protocols

Count and concentrate cells

Count cells

Cells can be counted using hemacytometers (available from most major laboratory supplier) or using the Countess™ Automated Cell Counter (Cat. No. C10227), which provides fast, easy and automated cell counting and eliminates the tedium and subjectivity of manual cell counting.

Trypan Blue exclusion

The following procedure enables quick and accurate determination of cell density and viability. Cell viability is calculated as the number of viable cells divided by the total number of cells counted. Cells that stain blue are dead and cells that are clear are viable.

1. Transfer 250 µL to 1 mL of cell suspension into a test tube, add an equal volume of 0.4% trypan blue solution, and gently mix.

   Note: The dilution may need to be adjusted based on the density observed on the hemacytometer. There should be 10–50 cells per square mm^2 (one large square of hemacytometer) to achieve accurate counts.
2. Add a sufficient volume of cell/trypan blue mixture to the hemacytometer and count the cells under an inverted microscope with a 10X ocular and 10X objective.
3. 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.
4. Use the formulas below to calculate the dilution factor and the number of cells per mL.

$$\text{dilution factor} = \frac{\text{volume of cell suspension + volume of stain}}{\text{volume of cell suspension}}$$

$$\text{cells/mL} = \frac{\text{total \# of cells counted} \times \text{dilution factor} \times 10^4}{\text{\# of squares counted}}$$

Concentrate cells

1. Transfer the cell suspension to an appropriately sized sterile centrifuge tube and centrifuge for 3–4 minutes at 100–200 × *g*.
2. Carefully remove the supernatant without disturbing the cell pellet and cap the tube containing only cell pellet.
3. Flick the bottom of the tube with a finger a few times to dislodge the cell pellet and gently resuspend the cells in the desired volume of fresh medium by slowly pipetting up and down a few times to break up the cell clumps.

   Note: These cells are very sensitive to physical force and agitation. Pipetting harshly or too much will lower cell viability.
4. Remove a small amount of the cell suspension to determine cell density and viability.
5. Transfer the cells into a new culture vessel based on recommended seeding densities for adherent and suspension culture conditions.

Adapt cells to serum-free medium

Adapt sequentially to serum-free medium

Insect cells cultured in serum-supplemented media may be adapted to serum-free formulations. Sequential adaptation to serum-free medium, which involves gradually decreasing the percentage of serum-supplemented medium in the cell culture, provides a higher success rate.

The following sequential adaptation protocol applies to cells in both adherent and suspension cultures. It may also be used to adapt cells from one serum-free formulation to another.

1. Take a small aliquot of the cells and determine the cell density and viability.

   Note: Cells to be adapted should be in mid-log phase of growth.
2. Prepare a mixture of 75% serum-supplemented medium and 25% serum-free medium. This is the 75:25 media mixture.
3. Seed the cells in 75:25 media mixture at double the recommended seeding density (i.e., 4×10^5 to 10×10^5 viable cells/cm^2 for adherent cells or 6×10^5 to 10×10^5 viable cells/mL for suspension cells).
4. When the cells reach mid-log phase of growth (at ~90 confluency for adherent cells or in the appropriate cell density range for suspension cells), passage them into two separate flasks. One flask should contain a 75:25 and the second a 50:50 media mixture of serum-supplemented medium to serum-free medium.
5. When the cells reach mid-log phase of growth, passage them into two separate flasks, containing a 50:50 and a 25:75 media mixtures of serum-supplemented medium to serum-free medium, respectively.
6. Continue to lower the serum-supplemented medium concentration and increasing the serum-free medium concentration through successive passaging.
7. Cells are considered 100% adapted to serum-free medium when routine culture shows healthy, growing cells displaying normal morphology and doubling times of 24–30 hours for Sf9 and Sf21 cells or 18–24 hours for High Five™ cells.

Note: At each step of the sequential adaptation procedure, continue a backup culture using the previous media dilution mixture until it has been determined that the cells in the media mixture with the lower concentration of serum-supplemented medium grow well.

If cells in the lower concentration of serum-supplemented medium mixture show poor growth, use the backup cells in the previous dilution and passage them at 40:60 instead of 25:75.

Cells may need to be passaged a few times at the same serum-supplemented and serum-free mixture before they can be passaged into the media mixture with a lower concentration of serum-supplemented medium.

Generate a growth curve

Generate a growth curve

Information collected from a growth curve is useful for troubleshooting potential problems with the cell culture, and determining the doubling time and growth phase based on the culture conditions.

The following protocol applies to the culture of insect cells in suspension (i.e., cells in shake or spinner flasks).

Note: The protocol requires counting cell growth for 7 consecutive days. We recommended starting this protocol early in the week (i.e., Monday).

1. Remove the cells from the incubator and determine the cell density and viability using a hemacytometer or other appropriate method of choice.

   Note: Cell viability should be >90% and cells should be actively dividing to proceed with this protocol.
2. Passage the cells into two separate flasks at a seeding density of 3×10^5 viable cells/ml. One flask will be used for generating the growth curve and the second flask will be used to continue culturing stock cells. Label each flask appropriately.
3. Incubate the cells in a 26–28°C, non-humidified incubator on a shaker apparatus or a spinner platform. Record the time when the cells are placed into the incubator.

   Note: Time of incubation is important to generate accurate data.
4. The next day, remove the flask used for generating the growth curve ± 1 hour from the initial culture incubation time recorded in step 3.

   Example: Cells that were placed in the incubator at 9:00 am in step 3 should be counted between 8 am and 10 am the next day to generate accurate data.
5. Remove 0.5 L of the cell suspension from the flask and return the flask to the 26–28°C incubator. Perform a cell count to determine and record cell density and viability.
6. Repeat steps 4 to 6 for seven days.
7. Graph the data generated from day 0 to day 7, with days on the X-axis and the viable cell counts on the Y-axis on the graph.

Clean glass culture vessels

Clean spinner flasks

Proper care of glass culture vessels is important for maintaining healthy growing cells that are free from cross-contamination with different viral stocks. Baculovirus particles can survive through an autoclaving cycle. We recommend using two autoclaving cycles to eliminate potential virus contamination of future cultures.

1. Wash glass culture vessels with 7X Cleaner (Bellco) or 10% acetic acid by spinning or shaking the vessel for approximately 2 hours. The 7X glass cleaner is preferable as trace amounts are not detrimental to cells. Acetic acid can be detrimental if not rinsed away completely. **Do not use detergents.**
2. Rinse five times with tap water.
3. Rinse five times with distilled water.
4. Cap culture vessel loosely.
5. Autoclave once wet with deionized water for 45 minutes on liquid cycle.
   Note: This step can be repeated, if desired.
6. Autoclave once dry for 45 minutes on dry cycle.

Cell Log

Cell line: Cell Lot no./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				
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Record any changes in medium and medium lot number in the "Comment" column as well as any notes about cell appearance.

Appendix B: Ordering information

Cells and media

Insect Cell Lines

See the table below for ordering information for frozen cell lines available from Thermo Fisher Scientific. For more information, refer to **thermofisher.com** or contact Technical Support (see page 39).

Cells	Amount	Cat. No.
Sf9	1 × 10^7 cells in 1 mL 60% Grace's Media, 30% FBS, heat-inactivated, and 10% DMSO	B825-01
Sf21	1 × 10^7 cells in 1 mL 60% Grace's Media, 30% FBS, heat-inactivated, and 10% DMSO	B821-01
High Five™	3 × 10^6 cells in 1 mL 92.5% unconditioned Express Five™ SFM, with 90 mL/L of L-glutamine and 7.5% DMSO	B855-02

Insect Growth Media

Gibco™ insect culture media are available from Thermo Fisher Scientific to culture Sf9, Sf21, and High Five™ insect cells. See the table below for ordering information. For more information, refer to **thermofisher.com** or contact Technical Support (see page 39).

Product	Amount	Cat. 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 1 L	10902-096 10902-088
Sf-900™ III SFM	500 mL 1 L	12658-019 12658-027
Express Five™ SFM	1 L	10486-025

Antibiotics/ Antimycotics

See the table below for ordering information for antibiotics and antimycotics suitable for use with insect cell lines. For more information, refer to **thermofisher.com** or contact Technical Support (see page 39).

Product	Amount	Cat. No.
Gentamicin Reagent Solution (10 mg/mL), liquid	10 mL 10 × 10 mL	15710-064 15710-072
Gentamicin Reagent Solution (50 mg/mL), liquid	10 mL 10 × 10 mL	15710-060 15710-078
Gentamicin/Amphotericin Solution (500X)	10 × 1 mL	R-015-10
Fungizone™ Antimycotic, liquid (Amphotericin B)	20 mL	15290-018
Penicillin-Streptomycin, liquid	20 mL 100 mL	15140-148 15140-122

Documentation and support

Customer and technical support

Visit thermofisher.com/support for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at: **www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf**
- World Health Organization, Laboratory Biosafety Manual, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at: **www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf**