EDVOTEK®

THE **BIOTECHNOLOGY**
EDUCATION COMPANY®

Revised and Updated

Edvo-Kit #
1001

Edvo-Kit #1001

Eukaryotic Cell Biology Using Insect Cell Culture

Experiment Objective:

The objective of the experiment is familiarize students with a simple and robust cell culture system. Students will be introduced to the basic principles of cell culture and will utilize sterile technique to examine the cell growth and viability of Sf9 insect cells.

See page 3 for storage instructions.

1.800.EDVOTEK • Fax 202.370.1501 • www.edvotek.com • info@edvotek.com

Table of Contents

Safety Data Sheets can be found on our website: **www.edvotek.com/safety-data-sheets**

Experiment Components

	Component	Storage	Check (✓)
A	Sf9 Insect Cells	Room temp.	❑
B	Insect Cell Media	4° C Refrigerator	❑
C	Sterile Technique Practice Media	4° C Refrigerator	❑
D	Trypan Blue Dye	Room temp.	❑
E	1x PBS	Room temp.	❑
F	Giemsa Stain	Room temp.	❑

Store the following components at room temperature.

- Cell culture flasks, 25 cm^2 (Sterile) ❑
- Cell culture dishes, 35 mm diameter (Sterile) ❑
- Cell counting Chambers ❑
- Large transfer pipets (Sterile) ❑
- Small transfer pipets ❑
- 10 ml and 25 ml disposable pipets (Sterile) ❑
- 15 ml conical bottom tubes ❑
- 50 ml conical bottom tubes ❑
- 1.5 ml microcentrifuge tubes ❑

This experiment is designed for 6 student groups.

Sf9 Insect Cells should be requested 2 weeks before starting the experiment. Culture cells immediately upon receipt.

Immediately store the Insect Cell Media and Sterile Technique Practice Media in the refrigerator (4° C).

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Requirements *(not included with this kit)*

- Incubator, covered plastic container, or cardboard box to grow cultures (the EDVOTEK box will work as an incubator)
- 70% Ethanol in spray bottles
- Methanol
- Pipet pumps or bulbs
- Phase contrast or bright field microscope suitable for cell culture (see note box)
- 10-100 µl micropipet and tips
- Safety goggles, disposable laboratory gloves, and lab coats
- Waste containers
- Marking pens

NOTE:
The cell culture flasks used in this experiment are approximately 2.5 cm tall. Please ensure that there is sufficient clearance between the stage and objectives to view the cells.

Background Information

Eukaryotic Cell Culture

Cell culture, the ability to grow and study bacteria, viruses, and eukaryotic cells, is a cornerstone of modern biology. In cell culture experiments scientists recreate the natural environment of the cells in a laboratory to answer important biological questions. This can include studies examining cellular architecture, behavior, or disease. Cell culture has increased the understanding of cellular functions and has become an important platform for studying both normal development and disease.

Although scientists performed the first cell culture experiments in the mid-1800's, the techniques did not truly develop until the 20th century. Since then cell culture has allowed cells from dozens of species to be grown and studied. The earliest of these experiments involved crude preparations of tissues that were placed into a buffer solution. Many early cell culture trials were unsuccessful, and even the most promising studies could only keep cells alive for a few days. Fortunately, through the use of improved reagents and techniques it is now possible to culture cells for months, years, and even decades. Many cultured cells have been selected for mutations that allow them to grow in culture indefinitely, producing so-called "immortalized cell lines".

Cell culture has lead to advances in the fields of life science, biotechnology, and pharmaceutical research. For example, early vaccine research relied heavily on the use of animals for testing and virus production. However, the development of cell culture strains has allowed for the transition away from live animals. In addition to reducing animal testing, cell culture has increased the reproducibility and decreased the costs associated with vaccine production. Cell culture is also used to study many common illnesses, including genetic disorders, viral and bacterial infections, and cancer. These experiments can examine healthy and diseased cells, monitor the effects of gene additions or deletions, or screen for effective therapeutics.

Sf9 Insect Cell Culture

Insect cell culture originated as an approach to better understand insect biology. Many early studies with insect cells were designed to analyze basic biological questions. These experiments provided valuable information about the development and pathology of insects. In addition, insect cell culture has been used to develop novel insecticides and other deterrents to agricultural pests. One of the most popular of these insect strains has been the Sf9 cell line, which was derived from the ovarian cells of the fall armyworm, *Spodoptera frugiperda* (Figure 1). Sf9 cells are an important model for examining basic cellular processes, many of which are present in higher eukaryotes.

Importantly, Sf9 cells grow rapidly and are easy to maintain. The cells are grown in standard atmosphere and at room temperature, unlike mammalian cell culture that requires complicated incubators to control temperature, CO_2, and humidity (Figure 2). These traits simplify growing conditions and reduce the cost of culturing the cells. The ease of growing Sf9 cells has made them an essential part of the biotech industry, where cells are commonly used in the production of recombinant proteins and viruses. Importantly, the simplicity of culture also makes insect cells a useful model system for the classroom.

Figure 1: Adult *Spodoptera frugiperda* (top) and Sf9 cells (bottom).

Cell Culture Techniques

Many tools and techniques have been developed to maintain cultured cells. For example, researchers use sterile flasks and plates that have been treated to allow adherent cells to attach and grow. Most non-human cell lines are non-pathogenic and can be cultured in simple culture hoods. These hoods help to prevent contamination of the cells by bacteria, fungi, yeast and mold, but are not designed to protect the scientist (Figure 3). In contrast, infectious cells and viruses can require elevated levels of personal protective equipment, dedicated culture hoods, and even specialized rooms and facilities. Sterile conditions are maintained by decontaminating all surfaces and equipment with ethanol, and by using "barrier" pipettes that contain a filter.

Figure 2: Cell culture incubators for standard or controlled atmospheric conditions.

Cells are cultured in growth media, a chemically complex solution that provides the nutrients necessary for cells to grow. Media typically contains essential amino acids, buffers, salts, and a carbon source like glucose. This mixture is carefully balanced for use with specific cell lines and the choice of media is essential for proper growth and behavior. In addition, many cell lines are supplemented with animal serum, to provide essential growth factors, and antibiotics to help reduce the chances of bacterial infection. The insect cell media provided for this lab contains both serum and antibiotics, which is referred to as a "complete media".

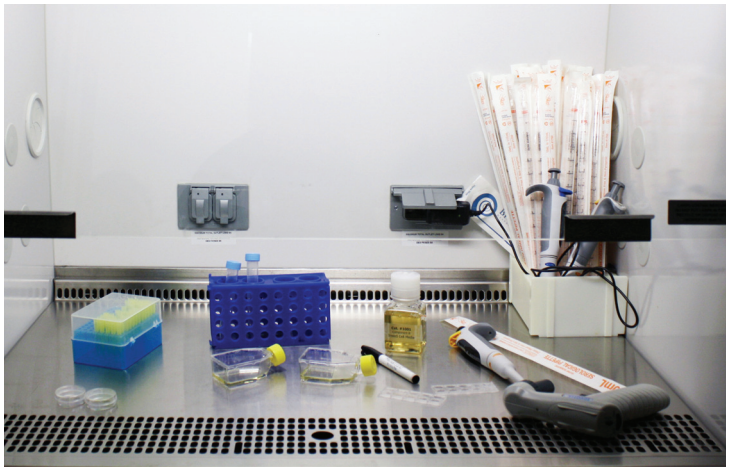


Figure 3: Cell culture hood and standard equipment.

Many cellular features are visible using a modest compound microscope. Special stains are available to accentuate cellular structures and improve observations. For example, trypan blue, a vital dye, is commonly used to enhance cell counting and to monitor the health and growth rate of cultured cells. Trypan blue will not stain healthy, living cells, but is quickly absorbed by dead cells. Therefore, when a mixture of cells is treated with trypan blue only the dead cells will stain blue. Other stains, such as Giemsa stain, can be used to examine cellular structure, stages of the cell cycle, or to distinguish between multiple types of cells within a population. Giemsa will stain the DNA of Sf9 cells dark blue, while the cytoplasm stains light blue or purple. Additional stains can be used to label specific cells or features with different colors, allowing a pathologist to differentiate between types of cells in a mixture.

Growth and Maintenance of Cultured Cells

Sf9 cells are immortalized, allowing them to proliferate for many generations under optimal conditions. The cells will continue to divide in a culture flask until they have either depleted the growth media of essential components or occupied all available space. In the later case, cells will become contact-inhibited and will stop dividing. Therefore, it is necessary to passage the cells, removing a small portion of the cells and their existing media and transferring it to a new container with new media. Passaging the cells provides more space for cells to proliferate and fresh media to promote growth.

After cells are passaged they go through three distinct phases of growth (Figure 4).

- **Lag Phase:** Immediately after being passaged into a new flask the cells may enter a lag phase of growth where there is little increase in the cell number. During this time the cells will "condition" the media by secreting proteins that enhance growth. The cell density is low, with cells covering less than 50% of the surface (known as 50% **confluent**). The lag phase can last about 1-2 days.

- **Log Phase:** The cell number increases exponentially during this phase, and cell growth will continue as long as there is enough nutrients to sustain the increasing cell number. The cells are approximately 50 – 80% confluent.

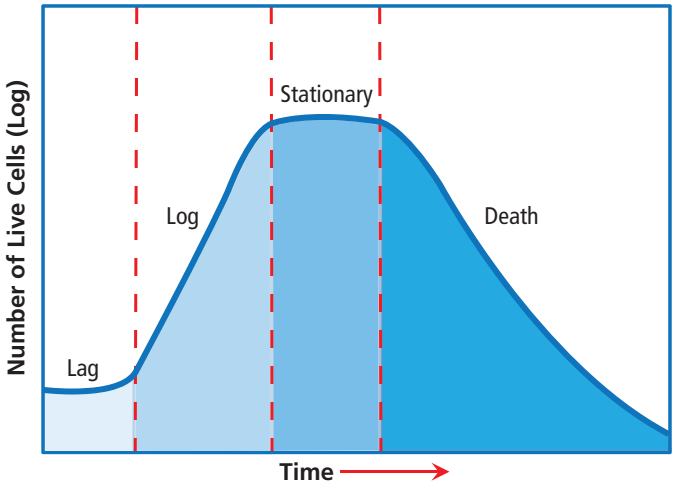


Figure 4: Phases of cell culture growth.

- **Stationary Phase:** During this phase, the number of cells remains constant, as some cells die and others slowly divide. Eventually, all of the cells will die unless subcultured or fresh media is added. At this point the cells are 90 – 100% confluent.

The phases of cell growth can be estimated by examining the confluency of a culture. For a more precise measurement scientists will determine the growth rate of the cells by performing repeated cell counts over a few hours or days. Changes in the growth rate of cells might indicate a problem with the health of the culture that is not immediately apparent.

The Future of Cell Culture Research

Cell culture experiments are essential for both academic and industrial research. Scientists routinely use cell lines to address questions about the behavior of cells, tissues, and even whole organisms. For example, cell culture has improved our understanding of evolution, cell function and behavior, and early development in animals. These experiments are also valuable for examining DNA, RNA, and protein function at a cellular level. Cell culture is also commonly used to develop and screen potential drugs, allowing for the rapid discovery of novel therapeutics. In these experiments scientists culture both healthy and diseased cells to understand the biological nature of the disease and to discover effective treatments for existing patients. Other applications of cell culture include stem cell research, organ transplantation, gene therapy, and neurological research. These types of cell culture screens are important for minimizing the use of research animals and preventing animal suffering. Future research will continue to enhance our understanding of cellular functions and improve human health.

In this experiment, students will develop practical skills in the manipulation and maintenance of Sf9 insect cells. Groups of students culture the cells using sterile techniques. The cells are examined by microscopy to determine the cellular morphology, growth rate, and viability. Using the data they collect, students create and analyze a growth curve.

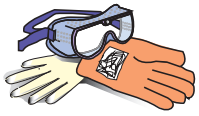
Experiment Overview

EXPERIMENT OBJECTIVE

The objective of the experiment is familiarize students with a simple and robust cell culture system. Students will be introduced to the basic principles of cell culture and will utilize sterile technique to examine the cell growth and viability of Sf9 insect cells.

LABORATORY SAFETY

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
4. Always wash hands thoroughly with soap and water after working in the laboratory.
5. If you are unsure of something, ASK YOUR INSTRUCTOR!



Wear gloves and safety goggles

STERILIZATION OF EQUIPMENT AND CONSUMABLES

1. Sterilize the lab bench with a 10% bleach solution, 70% Ethanol or a laboratory disinfectant.
2. All materials that come in contact with cells should be disinfected before disposal, including culture dishes, pipets, transfer pipets, and tubes.
   - Autoclave at 121 °C for 20 minutes.
     Remove media from flasks and plates, then collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium from spilling into the sterilizer chamber.
   - Soak in 10% bleach solution.
     Submerge dishes, tubes and other contaminated materials into a 10% bleach solution. Soak the materials for at least 1 hour and then discard. Wear gloves and goggles when working with bleach.

NOTE:
This experiment contains antibiotics to keep cultures free of contamination. Students who have allergies to antibiotics, such as PENICILLIN or STREPTOMYCIN, should not participate in this experiment.

LABORATORY NOTEBOOKS

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:
- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:
- Record your observations.

After the Experiment:
- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Module I: Basic Aseptic Technique

It is important to prepare a designated clean area before beginning the cell culture experiments. Start with a completely clear, non-porous surface that has been cleaned and disinfected. Follow the procedures below to maintain aseptic conditions throughout the experiments.

A. PREPARING STERILE INCUBATION CHAMBERS

Specialized culture incubators are widely used in microbiology and cell biology to culture bacteria and eukaryotic cells. The incubators maintain control of temperature, humidity, and other conditions such as the carbon dioxide and oxygen content of the atmosphere inside. The advantage of working with Sf9 insect cells is that they can be grown at room temperature and do not require a complicated growth environment.

If you have an incubator it can be set to maintain a temperature of 27°C. Before starting the experiment, all internal surfaces should be wiped with 70% Ethanol to disinfect. If an incubator is unavailable, one can be created by selecting an appropriately sized cardboard box or plastic container with lid (Figure 5). One container can be used to store the flasks for an entire class.

1. Insect cells prefer to grow in a dark environment and will not grow under direct light. If necessary, **COVER** the incubation chamber with aluminum foil to avoid light.
2. **SWAB** the inside of the container with 70% Ethanol and allow the surfaces to completely dry.
3. **PLACE** the chamber in a draft-free area that will maintain a temperature between 24-27° C. It is best to avoid windows or air vents that might alter the temperature of the chamber.

Figure 5: The EDVOTEK shipping box makes an excellent incubation chamber.

B. LEARNING BASIC ASEPTIC TECHNIQUES

Successful cell culture depends on keeping the cells free of contamination from microorganisms such as bacteria, fungi, and viruses. All materials that come into contact with the cell culture must be sterile and manipulations must not allow the non-sterile surroundings to contaminate the culture. Carefully read the following guidelines before starting any cell culture experiments.

Personal Hygiene

1. Lab gowns and face masks are **STRONGLY RECOMMENDED** to minimize the risk of contamination of your cell cultures. Tie back long hair and keep talking to a minimum.
2. Disposable gloves should be worn at all times. **SPRAY** disposable gloves with 70% Ethanol and spread between both gloves to disinfect. This should be done frequently while working with cells to prevent contamination. Change gloves as needed and immediately disinfect the new pair with 70% Ethanol.

Module I: Basic Aseptic Technique, continued

Prepare the work area and supplies

3. **STERILIZE** all bench surfaces with 70% Ethanol and a clean paper towel.
4. **GATHER** any necessary components and wipe any bottles or tubes with 70% Ethanol.
   NOTE: Bring only the items required for a particular procedure to the cell culture area.
5. **ARRANGE** your work area (a) to have easy access without having to reach over one item to get at another and (b) to leave a wide, clear space in the center of the bench. If you have too many things close to you, you will inevitably brush the tip of a sterile pipette against a non-sterile surface.
6. On completion of a specific procedure, **REMOVE** unnecessary solutions and equipment from your work surface, keeping only the materials that you will require for the next steps.

Pipetting

7. **TRANSFER** large volumes of liquids using disposable sterile plastic pipettes (10 ml or 25 ml) together with portable pipet pumps, either motorized or hand pumped. Hold the pipet pump comfortably to allow one-handed operation.
8. **WORK** only within your range of vision and ensure that the pipet is in your line of sight continuously and not hidden by your arm. Make sure the pipet is tilted away from you, or to the side, so your hand is never over an open bottle or flask.
9. **TRANSFER** small volumes with sterile transfer pipets. These should be removed from their plastic sleeve immediately before use.
10. **CLEAN** any spillage immediately and swab the area with 70% Ethanol to reduce contamination.

Handling Bottles and Flasks

11. Bottles should not be vertical when open, but should be kept at an angle as shallow as possible without risking spillage (Figure 6, top). Do not leave reagent/media bottles open and do not work immediately above an open bottle or flask.
12. Culture flasks should be laid down horizontally when open and held at an angle during manipulations (Figure 6, bottom).
13. **DO NOT POUR** from one sterile container into another unless the bottle you are pouring from will be used only once and will deliver all its contents (premeasured) in one transfer. Pouring causes a bridge of liquid to form between the inside and outside of the bottle, which could cause contamination.

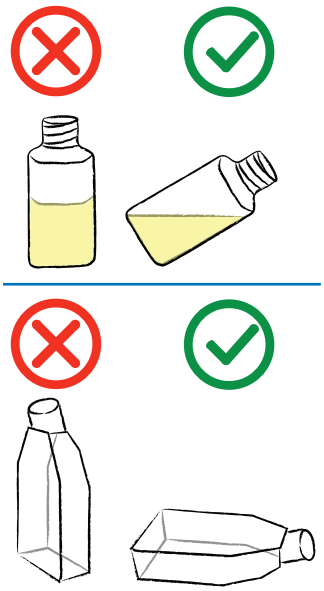


Figure 6: Properly orient your bottles and flasks.

FOR MODULE I
You will need:
Gloves, lab gown, face mask, 70% Ethanol, sterile 10 ml pipet, Sterile Technique Practice Media, 35 mm cell culture plate.

C. PRACTICING STERILE TECHNIQUE

1. **REMOVE** the "Sterile Technique Practice Media" from the refrigerator and allow it to **WARM** on the bench for 10 minutes.
2. **SPRAY** media tube, pipet pump, and gloves with 70% Ethanol to disinfect and prepare work area using sterile technique.
3. Carefully but quickly **TRANSFER** 3 ml of Sterile Technique Practice Media to a 35 mm cell culture plate.
   NOTE: Minimize the time that the media and plate are exposed to the environment to decrease the risk of contamination.
4. **COVER** the plate and carefully place it in the prepared cell culture incubator. **INCUBATE** the plate overnight.
5. **REMOVE** all used and unused pipettes, flasks, or other materials from the work area and swab down the work surface with 70% Ethanol.

Module I: Basic Aseptic Technique, continued

6. **RETURN** all media and stock solutions to the refrigerator.
7. The following day, **RETRIEVE** the plate from the incubator and **EXAMINE** under a microscope for contamination. The media should be clear and light yellow.

Microscopic observation can reveal the source of contamination in cell culture (Figure 7).

- Bacteria: media will appear cloudy and may have a white film on the surface. Under the microscope small, granular cells will be visible as black dots.
- Fungi: thin filamentous mycelia that overtake a culture as fuzzy growth (typically either white or black) that is visible to the naked eye.
- Yeast: round particles that are smaller than insect cells. Usually seen in chains of two or more.

NOTE: If contamination is seen it is important to promptly and safely dispose of any contaminated reagents and plates to prevent further spread of the infection. Review your proper sterile technique and analyze potential sources of contamination. Remember to disinfect the cell culture work area and all materials that will be used, including your gloves. Minimize the time that containers are open to the air and ensure that the pipet does not contact any surfaces other than the media and cell culture plate.

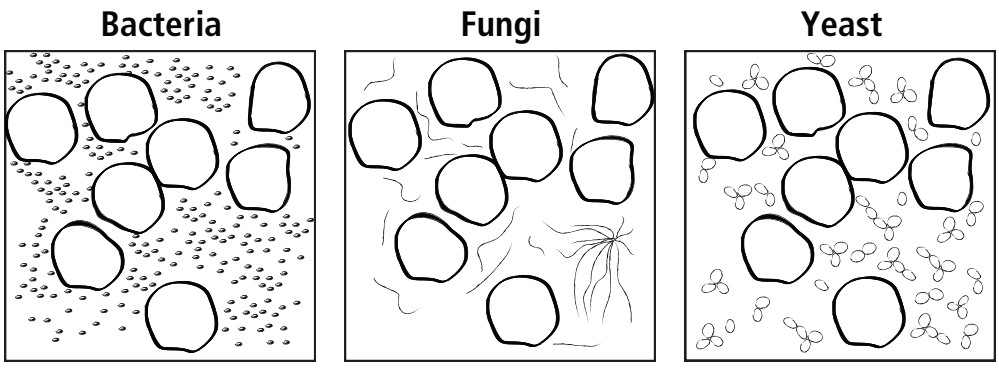


Figure 7: Types of common contaminants in cell culture.

Module II: Examination of Insect Cell Cultures

MONITORING THE HEALTH OF CULTURES

It is important to examine the insect cells before every cell culture experiment to ensure that they are healthy and free from contamination. Unhealthy and apoptotic cells will show an increase in small particles (called granules), vacuole formation, cell shrinkage, cell membrane "blebbing" and nucleus fragmentation (Figure 8A).

1. **RETRIEVE** a flask of cells from the cell culture incubator and return to your lab station. Remember to practice basic aseptic technique when working with the cells.
2. Hold flask up against a light source and **CHECK** if the medium is clear. Insect cells should be visible as a pale haze or cluster of cells on the bottom surface of the flask and the medium in the flask should be clear. A cloudy cell culture medium indicates microbial contamination.
3. **EXAMINE** the cells under a microscope. Look for signs of unhealthy cells which might indicate that the cell media needs to be changed and the cells need to be subcultured.
   NOTE: If the cell culture is contaminated, immediately add 1 ml of 10% bleach solution to the flask, incubate at least one hour, and discard the culture. Swab the cell culture incubator with 70% Ethanol to prevent the spread of contamination to other flasks.
4. **RECORD** your initial observations in the Cell Culture Data Record: appearance of cells, clarity of medium, and the presence or absence of contamination. **DRAW** a picture of the cell morphology, including the shape of individual cells and the size and distribution of cell clusters.
   NOTE: If possible, take photographs with a digital camera attached to the microscope. Include the digital images or printed photos with your cell culture records.
5. **DETERMINE** if the cells are ready to be fed or subcultured, or if they require additional time to grow. Cells should be fed every 5 days and subcultured every 10 days or when they reach 80-90% confluence, whichever occurs first (Figure 8B). To feed or subculture cells, continue immediately to Module III: Maintenance of Insect Cell Cultures.
6. If the cells are not ready to be subcultured, **RETURN** the flask to the incubator. Check cells daily to monitor growth, recording the data in your Cell Culture Data Record. Observe any changes in cell morphology as the cells increase in confluency.

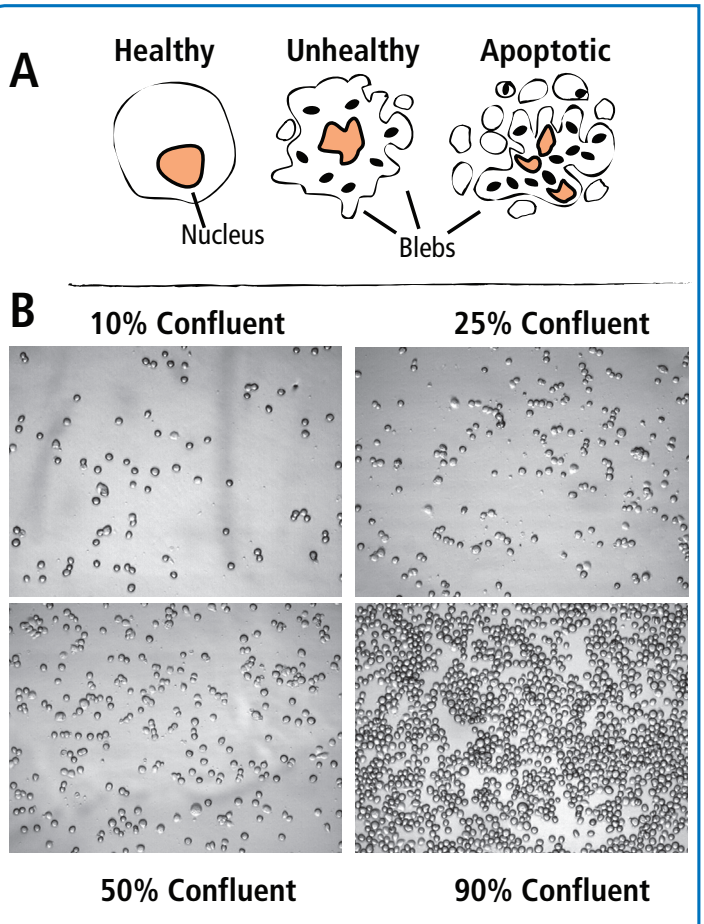
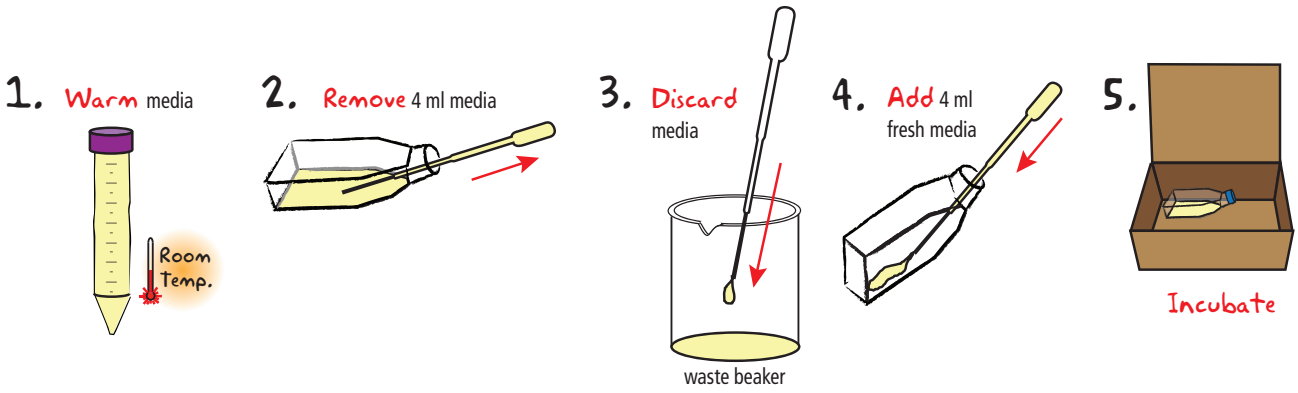


Figure 8: Insect cell characteristics and confluency.

Module III: Maintenance of Insect Cell Cultures



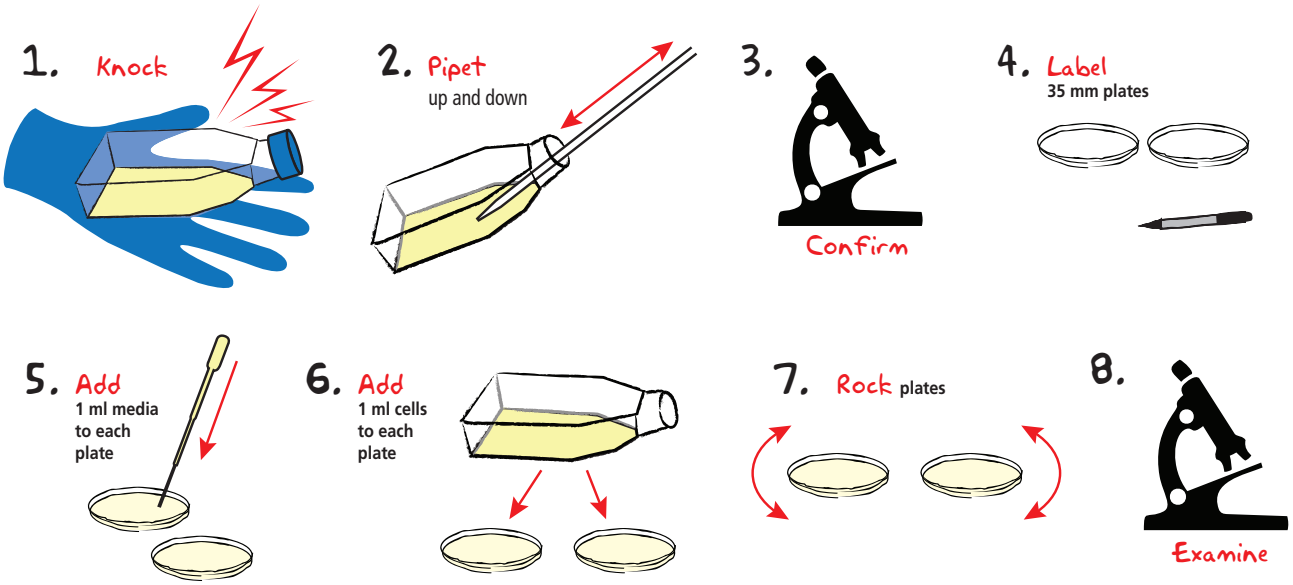
A. FEEDING THE INSECT CELLS

Cultured insect cells require a consistent source of nutrients to grow and divide, all of which are provided in the Insect Cell Media. Over time, the cells will deplete the media of nutrients and growth factors. In addition, media will accumulate toxic waste products from the cells. Cells left in these conditions will eventually die, so it is important to regularly change the media to maintain a healthy culture. ***Don't forget to follow the aseptic techniques!*** ***(See page 8.)***

1. **RETRIEVE** your tube of Insect Cell Media from the refrigerator and allow it to **WARM** to room temperature before using.
2. **REMOVE** 4 ml of the medium in the flask using a sterile transfer pipet, being careful to not disturb the cells on the bottom of the flask.
   NOTE: For optimal growth leave 1 ml of the old medium in the flask. This media contains growth factors secreted by the cells that are important for maintaining the health of the cells.
3. **DISCARD** of the media into a designated waste beaker containing a few ml of 10% bleach.
4. **ADD** 4 ml of fresh Insect Cell Media using a fresh sterile transfer pipet.
5. **RETURN** the flask of insect cells to the incubation chambers for 2-4 days before proceeding to Module III-B.

NOTE:
Remember to check the insect cells often to monitor the health of your culture.

Module III: Maintenance of Insect Cell Cultures, continued



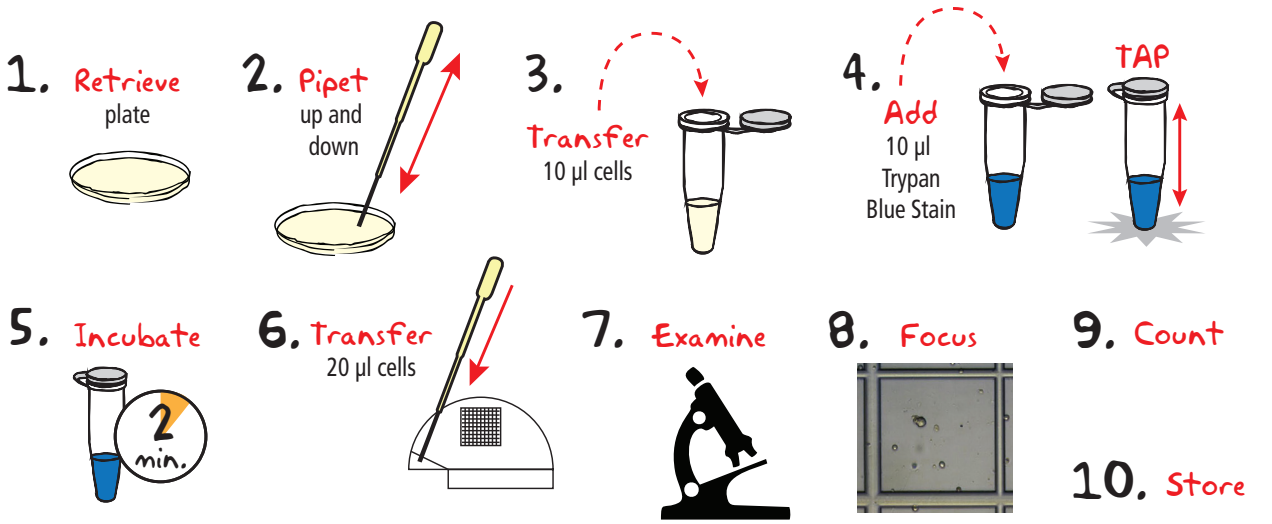
B. SUBCULTURING INSECT CELLS

When properly fed with Insect Cell Media the cells will grow and multiply on the surface of the flask until they reach 100% confluency. At this point, the cells will stop dividing because there is no more room to spread, which is known as contact inhibition. If left in this condition for too long the cells will become unhealthy and die. Instead, once the cells have reached the late log phase of growth and are about 70-80% confluent it is time to subculture the cells into new flasks.

1. **KNOCK** the bottom of the flask against the palm of your hand to shake the cells loose.
2. Using a sterile 10 ml pipet and a pipet pump, **PIPET** the cell suspension up and down several times to detach the remaining cells. Pipetting up and down also disperses cells into a single cell suspension (removing cell clumps), to promote uniform growth on the next plates.
   Note: Use enough force to lift the cells off the flask but avoid bubbles or foaming in the media.
3. Use a microscope to **CONFIRM** the detachment of the insect cells from the bottom of the flask.
4. **RETRIEVE** two new 35 mm cell culture plates and **LABEL** both with your initials. Label one plate "Module IV" and the other plate "Module V".
5. Use a sterile transfer pipet to **ADD** 1 ml of Insect Cell Media to each plate.
6. Using the same pipet, **TRANSFER** 1 ml of the insect cell suspension into each 35mm plate.
7. Gently **ROCK** the plates back and forth to **DISTRIBUTE** the cells evenly. This represents a 1:5 split of the cells into each of the new containers.
8. **EXAMINE** the cells under the microscope. **CONFIRM** that you have cells in your plates and that your cells are round and clear, not shriveled and dark.
   NOTE: At this point the flask can be returned to the incubation chamber and monitored for continued growth. Alternatively, the cells can be terminated by adding 1 ml of bleach and incubating for 1 hour before disposing.
9. **ENTER** the necessary data in your Cell Culture Data Record.
10. After 24 hours, the insect cells should have attached to the surface of the flask and plates. **CONFIRM** attachment of cells under the microscope.

Once cells have been subcultured to new plates you can proceed to Module IV: Cell Viability Assays Using Trypan Blue Staining (page 14) and Module V: Differential Staining Assay Using Giemsa Stain (page 16).

Module IV: Cell Viability Assays Using Trypan Blue Stain



The cell counting chamber, or "hemocytometer", is a device widely used to count the cells in a specific volume of fluid. In this specific case, the chamber will also be used to differentiate dead from live cells. Live, viable cells exclude Trypan Blue stain whereas dead cells take up the dye and stain blue. Cells will be counted daily for multiple days in a row (up to one week) to determine the growth rate and viability of your culture.

A. COUNTING LIVE AND DEAD CELLS

1. **RETRIEVE** your "Module IV" cell culture plate from the incubation chamber. Note the confluency in your Cell Culture Data Record.
2. **PIPET** the cells up and down three times with a sterile transfer pipet to disperse the culture.
3. Using an adjustable volume pipet, **TRANSFER** 10 µl of cell suspension into a microcentrifuge tube. **RETURN** the plate of cells to the incubator.
   NOTE: It is important to keep the plate of cells sterile. Remember your sterile technique! (See page 8.)
4. **ADD** 10 µl of Trypan Blue stain to the cells in the tube and gently **MIX** by tapping the tube on the lab bench or pipetting up and down. This is a 2-fold dilution of the cells.
5. **INCUBATE** the cells for 2 minutes at room temperature.
6. Slowly **TRANSFER** 20 µl (approximately one drop) of Trypan Blue-stained cell suspension to a notch on the bottom left side of one counting area of the cell counting chamber. The chamber will fill by capillary action.
7. **EXAMINE** the counting chamber on the microscope using the lowest objective.
8. **FOCUS** on the grid lines in the chamber. Move the slide until the field you see is the outer grid – this might also require changing to a higher-powered objective. Note: The grid area is 3 mm x 3 mm, while each small grid (area not divided by any additional lines) is 0.33 mm x 0.33 mm.
9. **COUNT** all of the cells (living and dead) within each of the small grids. **RECORD** the number of viable (clear and bright) and nonviable (deep blue) cells in the Cell Culture Data Record.
10. **STORE** the hemocytometer at room temperature until needed for the next cell count experiment. ***NOTE: Do not attempt to clean the cells out of the used well!***

Module IV: Cell Viability Assays Using Trypan Blue Stain, continued

Compute cells/ml and percent cell viability of the cell cultures as follows:

Calculating the Total Number of Cells/ml

Cells/ml = Average number of cells x Dilution x Multiplication factor
- Average number of cells = Count the number of live cells in each small grid, then take the average
- Multiplication factor = To convert into cells/ml (for this hemocytometer use 90,000)
- Dilution = The fold dilution of the cell suspension before pipetting into hemocytometer

For example: You dilute 10 µl of cells into 10 µl of Trypan blue and count 75 cell in 9 small grids
- Average number of cells = 75/9
- Multiplication factor = 90,000
- Dilution = 10 µl / 20 µl final volume = ½ = 2 fold dilution

To calculate cells/ml = 75/9 x 90,000 x 2 = 1.5 x 10^6 cells/ml

Calculating the Percent Viability of Cells

% Viability = Number of viable cells / total no. of cells counted x 100

For example: Of the 75 cells you count, 70 are bright and 5 are deep blue
To calculate viability = 70/75 x 100 = 93.3% Viability

B. PLOTTING CELL GROWTH CURVES

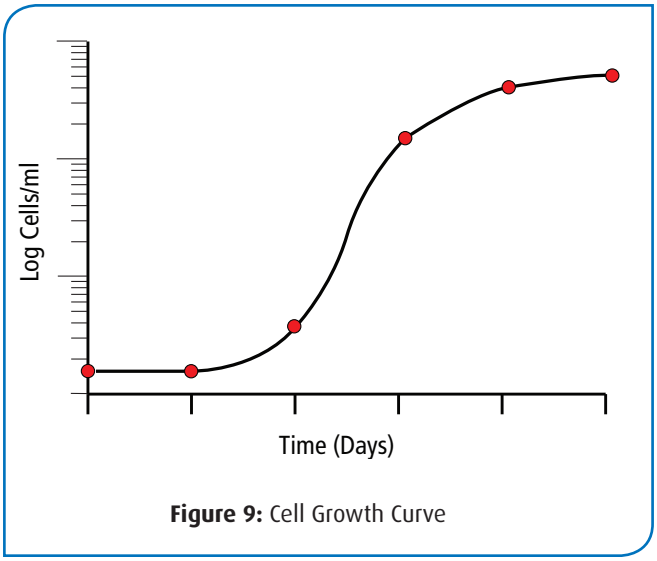
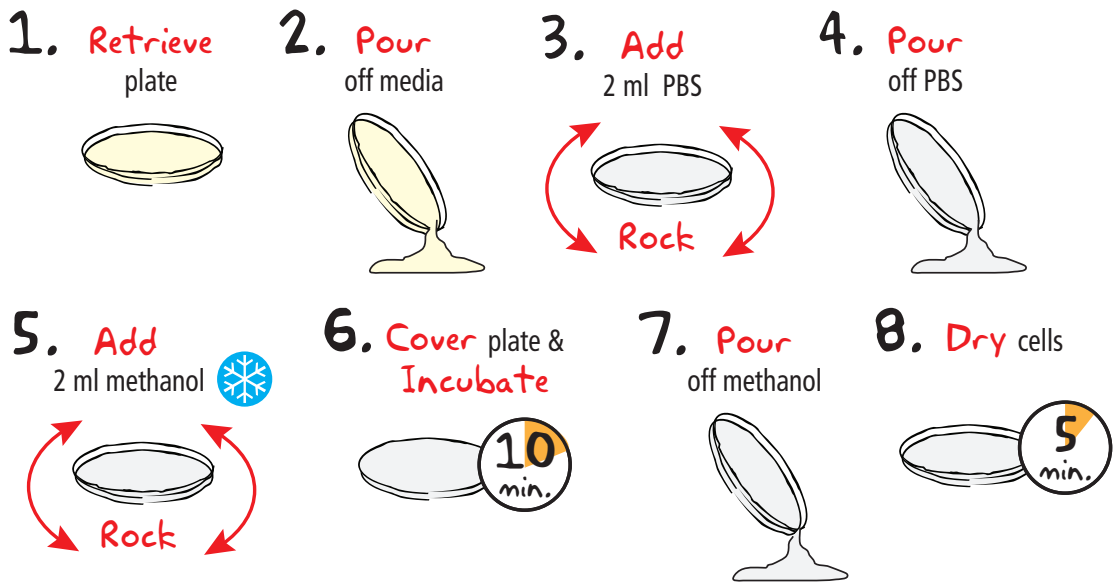


Figure 9: Cell Growth Curve

1. **PERFORM** a cell count and viability assay as described in the previous section every 48-72 hours until there is no change in the number of cells/ml of the culture (stationary phase). Use a new well in the hemocytometer for each measurement. ***NOTE: This will typically take 7-10 days, although growth rates can vary from experiment to experiment due to changes in temperature and starting cell number.***
2. Using a sheet of logarithmic graph paper or a computer graphing program **PLOT** your cell concentrations (cells/ml) on a log scale against time (in days) of culture.
3. **IDENTIFY** and label the Lag, Log and Stationary growth phases for your cell culture.
   NOTE: The Lag phase is not always seen in rapidly growing cultures.
4. Select a time interval during the Log Phase and **CALCULATE** the doubling time for your culture, the time required for the number of cells/ml to double. The doubling time can be determined by identifying a cell number along the exponential phase of the curve, tracing the curve until that number has doubled, and calculating the time between these two points.

Module V: Differential Staining Assay Using Giemsa Stain

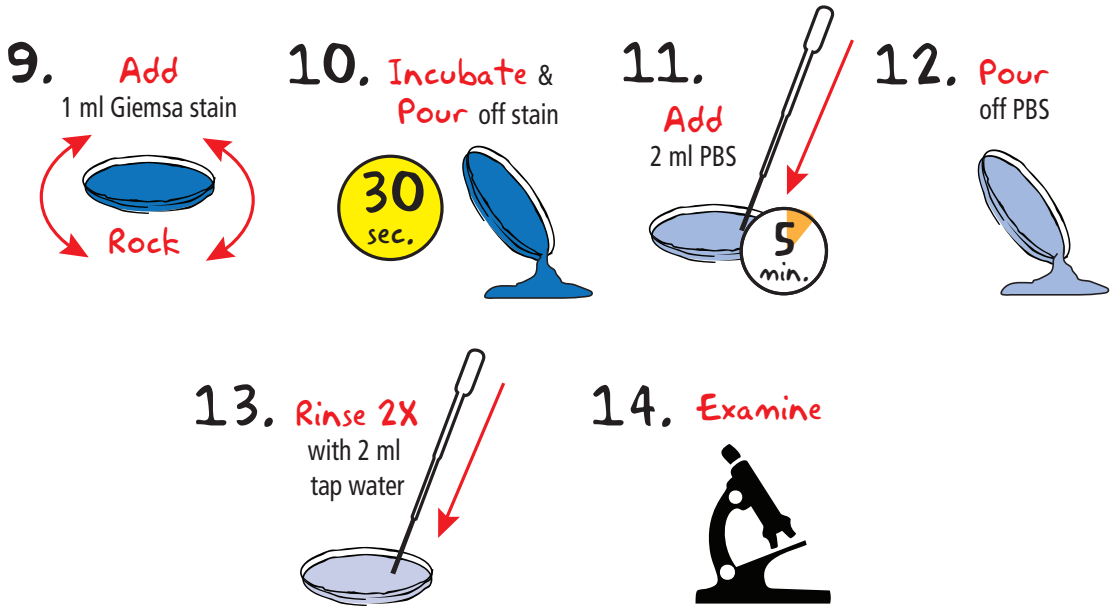


Cells are commonly treated with dyes to stain features within the cell, making it possible to distinguish finer details. Giemsa stain, a mixture of methylene blue and eosin, stains the membrane and nucleus blue and the cytoplasm light blue or purple. This Module should be performed when cells on the "Module V" plate are at least 70% confluent, typically 3-4 days after plating the cells in Module III.

OBSERVING STAINED CELLS UNDER THE MICROSCOPE

1. **RETRIEVE** your plate of Module V cells from the incubator and observe the cells using a microscope. The cells should be healthy and attached to the plate.
   Note: For this procedure, there is no need for aseptic technique but it is important to maintain standard lab safety practices.
2. **POUR** the culture medium out of the plate into a waste container.
3. **ADD** 2 ml of PBS and gently rock to rinse the cells.
4. **POUR** the PBS out of the plate into a waste container.
5. Using a transfer pipet, gently **ADD** 2 ml of ice-cold methanol to the plate and gently **ROCK** to coat the entire surface.
6. **COVER** the plate and **INCUBATE** the cells to fix for 10 minutes at room temperature.
7. **POUR** the methanol into a waste beaker.
8. **DRY** the cells uncovered for 5 minutes on the lab bench.

Module V: Differential Staining Assay Using Giemsa Stain, continued



9. To stain the cells, **ADD** 1 ml of Giemsa stain to the plate and gently rock to cover cells.
10. **INCUBATE** the plate for exactly 30 seconds and then **POUR** the Giemsa stain into a waste container. ***Note: It is possible to over-stain the cells if the Giemsa is left for more than 30 seconds.***
11. **ADD** 2 ml of PBS to the plate and **INCUBATE** for 5 minutes.
12. **POUR** the PBS out of the plate into a waste container.
13. **RINSE** the plate twice with 2 ml of tap water. **REMOVE** the water and immediately transfer the plate to a microscope.
14. Using a bright-field microscope, **EXAMINE** the morphology of the cells while still wet. Note the differential staining of the nucleus and the cytoplasm. Draw a representative image of the cells in your lab notebook, taking note of the color and intensity of the staining.

Optional Stopping Point: The plates can be allowed to air dry and then stored at room temperature for up to a week. To visualize, gently wet the plate with tap water as in step 13 and analyze.

Study Questions

1. Why has cell culture become such an important tool for researchers?
2. What are the advantages and applications of insect cell culture?
3. Why is it recommended to passage the cells at 70-80% confluency?
4. What is the rationale for leaving a small amount of media behind when feeding or splitting the cells?
5. Describe the common symptoms of bacterial contamination.
6. Why would it be important to determine the doubling time of a cell line? What information does the doubling-time tell you about the cells?

Instructor's Guide

IMPORTANT - READ ME!!

Cell Culture experiments contain antibiotics that are used to keep cultures free of contamination. Students who have allergies to antibiotics, such as PENICILLIN or STREPTOMYCIN, should not participate in this experiment.

ORGANIZATION AND IMPLEMENTING THE EXPERIMENT

Prior to starting this experiment, carefully check the list of Components and Requirements on page 3 to ensure that you have all the necessary components and equipment.

The guidelines that are presented in this manual are based on six laboratory groups. The experiment is divided into five modules and should take approximately two weeks to perform. The following are implementation guidelines, which can be adapted to fit your specific set of circumstances.

IMPORTANT:
Cells must be cultured immediately upon receipt!

Approximate Time Requirements

Module	PreLab	Experiment
I	30 min.	1 hour
II	15 min.	15-20 min.
III	30 min.	45-60 min.
IV	15 min.	30 min.
V	30 min.	1 hour

NOTE:
The experiment in Module IV will be performed multiple times to collect data for a growth curve. We recommend a minimum of 3 separate days for best results.

Pre-Lab Preparations

ADVANCE PREPARATION:

Preparation for:	What to do:	When?	Time Required:
Establish Cell Culture	Inoculate flask with insect cells	Immediately upon receipt of cells – approx. one week before starting the experiment	15 min.
Module I: Basic Aseptic Technique	Prepare and aliquot reagents	One hour before performing the experiment	30 min.
Module II: Examination of Insect Cell Cultures	Prepare compound microscopes	Anytime before the lab period	15 min.
Module III: Maintenance of Insect Cell Culture	Prepare materials	One hour before performing the experiment	30 min.
Module IV: Cell Viabiity Assays Using Trypan Blue Stain	Aliquot Trypan Blue and distribute supplies	Anytime before the first viability count	15 min.
Module V: Differential Staining Assay Using Giemsa Stain	Aliquot Giemsa stain	One hour before performing the experiment	15 min.
	Pre-chill Methanol	Anytime before the lab period	15 min.

NOTE: For best results, be sure to follow the basic aseptic technique (page 8) before starting any lab experiment or reagent preparation.

We recommend preparing the equipment and reagents for Modules I, II, and III before starting the experiment with the students. The reagents for modules IV and V can be prepared as needed once student groups have progressed to those experiments. Have ready a microscope for analysis of the cells throughout all modules. ***NOTE: The cell culture flasks used in this experiment are approximately 2.5 cm tall. Please ensure that there is sufficient clearance between the stage and objectives to view the cells.***

Pre-Lab Preparations

ESTABLISHMENT OF INSECT CELL CULTURE

Preparation of Incubation Chamber

It is necessary to prepare an incubator chamber to hold the cells. Incubators should be held between 24-27° C and standard atmosphere. A large plastic container or cardboard box can serve as a great incubator for the class. Insect cells prefer to grow in the dark, so any transparent containers should be covered in aluminum foil.

NOTE: It is recommended that the incubator chambers are sterilized by swabbing with 70% ethanol before starting the experiment.

NOTE:
Be sure your classroom maintains at least 24° C over nights and weekends.

Aliquot the Insect Cell Culture Media

1. Aseptically **ALIQUOT** 15 ml of Insect Cell Media (B) into six 50 ml conical tubes. Reserve the remaining media to initiate the Insect Cell Culture.
2. **LABEL** each tube as Insect Cell Media.
3. **STORE** at 4° C until needed by students in Module III.

Initiation of Insect Cell Culture

The Sf9 insect cells (A) are shipped in a 5 ml tube and should be transferred to a flask as soon as they are received.

1. **WARM** the Insect Cell Media to room temperature.
2. Gently **INVERT** the tube of cells to mix.
3. Using a sterile transfer pipet or sterile micropipet tip, **TRANSFER** the entire volume of Sf9 insect cells (A) to a sterile cell culture flask.
   NOTE: Do NOT pour the cells into the flask as this greatly increases the risk of contamination.
4. Using a fresh transfer pipet, **ADD** 2 ml of fresh Insect Cell Culture Medium to the flask.
5. **INCUBATE** the cell culture flask in the incubation chamber.
6. After 24 hours the insect cells should have attached to the surface of the flask. **CONFIRM** attachment of the cells under a microscope.
7. **ALLOW** the cells to grow for an additional 24-72 hours, checking health and confluency daily. The cells should be at least 80% confluent before passaging in the following steps.
8. Using the 25 ml disposable pipet and a pipet pump **ADD** 4 ml of Insect Cell Culture Medium to six T25 flasks, one for each student group.
9. **TAP** the side of the flask and gently pipet up and down with a sterile 10 ml pipet to loosen the cells. Add 1 ml of cells to each prepared flask.
10. Carefully **PLACE** the student flasks into the incubators until needed. The initial flask and any remaining cells can be maintained or discarded.

Pre-Lab Preparations

MODULE I: LEARNING BASIC ASEPTIC TECHNIQUE

1. Aseptically **ALIQUOT** 5 ml of Sterile Technique Practice Media (C) into six 15 ml tubes using a sterile 10 ml pipet. Each group should have its own tube of media to reduce the chance of contamination.

MODULE II: EXAMINATION OF INSECT CELL CULTURES

Prepare microscopes for analysis of insect cells. Phase contrast or brightfield microscopes will work for the observations. The cell culture flasks used in this experiment are approximately 2.5 cm tall, please ensure that there is sufficient clearance between the stage and objectives to view the cells.

MODULE III: MAINTENANCE OF INSECT CELL CULTURE

1. **REMOVE** aliquots of Insect Cell Media from 4° C and allow to warm to room temperature.
2. **GATHER** the required components for each group.

MODULE IV: CELL VIABILITY ASSAYS USING TRYPAN BLUE STAINING

1. **ALIQUOT** individual tubes of 250 µl Trypan Blue (D) for the 6 groups.

   NOTE: Each group will receive one aliquot of Trypan Blue and one counting chamber. These should be saved after the experiment for use in subsequent counting assays.

MODULE V: DIFFERENTIAL STAINING ASSAY USING GIEMSA STAIN

Enough supplies and reagents are provided to stain 6 plates of cells.

1. **ALIQUOT** 10 ml PBS (F) into 15 ml tubes and 1 ml Giemsa Stain (G) into 1.5 ml snap cap tubes for each group (6 total).
2. **PREPARE** ice-cold methanol by placing small containers of 100% methanol into a -20° C freezer at least 1 hour before the lab period.

FOR MODULE I
Each Group Requires:

Sterile disposable pipet, Sterile Technique Practice Media, 35 mm cell culture plate, gloves, lab gown, face mask, 70% Ethanol.

FOR MODULE III-A
Each Group Requires:

Tube of Insect Cell Media, Two sterile disposable pipets, waste beaker, gloves, lab gown, face mask, 70% Ethanol.

FOR MODULE III-B
Each Group Requires:

Tube of Insect Cell Media, 10 ml pipet, pipet pump or bulb, Two 35 mm cell culture plates, Two sterile disposable pipets, waste beaker, gloves, lab gown, face mask, 70% Ethanol.

FOR MODULE IV
Each Group Requires:

Microcentrifuge tubes, transfer pipets, micropipet and tips, gloves, trypan blue, counting chamber, waste beaker*.

NOTE: Add a small amount of bleach to each waste beaker to disinfect cell culture waste.

FOR MODULE V
Each Group Requires:

Transfer pipets, PBS, Giemsa stain, ice-cold methanol, waste beaker, gloves.

Experiment Results and Analysis

The expected results will vary depending on the growth characteristics of the cells. The viability and growth rate of the cells depends heavily on the conditions that the cells are grown in, including temperature, confluency, and accuracy of pipetting.

MODULE IV – A. COUNTING LIVE AND DEAD CELLS

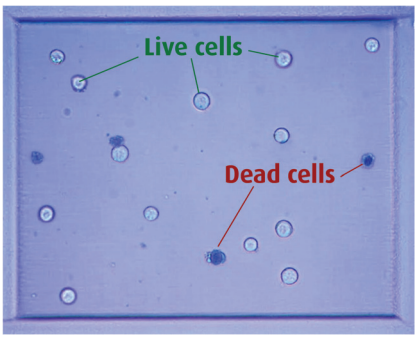
Calculating the Total Number of Cells per mL Culture

Cells/ml = Average number of live cells x Dilution x Multiplication factor

Using the image in Figure 10:
- Average number of live cells = 13
- Dilution = 10 µl / 20 µl final volume = ½ = 2 fold dilution
- Multiplication factor = 90,000

To calculate cells/ml = 13 x 90,000 x 2 = 2.34 x 10^6 cells/ml

Figure 10: Trypan blue staining of cells



Calculating the Percent Viability of Cells

% Viability = Number of viable cells / total no. of cells counted x 100

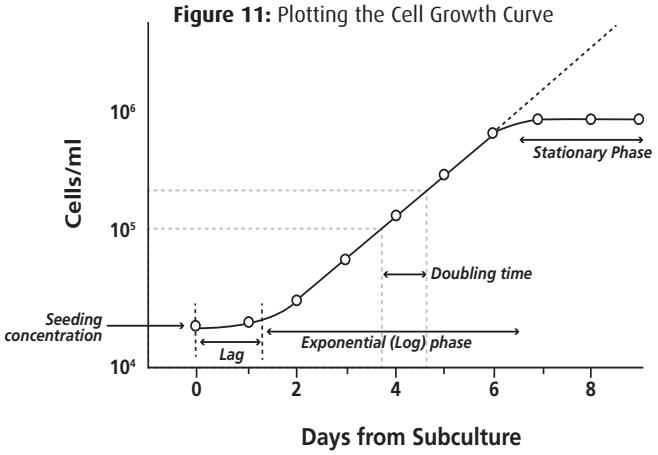
Once again using Figure 10 for the example:
- Live (bright) cells = 13
- Total cells counted = 17

To calculate viability = 13 / 17 x 100 = 76.5% Viability

MODULE IV – B. PLOTTING CELL GROWTH CURVES

The cell growth curves will vary depending on many factors, including the starting density of cells, temperature of culture, and health of the cells. Healthy cells might not experience a lag phase after passaging and will immediately enter the log phase of growth.

Figure 11: Plotting the Cell Growth Curve



MODULE V

A sample image of Giemsa stained cells can be seen below. These images represent the typical results achieved from healthy Sf9 cells; student results will vary due to variations in the cell preparation and the intensity of staining.

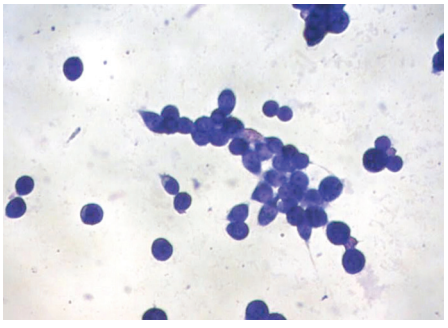


Figure 12: Giemsa stained Sf9 cells

Please refer to the kit insert for the Answers to Study Questions

CELL CULTURE DATA RECORD

Week of _____

Group # _____ **Names:** _____

	Date					
Health of Cell Culture	Appearance of cells					
	Clarity of media					
	Confluency (Density of cells)					
Subculturing	Final volume in new flask (ml)					
	Volume of Cells (ml)					
	Split ratio of cells					
Cell Counts	Average number of live cells per grid					
	Average number of dead cells per grid					
	Total cells per grid					
	Live cells in flask (# live x volume)					
	Percent viability (# live/# dead)					
	Phase of growth cycle