6 groups of students

BIOLOGY AND CULTURE OF EUKARYOTIC CELLS

1. OBJECTIVE OF PRACTICE

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

2. MATERIAL AND REAGENTS INCLUDED IN THE KIT

Components	Ship	Conservation
Insect cells (Sf9).	6 vials	R.T.
Culture medium of insect cells (A).	125 ml	4 ºC
Culture medium for sterility practice (B).	30 ml	4 ºC
Phosphate Buffer Saline (PBS 1x).	30 ml	4 ºC
Trypan blue dye.	1 ml	Room temperature
Cell culture flask (sterile, 25 cm^2).	6 units	
Cell culture plates (sterile, 35 mm).	36 units	
10 ml pipettes (sterile).	18 units	
Pasteur pipettes (sterile).	70 units	
Scrapers (sterile).	6 units	
Cell counting chambers.	6 units	
15 ml conical bottom tubes (sterile)	18 units	
50 ml conical bottom tubes (sterile)	6 units	
1,5 ml centrifuge tubes	90 units	

This practice is designed for 6 groups of students and contains enough reagents and materials for its successful development.

> **NOTE:** Sf9 insect cells must be ordered a minimum of 2 weeks before the planned date to start the experiment. Insect cells must be **cultured immediately** after receipt (see annex 3 in this protocol).
>
> **NOTE: Insect Cell Culture Medium, Sterile Practice Culture Medium** and **Phosphate Buffered Saline (PBS)** should be stored in a refrigerator.

All components of this practice are intended for educational research only. They must not be used for diagnostic or pharmacological purposes, or to be administered or consumed by humans or animals.

2.1 Material required and not included in this kit

• Incubation chamber. If it isn't available, use a large container with a plastic cover or a cardboard box with a lid (the BIOTED box containing the kit materials can be used as an incubation chamber).

• 70% ethanol in aerosol bottles.

• Methanol.

• Pipette with suction bulb or automatic pipette.

• Inverted microscope phase contrast/bright field capability (cells can be viewed with a student's microscope in an upright position, but this is limited by the height of the culture plates).

• 10-1000 µl micropipette and tips.

• Marker pens.

• Safety glasses, mask (optional), disposable lab gloves and lab coats.

• Waste containers (beakers).

NOTE: The inverted microscope is used for the observation of cells in culture (in flask or plate). For the observation and counting of the cells in the cell counting chamber, use the student's microscope (vertical position).

3. INTRODUCTION

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 cells in a laboratory to answer important biological questions. This may include studies of the structure, behavior, or disease of cells. Cell culture has increased the understanding of cell functions and has become an important platform for the study of both the normal development and the disease of cells.

Although scientists performed the first cell culture experiments in the mid-1800s, the techniques were not really developed until the 20th century. Since then, cell culture has allowed to grow and study cells from dozens of species. One of the first of these experiments involved crude tissue preparations that were placed in a buffer solution. Many of the early cell culture trials were unsuccessful, and even the most promising studies were only able to keep cells alive for a few days. Fortunately, through the use of improved reagents and techniques, it is now possible to grow cells for months, years, and even decades. Cell cultures have been selected, whose DNA contains numerous mutations that allow them to grow indefinitely, producing the so-called *immortalized cell lines*.

Cell culture has given rise 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 their development without the need to use live animals. In addition to reducing animal testing, cell culture has increased reproducibility and reduced costs associated with vaccine production. Cell culture is also used to study many common diseases, including genetic disorders, viral and bacterial infections, and cancer. These experiments make it possible to examine healthy and diseased cells, monitor the effects of gene additions or deletions, or detect effective therapies.

Sf9 insect cell culture

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

Insect cell culture originated as an approach to better understand the biology of insects. Many of the early studies with insect cells were designed to explore basic biological questions. These experiments provided valuable information on the development and pathology of insects. In addition, insect cell culture has been used to develop new insecticides and other deterrents elements against agricultural pests. One of the most popular insect strains has been the Sf9 cell line, which was derived from the ovarian cells of the common bollworm, *Spodoptera frugiperda* (corn moth) (Figure 1).

Sf9 cells are an important model for examining basic cellular processes, many of which are present in higher eukaryotes.

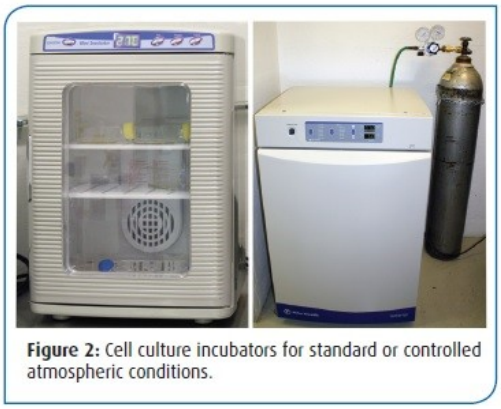

Figure 2: Cell culture incubators for standard or controlled atmospheric conditions.

Importantly, Sf9 cells grow rapidly and are easy to maintain. Cells are grown in standard atmosphere and at room temperature, unlike mammalian cell culture which requires complicated incubators to control temperature, CO2, and humidity (Figure 2). These traits simplify growth conditions and reduce the cost of culturing the cells. The ease of growth of Sf9 cells has made them an essential part of the biotechnology industry, where the cells are commonly used in the production of recombinant proteins and viruses. Moreover, the simplicity of the culture makes insect cells a useful model for students.

Cell culture techniques

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

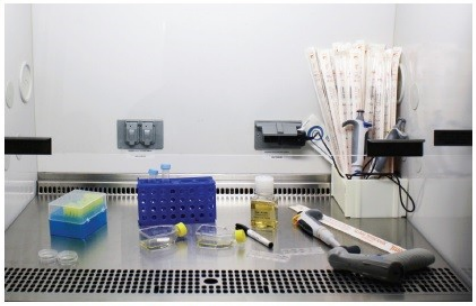

Figure 3: Cell culture hood and standard equipment.

The cells are cultured in a growth medium whose chemically complex solution provides the necessary nutrients for the cells to grow. Media typically contain essential amino acids, buffers, salts, and a carbon source such as glucose. This mix is carefully balanced for its use with specific cell lines and the choice of media is essential for proper growth and performance. 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 medium provided for this practice contains serum and antibiotics, which is known as a *complete medium*.

Many cellular features are visible using a modest compound microscope. The use of special stains, available to accentuate cellular structures, improves observations. For example, trypan blue, a vital dye, is commonly used to increase cell counts and to monitor the health and growth rate of cultured cells. Trypan blue will not stain living cells, but is rapidly 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 cell structure, cell cycle stages, or to distinguish between multiple cell types within a population. Giemsa stain will stain the DNA of Sf9 cells dark blue, while the cytoplasm will stain light blue or purple. Additional dyes can be used to identify specific or characteristic cells with different colors, allowing a pathologist to differentiate between cell types in a mixture.

Growth and maintenance of cultured cells

Sf9 cells are immortalized, allowing them to proliferate for many generations under optimal conditions. Cells will continue to divide in a culture flask until the growth medium is depleted of essential nutrients or until they have filled all available space. In the latter case, the growth of the cells is inhibited by contact and, as a consequence, they will stop dividing. Therefore, it is necessary to dilute the cells (decrease cell density) and

culture them in another flask. The operation is simple, a small portion of the cells must be removed and transferred to a new container with new medium containing the necessary nutrients. Once this procedure has been carried out, the cells will proliferate again and promote their growth, going through three different phases (Figure 4).

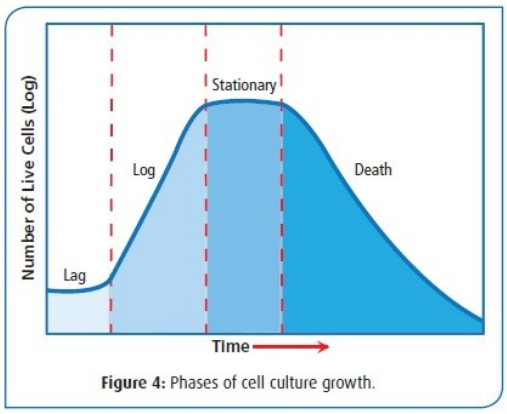

Figure 4: Phases of cell culture growth.

• *Lag phase*: Immediately after cells have been transferred to a new flask, they may enter a lag phase of growth where there is little increase in cell number. During this time, the cells "condition" the media by secreting proteins that stimulate growth. Cell density is low, with cells covering less than 50% of the surface (*confluent to 50%* or *50% of confluence*, see Figure 8). The lag phase can last around 1-2 days.

• *Growth (or exponential) phase*: During this phase, the number of cells increases exponentially and cell growth continues as long as there are enough nutrients to keep the number of cells growing. Cells are approximately confluent to 50-80%.

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

Cell growth phases can be estimated by examining the confluence of the culture. For a more precise measurement, scientists determine the rate of cell growth by repeating the cell count over a few hours or days. Changes in cell growth rate could 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 answer questions about the behavior of cells, tissues, and even entire 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 the cellular level. Cell culture is also commonly used to develop and screen potential drugs, allowing rapid discovery of novel therapies. In these experiments, scientists grow healthy and diseased cells to understand the biological nature of disease and discover effective treatments for existing patients. Other cell culture applications include stem cell research, organ transplantation, gene therapy, and neurological research. The use of cell cultures is important to

minimize the use of research animals and prevent animal suffering. Future research will continue to improve our understanding of cell functions and improve human health.

In this practice, students will develop skills in the manipulation and maintenance of Sf9 insect cells. Student groups are to culture the cells using sterile techniques. Cells should be examined microscopically to determine cell morphology, growth rate, and viability. Using the data they collect, students are to create and analyze a growth curve.

4. PRACTICE

The practice will be divided into 4 modules:

- **Module I:** *Basic aseptic technique*.

  In this module the student will learn how to maintain a work area in aseptic conditions, some practical advice on how to achieve sterility of the materials and how to perform a sterile pipetting procedure. In addition, a small practice will be carried out to see if this knowledge has been acquired correctly.

- **Module II:** *Observation and evaluation of the cellular state*.

  In this module, students will learn to observe insect cells under a microscope and determine if they are growing under optimal conditions. They will also be able to know when is the best time to make subcultures or to change the culture medium for a new one that contains the necessary essential nutrients. Normally, cells should be fed every 3-5 days and reseeded (subcultured) every 10 days, preferably when there is 80-90% of confluence.

- **Module III:** *Handling of Insect cell culture.*

  In this module, students will learn to maintain a cell culture applying, at all times, the asepsis technique (**Module I**). Cultured insect cells require a certain source of nutrients, present in the culture medium, which allows them to grow and divide. Over time, cells deplete nutrients and growth factors from the medium and require their replacement. In addition, culture media accumulate toxic waste products derived from cellular activity. The cell culture maintained under these conditions will eventually die, to avoid this it is necessary to regularly change the media to maintain a healthy culture.

- **Module IV:** *Viability and Kinetics of cell growth.*

  In this section, students will learn how to count live and dead cells, as well as how to calculate culture viability. Cell counting is done using a special cell counting chamber (Neubauer's chamber) or *hemocytometer*, a widely used device for counting cells present in a specific volume of fluid. In addition, it can also be used to distinguish living cells from samples, with the help of a dye. Living cells exclude the dye, while dead cells incorporate it, staining with the dye used.

Module I: Basic aseptic technique

NECESSARY MATERIAL

To carry out this practice, it is necessary to have the following materials before its start:

• Gloves	• Face Mask
• Lab coat	• 70% ethanol
• Sterile 10 ml pipettes	• Medium for practicing sterile technique
• Pipettes dispenser	• 35mm cell culture plate

BASIC ASEPTIC TECHNIQUES

A. Overview

Successful cell culture depends on maintaining cells in an environment free from contamination by microorganisms such as bacteria, fungi, and viruses. All materials that come into contact with the cell culture ***must be previously disinfected so that the manipulations do not allow*** the non-sterile environment to contaminate the culture. For this it is very important to:

1. **Use lab coats and face masks**. Their use minimizes the risk of contamination of cell cultures. Long hair should be kept tied up and talking should be avoided as much as possible during the cell culture manipulation.

2. **Wear disposable gloves** at all times. Prior to their use, **SPRAY** the disposable gloves with 70% ethanol and rub both gloves to disinfect them (specially the interdigital area). This step should be performed frequently while working with the cells to avoid contamination. Whenever we think we have touched any non-sterile area, or when in doubt, **change** the gloves and immediately **disinfect** the new pair with 70% ethanol.

B. Work area. Necessary material

We must take into account, before starting to manipulate the culture cell, the following methodology:

1. **STERILIZE** all work bench surfaces with 70% ethanol using a clean paper towel.

2. **PREPARE IN ADVANCE** all the necessary material and clean it with 70% ethanol.

3. **ORGANIZE** the work area to (a) have easy access to all the material that we are going to use, with the minimum possible manipulation to reach what we need and (b) leave a wide and clear space in the center of the work area in which we can work, avoiding contact with the rest of the material that we are not going to use at that moment. If we have too much stuff around us, we can inevitably touch or brush a sterile pipette tip against a non-sterile surface and contaminate our culture.

4. At the end of a specific procedure, **REMOVE** unnecessary solutions and equipment from the work area, keeping only the materials that are necessary for the next steps and that have been cleaned with 70% alcohol.

C. Pipetting

The moment of handling pipettes for the use of liquids is one of the most common actions in which cell cultures and/or culture media are contaminated. To avoid this, we have to:

1. **TRANSFER** large volumes of liquids using disposable sterile plastic pipettes (10 mL or 25 mL) with pipettes dispensers or portable pipettes suction bulbs, either automatic or manual. Hold the pipette bulb comfortably to allow one-handed operation.

2. **WORK** only within your line of sight and make sure the pipette is in your line of sight at all times and not hidden by your arm. Make sure the pipette is angled towards you, or to the side, so that no hand is on an open bottle or vial.

3. **TRANSFER** small volumes with sterile transfer pasteur pipettes. These must be removed from their protective plastic cover, in a sterile area (under the hood) and immediately before use.

4. **CLEAN UP** any spill immediately, trying not to enlarge the affected area, and then clean again the work area with 70% ethanol to reduce contamination.

D. Bottles and flask

1. The bottles must be vertical when they are open, avoiding the risk of spilling the liquid they contain. Do not leave reagent/media bottles open and do not work directly above an open bottle or vial.

2. Flasks that contain cell culture should be placed horizontally when open and kept at an angle during manipulations. When it is necessary to aspirate the culture medium contained in the bottle, it must remain inclined, reducing contamination (Figure 6).

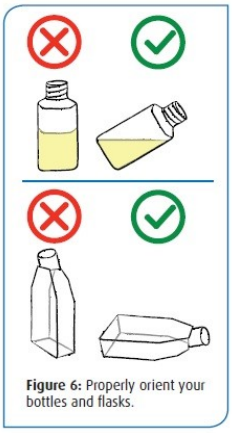

Figure 6: Properly orient your bottles and flasks.

3. **AVOID** pouring from one sterile container to another unless the bottle you are pouring from is used only once and empties its entire contents in the transfer. The pouring procedure causes the formation of a liquid bridge between the inside and outside of the bottle, which could cause contamination.

E. Use of incubation chambers

Cell culture incubators are widely used in microbiology and cell biology to cultivate bacteria and eukaryotic cells. These incubators keep control of temperature, humidity, and other conditions such as the carbon dioxide and oxygen content of the indoor atmosphere.

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 an incubator exists in the laboratory, it can be set to maintain a temperature of 27°C.

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

Before starting the experiment, all existing surfaces around the work area should be cleaned with 70% ethanol to disinfect them. If an incubator is not available, an appropriately sized cardboard box (such as the box containing the BIOTED kit materials) or a plastic container with a lid can be used (Figure 5). A single container can be used to store the flasks of all the groups in the class (properly labeled to recognize the group to which they belong).

We have to take into account that:

1. Insect cells prefer to grow in a dark environment and will not grow under direct light. If it is necessary, **COVER** the incubation chamber with aluminum foil to prevent light.

2. We have to **CLEAN** the inside of the incubation chamber with 70% ethanol and let the surfaces dry completely.

3. The camera must be **PLACED** in an area free of airstream that maintains a temperature between 24-27°C. **Avoid** windows or vents that can alter the temperature of the chamber and, in addition, that facilitate the contamination of the crop.

PRACTICE OF THE STERILE TECHNIQUE

1. **REMOVE** the **Sterile Technique Practice Medium** from the refrigerator and let it **WARM** on the bench, at room temperature, for 10 minutes.

2. **SPRAY** the tube containing the medium for this practice with 70% ethanol, in addition to the suction bulb, the gloves, and the work area. Once the materials are disinfected, we can continue with the development of the sterile technique.

3. Next, **TRANSFER** 2 ml of sterile technique practice medium to a 35 mm cell culture plate.

> **NOTE:** We recommend opening the tube with the medium, but leaving the lid on it, take the pipette and load the 2 ml, recap the tube and open the lid of the culture plate, deposit the 2 ml of medium and close it as quickly as possible. **AVOID** the pipette touching the edges of the plate or tube to avoid contamination.

4. **COVER** the plate and carefully place it in the prepared cell culture incubator chamber. **INCUBATE** the plate overnight at room temperature.

5. **REMOVE ALL** pipettes, both used and unused, vials, and other materials from the work area and clean the work surface with 70% ethanol.

6. **STORE** in the refrigerator at 4ºC all media and stock solutions used.

7. The next day, **RECOVER** the plate from the incubator and, **AVOIDING** that the culture medium protrudes from the plate, observe it under a microscope to verify that there is no contamination. Media should be clear and light yellow in color.

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

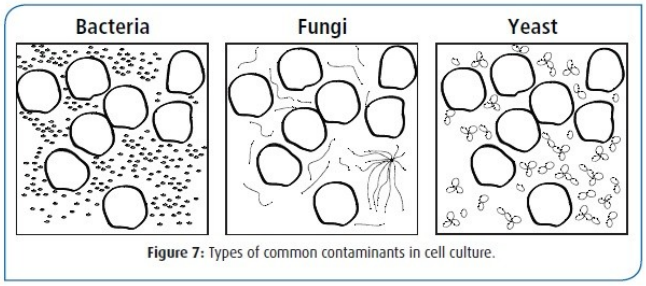

Figure 7: Types of common contaminants in cell culture.

• Bacteria: Media appears cloudy and may have a white film on the surface. Small, granular cells, like black dots, will be seen under the microscope.

• Fungi: In the culture medium, thin filamentous mycelia appear covering the cell culture as a fuzzy growth (typically white or black). It is also visible to the naked eye.

• Yeast: Round particles that are smaller than insect cells. Chains of two or more cells are usually seen.

NOTE: If contamination is observed, **it is important** to quickly and safely remove contaminated reagents and plates to prevent the spread of contamination. Check if the sterile technique has been applied properly and analyze the possible sources of contamination. **Remember** that before starting the practice, it is necessary to disinfect the work area and all the materials that will be used, including gloves. Minimize the time that the vessels are open to air and ensure that the pipette does not come into contact with any surface other than the support and cell culture plate.

Module II: Observation and evaluation of cell status

NECESSARY MATERIAL

To carry out this practice, it is necessary to have the following materials before its start:

● Gloves	● Face mask
● Lab coat	● 70% ethanol
● Sterile 10 ml pipettes	

OVERVIEW

It is important to examine the insect cells prior to each cell culture experiment to ensure that they are healthy and free from contamination (optimal conditions). Unhealthy and apoptotic cells will show an increase in small particles (called granules), the formation of vacuoles, cell shrinkage, the appearance of cell membrane of *blebbing* (formation of small bubbles at the edges of cells), and fragmentation of the core (Figure 8A).

METHODOLOGY

1. **RECOVER** a flask of cells from the cell culture incubator and bring it to the laboratory work area. Remember to keep the area clean applying 70% ethanol.

2. **CHECK** that the medium is clean and transparent. Insect cells should be visible as a pale haze or clump of cells on the bottom surface of the flask and the medium inside the flask should be clear. A cloudy cell culture medium indicates microbial contamination.

3. **EXAMINE** the cells under a microscope. Look for signs of diseased cells that could indicate that the cell medium has drastically decreased in nutrients and needs to be changed or the cells have to be sub-cultured if confluence is greater than 80%.

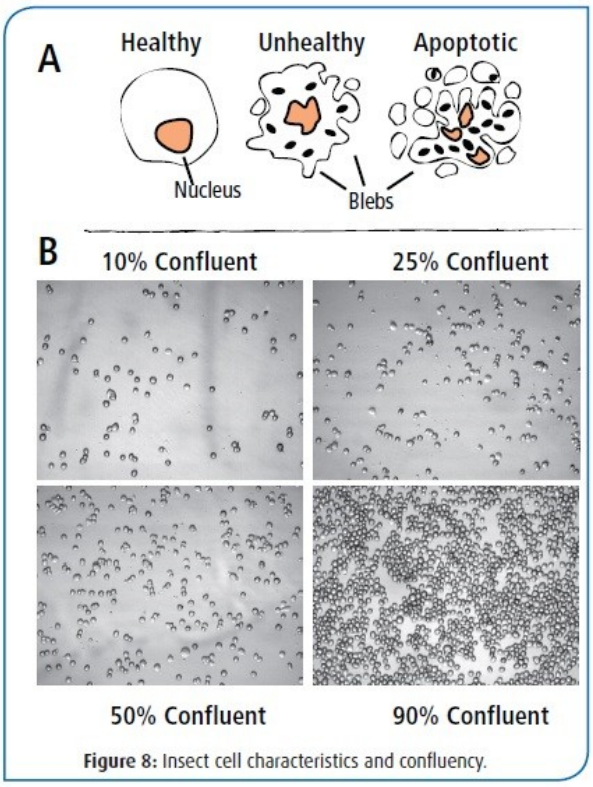

Figure 8: Insect cell characteristics and confluency.

NOTE: If the cell culture is contaminated, immediately add 1 mL of 10% bleach solution into the flask. After at least one hour, discard the culture. Clean the cell culture incubator with 70% ethanol to prevent the spread of contamination.

4. **RECORD** in the data log: the appearance of the cells, the clarity of the medium, and the presence or absence of contamination. **DRAW** a picture of cell morphology, including the shape of individual cells and the size and distribution of groups of cells.

> **NOTE:** If it is possible, take photos with a digital camera, print them, and include them in the cell culture data record.

5. **DETERMINE** if cells require additional time to grow and need to be fed or if they reach 80%-90% of confluence and need to be amplified (spread to other plates to get more cells). Follow the instructions of module III.

6. If cells are not ready to be amplified, **RETURN** flask to incubator. Check cells daily to monitor growth, recording the data in the cell culture data log. Observe any changes in cell morphology as cells increase of confluence.

Module III: Insect cell culture manipulation

NECESSARY MATERIAL

To carry out this practice, it is necessary to have the following materials before its start:

● Gloves	● Face mask
● Lab coat	● 70% ethanol
● Beaker for waste disposal	● Insect cell culture medium
● Dispenser/pipette bulb	● 35mm cell culture plates
● 10 ml sterile pipettes	● Plates scrapers
● Variable volume pipettes	● Pipettes tips (sterile)
● Sterile pasteur pipettes	● Cell counter chambers (Neubauer)
● 15 ml sterile tube	

OVERVIEW

Insect cells can be kept alive for long periods of time without DNA degeneration affecting cell physiology and viability.

To obtain a high number of cells that allows us to carry out the experiments we want, we have to feed and distribute the cells in new cultures (a process commonly known as **passages**).

The basic nutrients that allow optimal cell growth are depleted during the culture days, so they must be replenished. This involves **replacing the old growing medium with fresh growing medium**.

The process that is carried out to obtain a greater number of cells requires separating a certain number of cells and distributing them in different culture flasks (**passages**). In most cultures, this process is carried out when cell exceeds 80% of clonfuence.

CULTURE MEDIUM REPLACEMENT

The replacement of the culture medium should be done every 2-3 days. The need to change the medium will be indicated by microscopic observation of the culture's state.

Once we have disinfected the work area and the necessary material that we will use (see Module I), we proceed to:

1. **RECOVER** the tube of **Insect Cell Culture Medium** from the refrigerator (at 4ºC) and allow it to warm to room temperature before its use.

2. **REMOVE** 4 ml of the medium from the flask containing the cells in culture using a sterile pasteur pipette, gently aspirating. Take care that the pipette does not contact the surface of the flask to which the cells are attached (a small volume of the old medium can be kept, as it contains growth factors that have been secreted by the cells.)

3. **DISPOSE** the media removed from the flask into a disposal container (beaker) designated for this purpose.

4. **ADD 4 ml** of fresh insect cell culture medium using a new sterile pasteur pipette. The pipette used to aspirate the old medium must not come into contact with the fresh new medium.

5. **RETURN** the flask with the insect cells to the incubation chamber for as long as necessary before changing again the culture medium. It is necessary **to observe** the cell culture daily, under the microscope, and determine its condition. If the culture requires nutrient replacement, change the culture medium to a fresh one and if the confluence of cell growth is greater than 80%, amplify the cell culture (pass the culture).

CELL CULTURE AMPLIFICATION (CELL CULTURE PASSAGES)

The cells can continue to grow continuously, without cell death occurring. For this, it is necessary to carry out successive passages or amplifications of the cell culture.

When the cells reach a confluence of 80-90%, they are ready to carry out this process. If we let them grow to 100% of confluence and remain under these conditions for a long time, there would be contact inhibition between the cells and therefore they would stop growing and enter the process of senescence and cell death.

Once we have disinfected the work area and prepared the necessary material that we will use (see Module I), we proceed to:

1. With the help of the **sterile scraper** gently **RUB** repeatedly the surface to which the cells are adhered, taking care that it does not touch the outside of the flask.

2. Using a 10 ml sterile pipette and pipette dispenser (suction bulb for pipette), **PIPET** the cell suspension up and down several times against the surface of the flask to which the cells are attached. This process helps to disperse the cells and prevent them from forming cell aggregates, facilitating uniform growth in the following plates.

This procedure must be carried out gently and without sucking air in to **AVOID** the formation of foam.

3. Use a microscope to **CONFIRM** detachment of insect cells from the inside of the flask.

3A. Cellular amplification. New pass (Methodology not applicable and not included in the development objectives of this kit)

DILUTE the cell homogenate with fresh culture medium 1:1 (in this case 2 ml of homogenate and 2 ml of fresh culture medium), obtaining 2 flasks of cells.

PLACE the flasks of cells in the incubator and observe them periodically to check their physiological status. Change the medium when necessary to obtain optimal growth.

3B. Preparation of the crop to carry out the practice corresponding to Module IV

For the development of the practices corresponding to Module IV, we need a total of 5 new 35 mm cell culture plates.

Label the plates with the identification of the corresponding student group. **Label** 5 plates with the *Module IV* label.

WARNING: For the development of these practices, we will add from 0.075 to 0.1 million cells in each culture plate.

We will use a total of 5 culture plates to carry out the practices of Module IV, therefore, in the event that we decide to seed 0.075 million cells per plate, for a total of 5 plates x 0.075 million/plate → we will need a total of 0.375 million of cells.

Protocol for obtaining the desired cell density:

We need to **COUNT** the number of cells present in the homogenate obtained in the previous section:

1. **Add** 15 µl to the tab of one of the 10 areas of the cell counting chamber (Neubauer chamber).

2. **Count** the number of cells present in each of the 9 counting zones (each of them contains a total of 9 squares).

As an equally representative alternative, the number of cells present in each of the zones present in the corners of the 3x3 square of the Neubauer chamber can also be counted.

If we count the four zones: 1, 2, 3, and 4, then the average number of cells would be:

Average number = Number of counted cells / Number of counting zones used.

Average number = Number of cells counted / 4

The formula to determine the number of cells present in the culture is as follows:

> **Total nº cells present in 1ml (cell density)** = average nº cells x multiplication factor x dilution factor.
>
> Multiplication factor = 4500.
>
> Dilution factor = 1 (in this case, we have not diluted the cells before depositing them in the counting chamber).

The value obtained is the number of cells per milliliter. Our **cell homogenate** is in a volume of culture medium of 4 ml.

NOTE:

To know the total number of cells in the plate, we must perform the following operation:

Total nº cells plated = nº cells counted / 1 ml x 4 ml

3. **Dilute** the cell homogenate **in a 15 ml sterile tube** to bring their concentration to a density of 0.075 million/ml.

For example, if in our culture we have obtained a cell density of 0.8 M/ml, to bring it to a density of 0.075 M/ml, which we will seed in each of the 35 mm plates, we have to know the dilution factor to obtain the desired density

$$0.8M/ml: 0.075M/ml \sim 10.6$$

This means that, to dilute the initial density from which we start and obtain the density at which we will plant the cells in the 35 mm plates, we have **to transfer** 1 ml of cells from the culture to a 15 ml sterile tube and **add**, with the help of from a 10 ml sterile pipette, 9.6 ml of cell culture medium to bring them to a total volume of 10.6 ml.

4. **Add**, with the help of a sterile pasteur pipette, 1 ml of fresh culture medium to each of the previously identified 35 mm plates.

5. **Add**, with the help of a sterile pasteur pipette, 1 ml of the cell homogenate, δ=0.075M/ml, to each of the culture plates.

Warning: Before adding the cells, with the help of the sterile pasteur pipette, gently resuspend the homogenate.

6. Gently **move** each of the plates forward and back, making a cross, to evenly **distribute** the cells present on the plate.

7. **Examine** the cells under the microscope and **confirm** the presence of cells in the plates and that the cells are round and clear, not shriveled or dark.

> **NOTE:** Unused cells can be removed by adding 1 ml of 10% bleach to the 15 ml tube containing the homogenate. After at least 1 hour of adding the bleach, this can be discarded.

8. **Record** the observed data in the cell culture data log.

9. Within 24 hours of adding the cells to the plates, they should have adhered to their surface. **Confirm**, under the microscope, the fixation of the cells.

The cell plates will be used for the development of the practice corresponding to Module IV.

> **NOTE:** If the laboratory practices are carried out every day, the cell density used in each culture plate should be about 100,000 cells.

Module IV: Viability and Kinetics of Cell Growth

NECESSARY MATERIAL

To carry out this practice, it is necessary to have the following materials before its start:

• Gloves	• Face mask
• Lab coat	• 70% ethanol
• Beaker for waste disposal	• Phosphate buffer saline (PBS 1x)
• Trypan blue dye	• Plates scrapers
• Variable volume pipettes	• Pipettes tips (sterile)
• Sterile pasteur pipettes	• Microcentrifuge tubes

Cell viability will be determined by the relationship between the number of viable cells, living cells present in the cell culture, and the total number of cells, obtained from the addition of the number of viable cells and the number of non-viable cells.

The data obtained each day, along different days, both the number of **viable cells** and the number of **non-viable cells**, will be used *to determine the kinetics of insect cell growth throughout the culture*.

DISCRIMINATION OF VIABLE AND NON-VIABLE CELLS

In order to be able to distinguish viable (alive) from non-viable (dead) cells and to obtain the amount present in the cell culture of each of them, we need to identify them.

In this practice we will use *trypan blue dye*, a dye that penetrates through the membranes of *non-viable cells*, staining them an intense blue color. *Viable cells* do not take up the dye and therefore do not show the blue color.

Once we discriminate some cells from the others, **we will be able to establish the present number of each of them in the culture**.

Cell staining protocol with trypan blue dye:

The application of this protocol does not require the use of sterile material, so we can do it on a clean bench with enough space, on which we have previously arranged the necessary material.

1. **RECOVER**, 24 hours after culture, one of the 35 mm plates corresponding to *Module IV*. **Identify** the plate as day 1 and **note** the confluence in the cell culture data log.

2. With the help of the scraper, gently **SCRATCH** the cell culture plate, making sure that we reach the entire surface where the cells are adhered with the scraper.

3. Gently **PIPET** the cells up and down three times with the aid of a pasteur pipette to disperse the culture.

4. **IDENTIFY** two eppendorf tubes with the name of the student group and **ADD** 1 ml of cell homogenate to each tube.

5. **CENTRIFUGE** tubes at 1000 rpm for 5 minutes.

6. **SUCK UP** the supernatant (culture medium) with the help of a variable volume pipette and **AVOID** aspirating the cells present.

7. **ADD** 500 µl of phosphate buffer (PBS 1x) to each of the two microcentrifuge tubes using the adjustable volume pipette and **gently** resuspend the cells, **avoiding** foaming.

8. **ADD** to one of the microfuge tubes the 500 µl of cell homogenate present in the other microfuge tube. We will now have a single microcentrifuge tube with a volume of 1 ml of the cell suspension.

9. **TRANSFER** 10 µl of trypan blue dye into a microcentrifuge tube. **ADD** 10 µl of the cell suspension and using the same adjustable-volume pipette tip, pipette the volume up and down repeatedly to make the cell suspension homogeneous. We have diluted the volume 2 times. *Dilution factor = 2*.

10. **INCUBATE** the cells in the tube for 1 minute at room temperature.

11. Slowly **TRANSFER** 12 µl (approximately one drop) of the trypan blue dye stained cell suspension to a well in the upper center side of one of the ten counting chambers containing each cell counting slide. The chamber will filled by capillary action.

12. **EXAMINE** the counting chamber under the microscope using the lowest magnification objective.

13. **FOCUS** the grid lines on the camera. Move the slide until the field you see is the outer grid. Use a higher magnification objective if it is more comfortable to observe the field of cells to be counted.

14. **COUNT** all the living cells (not incorporating the dye and appearing bright and whitish in color) and all the dead cells (incorporating the dye and appearing blue in color) within each of the small grids. **RECORD** the number of viable (clear and bright) and non-viable (stained deep blue) cells on the cell culture data log.

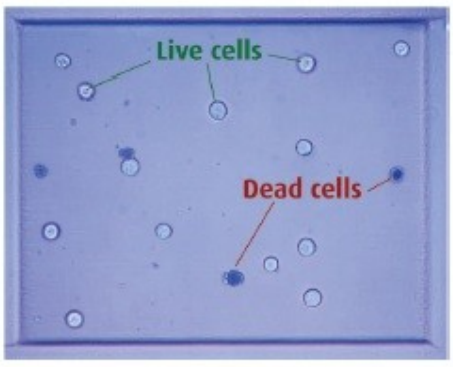

Figure 10: Trypan blue staining of cells

15. **STORE** the hemocytometer at room temperature until needed for the next cell count in the experiment.

> **NOTE:** Do not attempt to wipe cells out of the used well as this may render unused counting chambers unusable.

CALCULATION OF THE NUMBER OF VIABLE AND NON-VIABLE CELLS

The cell viability of the culture is expressed as the ratio of the number of viable cells with respect to the total number of cells.

The formula for calculating viable cells/ml and non-viable cells/ml is as follows:

Total nº viable (or non-viable) cells present in the total of the 9 counting zones of the slide (Neubauer chamber) = nº viable (or non-viable) cells counted x multiplication factor x dilution factor.

Multiplication factor = 4500.

Dilution factor = 2 (in this case, we have diluted the cells twice before depositing them in the counting chamber).

For example: 10 µl of cells are diluted in 10 µl trypan blue dye and 75 cells are counted on 9 small grids

- Total number of viable cells = 75
- Multiplication factor = 4500
- Dilution factor = 2

To calculate viable cells/ml = 75 x 4500 x 2 = 6.75 x 10^5 cells/ml

Note: It should also be done with the non-viable cells that we have counted.

CALCULATION OF CELL VIABILITY. PERCENTAGE OF VIABLE CELLS

Cell viability can be determined by calculating the percentage of viable cells present in the culture relative to the total number of cells in the culture (viable + non-viable).

The formula to apply is the following:

Viability = Nº of viable cells/Total nº of cells (viable + NOT viable) x 100

For example: Of the cells we have counted, 75 are bright (viable) and 15 deep blue (non-viable).

To calculate viability = 75/90 x 100. **Viability** is 83.3%.

CELL GROWTH CURVE

To make the cell growth curve, ***we need to obtain the data of the viable and non-viable cells present in the culture, during successive days (for example, days 1, 2, 3, 4, 7 after the start of the culture)***.

1. **PERFORM** a cell count and viability assay as described in the previous section every 24 hours until no change in the number of cells/ml of the culture is detected (stationary phase). Use a new well in the hemocytometer for each measurement.

NOTE: Usually, *considering our cell culture conditions*, after 5 days, the culture reaches the stationary phase, although growth rates may vary from one experiment to another due to changes in temperature and the number of initial cells.

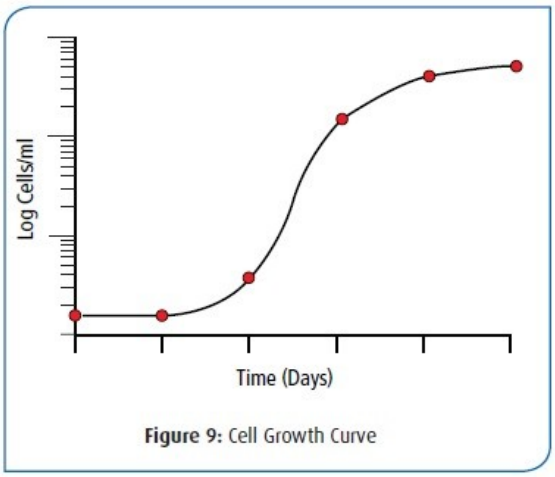

Figure 9: Cell Growth Curve

2. Using a sheet of logarithmic graph paper or a computer graphing program, **RECORD/PLOT** cell concentration data (cells/ml) on a logarithmic scale as a function of time (in days) in culture.

3. **IDENTIFY** and label each of the growth phases: lag phase, exponential growth phase, and stationary phase for cell culture.

NOTE: The lag phase is not always observed in rapidly growing cultures.

4. Select a time interval during the growth phase and **CALCULATE** the **doubling time** for the culture, the time required for the number of cells/ml to double. The doubling time can be determined by identifying a number of cells along the growth phase of the curve, drawing the curve until that number has doubled, and calculating the time between these two points.

ANNEX 1: PRACTICE 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 subculture cells at 80-90% of confluence?

4. What is the reason for leaving a small amount of old culture medium in the flask when feeding or dividing cells?

5. Describe the common symptoms of bacterial contamination.

6. Why is it important to determine the doubling time of a cell line? What information does the doubling time of cells indicate?

ANNEX 2: RESULTS OF THE PRACTICE AND ANALYSIS

Expected results will vary depending on the growth characteristics of the cells. The viability and growth rate of cells is highly dependent on the conditions under which the cells are cultured, including initial cell density, temperature, confluence, and pipetting precision.

Cell growth curves will vary depending on many factors, including initial cell density, culture temperature, and cell health. Healthy cells may not experience a lag phase after transfer to a new flask and will immediately enter the exponential growth phase.

The results obtained with cells stained with Giemsa stain may vary between groups of students, due to variations in cell preparation and intensity of staining.

ANNEX 3: PREPARATION OF THE PRACTICE BY THE TEACHERS

It is very important that the practice teacher **takes into account** the following considerations:

a) When receiving the practice kit

Check that the material received is the one indicated on the delivery sheet.

Cultivate each of the 6 tubes of cells in each culture flask of 25 cm^2 (with blue lid) corresponding to each group of students, previously identified.

b) Before the start of practices

Disinfect the work area with 70% ethanol (*preferably work in a flow hood*).
Prepare and distribute the necessary material for the development of each practice before its start.
Disinfect the material with 70% ethanol before incorporating it into the work area.
Insist on the need for all the material used to be disinfected to avoid contamination.
Indicate that *sterile cell scrapers must be washed and stored for successive uses* that do not require sterility since the culture is not maintained and is discarded (Module IV).

c) During practices

Remember that students *have to change the cell culture medium 3 days after growing* them in the plates.

Prior organization and implementation of the practice

The instructions presented in this protocol are those indicated to carry out the practices with six groups of students.

Before starting each practice, carefully review the list of the *Necessary Material* that appears in the header at the beginning of each module. Make sure you have all the necessary components and equipment.

> **IMPORTANT: The cells must be cultured immediately after receiving them.** ***See the Starting the culture section of this annex.***

Cautions

The media contain antibiotics to keep the cultures free from contamination. **Students who have allergies to antibiotics such as penicillin, streptomycin, or gentamicin should NOT participate in this experiment**.

Sterilization of equipment and materials

1. Sterilize the laboratory bench with a 70% ethanol solution or any other commercial laboratory disinfectant.

2. All materials, both solid and liquid, that come into contact with cells and are to be discarded, have to be disinfected prior to disposal, including culture plates and flasks, culture media, pipettes, transfer pipettes, and tubes.

Liquids:

Whether we want to eliminate the culture medium that we have discarded and have deposited in the beakers, or we want to eliminate the cell cultures, we have to add a few milliliters of 10% bleach for a minimum of 1 hour and then discard it. Deposit plastic bottles, jars, and plates in an autoclave bag and treat it as solid material for disposal.

Solids:

Collect all contaminated materials in an autoclavable disposable bag. Seal the bag and place it on a metal tray to avoid any chance of any remaining liquid spilling into the sterilizer chamber.

Autoclave at 121 ºC for 20 minutes.

Approximate Time Guidance for Practice Procedures

The practice is divided into five modules and should last approximately two weeks. The following Tables are a guide for the implementation of this practice, which can be adapted to the specific circumstances of each class.

Module	Previous preparations	Practice
I	15 min.	30-45 min
II	15 min.	15-30 min.
III	20-25 min	1. Medium change: 10 min. 2. Amplification: 50-60 min
IV	15 min.	20-30 min.

NOTE: The experiment in Module IV **will be performed multiple times** to collect the data used in plotting a growth curve. We recommend repeating them on the indicated days to obtain results that are indicative of the process.

Table/Summary of previous preparations

Preparation for:	What to do:	When?	Required time
Start the cell culture	Transfer the received cells the culture flasks	Immediately after receiving the cells (*)	15 min.
Module I: Basic aseptic technique	Prepare and distribute aliquots	One hour before the experiment	15 min.
Module II: Cell cultura observation and evaluation	Prepare compound microscopes	Anytime before the lab practice	15 min.
Module III: Insect cell cultura handling	Prepare and distribute materials	One hour before practice	20-25 min.
Module IV: Viability and kinetics of cell growth	Aliquot trypan blue and distribute materials	Any time before the first viability count	20-30 min.

> **NOTE:** For best results, review basic aseptic technique (Module I: Basic Aseptic Technique) before beginning any laboratory experiments or reagent preparation.

We recommend preparing the equipment and reagents for Modules I, II and III before starting the practice with the students. The reagents for Module IV can be prepared as needed once student groups have progressed to those sections of the practice (we minimized the expected time for lab preparation). Have a microscope ready for cell observation and analysis throughout all the Modules.

> **NOTE:** The observation of the cell culture in plates is highly recommended to be carried out with the help of an inverted microscope. **If not available, check that the plate containing the cells has, at most, the height between the stage and the microscope objectives.**

BEGINNING OF THE CULTURE OF INSECT CELLS

The preparations to be carried out as soon as possible once the kit is received are detailed:

a) Preparation of the incubation chambers

It is necessary to prepare an incubator chamber to contain the cells. Incubators should be kept at 24-27°C in a standard atmosphere. A large plastic container or cardboard box can serve as a large incubator for the whole class (the same BIOTED box in which the kit is shipped can be used). Insect cells prefer to grow in the dark, so transparent containers should be covered with aluminum foil.

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

b) Preparation of aliquots of the sterile practice medium (Module I) and the cell culture medium (Module III).

Sterile Practice Medium:

1. Aseptically **ALIQUOTE 4 ml** of **Sterile Practice Medium (B)** into each of six 15 ml conical tubes, previously identified by student group.

2. **LABEL** each tube **Sterile Practice Medium**.

3. **STORE** at 4°C until needed by students in Module I.

Cell culture medium:

1. Aseptically **ALIQUOTE 20 ml** of **Cell Culture Medium (A)** into each of six 50 ml conical tubes, previously identified by student group. Reserve the remaining medium to start the insect cell culture.

2. **LABEL** each tube **Insect Cell Culture Medium**.

3. **STORE** at 4°C until needed by students in Module III.

c) Initiation of the culture of the cells received

Sf9 insect cells (A) are shipped in a 3 ml tube and should be transferred to a 25 cm^2 culture flask as soon as they are received.

1. **TEMPER** the insect cell culture medium to room temperature.

2. **ADD** 1ml of insect cell culture medium to each of the 6 Culture Flasks, identifying them according to the group of students to which they correspond, keeping them in a vertical position.

3. Gently **REVERSE** the tube of cells to mix the contents.

4. Using a sterile transfer pipette or sterile micropipette tip, **TRANSFER** the entire volume of Sf9 insect cells (A) to each of the (sterile) cell culture flasks.

> **NOTE: DO NOT pour cells directly by decanting the tube onto the culture flask, as this increases the risk of contamination.**

5. **INCUBATE** the cell culture flask in the incubation chamber.

6. After 24 hours, the insect cells should have adhered to the surface of the flask. **CONFIRM** cell adherence under a microscope.

7. **ALLOW** cells to grow for additional 24-72 hours, checking cell health and the confluence daily. It is recommended that, if after 72 hours the confluence is not yet 80%-90%, they change the culture medium, removing the culture medium contained in the culture flask with a pasteur pipette, always leaving a remnant, and adding 4ml of fresh insect cell culture medium prewarmed to 27°C or temper to room temperature. **Cells should be at least 80% of confluence before starting the following experiments**.

MODULE I PREPARATION: BASIC ASEPTIC TECHNIQUE

1. Aseptically **ALIQUOTE** 4 ml of **Sterile Technique Practice Medium (B)** into six 15 ml tubes using a 10 ml sterile pipette. Each group should have its own tube of medium to reduce the possibility of contamination.

Material needed for each group:

● Gloves	● Face mask
● Lab coat	● 70% ethanol
● Sterile 10 ml pipettes	● Medium for practicing sterile technique
● Pipettes dispenser	● 35mm cell culture plate

MODULE II PREPARATION: OBSERVATION AND ASSESSMENT OF CELL CULTURE STATUS

PREPARE inverted microscopes for analysis of insect cells. Inverted microscopes will preferably be used, but if they are not available, phase contrast or bright field microscopes can be used for observations. In this case, the cell culture samples used in this experiment are approximately 2.5 cm tall, please make sure there is enough space between the stage and the objectives to see the cells. In the case of not fitting, 35 mm culture plates can be used, passing the cells from the flask with 1 ml of the cells and 2 ml of culture medium.

Each group needs:

● Gloves	● Face mask
● Lab coat	● 70% ethanol
● Sterile 10 ml pipettes	

MODULE III PREPARATION: HANDLING OF CELL CULTURE

1. **REMOVE** aliquots of insect cell culture medium from the refrigerator and allow temper to room temperature.

2. **DISTRIBUTE** the necessary components to each group.

For Module III-A

Each group needs:

● Gloves	● face mask
● Lab coat	● 70% ethanol
● Beaker for waste disposal	● Culture medium of insect cells
● Sterile pasteur pipettes	

For Module III-B

Each group needs:

● Gloves	● Face mask
● Lab coat	● 70% ethanol
● Beaker for waste disposal	● Culture medium of insect cells
● Dispenser/pipette bulb	● 35mm cell culture plates
● Sterile 10 ml pipettes	● Plates scrapers
● Variable volume pipettes	● Pipettes tips (sterile)
● Sterile pasteur pipettes	● Cell counter chamber (Neubauer)
● 15 ml sterile tube	

MODULE IV PREPARATION: VIABILITY AND KINETICS OF CELL GROWTH

1. **ALIQUOTE** into microcentrifuge tubes 250 µl of **trypan blue (D)** for the 6 groups.

> **NOTE:** Each group will receive an aliquot of trypan blue and a counting chamber. These should be saved after the experiment to use in subsequent counting trials.

Each group needs:

● Gloves	● Face mask
● Lab coat	● 70% ethanol
● Beaker for waste disposal	● Phosphate buffer (PBS 1x)
● Trypan blue die	● Plates scrapers
● Variable volume pipettes	● Pipettes tips (sterile)
● Sterile pasteur pipettes	● Centrifuge tubes

> *__NOTE:__ Add a few milliliters of 10% bleach to each beaker to disinfect cell culture waste.

Recording of cell culture (subculture) data

Group:	Names of students:										
Date											
Health status of cell culture	Appearance of cells										
	Culture medium clarity										
	Confluence (density of cells)										
Subcultures	Final volume in new bottle (ml)										
	Cell volume (ml)										
	Cell dilution ratio										
Cell count	Average live cells per grid										
	Average number of dead cells per grid										
	Total cells per grid										
	Live cells in the flask (#live x volume)										
	% viability (#alive/#dead)										
	Growth cycle phase										

OBSERVATIONS:

RESUME