4 groups of students

TOXICITY STUDY IN INSECT CELLS

1. PRACTICE'S OBJECTIVE

The goal of these practices is to introduce the students to the experimental design of a toxicity study in cell cultures.

2. MATERIAL AND REAGENTS INCLUDED IN THE KIT

Components	Ship	Conservation
Insect cells (Sf9)	2 vials	R.T.
Insect cell culture medium	62 ml	4 ºC
Reaction medium	56 ml	4 ºC
Phosphate-buffered saline (PBS 1x)	28 ml	4 ºC
CuSO4	2x6 ml	Room temperature
ZnSo4	2x5 ml	
Trypan blue dye	1 ml	
Cell culture flask (sterile, 25 cm^2)	2 units	
12-well cell culture plates (sterile)	4 units	
10 ml pipette (sterile)	10 units	
Pasteur pipette (sterile)	10 units	
Scrapers (sterile)	5 units	
Cell counting chamber	7 units	
15 ml conical bottom tube (sterile)	21 units	
1,5 ml centrifuge tube	104 units	

This practical is designed for 4 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 receiving them (see annex 3 of this protocol).
>
> **NOTE: Insect Cell Culture Medium**, **Reaction Medium**, and **Phosphate-buffered saline (PBS)** must be stored in a refrigerator (4ºC) immediately after receiving them.

All components of this practice are intended to be for educational research only. They must not be used for diagnostic or pharmacological purposes, nor 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 and isopropanol bottles
- Pipette with suction bulb or automatic pipette
- Phase contrast/bright field capability inverted microscope (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 cells observation and counting in the cell counting chamber, use the student's microscope (vertical position).

3. INTRODUCTION

El cultivo de células eucarióticas

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.

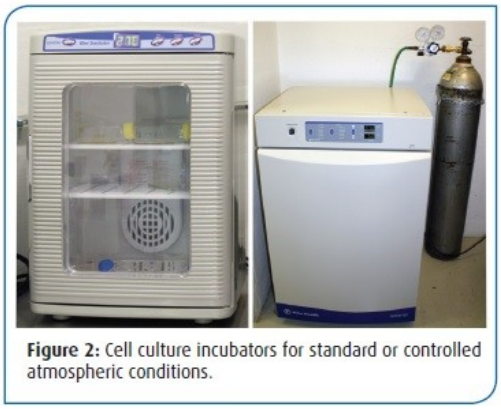


Figure 2: Cell culture incubators for standard or controlled atmospheric conditions.

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.

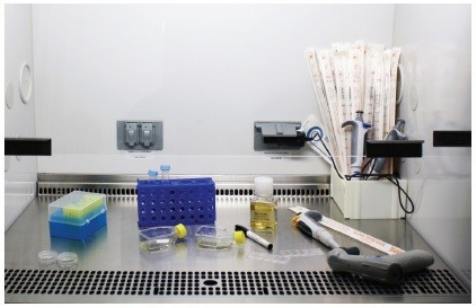


Figure 3: Cell culture hood and standard equipment.

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 and 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.

Toxicity tests in cell cultures

Toxicity tests are of huge importance to evaluate the safety of different compounds and prevent possible alterations that can originate in living organisms, assuring their health and well-being.

One of the most used experimental approximations has been the use of cell cultures to evaluate the toxic potential of certain chemical products, drugs, or pesticides. The results obtained in these tests with cell cultures help optimizing the design of the study of these toxic agents in the experimentation with animals and their application in agricultural exploitations, rationalizing their use and minimizing their derivative effects on the animal well-being.

The incidence of toxic substances in the cell culture is highly variable and related to the toxic potential of these chemical compounds. Thus, an extremely toxic substance can cause aggressive and fast cell death. However, the toxicity study in lower concentrations or with other less toxic substances can damage the cell without causing its death. The study of these toxic agents will determine their quality as more or less dangerous elements.

The cell culture exposure to diverse toxic compounds can affect cell viability in different ways. Microscope observation of the cells reveals changes in the cell shape or structure, indicating that these cells show affectations in their health status, including cell growth and reproduction.

One of the assays used to assess the potential of toxicity of determined substances and be able to compare between them is based on the determination of the concentration of the substance that causes 50% of the mortality in the cells present in the cell culture **(LD50)**. In organisms, this value is expressed as the milligrams of the substance per kilogram of the animal's body weight that **causes 50% of animal mortality**.

A substance's LD50 that is lower than another substance's LD50 would indicate the lower toxicity of the former. If in a rat study the obtained LD50 for the sodium chloride is 300 mg/kg and the cyanide's LD50 is 10 mg/kg, this means that, in these animals, cyanide's toxicity is much greater than that of the sodium chloride (salt).

On the other hand, the comparison of LD50s of certain substances obtained in the study of a determined cell line does not have to be extrapolated to another different cell line. Each cellular type's physiology can cause large variations in the LD50 of each of these toxic substances. Similarly, one cannot extrapolate either the obtained results in animals of a certain species to a different one.

One of the experimental approaches used in the toxicity study of a substance in a cell culture is based on the determination of the residual cell viability resulting after exposure to the substance. **In the case of cell cultures**, *LD50 indicates the toxic agent's concentration that causes the death of 50% of the cells present in the culture.*

A large number of experiments on cell viability are based on the ability of healthy cells to prevent the incorporation of trypan blue dye. Nonviable cells incorporate this dye and their cytoplasm appears dyed blue under the microscope (Figure 4).

In this toxicity kit, students will have to design and develop a toxicity assay in Sf9 insect cells. Each group must use one of the potentially toxic substances that are provided for their study.

The chemical products that will be used must be applied taking into account the corresponding dilution factor in each case.

The trypan blue staining will be used to count the number of viable cells and the number of nonviable ones. Calculating the corresponding LD50s will be performed by determining the percentage of nonviable cells present in the culture:

$$\frac{viable\,cells}{total\,cells\,(viable + nonviable)}\,x\,100$$

4. PRACTICAL TO CARRY OUT WITH THE KIT:

Practices will be divided into 4 modules:

- **Module I:** *Observation and evaluation of the cell culture status*

  In this module, students will learn to observe insect cells in the microscope and determine whether they are growing in optimal conditions. Also, they will be able to know when it is the best moment to do the subcultures or change the culture medium for another fresh one containing the necessary essential nutrients. Usually, cells should be fed every 3-5 days and reseeded (subculture), preferably when there exists a confluence of 80-90%.

- **Module II:** *Experimental design of toxicity in insect cells caused by environmental agents*

  In this module, students will learn to formulate the experimental design for determining the cellular toxicity induced by an environmental agent.

- **Module III:** *Cell culture manipulation. Reseeding.*

  In this section, students will manage insect cell cultures and learn to do reseedings. Likewise, they will learn to assess the physiologic suitability of the culture to get reliable experimental data.

- **Module IV:** *Study of toxin exposition in insect cell cultures. Cellular viability.*

  Students will learn to develop in the lab the experimental planning that has been designed to answer the raised hypothesis.

  Similarly, they will learn to execute serial dilutions of the substances under study, focused on assessing their toxicity in the insect cell cultures.

  Moreover, they will put into practice the cellular viability assays using the trypan blue dye and the counting of cells that do not incorporate it in their cytoplasm (viable) and those who incorporate it (nonviable) staining in blue. They will obtain the LD50 values as a measure of toxic potential.

PRACTICES LAB

A. General Considerations

The successful cell culture depends on the cells being kept in an environment free of contamination by microorganisms such as bacteria, fungi, and viruses. All materials that are in contact with the cell culture **must be previously disinfected so that the manipulations do not let** the non-sterile environment contaminate the culture. For this, it is very important to:

1. Make use of lab coats and masks. Their use minimizes the risk of contamination of cell cultures. Long hair should be kept tied up -preferably under a lab hat- and talking should be avoided as much as possible during the cell culture manipulation.

2. Make use of disposable gloves at all times. Prior to its 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 flasks**

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.

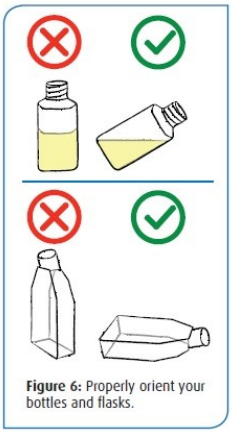


Figure 6: Properly orient your bottles and flasks.

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).

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.

MODULE I: Observation and evaluation of cell culture status

OVERVIEW

It is necessary to get used to examine the cell culture before each experiment. During the microscopic observation of cells, we will we able to determine their health state and check that the culture is free from contamination.

ONLY when we are sure that the cells are growing in optimal conditions, we will be able to start the experiment.

The microscopic observation can reveal us the source of contamination in the cell culture (Figure 7).

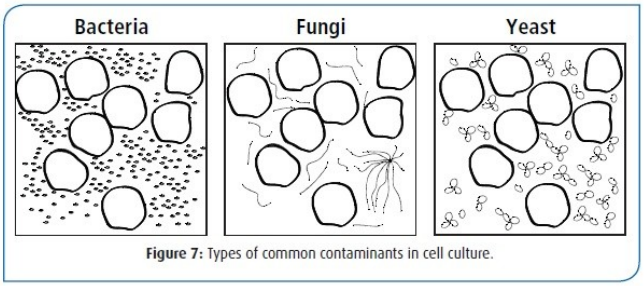


Figure 7: Types of common contaminants in cell culture.

• Bacteria: media seems cloudy and can have a white surface. Under the microscope, small and granular cells will be seen, resembling black dots.

• Fungi: there appear thin filamentous mycelia covering the cell culture, as a blurry growing (typically white or black). It is visible with naked eye.

• Yeast: round particles that are smaller than the insect cells. Often, chains of two or more cells are observed.

NOTE: If contamination is observed, **it is important** to eliminate, quickly and with confidence, the reagents and the contaminated plates to prevent the contamination propagation. Check if the sterile technique has been applied correctly and analyze the possible sources of contamination. **Remember** that before initiating the practice, it is necessary to disinfect the work area and all the materials that are going to be used, including the gloves. Minimize the time in which the recipients are open to the air and make sure that the pipette does not come into contact with any other surface that it is not the support and the cell culture plate.

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 *blebbing* (formation of small bubbles at the edges of cells) in the cell membrane, and fragmentation of the core (Figure 8A).

METHODOLOGY

Remember that is needed to previously clean the area by applying 70% alcohol.

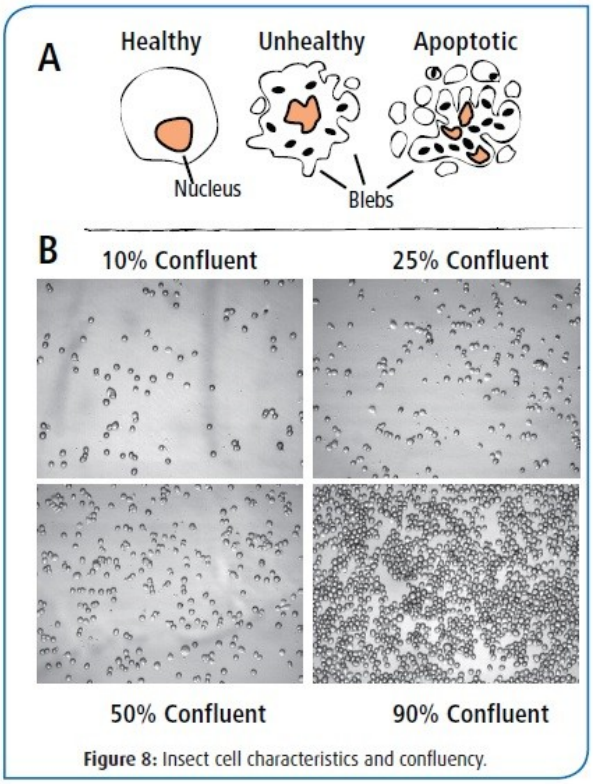


Figure 8: Insect cell characteristics and confluency.

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%.

> **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 II: *Experimental design of toxicity in insect cells caused by environmental agents*

The execution of an experiment is the culmination of a process that wants to give an answer to an intellectual proposition through an ordered planning of logical events, the experimental design.

The ordered sequence of all experimental designs is based on:

1. **Establishing** a question to which we intend to provide an answer.
2. **Formulating** a working hypothesis.
3. **Planning** the experiments to carry out.

It is very important to do a concise and quality bibliographic search before making any experimental design. Only through prior knowledge, we can be successful in our experiments. It depends on this that, after the analysis of our experimental results, we can give an answer to the proposed question.

The evaluation of the toxicity in a cell culture caused by the action of some agents present in the medium is done by observing the effects of these on the cellular viability.

As indicated, before initiating any planning in the lab, it is essential to make a rational and objective design of the development of the experiments.

Bibliographic information:

According to the information we can find in the literature about the environmental substances under study in the kit, copper sulfate (CuSO4) is used as a fungicide in fungus elimination and sulfate zinc (ZnSO4) is used as an agricultural fertilizer.

Specifically, we ask ourselves if a certain environmental substance can be harmful to the survival of insect cells and we will design the experimental conditions needed to answer this proposition.

There are two compounds in this kit, CuSO4 and ZnSO4, that are chosen to be used as possible toxic agents in the resolution of the raised question.

There is a chance that both agents or environmental substances have some type of impact on the life of the eukaryotic cells.

1. We establish a question:

CuSO4 or ZnSO4 cause toxicity in insect cells?

To answer this, we chose as biological model the insect cell culture.

You can search for potential environmental toxins and make questions about which of them would be interesting for doing a study:

2. We formulate a working hypothesis:

In the case of CuSO4 and ZnSO4 exerting a toxic action over these cells, then

Variable concentrations of each of these molecules produce variability in the insect cells' viability.

The hypothesis formulation can be diverse, even though we have to choose one that adjusts to the practical demonstration that entails a clear answer to our question. We should take into account the prediction of the results we are going to obtain.

Formulate some hypotheses about the questions made about your search.

3. Plan the experiments to perform:

To determine the effects of the toxic agents over the cellular viability, we are going to carry out a series of dilutions for each of the toxic agents, choosing three different concentrations of CuSO4 and another three of ZnSO4. The obtained data will let us compute the LD50 for CuSO4 and ZnSO4.

Usually, to obtain the best results in the studies of any molecule's sensitivity to a biological activity, serial dilutions are used with a 1:10 ratio in each dilution.

In our experiment, the experimental conditions in which we will incubate the cells are the following:

CuSO4 0.5 mM	
CuSO4 2 mM	
CuSO4 4 mM	

ZnSO4 0.5 mM	
ZnSO4 1.5 mM	
ZnSO4 3 mM	

We should establish an experimental condition in which the cell culture has not been in contact with the toxic agent but has been manipulated the same way as the treated cultures.

Control 0 mM	

Since we carried out the serial dilutions using the reaction medium, in the experimental condition corresponding to a toxic agent's concentration equal to 0 mM (**or control treatment**) the culture medium must be replaced by reaction medium. This way the cellular viability results obtained in the cellular incubations with any of the toxic agents will be only and exclusively due to the agent's action and not existing differences in the media used for incubating the cells.

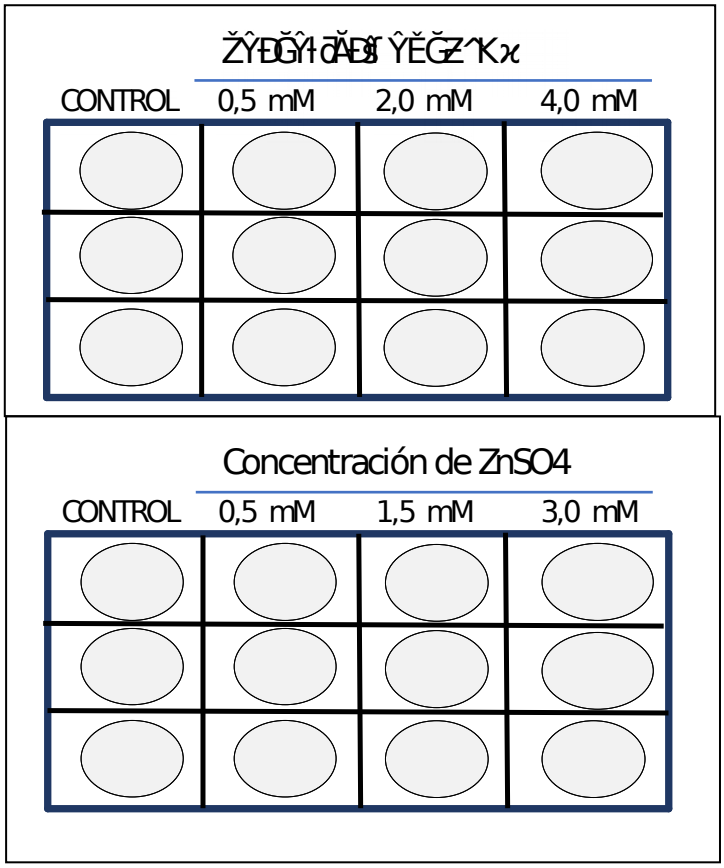
Lastly, we have to decide on the number of replicas for each of the experimental conditions. The goal is to be able to do a statistical study with the results to determine whether the obtained differences in each of the experimental conditions are significantly different or not.

In our planning, we choose to perform each experimental condition -including the controls- three times.

The experimental design should be aimed to know a toxic agent's potential, related to the value of the lethal dose needed to provoke 50% of cell death (LD50). Analyzing this value, it is possible to know a substance's toxic potential and compare it against another one.

In short, in our experimental planning to respond to our hypothesis, we will use three different concentrations of each of the toxic agents, using 3 wells for each concentration -triplicated-. We must operate the same way for the control condition.

Remember that we have to identify in the cell culture plates, with the help of a maker pen, each of the experimental situations. One option is the one shown in the image, where a horizontal line can be drawn to separate the triplicates and identify each of the concentrations of the molecule under study:



Module III: *Cell culture manipulation. Reseeding*

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	• Sterile pasteur pipettes
• Culture medium	• Scrapers
• Variable volume pipettes	• Pipette tips (sterile)
• Neubauer chamber	• 15 ml tubes (sterile)
• 12 wells culture plates	• 10 ml pipettes (sterile)

The toxicity practical will be developed using one culture plate of 12 wells per group. In each of the four plates, the cells contained in the two 25 cm^2 flasks will be seeded, at the specified density.

Before proceeding to count the cells, **IT IS IMPORTANT** to check that the culture is approximately at 80-90% confluence.

Students must be aware of the relevance of **keeping a sterile environment at all times**. We must use 70% alcohol to clean all the surfaces and protect the coat's sleeves when touching any sterile area, including the culture plates, to avoid any contamination. Before the start of the practice, **WE MUST wash our hands** with soap and avoid contact with any nonsterile surface.

1. **Spray** the flask containing the cell culture with 70% alcohol.

2. Softly **lift** the cells adhered to the floor of the green lid flask (25 cm^2 surface area) ***-the culture is approximately at 80-90% confluence-***, with the help of the sterile scraper.

3. Softly **suspend** the cells using a sterile pasteur pipette and deposit them in a 15 ml tube. Gently invert the tube 3-4 times so that the cell density is homogeneous.

4. **Distribute** in four 15 ml tubes -a tube for each group- the cellular volume from the tube where we have collected the cells from the two flasks.

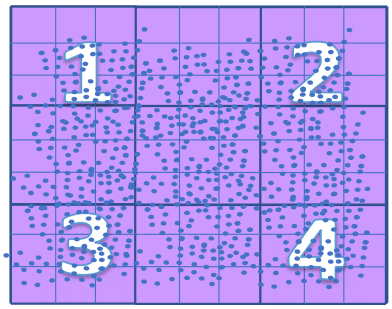
5. **Add** 12-15 microliters of the cell suspension to one of the Neubauer chamber's wells.

6. With the help of the microscope, **count** the cells in each of the edge areas of the chamber (there are 9 small squares in each of the four corners of the chamber).

7. **Obtain** the number of cells per milliliter of the homogenate:

$$average\ number\ of\ cells = ¿$$

$$\frac{total\ number\ of\ cells\ of\ the\ 4\ areas}{4\ areas}$$



$$\frac{number\ of\ cells}{ml} = average\ number\ of\ cells\ x\ dilution\ factor\ x\ multiplication\ factor$$

Example:

Average number of cells = 115

Dilution factor = 1

Multiplication factor = 4500

(0,52 Million cells) / ml

8. **Adjust the volume of the homogenate** by adding culture medium til reaching the necessary density. Before proceeding, **READ** the example described below.

9. **Seed** the culture plates of 12 wells under the cabinet at a *300000 cells/well density.* The final volume of culture medium in each well should be 1 ml.

Example:

We have 12 wells in each culture plate, meaning that we need:

0.3 million cells / well x 12 wells = 3.6 million cells per plate

1 ml of culture medium / well x 12 wells = 12 ml of culture medium per plate.

IMPORTANT: In our example, if we have a concentration of 0.52 M cells / ml and we need a number of 3.6 M for each plate, we should take approximately 6.9 ml of the cellular homogenate.

Besides, since we need 12 ml of culture medium in each plate, 6.9 ml correspond to the cell volume.

$$Volume\ of\ medium\ we\ add = 12\,ml\,(Vtotal) - 6.9\,ml\,(Vhomogenate)$$

$$Volume\ of\ medium\ we\ add = 5.1\,ml\ of\ complete\ medium$$

Once we have the 12 ml that include the 3.6 M cells, we gently homogenate them with the help of a pasteur pipette and deposit 1 ml in each of the 12 wells of the plate. We softly shake the plate to distribute the cells in each well and deposit the plate in the incubator.

TAKE INTO ACCOUNT that, to use this example's values, IT IS NECESSARY to adjust the initial cell concentration to 0.52 M / ml.

10. **Incubate** the cells at 27℃ until reaching a 80-85% confluence.

Module IV: Study of toxin exposition in insect cell cultures. Cellular viability

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 (PBS 1x)
• Trypan blue	• Scrapers
• Variable volume pipettes	• Pipette tips (sterile)
• 15 ml tubes (sterile)	• Toxic agents (CuSO4 and ZnSO4)
• 10 ml pipettes (sterile)	• Neubauer chamber
• Reaction medium	• Microcentrifuge tubes
• Sterile pasteur pipettes	

MODULE IV-A

PREPARATION OF THE SERIAL DILUTIONS OF THE TOXIC AGENT WHICH WE ARE GOING TO INCUBATE THE CELLS WITH

Each of the 4 groups will use ONLY ONE toxic agent for the study, at the concentrations indicated in each of the serial dilutions corresponding to that agent -**two groups** will use *CuSO4* and the other **two groups** *ZnSO4*-.

1. **MARK** each of the 4 sterile tubes of 15 ml the following way: mark 1 tube as *control* and the rest as CuSO4 or ZnSO4, depending on the toxic agent under study.

2. **IDENTIFY** the three tubes corresponding to CuSO4 as 0.5 mM – 2 mM and 4 mM. Do the same for the three tubes corresponding to ZnSO4 identifying them as 0.5 mM – 1.5 mM and 3 mM.

3. **CREATE, each of the 4 groups,** the serial dilutions that we are going to use, taking into account that we must shake the tubes to homogenate the content **before taking a volume and doing the next dilution:**

CuSO4 dilutions:

Control: Add 3 ml of Reaction Medium (RM)

4 mM: Shake the CuSO4 solution

2 mM: Add 2 ml of RM + 2 ml of 4 mM solution

0.5 mM: Add 3 ml of RM + 1 ml of 2 mM solution

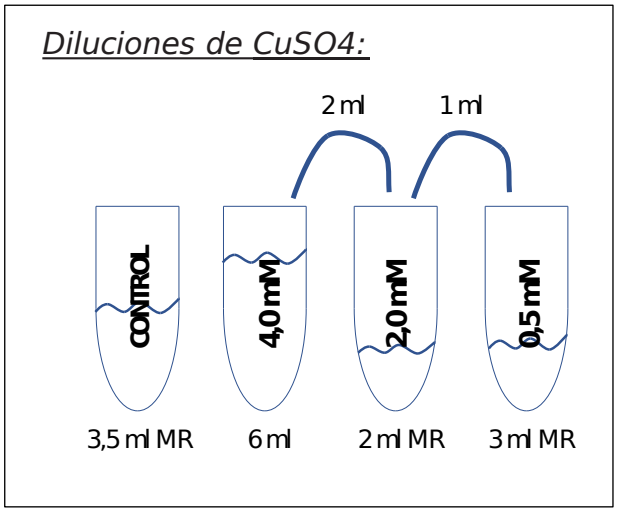
ZnSO4 dilutions:

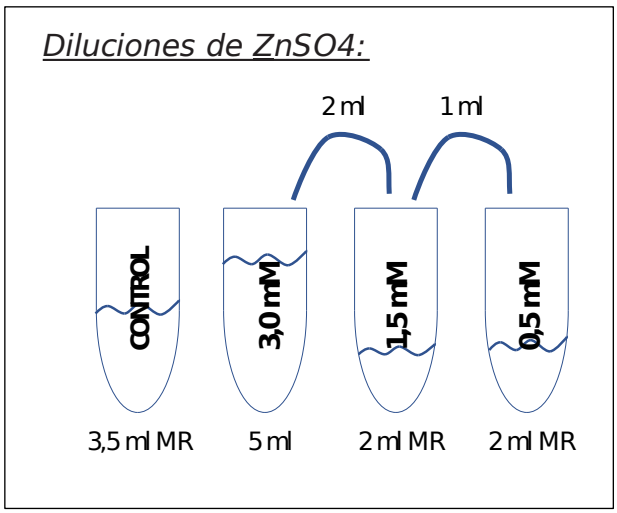
Control: Add 3 ml of Reaction Medium (RM)

3 mM: Shake the ZnSO4 solution

1.5 mM: Add 2 ml of RM + 2 ml of 3 mM solution

0.5 mM: Add 2 ml of RM + 1 ml of 1.5 mM solution



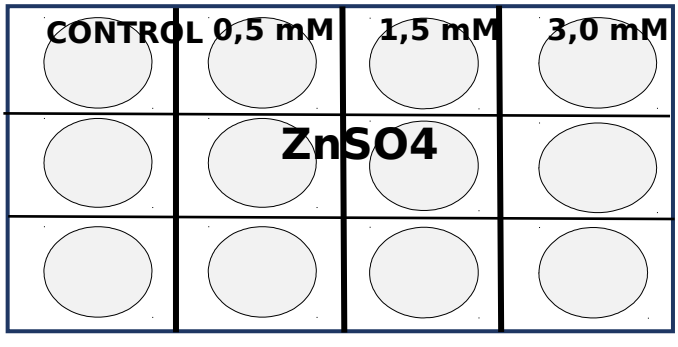


Schematically, the process to follow for the preparation of the serial dilutions of CuSO4 and ZnSO4 is the one above.

CELLULAR INCUBATION WITH THE TOXIC AGENT

The incubation of cells with the toxic agent's serial dilutions must be done when the cell culture's density reaches 80-85% confluence.

We have to **ENSURE** that the culture plates are well identified with the toxic agent to be used, the concentration used, and the corresponding triplicate. You can write these data on the lid of each plate, according to the scheme shown above. *Example of plate identification:*



REMEMBER that each tube in the serial dilutions contains enough volume to carry out the incubation of the toxic agent in three wells of cells (triplicate of the same toxic agent's concentration).

1. **ASPIRE** the medium contained in a well with a sterile pasteur pipette **being careful not to drag the cells adhered to the plate**, and gently **ADD** 1 ml of the tube that contains the toxic agent's concentration. **REPEAT** this process with the wells that are part of the triplicate.

2. **REPEAT** step 1 for each of the toxic agent's concentrations and the control as well.

3. **INCUBATE** the culture plates at 27ºC for 24 hours.

MODULE IV-B

DISCRIMINATION OF VIABLE CELLS REGARDING THE NONVIABLE ONES

To be able to distinguish the viable cells (alive) from the nonviable (dead) and obtain the number of each of them present in the cell culture we need to discriminate them.

We will use *trypan blue*, a dye that penetrates through the membrane of *nonviable cells* staining them with intense blue color. *Viable cells* do not incorporate the dye and therefore do not show the blue color.

Once we discriminate one type of cell from the other, **we will be able to establish the number of each of them present in the culture**.

Protocol of cellular viability through the trypan blue dye:

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

Before starting the practice, we must label the 12 eppendorf microtubes to identify the cells that we will deposit in them. For example, Control1, Control2, Control3, Cu0,5 1, Cu0,5 2, Cu0,5 3...

1. **TAKE** the 12-well plate after 24 hours of incubating the culture with the toxic agent and observe the cells under the microscope. **WRITE DOWN** in the practices notebook the observation's result and compare it with the results of the *CONTROL* triplicate. **IDENTIFY** the differences, if any.

2. With the help of the scraper, gently **SCRATCH** each of the wells of the cell culture plate making sure that we cover with the scraper the whole surface where the adhered cells are. *NOTE:* It is recommended to perform this task every 3 wells (in treatment groups), cleaning the scraper for each triplicate.

3. Softly **PIPET** the cells up and down with a pasteur pipette to disperse the culture.

4. **ADD** the cellular homogenate to each of the corresponding microtubes.

5. **CENTRIFUGE** the microtubes at 1000 rpm for 5 minutes.

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

7. **ADD** 500 µl of phosphate buffer (PBS 1x) to each of the two microcentrifuge tubes with a variable volume pipette and **gently** suspend the cells, **avoiding** the creation of foam.

8. **TRANSFER** 10 µl of trypan blue to a microcentrifuge tube. **ADD** 10 µl of the cell suspension and carefully pipette the volume up and down the repeatedly so that the cell suspension is homogeneous. We have diluted the volume twice. *Dilution factor = 2*.

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

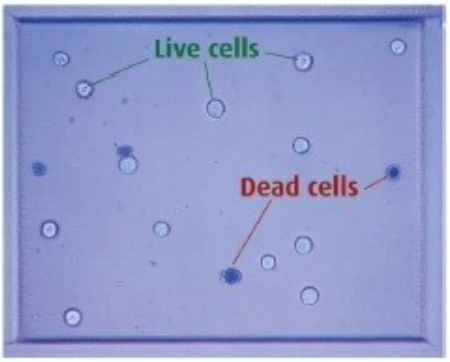
10. Slowly **TRANSFER** 10 - 12 µl (approximately one drop) of the trypan blue stained cell suspension to a hole placed in the middle of one of the ten counting chambers that each cell counting slide has. The chamber will be filled by capillary action.

11. **EXAMINE** the counting chamber in the microscope using the smallest objective.

12. **FOCUS** the lines on the grid square of the camera. Move the slide until the part you see is the outer grid. Use an objective with a higher augment if that helps to better observe the cell field to be counted.

13. **COUNT** all the living cells (not incorporating the dye and appearing bright and whitish in color) and all the dead (incorporating the dye and appearing blue in color) that are within each of the small grids. **RECORD** the number of viable cells (bright) and nonviable (dyed intense blue) in the cell culture data log.

Figure 10: Trypan blue staining of cells

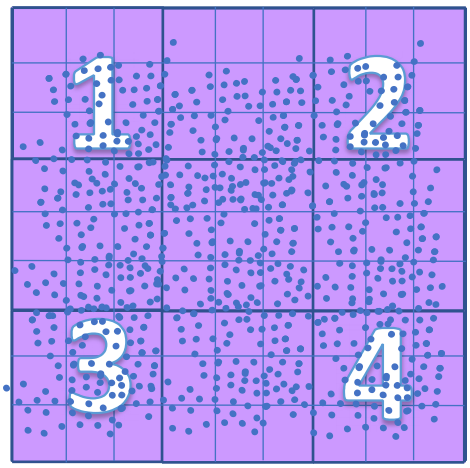


NOTE: This step can be done directly in the observation under the microscope or with a picture of the observation if the microscope is equipped with a photographic camera.

14. **STORE** the hemocytometer at room temperature until needed for the next cell count of the experiment.

NOTE: Do not try to clean the cells out of the used well as this could render the unused counting chambers unable to use.

NOTE: One of the options to obtain the **average number of cells** is to count the cells present in each of the four corner areas of 9 squares. Once the numbers of cells are obtained, they are summed up and the result is divided by four.



$$average\ number = \frac{n1+n2+n3+n4}{4}$$

NUMBER OF VIABLE AND NONVIABLE CELLS CALCULATION

Culture's cell viability is expressed as the relation between the number of viable cells and the total number of cells.

The formula for calculating the viable cells / ml and nonviable / ml is as follows:

average number of viable cells (or nonviable) / ml =

average number of viable cells (or nonviable) x multiplication factor x dilution factor

Multiplication factor = 4500

Dilution factor = 2 (in this case, we have diluted the cells in a volume twice the initial, 10 µl trypan blue + 10 µl, before depositing them in the counting chamber)

Example: 10 µl of cells are diluted in 10 µl of trypan blue dye and the average number of cells is 75

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

To **compute** the number of viable cells/ml = 75 x 4500 x 2 = 0.65 x 10^6 cells/ml

Note: It also has to be done with the nonviable cells we counted.

CELLULAR VIABILITY CALCULATION. VIABLE CELLS PERCENTAGE

Cell viability can be determined by calculating the percentage of viable cells present in the culture regarding the total number of cells in the culture (viable + nonviable).

The formula to apply is the following:

Viability = number of viable cells/total number of cells (viable + NONviable) x 100

Example: From the cells we counted, 75 are bright (viable) and 15 intense blue (nonviable).

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

NOTE:

Once the cell counting of a group's triplicate is finished, **initiate** the protocol scratching another triplicate, and **perform** the same procedure for the triplicate of the next group of experimental conditions to which the cells with the toxic agent were exposed.

Order the results in a table and represent them in a graph.

Record the discussion of results in the practices notebook, indicating the concentration that causes 50% of cell mortality, the one causing 100% cell death, and the minimum concentration at which the toxic agent starts to produce cell death.

Compare the results of the different toxic agents used.

ANNEX 1: PRACTICAL'S QUESTIONS

1. Why is it recommended to subculture the cells when reaching a 80-90% confluence?

2. Describe the common symptoms of the bacteria contamination.

3. Why are serial dilutions of a supposed toxic agent used in the incubation of cell cultures?

4. Can the toxicity caused by a toxic agent in a certain cell culture (e.g. insect cells) be extrapolated to another culture of different cells (e.g. mouse fibroblast cells)? Reason your answer.

5. Is it interesting to know the value of a determined toxic agent's LD50? Why?

6. Why the present cellular medium was changed by the reaction medium in the control triplicate? Reason your answer.

ANNEX 2: PRACTICAL'S RESULTS AND ANALYSIS

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

The differences in the toxicity curves are sensitive to the variations in the used concentrations due to errors in the pipetting and the confluence level of the cell culture when incubated with the toxic agent.

The small differences obtained in the serial dilutions of the toxic agents due to mistakes in the pipetting can be translated in the experimental observation to a higher or lower produced toxicity. **We must bear in mind** that cellular death is sensitive to small changes in the concentration of toxic agents.

ANNEX 3: PRACTICE PREPARATION PERFORMED BY THE TEACHERS

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

a) **When receiving the practice kit**

Check that the received material is the indicated in the shipping sheet.

Cultivate the tubes that contain the cells in the green-lid culture flasks of 25 cm^2, one vial per tube. The cellular content of the two flasks at 85% confluence is enough so that four student groups execute the practice (4 12-well plates).

b) **Before the start of the practices**
Disinfect the work area with 70% ethanol (*preferably, work in a flow cabinet*).
Prepare and distribute the material needed for the development of each practice before its start.
Disinfect the material with 70% ethanol prior to incorporating it to the work area.
Insist on the need that all the material that is going to be used is disinfected to avoid contamination.
Sterile cell scrapers must be washed and saved for successive uses that do not require sterility because the culture is not kept (and is discarded) (Module IV).

c) **Duraning the practices**
Remember that the students *have to observe the status of the cells, before and after the incubation with the toxic agent.*

Organization and implementation prior to the practical

The instructions presented in this protocol are suitable for performing the practices in six student groups.

Before the start of each practice, carefully check the Necessary material list that appears at the head of each module. Make sure to have all the necessary components and equipment.

> ***IMPORTANT:*** ***Cells must be cultivated immediately after receiving them.*** ***See the section Start of the culture in this annex.***

Precautions

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

Equipment and materials sterilization

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 neutralized* prior to its disposal, including culture plates and flasks, culture media, pipettes, transfer pipettes, and tubes.

Liquids:

Whether we want to eliminate the culture medium we have discarded and 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 remaining liquid spilling into the sterilizer chamber.

Autoclave at 121ºC for 20 minutes.

Approximate time guidance for practice procedures

The practice is divided into four modules and should last approximately from one to 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	Prior preparations	Practice
I	15 min	15-30 min
II	10-15 min	30-45 min
III	15 min	45-60 min
IV	30 min	1. Toxic agents' incubation: 45 min 2. Cellular viability: 70-90 min

Table/summary of prior preparations

Preparation for:	What to do:	When?	Required time
Start the cell culture	Transfer the received cells to the culture flasks	Immediately after receiving the cells	15 min
Module I: Observation and evaluation of cell culture	Prepare the compound microscopes	Anytime before the practice	15 min
Module II: Experimental design of toxicity in insect cells caused by environmental agents	Provide information	At the start of the practical	5 min
Module III: Cell culture manipulation. Reseeding	Prepare and distribute the material	One hour before executing the practical	15-20 min
Module IV: Study of toxin exposition in insect cell cultures. Cellular viability	Aliquot the trypan blue, do the serial dilutions, and distribute the material	Before executing the practical	20-30 min

We recommend preparing the correspondent equipment and reagents for Modules I, III y IV before initiating the practice with the students. Have a microscope ready for cellular observation and analysis throughout all 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.**

INICIO DEL CULTIVO DE LAS CÉLULAS DE INSECTOS

Se detallan las preparaciones a realizar lo antes posible una vez recibido el kit:

a) Preparation of 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 classroom (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 cell culture medium (Module III) and the reaction medium (Module IV).

1. Aseptically **ALIQUOT 15 ml** of **Cell culture medium** in four 15 ml tubes, previously identified according to the student groups. Keep the remaining medium to initiate the insect cell culture.

2**.** Aseptically **ALIQUOT 14 ml** of **Reaction medium** in four 15 ml tubes, previously identified according to the student groups.

3. **ALIQUOT** 7 ml of phosphate buffer in 4 15 ml tubes.

4. **LABEL** each tube as **Insect cell culture medium**, **Reaction medium**, or **phosphate buffer**, as appropriate.

5. **STORE** at 4°C until the students need them in Module III and Module IV.

c) Start of the culture of the received cells

Sf9 insect cells are sent in a vial and should be transferred to the 25cm^2 culture flasks, as soon as they are received.

1. **TEMPER** the culture of insect cells to room temperature.

2. **ADD 1 ml** of insect cell culture to each culture flask, keeping it in a vertical position.

3. Softly **INVERT** the cells tube to mix the contents.

4. Utilizando una pipeta de transferencia estéril o una punta de micropipeta estéril, **TRANSFER** all the volume of Sf9 insect cells to the (sterile) cell culture flask.

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

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

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

7. **ALLOW** the cells grow for 24-72 hours, checking their health and confluence every day. It is recommended that, if after 72 hours the confluence is not yet 80-90%, you perform a change of the culture medium, removing with a pasteur pipette the culture medium contained in the culture flask, always leaving a remnant, and adding 4 ml of a new medium of insect cells culture preheated at 27ºC or tempered at the same lab.

Cells should achieve at least 85% confluence before initiating the next experiments.

MODULE I PREPARATION: OBSERVATION AND EVALUATION 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.

Each group needs:

• Gloves	• Face mask
• Lab coat	• 70% ethanol

MODULE II PREPARATION: EXPERIMENTAL DESIGN OF TOXICITY IN INSECT CELLS CAUSED BY ENVIRONMENTAL AGENTS

1. **READ** the protocol in the Module II section.

2. **DESIGN** an alternative experiment.

Each group needs:

• Lab coat

MODULE III: CELL CULTURE MANIPULATION. RESEEDING

1. **TAKE OUT** from the refrigerator the aliquots of the insect cell culture medium and let them temper to room temperature.

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

Each group needs:

● Gloves	● Face mask
● Lab coat	● 70% ethanol
● Beaker for waste disposal	● Variable volume pipettes
● Culture medium	● Scrapers
● 10 ml pipettes (sterile)	● Neubauer chamber
● Sterile pasteur pipettes	● Pipette tips (sterile)
● 12-well plates	

MODULE IV PREPARATION: STUDY OF TOXIN EXPOSITION IN INSECT CELL CULTURES. CELLULAR VIABILITY

1. ALIQUOT 250 µl of **trypan blue (D)** in microcentrifuge tubes for the four groups.

2. PERFORM the serial dilutions with the toxic agent.

> **NOTE:** The counting chamber and the trypan blue aliquot that each group will receive should be stored for their use in later counting assays.

Each group needs:

● Gloves	● Face mask
● Lab coat	● 70% ethanol
● Beaker for waste disposal	● Phosphate buffer (PBS 1x)
● Reaction medium	● Scrapers
● Variable volume pipettes	● Pipette tips (sterile)
● 15 ml tubes (sterile)	● Toxic agents ($CuSO_4$ and $ZnSO_4$)
● 10 ml pipettes (sterile)	● Neubauer chamber
● Trypan blue	● Microcentrifuge tubes
● Sterile pasteur pipettes	

Data recording for cellular viability

GROUP
STUDENTS NAMES
DATE

RESULTS OF CELLULAR VIABILITY IN SF9 INSECT CELL CULTURES

TOXIC AGENT: ZnSO4	CONTROL			ZnSO4 (0.5 mM)			ZnSO4 (1.5 mM)			ZnSO4 (3 mM)		
	1	2	3	1	2	3	1	2	3	1	2	3
NUMBER OF VIABLE CELLS												
NUMBER OF NONVIABLE CELLS												
TOTAL NUMBER OF CELLS												

	AVERAGE VALUE							
	CONTROL		ZnSO4 0.5 Mm		ZnSO4 1.5 Mm		ZnSO4 3 Mm	
	Value	±SD	Value	±SD	Value	±SD	Value	±SD
NUMBER OF VIABLE CELLS								
NUMBER OF NONVIABLE CELLS								
TOTAL NUMBER OF CELLS								

SD Standard Deviation

	% OF CELLULAR VIABILITY			
	CONTROL	ZnSO4 0.5 Mm	ZnSO4 1.5 Mm	ZnSO4 3 Mm
AVERAGE VALUE				
STANDARD DEVIATION				

LD50:

GROUP
STUDENTS NAMES
DATE

RESULTS OF CELLULAR VIABILITY IN SF9 INSECT CELL CULTURES

TOXIC AGENT: CuSO4	CONTROL			CuSO4 (0.5 mM)			CuSO4 (2 mM)			CuSO4 (4 mM)		
	1	2	3	1	2	3	1	2	3	1	2	3
NUMBER OF VIABLE CELLS												
NUMBER OF NONVIABLE CELLS												
TOTAL NUMBER OF CELLS												

	AVERAGE VALUE							
	CONTROL		CuSO4 0.5 Mm		CuSO4 2 Mm		CuSO4 4 Mm	
	Value	±SD	Value	±SD	Value	±SD	Value	±SD
NUMBER OF VIABLE CELLS								
NUMBER OF NONVIABLE CELLS								
TOTAL NUMBER OF CELLS								

SD Standard Deviation

	% CELLULAR VIABILITY			
	CONTROL	CuSO4 0.5 Mm	CuSO4 2 Mm	CuSO4 4 Mm
AVERAGE VALUE				
STANDARD DEVIATION				

LD50: