6 student groups

DETECTION OF CYTOSKELETON FIBERS THROUGH FLUORESCENCE

1. OBJECTIVE OF PRACTICE

The objective of these practices is to offer the students an experimental tool for the detection of cytoskeleton fibers through the use of fluorescence.

2. MATERIAL AND REAGENTS INCLUDED IN THE KIT

Components	Ship	Conservation
Insect cells (Sf9).	1 vial	**R.T.**
Culture medium of insect cells	16 ml	**4 ºC**
Fixation medium	6 vials	**4 ºC**
Permeability medium	6 vials	**R.T.**
Cell culture flask (sterile, 25 cm^2)	1 unit	
Treated coverslips (sterile)	6 units	
35 mm cell culture plates (sterile)	6 units	
Phosphate buffer	18 ml	**4 ºC**
Glass slides	6 units	
10 ml pipettes (sterile)	4 units	
Pasteur pipettes	42 units	
Scrapers (sterile)	1 unit	
Cell counting chambers	1 unit	
TR-phalloidin	1 unit	**4 ºC**
Mounting medium	1 unit	
15 ml tubes	1 unit	
50 ml tubes	1 unit	

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**, **Fixation Medium**, **phosphate buffer** and the **vial containing the TR-phalloidin** should be stored in a refrigerator (4ºC) immediately after its reception.

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 and isopropanol bottles.
- 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).
- Fluorescence microscope with filter for TRITC.
- 10-1000 µl micropipette and tips.
- Marker pens.
- Safety glasses, mask (optional), disposable lab gloves and lab coats.
- Waste containers (beakers).
- Fine-tipped tweezers -sterile-.
- Sleeved needles -sterile-.

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.

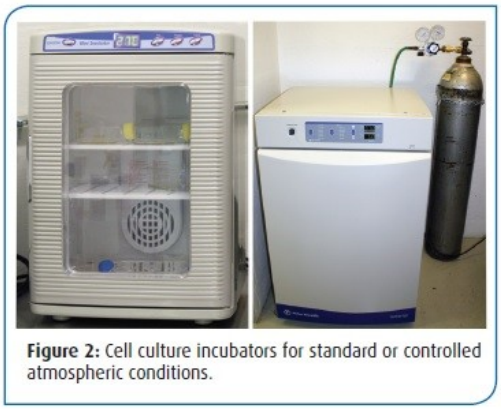


Figure 2: Cell culture incubators for standard or controlled atmospheric conditions.

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.

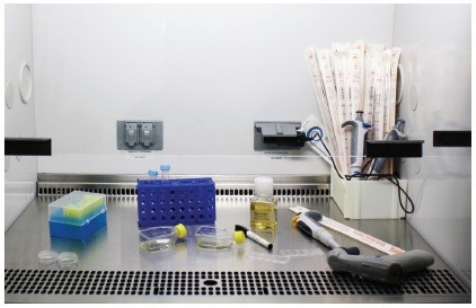


Figure 3: Cell culture hood and standard equipment.

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 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.

Cytoskeleton

The cytoskeleton is composed of different types of filamentous proteins. One of these is **actin**, a cytosolic protein that represents close to 10% of the proteins present in the cellular cytosol. There are two types of actin protein, the so-called G -globular and depolymerized- actin and, when this one is polymerized, F or filamentous actin, involved in diverse cellular processes, being the one responsible for constituting the cell shape.

The cytoskeleton is a dynamic structure and this dynamism is what allows cellular movement, cellular signaling, cellular division, endocytosis, phagocytosis, communication between cellular organelles, etc. The polymerization and depolymerization processes of actin appear in all the aforementioned cellular processes. This means that filamentous actin is being created continuously and is depolymerized to the globular shape of actin. The actin filaments are either shortened or lengthened through these dynamic processes of polymerization/depolymerization.

Detection of filamentous actin (actin fibers or stress fibers) is carried out through the use of a toxin present in the *Amanita phalloides* fungus.

Isolated for the first time in the 1930s, phalloidin is a phallotoxin produced by this fungus. Its intoxication produces the so-called acute hunger, due to the destruction it causes to the liver cells. The intoxication mechanism of phalloidin resides in its union in a specific way with the actin filaments in the cytoskeleton. This characteristic is useful for the detection of actin fibers.

There are phalloidin derivatives that are conjugated with chromophores, substances prone to absorb and emit light in a determined range of wavelength. These phalloidins are used in the detection and study of the cytoskeleton through the use of fluorescence microscopes.

4. PRACTICE TO CARRY OUT WITH THE KIT:

Practices will be divided in 3 modules:

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

  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:** *Seed of Sf9 cells in coverslips*

  In this module, students will learn to manage a cell culture, do a reseed in coverslips that allows cell culture's growth, and take the culture to optimal conditions to execute immunocytology tests.

- **Module III:** *Detection of actin fibers through fluorescence*

  In this section, students will learn the basic technique to perform detections through fluorescence, using the microscope.

THE 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.

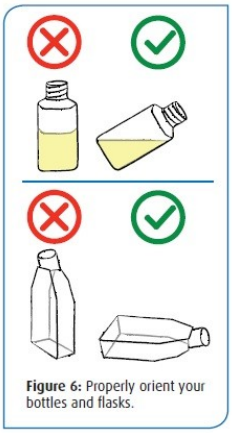


Figure 6: Properly orient your bottles and flasks.

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 state

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).

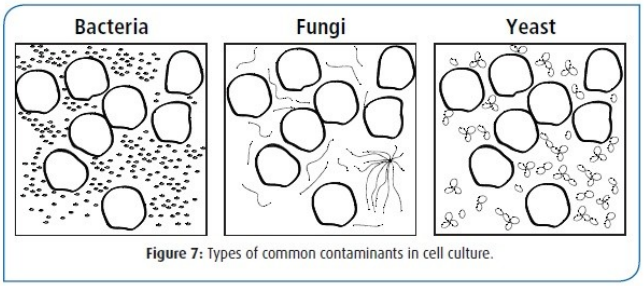


Figure 7: Types of common contaminants in cell culture.

• 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.

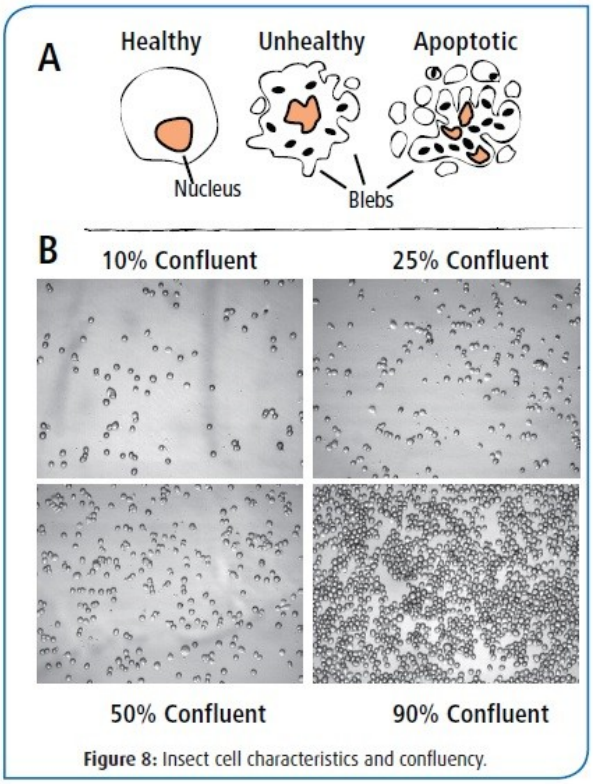


Figure 8: Insect cell characteristics and confluency.

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: Seed of Sf9 cells in coverslips

MATERIAL NECESSARY FOR MODULE II-1

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	• Coverslips pretreated for the Sf9 cells' adhesion (sterile)
• Complete culture medium	• Plates scrapers
• Variable volume pipettes	• Pipettes tips (sterile)
• Neubauer chamber	• 15 ml tubes (sterile)
• 35 mm cell culture plates	• 10 ml pipettes (sterile)
• Fine-tipped tweezers (sterile)	• 50 ml tubes (sterile)

Once we observe the cell culture and see that there is not cellular contamination, we take the cell flask (25 cm2, green cap) and proceed to reseed them in sterile pretreated coverslips, contained in 35 mm plates.

Protocol:

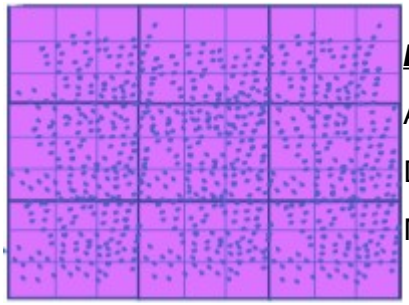
1. **Lift** the cells attached to the culture flask with a sterile scraper.

2. **Resuspend** the cells with a sterile 10 ml pipette and add them to a sterile 15 ml tube.

3. **Take** around 12 µl of cell homogenate and deposit them in one of the 10 Neubauer chambers.

4. With the use of the microscope, **count** the cells that are in each of the areas in the chamber's corners (in each of the 4 corners of the chamber there are 9 small squares).

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



Example:

Average number of cells = 110

Dilution factor = 1

Multiplication factor = 4500

(495000 cells) / ml

6. **Adjust the volume of the homogenate** by adding culture medium til reaching the necessary density, attending to the following information:

We have 6 35 mm plates between the 6 groups, meaning that we **need**:

0.25 millions of cells/plate x 6 plates = **1.5 million of cells**

3 ml of culture medium/plate x 6 plates = a total of **18 ml of culture medium**.

We will use a 50 ml tube in the preparation of the cellular homogenate. The tube will contain a final volume of homogenate of 18 milliliters of culture medium with a total of 1.5 million of cells, **needed to execute the practices**.

IMPORTANT: In our example, if we have **in a tube** a concentration of 0.495 M cells / ml and we need a total of 1.5 million of cells, we have to take, approximately, 3 ml of the cellular homogenate.

With the help of a sterile 10 ml pipette, we will put 3 ml of the cellular homogenate in a 50 ml tube and add 15 ml of the culture medium, with another sterile 10 ml, to obtain the 18 ml of the cellular homogenate that we need for the 6 plates that contain the pretreated coverslips.

$$Volume\ of\ the\ medium\ that\ we\ will\ add = 18\,ml\,(Vtotal) - 3\,ml\,(Vhomogenate)$$

$$Volume\ of\ the\ medium\ that\ we\ will\ add = 15\,ml\ of\ complete\ medium$$

Once we have the 18 ml that include 1.5 M of cells, we softly homogenate them with the help of a sterile 10 ml pipette, deposit 3 ml in each of the culture plates and put them in the incubator.

TAKE INTO ACCOUNT that, to use the data from the example, IT IS NECESSARY to adjust the initial cell concentration to 0.5 M / ml, approximately.

7. With the help of sterile tweezers, **place** a sterile pretreated coverslip for the Sf9 cells culture in a sterile 35 mm plate.

8. **Seed** the 35 mm culture plates, in a sterile environment / under the cabinet, adding 3 ml of the cellular homogenate in each of the plates. **Carry out** this step softly, adding drop by drop in the coverslip, and let the homogenate expand in the plate. The cellular density is 0.25 millions of cells/plate.

9. **Incubate** the cells at 27ºC til reaching a 75-80% confluence.

Module III: Detection of the actin fibers through fluorescence.

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
• Phosphate buffer	• Fixation medium
• Variable volume pipettes	• Pipette tips (sterile)
• Permeabilization medium	• TR-phalloidin
• Petri plates	• Parafilm
• Slide	• Mounting medium
• Tweezers	

Proceeding to obtain the cellular homogenate, we have to observe each one of the cell culture plates in the microscope and take notes about their state in our lab notebook.

This section of the practice can be done in the lab table since it is not necessary to keep the sterility conditions.

Protocol:

1. **Place** the Parafilm inside the 10 cm Petri plate. For this, cut the Parafilm long enough so that the coverslip where we have the cells does not stand out.

2. Softly **pick up**, very carefully and with the use of fine-tipped tweezers, the coverslip. **Place** it over the Parafilm we put in the 10 cm Petri plate, very carefully. **The side of the coverslip in which the cells have adhered SHOULD NOT be placed facing the Parafilm.**

3. With the help of a pasteur pipette settled in a vertex of the coverslip, **aspire** the culture medium and discard it.

4. **Add 200 microliters**, approximately, **of phosphate buffer** -until the coverslip is covered- and leave it there for 5 minutes.

5. **Perform this procedure of medium aspiration and phosphate buffer adhesion for 5 minutes, *a total of 3 times*.**

6. After having aspirated the phosphate buffer for the last time, **ADD 200 microliters**, approximately, **of fixation medium covering all the cells present in the coverslip. Leave it there for 10 minutes.**

7. **Aspire** the fixation medium and discard it.

8. **Add 200 microliters**, approximately, **of phosphate buffer** -until the coverslip is covered- and leave it there for 5 minutes.

9. **Perform this procedure of medium aspiration and phosphate buffer adhesion during 5 minutes, *a total of 3 times*.**

10. After having aspirated the phosphate buffer for the last time, **ADD 200 microliters**, approximately, **of permeabilization medium covering all the cells present in the coverslip. Leave it there for 5 minutes.**

11. **Aspire** the permeabilization medium and discard it.

12. **Add 200 microliters**, approximately, **of phosphate buffer** -until the coverslip is covered- and leave it there for 5 minutes.

13. **Perform this procedure of medium aspiration and phosphate buffer adhesion during 5 minutes, *a total of 3 times*.**

14. During the last incubation with the phosphate buffer, take out of the fridge the microtube containing the phalloidin and centrifuge it a little bit. Next, **add** 200 microliters of phosphate buffer inside the eppendorf microtube. Softly **resuspend** the phalloidin, with the help of the micropipette.

15. **Aspire** the phosphate buffer and carefully **ADD** the 200 microliters of the solution containing the phalloidin, drop by drop. **Leave** it for 10 minutes.

16. **Aspire** the medium containing the phalloidin and discard it.

17. **Add 200 microliters**, approximately, **of phosphate buffer** -until the coverslip is covered- and leave it there for 5 minutes.

18. **Perform this procedure of medium aspiration and phosphate buffer adhesion during 5 minutes, *a total of 3 times*.**

19. **Add** 1-2 drops of mounting medium to the slide. **Take** the coverslip with the tweezers and carefully put one of the corners over the filter paper so that there is no buffer left in the coverslip. **Softly deposit** the coverslip over the slide, in a way that the cells are in contact with the mounting medium.

NOTE: The slide should be clean from impurities. Wash it with dishwasher and clear it up with tap water. Add 70% ethanol and then distilled water. Lastly, decant the water excess and clean the humidity of the slide with paper.

20. Observation with the fluorescence microscope with the filter for TRITC.

21. **Write** the results **down** in the practice notebook -supported with pictures-.

ANNEX 1: PRACTICE 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. What is the Parafilm that we placed in the Petri plate for?
4. Why do we use a cellular fixation solution?
5. Why do we use a permeabilizer solution?
6. Why do we use TR-phalloidin?

ANNEX 2: PRACTICE RESULTS AND ANALYSIS

The fluorescence microscope observation requires the elimination of strange bodies to which the chromophore we are using can be united, whose result would be an unspecific fluorescence and would mask the observation of the specific target for which we use the marker.

The diffuse observation in the fluorescence microscope can be due to not having respected the protocol of washing with the phosphate buffer, even though not having incubated the cells with the fixation or the permeabilization medium could have as a consequence the visualization of an unspecific fluorescence.

It is important that the fat and impurity have been eliminated from the slide, as they could mask the sign of specific fluorescence.

An effect that could cause the marking with fluorescence not being observed could be due to the lack of cells -because of having incubated the chromophore on the side of the coverslip where there are no cells- or because of having diluted the phalloidin excessively. IT IS IMPORTANT to respect the protocol.

ANNEX 3: PRACTICE PREPARATION CARRIED OUT 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 tube containing the cells in a 25 cm^2 culture flask (with a green lid).

The cellular content in a flask with 80-90% confluence is enough for the six student groups to execute the practice.

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.

c) **During the practices**

Remember that students *have to observe the state of the cells, before and after incubation, as a step prior to executing the protocol of actin detection.*

Organization and implementation prior to the practice

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**.

Students must be careful with the use of fixation medium, permeabilization medium, and phalloidin. They always must use gloves to avoid direct contact with these media.

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 disinfected 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 121 ºC for 20 minutes.

Approximate Time Guidance for Practice Procedures

The practice is divided into three 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 min
III	15 min	90-120 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: Sf9 cells seed in coverslip	Prepare and distribute the materials	At the start of the practice	5 min
Module III: Detection of actin fibers through fluorescence	Prepare and distribute the materials. Have the fluorescence microscope ready	Thirty minutes before executing the practice	15-20 min

We recommend preparing the correspondent equipment and reagents before initiating the practice with the students. Have a microscope ready for cellular observation and analysis throughout all Modules. Have the fluorescence microscope at the end of Module III.

> **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.**

START 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 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) Start of the culture of the received cells

The Sf9 insect cells are sent in a vial that should be transferred in a 25 cm^2 flask as soon as they are received.

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

2. **ADD** with a sterile 10 ml pipette a volume of **1 ml** of insect cells culture to the 25 cm2 culture flask, keeping it in a vertical position.

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

4. Using a sterile transfer pipette or a tip of a sterile micropipette, **TRANSFER** the volume of the vial of the Sf9 insect cells to the (sterile) cell culture flask to which we have already added 1 ml of culture medium.

> **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 STATE

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: SEED OF SF9 CELLS IN COVERSLIPS

1. **TAKE OUT** from the refrigerator the insect cells culture medium and allow it to temper to room temperature.

2. **DISTRIBUTE** the material necessary for the execution of this module.

Each group needs:

● Gloves	● Face mask
● Lab coat	● 70% ethanol
● Beaker for waste disposal	● Sterile pasteur pipettes
● Complete culture medium	● Plate scrapers
● Variable volume pipettes	● Pipette’s tips (sterile)
● Neubauer chamber	● 15 ml tube (sterile)
● 35 mm culture plates	● 10 ml pipettes (sterile)
● 50 ml tube (sterile)	● Coverslips (sterile)
● Tweezers (sterile)	

MODULE III PREPARATION: DETECTION OF ACTIN FIBERS THROUGH FLUORESCENCE

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

Each groups needs:

• Gloves	• Face mask
• Lab coat	• 70% ethanol
• Beaker for waste disposal	• Sterile pasteur pipettes
• Fixation medium	• Permeabilization medium
• Variable volume pipettes	• Pipette’s tips
• Phosphate buffer	• TR-phalloidin
• Mounting medium	• Tweezers
• Petri plates	• Parafilm

Data recording

GROUP
STUDENTS NAMES
DATE