6 groups of students

DETERMINATION OF APOPTOSIS BY DNA DETECTION

1. PRACTICE'S OBJECTIVE

The objective of these practices is to offer the students an alternative experimental tool in the identification of the process of cellular death by apoptosis.

2. MATERIALS AND REAGENTS INCLUDED IN THE KIT

Components	Ship	Conservation
Insect cells (Sf9)	4 vials	**R.T.**
Insect cells culture medium	80 ml	**4 ºC**
Reaction medium	60 ml	**4 ºC**
Lysis buffer	11 ml	**R.T.**
Cell culture flask (sterile, 75 cm^2)	2 units	
50 ml tube (sterile)	2units	
Ethanol solution in reaction medium	9 ml	**4 ºC**
Actinomycin solution (reaction medium)	2 ml	**4 ºC**
60 mm cell culture plates (sterile)	18 units	
10 ml pipette (sterile)	11 units	
Pasteur pipette (sterile)	42 units	
Scrapers (sterile)	8 units	
Cell counting chamber	1 unit	
15 ml conical bottom tube (sterile)	26 units	
5 ml conical bottom tube (sterile)	18 units	
1,5 ml centrifuge tube	18 units	

This practical 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 receiving them (see annex 3 of this protocol).
>
> **NOTE: Insect Cell Culture Medium and Reaction Medium** 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)
- Bath, centrifuges, and electrophoresis cells
- Agarose
- 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

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.

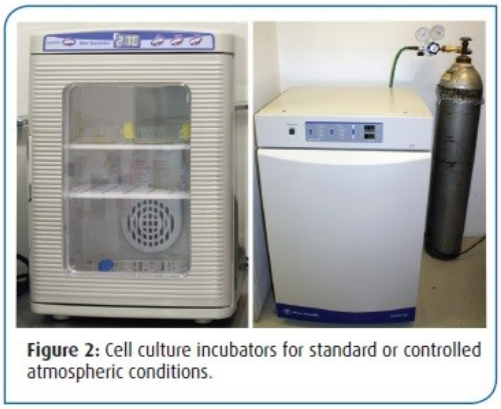


Figure 2: Cell culture incubators for standard or controlled atmospheric conditions.

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.

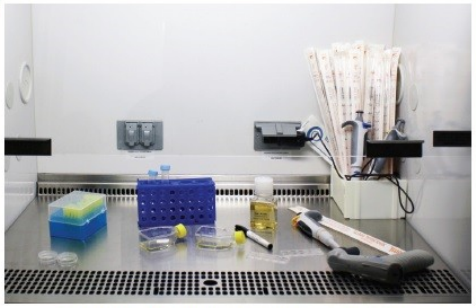


Figure 3: Cell culture hood and standard equipment.

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.

Apoptosis

The term apoptosis was introduced in 1972 by Kerr, Wyllie, and Currie to describe a mechanism of programmed cell death in which cells are auto-destroyed without the inflammation processes being activated as a response to cell death.

Apoptosis is considered a process of physiological natural death that takes place in the stages of non-desired cells elimination. During the embryonic development, an excess of cells is produced, whose amount is being reduced in the different stages of formation and maturation of the embryo. The decrease in this number of cells is produced through the activation of a cellular signaling mechanism.

Death by apoptosis is evident in physiologic processes such as the bones and cartilage remodeling, the fingers morphogenesis in the elimination of the interdigital areas, the elimination of neuron excess observed in the development of the nervous system, the organogenesis -e.g., livers formation-...

Apoptosis is characterized by an ordered set of changes that start with a condensation of the nuclear chromatin followed by nuclear fragmentation, emergence of cytoplasmic protrusions in the cellular surface, and, finally, cellular degradation, generating multiple apoptotic bodies wrapped in cytoplasmic membrane. These apoptotic bodies will be phagocytosed by specialized cells (macrophages) or, in some cases, neighboring cells.

One of the main biochemical elements in the apoptotic process is the initial DNA degradation in large fragments of 50 to 300 kilopairs of bases that later originate the internucleosomal fragments, in which the endonuclease interferes, creating 200 base pairs fragments.

These fragments' separation by agarose gel electrophoresis reveals the apoptosis' characteristic pattern being observed that the DNA appears in the gel as a scaled gradient. This pattern contrasts with the observed in the apoptotic death, since the DNA appears in the gel as a diffuse stain, due to the complete DNA degradation without the internucleosomal fragments typical of apoptosis being appreciated.

Similarly, there exist different experimental approximations that are used to show up the apoptotic death, among which we find:

The use of fluorochromes that are inserted in the DNA and allow to visualize the chromatin morphology. The most used are the ethyl bromide or the Hoechst, which let us quantify the apoptotic cells in a culture.

The TUNEL staining, based on the ability to join a dUTP digoxigenin that the DNA ends, hydrolyzed by the endonuclease, present and the posterior application of immunological techniques, allowing their identification.

Phosphatidylserine labeling, in the extracellular side of the cytoplasmic membrane, by the annexin V protein.

In this apoptosis detection kit, you will obtain the DNA of cells treated with an apoptotic agent that can be detected in the agarose gel electrophoresis.

4. PRACTICAL TO CARRY OUT WITH THE KIT:

Practices will be divided into 3 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:** *Apoptosis induction in Sf9 cells*

  In this module, students will learn to manage a cell culture, reseed it, and bring it to the optimal conditions for stimulating it with an apoptotic agent.

- **Module III:** *Obtainment of the cellular homogenate. DNA extraction and detection*

  In this section, students will manipulate the insect cells culture and obtain the homogenates of apoptotic cells in which there will be the DNA. Students will learn to do DNA extraction and visualize it by agarose gel electrophoresis.

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.

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

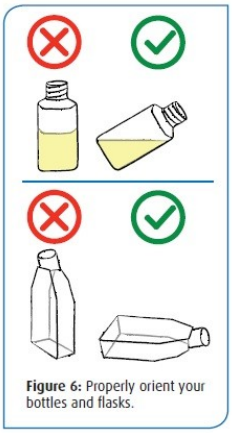


Figure 6: Properly orient your bottles and flasks.

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

E. **Use of incubation chambers**

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

The advantage of working with Sf9 insect cells is that they can be grown at room temperature and do not require a complicated growth environment. If an incubator exists in the laboratory, it can be set to maintain a temperature of 27°C.

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

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

We have to take into account that:

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

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

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

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).

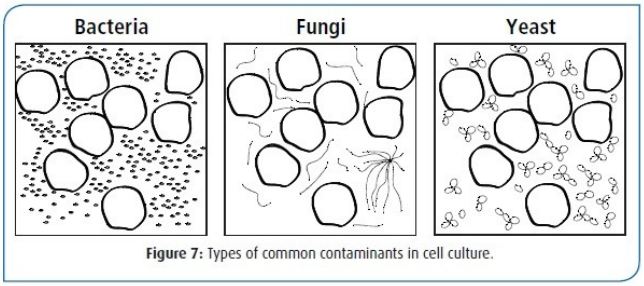


Figure 7: Types of common contaminants in cell culture.

• 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.

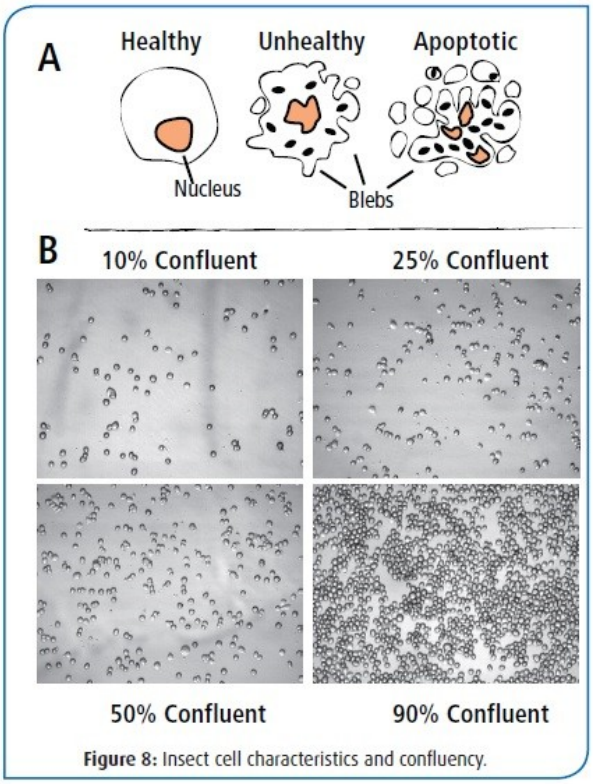


Figure 8: Insect cell characteristics and confluency.

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: Apoptosis induction in Sf9 cells

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	• Sterile pasteur pipettes
• Complete culture medium	• Scrapers
• Variable volume pipettes	• Pipettes tips (sterile)
• Neubauer chamber	• 15 ml tubes (sterile)
• 60 mm culture plates	• 10 ml pipettes (sterile)
• 50 ml tubes (sterile)	

MODULE II-1

Once we observe the cell culture and see that there is no cellular contamination, we take each one of the 2 cells flasks (75 cm^2, green cap) and proceed to reseed the cells in 60 mm plates.

*Remember that the contents of each cell culture flask have enough cells for **three groups and each group will have three 60 mm plates to do the cell subculture and proceed to the practice.***

Protocol:

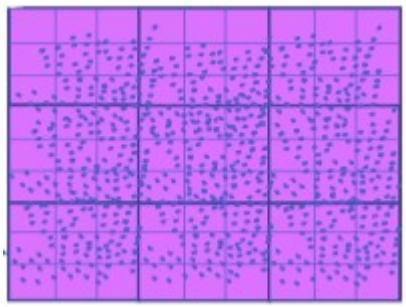
1. **Lift** the cells attached to the culture flask with a sterile scraper.

2. **Suspend** 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 = 275

Dilution factor = 1

Multiplication factor = 4500

(1.23 Million cells)/ml

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

Each tube of cells will be used for three groups:

We have 9 60 mm plates between the three groups, meaning that we **need**:

1.2 million cells x 9 plates = **10.8 million cells**

3 ml of culture medium x 9 plates = a total of **27 ml of culture medium**.

We will use two 50 ml tubes in the preparation of the cellular homogenate. Each one will contain a final volume of homogenate corresponding to *27 milliliters of culture medium with a total of 10.8 million cells,* **needed for 3 groups**.

IMPORTANT: In our example, if we have **in a tube** a concentration of 1.23M cells / ml and we need a total of 10.8 million cells (**needed for three groups**), we should take, approximately, 8.8 ml from the cellular homogenate.

With the help of a sterile 10 ml pipette, we will put 8.8 ml of the cellular homogenate in a 50 ml tube and add 18.2 ml of the culture medium, with another sterile 10 ml pipette, to obtain the 27 ml of cellular homogenate that we need for the 9 plates corresponding to the 3 groups.

Repeat *the process with the other flask of cell culture.*

$$Volume\ of\ the\ medium\ that\ we\ will\ add = 27\ ml\ (Vtotal) - 8.8\ ml\ (Vhomogenate)$$

$$Volume\ of\ the\ medium\ that\ we\ will\ add = 18.8\ ml\ of\ complete\ medium$$

Once we have the 27 ml that include 10.8 M of cells, we softly homogenate them with the help of a sterile 10 ml pipette and deposit 9 ml in a 15 ml tube for each of the student groups. We will add 3 ml with a sterile pasteur pipette in each of the three plates that every group has, we softly shake them to distribute the cells, and deposit 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 1.23 M / ml.

7. **Distribute** the cellular homogenate, 9 ml in each of the 15 ml tubes.

8. **Seed** the 60 mm culture plates under the cabinet by adding 3 ml of the cellular homogenate in each of the plates, with the help of a sterile pasteur pipette. *The cellular density is 1.2 million cells/plate.*

9. **Incubate** the cells at 27°C until reaching a 75-80% confluence.

10. **Carry on** with module II-2.

MATERIAL NECESSARY FOR MODULE II-2

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
• Reaction medium	• Ethanol solution
• Variable volume pipettes	• Pipettes tips (sterile)
• Actinomycin solution	• 15 ml tubes (sterile)
• 10 ml pipettes (sterile)	

MODULE II-2

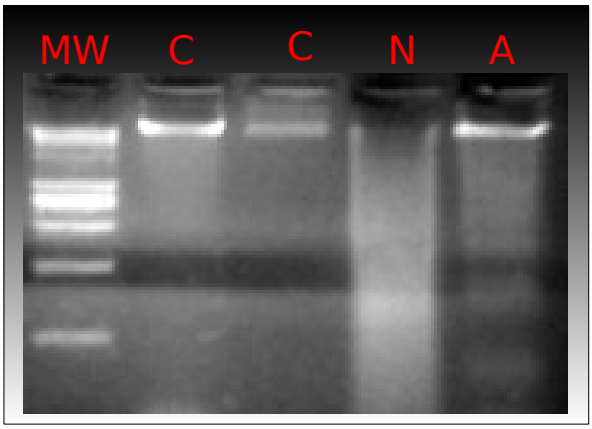
Remember that you must work in sterile conditions to not contaminate the cultures, so you must work under the laminar flow cabinet as much as possible and keep sterile all material we introduce in it.

The experiment's planning requires that we have controls to which we compare the apoptotic effects of the agent.

In order to analyze the effect of the apoptotic agent, we should consider three possibilities in our results:

1) the agent to study **does not have any effect over our culture**. Then the result obtained in the agarose gel should be similar to the obtained in the culture that has not been incubated with the apoptotic agent.

2) the effect of **cellular death is necrotic** and not apoptotic. Then the result obtained in the agarose gel should be similar to the obtained in the culture that has been incubated with the necrotic agent.



3) the effect produced by the agent is **apoptotic** and the result obtained in the agarose gel is different from the two previous ones and corresponds to the ladder type pattern typical of DNA strands (see figure).

To identify the results gathered from the incubation of the agent (object of study), we must have:

a) a cell culture that has been incubated with the reaction medium in which the necrotic and the apoptotic agents were NOT included.

b) a cell culture that has been incubated with a known agent that causes necrotic cellular death.

c) a cell culture that has been incubated with the apoptotic agent, object of study.

Protocol:

Before proceeding to incubation, each group should have 3 15 ml tubes in the tube rack and identify them the following way:

a) C*ontrol tube*

b) *Necrosis tube*

c) A*ctinomycin tube -its apoptotic effect is to be determined-*

1. **Add** 3 ml of reaction medium to the sterile 5 ml tube marked as control.

2. **Add** 1.5 ml of reaction medium to the sterile 5 ml tube marked as necrosis.

3. **Add** 1.5 ml of the received ethanol solution to the necrosis tube -to reach the final volume of 3 ml-.

4. **Shake** the tube with the actinomycin solution received (so that it homogenizes). Pipet 300 microliters of this solution and add them to a 5 ml tube which we label as actinomycin. Add 2.7 ml of reaction medium to this tube.

5. **Homogenize** the medium of each of the tubes with the pasteur pipette.

6. **Mark** each of the 3 plates as control, necrosis, and actinomycin.

7. **Aspire** the medium contained in the control plate with the help of a pasteur pipette, being careful not to drag the cells adhered to the plate.

8. **Add** again to the control plate and with the help of a pipette pasteur 3 ml of the control tube.

9. **Repeat** the same process with each of the remaining plates.

10. **Incubate** the cell plates at 27ºC for 24 hours.

MODULE III: *Obtainment of the cellular homogenate. DNA extraction and detection.*

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
• Lysis buffer	• Scrapers
• Variable volume pipettes	• Pipettes tips (sterile)
• 15 ml tubes (sterile)	• DNA extraction kit

Before proceeding to obtain the cellular homogenate, we must observe in the microscope each of the cell culture plates and write down in our lab notebook their current status.

This practice section can be executed in the lab table, since the sterility conditions are not necessary.

Protocol:

1. **Identify** three 15 ml tubes, as the way we did with the three culture plates.

2. Softly **lift** the cells adhered to the plate with the help of a scraper.

3. **Aspire** the homogenate with a pasteur pipette and deposit it in the corresponding tube that we have just identified.

4. **Centrifuge** the tubes at 1000 rpm for 10 minutes.

5. **Mark** three tubes the same way we marked the culture plates.

6. After centrifuging the 15 ml tubes, **aspire** the culture medium being careful not to drag cells.

7. **Add** 600 µl of lysis buffer to each of the tubes, suspend the cells with the help of a micropipette increasing and decreasing the volume. **Make sure** we do not leave cell clumps and deposit them in each of the marked microtubes.

***NOTE*:** **Proceed** to extract the DNA (with the Danagen Genomic Cell DNA kit) and **analyze** the extracted DNA by agarose gel electrophoresis (electrophoresis equipment supplied by Danagen).

8. **Incubate** at 55ºC for 15 minutes and observe the homogeneous solution. RNase treatment.

9. **Add** 2 µl of RNase to the lysed.

10. **Shake** the sample by tube inversion and incubate it at 37ºC for 15-60 minutes.

11. **Add** 360 µl of Solution of protein precipitation.

12. Strongly **shake** with Vortex for 20-30 seconds.

13. **Centrifuge** at 14,000 x g for 5 minutes. It will be observed that the protein precipitate creates a pellet.

14. **Take** the supernatant that the DNA has to a 1.5 ml tube containing 600 µl of isopropanol. Shake by inversion several times.

15. **Centrifuge** at 14.000 x g for 3 minutes.

16. **Eliminate** the supernatant. **Add** 600 µl of 70% ethanol and invert several times to wash the DNA pellet.

17. **Centrifuge** at 14.000 x g for 2 minutes. Carefully eliminate all the ethanol. Take care not to lose the DNA pellet.

18. **Invert** the tube and let it dry in paper towel for 15 minutes.

19. **Add** 100 µl of Solution of DNA Hydration.

NOTE: If the obtained pellet is too big, increase the Solution of Hydration.

20. **Incubate** at 65ºC for 1 hour with periodic shakings to help the DNA dispersion, or incubate overnight at either room temperature or 4ºC with shaking so that it rehydrates.

21. **Store** at 2-8ºC. For long storages, store at –20ºC or –80ºC.

22. **Visualize** the DNA in a 3% agarose gel electrophoresis.

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. If we want to determine the apoptotic effect of a certain molecule, why do we use cell cultures incubated at different experimental conditions?

4. Should we apply another technique to confirm the apoptotic effect observed in the DNA fragmentation?

5. Reason the mechanism in which the actinomycin produces cellular death by apoptosis -search for additional information-.

6. Indicate some alternative method to detect the cellular death by apoptosis.

ANNEX 2: PRACTICE RESULTS AND ANALYSIS

The intensity of the DNA strands obtained in the cells in which apoptosis has been induced will depend on the homogeneity in the DNA concentration obtained in the diverse stimulations. **It is important** to follow the instructions of the DNA extraction kit so that in the DNA hydration in the last stage of the extraction we have the DNA concentrated.

The DNA lanes observed in the agarose gel from the DNA samples of apoptotic cells can be similar to the lane with the control samples. In this case, make sure to have added the RNase enzyme in the extraction process and that the DNA concentrations of the apoptotic cell samples are similar to those of control samples.

The laddering effect of the fragmented DNA is diffuse and the fragments' size cannot be clearly determined. To know for sure their size, we should enable a well in the agarose gel in which we have to add the corresponding molecular weight marker. The obtained DNA could be too diluted.

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 two tubes that contain the cells in a 75 cm^2 culture flask (with a green lid).

The cellular content of a 80-90% confluence flask is enough for three student groups to execute the practical (9 60 mm 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).

c) **Duraning the practices**
Remember that the students *have to observe the status of the cells, before and after the incubation with the agent that causes cell death by apoptosis.*

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 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	30-45 min
III	15 min	45-60 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: Apoptosis induction in Sf9 cells	Provide information	At the start of the practical	5 min
Module III: Obtainment of the cellular homogenate	Prepare and distribute the materials	One hour before executing the practical	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.

> **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) Preparation of aliquots of the cell culture medium and the reaction medium

1. Aseptically **ALIQUOT**, for each group of students, **10 ml** of **Cell culture medium** in a 15 ml tube, previously identified. Keep the remaining medium to initiate the insect cell culture.

2. Aseptically **ALIQUOT**, for each group of students, **10 ml** of **Reaction medium** in a 15 ml tube, previously identified.

3. **ALIQUOT 2 ml of the lysis buffer** in each of the six, accordingly marked, 15 ml tubes.

4. CHECK the **LABEL** in each tube: **Culture medium**, **Reaction medium**, or **lysis buffer**.

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

c) Start of the culture of the received cells

Sf9 insect cells are sent in four vials from which two of them should be transferred to one of the 75cm^2 culture flasks and the other two to the other 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 **9 ml** of insect cell culture to each culture flask -of 75 cm^2-, 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 two vials of Sf9 insect cells to a (sterile) cell culture flask. Do the same with the other two received cell vials.

> **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 10 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: APOPTOSIS INDUCTION IN SF9 CELLS

Module II-1:

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	• Scrapers

• Variable volume pipettes	• Pipette tips (sterile)
• Neubauer chamber	• 15 ml tubes (sterile)
• Placas de cultivo de 60 mm	• Pipetas de 10 ml (estériles)
• 50 ml tubes (sterile)	

Module II-2:

1. **TAKE OUT** from the refrigerator the aliquots of the reaction medium and let them 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
• Reaction medium	• Ethanol solution
• Variable volume pipettes	• Pipette tips (sterile)
• Actinomycin solution	• 15 ml tubes (sterile)
• 10 ml pipettes (sterile)	

MODULE III PREPARATION: OBTAINMENT OF THE CELLULAR HOMOGENATE

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	• Sterile pasteur pipettes
• Culture medium	• Plate scrapers
• Variable volume pipettes	• Pipette tips (sterile)
• Neubauer chamber	• 15 ml tubes (sterile)
• 12-well culture plates	• 10 ml pipettes (sterile)

Data recording

GROUP
STUDENTS NAMES
DATE