Edvo-Kit # 1002

Edvo-Kit #1002

Cell Culture Toxicity Screening

Experiment Objective:

Toxicity screening is a powerful technique that allows scientists to determine the effect of potentially harmful substances on living cells. In this inquiry-based lab, students will plan and implement a toxicity screening experiment using insect cell culture. The results will be analyzed to determine cell viability and to estimate the LD_{50} of the toxic solution.

See page 3 for storage instructions.

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EDVOTEK®

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

Experiment Components

	Component	Storage	Check (✓)
A	Sf9 Insect Cells	Use Immediately (See Note)	❑
B	Insect Cell Media	4°C Refrigerator	❑
C	Dilution Media	4°C Refrigerator	❑
D	$CuSO_4$ Solution	4°C Refrigerator (in dark)	❑
E	$ZnSO_4$ Solution	4°C Refrigerator (in dark)	❑
F	50% Alcohol	4°C Refrigerator (in dark)	❑
G	Trypan Blue Dye	Room Temp.	❑

This experiment is designed for 6 student groups.

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

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

Store the following components at room temperature.

- Cell culture flask (sterile) ❑
- Cell counting chambers ❑
- Large transfer pipets (sterile) ❑
- Small transfer pipets ❑
- 10 mL disposable pipets (sterile) ❑
- 15 mL conical bottom tubes ❑
- 12 well cell culture plates (sterile) ❑
- Microcentrifuge tubes ❑
- Cell scraper (sterile) ❑
- 5 mL tubes (sterile) ❑

Requirements *(not included with this kit)*

- Incubator, covered plastic container, or cardboard box to grow cultures (the EDVOTEK box will work as an incubator)
- 70% Ethanol in spray bottles
- Pipet pump or bulb
- Inverted phase contrast or bright field microscope suitable for cell culture
- 10-100 µL micropipets and tips
- Safety goggles, disposable laboratory gloves, and lab coats
- Marking pens
- Containers for trash
- Bleach

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

Background Information

Eukaryotic Cell Culture

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

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

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

Sf9 Insect Cell Culture

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

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

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

Cell Culture Techniques

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

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

Cells are cultured in growth media, a chemically complex solution that provides the nutrients necessary for the cells to grow. Media typically contains essential amino acids, buffers, salts, and a carbon source like glucose. Because this mixture is carefully balanced for use with specific cell lines, the choice of media is essential for proper growth and behavior. In addition, many cell lines are supplemented with animal serum, to provide essential growth factors, and antibiotics to help reduce the chances of bacterial infection. The cell culture media provided for this experiment is supplemented for use in insect cells and does not require animal serums.

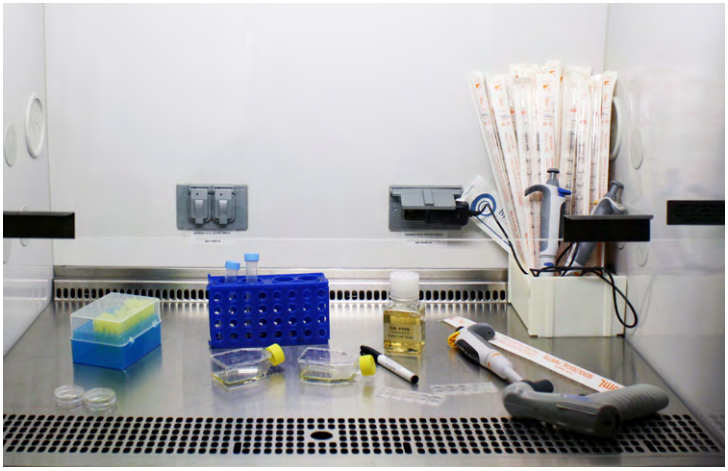


Figure 3: Cell culture hood and standard equipment.

Cell Culture Toxicity Testing

Toxicity testing, determining the potential safety of a substance on living organisms, ensures the health and well-being of the public. Cell culture experiments are essential for both academic and industrial toxicity screening. For example, scientists routinely use cell lines for initial *in vitro* studies on new chemicals, medicines, and pesticides. These experiments provide valuable information on the potential toxicity of a substance and can help to minimize animal testing.

Toxic substances affect cells in a variety of different ways. Extremely hazardous substances cause rapid cell death while less hazardous treatments might damage cells without killing them. Microscopic observation often reveals changes in these "sick" cells, including differences in cell shape or structure. In addition, toxins affect cell growth and reproduction.

Since chemicals will have different reactions in an organism it is important to compare responses across experiments. One way to do this is by measuring the lethal dose (LD_{50}), the amount of a substance that causes death in 50% of the tested organisms. LD_{50} measurements are normally expressed as the amount of a chemical in milligrams per kilogram of body weight. Therefore, a lower LD_{50} would indicate that it takes less of the substance to reach a lethal dose. For example, table salt (NaCl) has an LD_{50} of 300 mg/kg in rats, while cyanide has an LD_{50} of 10 mg/kg. Thus, comparing the LD_{50} values of NaCl and cyanide tells us that cyanide is significantly more toxic to rats. LD_{50} values are regularly tested in cells from

animals, including mice, rats, and primates, but extrapolating LD_{50} values between species can be difficult. Differences in the physiology of organisms can cause large variances in the LD_{50} of a substance. Because of this it is important to match the cell line being tested with the target organism.

Many cellular features in healthy and unhealthy cells are visible using a modest compound microscope. In addition, special stains are available to accentuate cellular structures and improve observations. For example, Trypan blue, a vital dye, is commonly used to enhance cell counting and to monitor the health and growth rate of cultured cells. Trypan blue will not stain healthy, living cells, but it is quickly absorbed by dead cells. This allows scientists to monitor the health of a cell population (Figure 4).

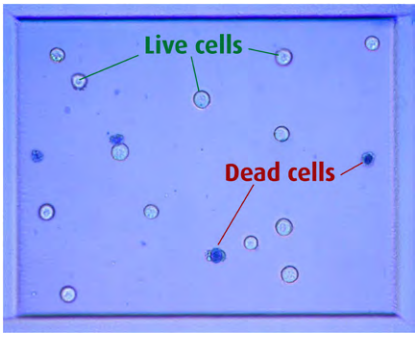


Figure 4: Trypan blue staining of cells

In this experiment, students will design and implement a toxicity test using Sf9 insect cells. Each group selects a potentially hazardous chemical for analysis. The chemicals will be serially diluted and then administered to the insect cells. Trypan blue staining will be used to quantify the percentage of live and dead cells in each treatment, allowing students to estimate the LD_{50} of their compound.

Experiment Overview

EXPERIMENT OBJECTIVE

Toxicity screening is a powerful technique that allows scientists to determine the effect of potentially harmful substances on living cells. In this inquiry-based lab, students will plan and implement a toxicity screening experiment using insect cell culture. The results will be analyzed to determine cell viability and to estimate the LD_{50} of the toxic solution.

LABORATORY SAFETY

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

Wear gloves and safety goggles

STERILIZATION OF EQUIPMENT AND CONSUMABLES

1. Sterilize the area you are doing the experiment on with 70% ethanol. If ethanol is not available, a 10% bleach solution or a laboratory disinfectant can be used.
2. All materials that come in contact with cells should be disinfected before disposal, including culture dishes, pipets, transfer pipets, and tubes.
   - Autoclave at 121 °C for 20 minutes.
     Remove media from flasks and plates, then collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium from spilling into the sterilizer chamber.
   - Soak in 10% bleach solution.
     Submerge dishes, tubes and other contaminated materials into a 10% bleach solution. Soak the materials for at least 1 hour and then discard. Wear gloves and goggles when working with bleach.

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

LABORATORY NOTEBOOKS

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

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

During the Experiment:
- Record your observations.

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

Module I: Preparing for Insect Cell Culture

It is important to prepare a designated clean area before beginning the cell culture experiments. Cell culture hoods, or laminar flow hoods, are typically used for cell culture as it provides an aseptic environment that maintains a contaminant-free work space. A laminar flow hood would be the most ideal work environment to complete this experiment. If this is not accessible to you, use a work space with a completely clear, non-porous surface that has been cleaned and disinfected. A lab bench can be used, for example, if sterilized thoroughly. Follow the procedures below to maintain aseptic conditions throughout the experiment.

A. PREPARING STERILE INCUBATION CHAMBERS

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

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

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

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

B. LEARNING BASIC ASEPTIC TECHNIQUES

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

Personal Hygiene

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

Module I: Preparing for Insect Cell Culture, continued

Preparing the Work Area and Supplies

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

Pipetting

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

Handling Bottles and Flasks

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

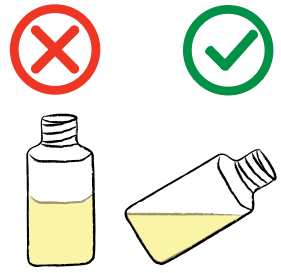


Figure 6: Properly orient your bottles when open.

Module II: Planning the Toxicity Screening Experiment

In this lab you will design and conduct a toxicity screening experiment to examine the effect of an environmental toxin on cell survival. Use the following worksheet to develop your experimental outline before beginning the experiment.

Ask a Question: This is the question that can be answered using experimentation and factual reasoning. In the question you should specify which toxin you are interested in testing. Research potential environmental toxins, or use one of the three provided with the kit – $CuSO_4$, $ZnSO_4$, or Ethanol.

Example: "Is $CuSO_4$ toxic to insect cells?"

Form a Hypothesis: This is one possible answer to your question and a prediction of what you think will happen. Remember that you will be observing the response of insect cells to an increasing dose of your chosen chemical.

Example: "Increasing concentrations of $CuSO_4$ will reduce the health and survival of insect cells."

Module II: Planning the Toxicity Screening Experiment, continued

Plan the Treatment: Test your chosen chemical at a minimum of 3 different concentrations to determine the toxicity. It is often convenient to construct a dilution series for the treatment groups. We have found that a 1:3 dilution series provides a good level of contrast between conditions for the provided chemicals and should allow for the calculation of an LD50. If you are using a different solution we recommend a 1:10 dilution series to provide the best chances of observing an effect on the insect cells. In all cases, the pollutants will be diluted in Insect Cell Media.

NOTE:
The heavy metal pollutants are provided by your instructor at a concentration of 6 µM, the Ethanol is provided as a 50% solution.

Example: "Experimental Condition #1 = 6 µM $CuSO_4$. Experimental Condition #2 = 2 µM $CuSO_4$. Experimental Condition #3 = 0.66 µM $CuSO_4$."

Experimental Condition #1	
Experimental Condition #2	
Experimental Condition #3	

Choose a Control: This is the condition that will serve as a standard of comparison. If the control group is treated very similarly to the experimental groups it increases our confidence that any difference in outcome is caused by the presence of the experimental treatments.

Example: "Control – Insect Cell Media, 0 µM $CuSO_4$."

Control Treatment	

Decide on the Number of Replicates: It is important to provide multiple replicates for each treatment condition to ensure that the results are statistically significant. In this experiment each well of a 12-well plate can be used to examine a treatment condition. We recommend that each condition is tested on 3 wells, providing 3 replicates for each group.

Example: "3 wells of insect cells for each concentration of $CuSO_4$, plus 3 wells for the control"

Module III: Examination of Insect Cell Cultures

A. MONITORING THE HEALTH OF CULTURES

It is important to examine the insect cells before every cell culture experiment to ensure that they are healthy and free from contamination. Microscopic observation can reveal the source of contamination in cell culture (Figure 7).

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

NOTE: If contamination is seen it is important to promptly and safely dispose of any contaminated reagents and plates to prevent further spread of the infection. Refresh your proper sterile technique and analyze potential sources of contamination. Remember to disinfect the cell culture work area and all materials that will be used, including your gloves.

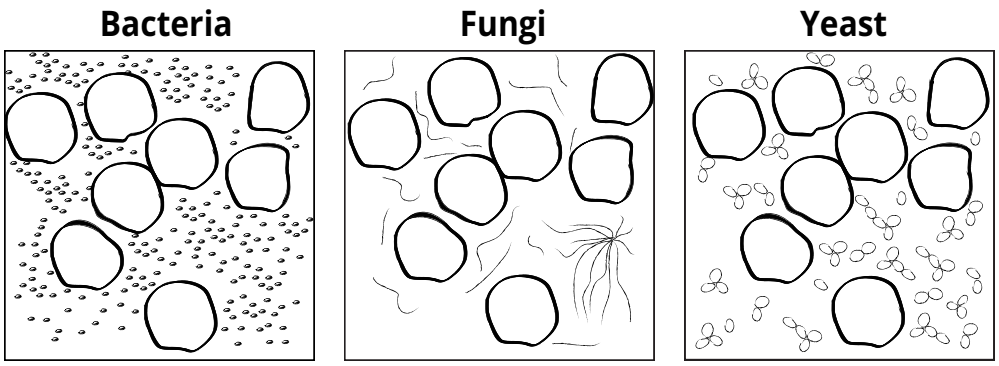


Figure 7: Types of common contaminants in cell culture.

1. **RETRIEVE** a plate of cells from the cell culture incubator and return to your lab station. Remember to practice basic aseptic technique when working with the cells.

2. Hold plate up against a light source and **CHECK** if the media is clear. Insect cells should be visible as a pale haze or cluster of cells on the bottom surface of the flask and the media in the flask should be clear. A cloudy cell culture media indicates microbial contamination.

3. **EXAMINE** the cells under a microscope. Look for signs of unhealthy cells which might indicate that the cell media needs to be replaced or that the culture has been contaminated. Unhealthy and apoptotic cells will show increased granularity, vacuole formation, cell shrinkage, cell membrane "blebbing", and nucleus fragmentation (Figure 8A).

NOTE: If contamination is observed, immediately add 1 mL of 10% bleach solution to the contaminated well, incubate for 5 minutes, and discard the culture. Swab the cell culture incubator with 70% Ethanol to prevent the spread of contamination to other plates. It is possible to continue the experiment even if a few wells become contaminated.

Module III: Examination of Insect Cell Cultures, continued

4. **RECORD** your initial observations in the Cell Culture Data Record or a separate lab notebook. Observe the appearance of cells, clarity of media, and the presence or absence of contamination. **DRAW** a picture of the cell morphology, including the shape of individual cells and the size and distribution of cell clusters.

NOTE: If possible, take photographs with a digital camera attached to the microscope. Include the digital images or printed photos with your cell culture records.

5. **DETERMINE** if the cells are ready for the toxicity screening experiment, or if they require additional time to grow. Cells should be 80-90% confluent for toxicity screening (Figure 8B.)

6. If the cells are not ready for toxicity screening, **RETURN** the plate to the incubator. Check cells daily to monitor growth, recording the data in your Cell Culture Data Record. Observe any changes in cell morphology as the cells increase in confluency.

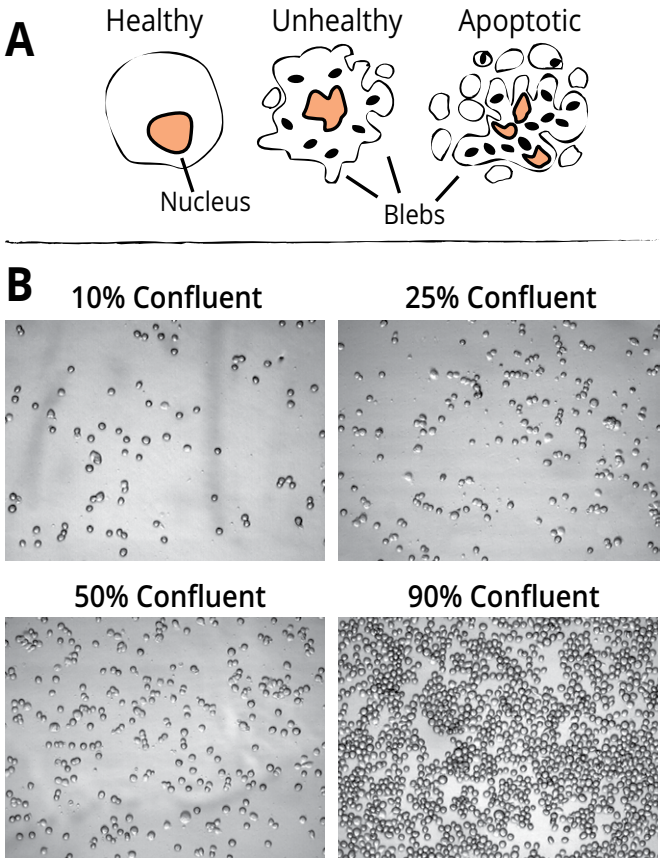
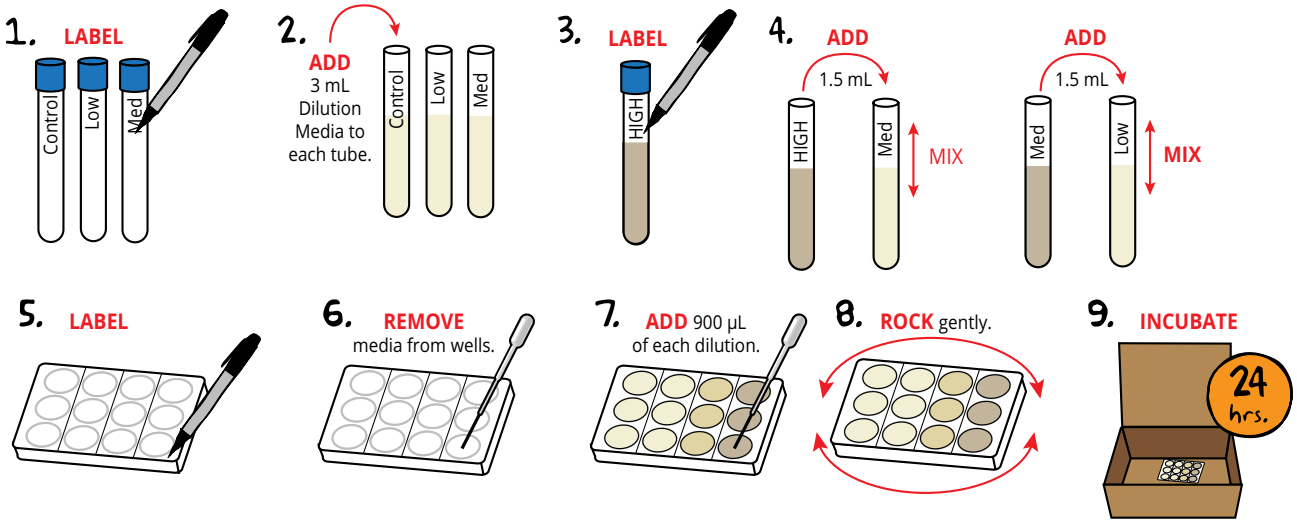


Figure 8: Insect cell characteristics and confluency.

Module IV: Exposure of Insect Cells to Toxins



1. **LABEL** three 5 mL tubes as "Control", "Low", and "Medium".
2. **ADD** 3 mL of Dilution Media to each tube. It is important to maintain sterile technique while working with the media to prevent contamination.
3. **OBTAIN** the concentrated toxin tube from your instructor. **LABEL** this tube "High".
4. **CREATE** solutions for each of the experimental concentrations.
   a. Using a sterile pipet tip or a sterile transfer pipet **ADD** 1.5 mL of the "High" solution to the "Medium" tube. **MIX** by pipetting up and down.
   b. Use the same tip or transfer pipet to **ADD** 1.5 mL of "Medium" to the tube labeled "Low". **MIX** by pipetting up and down.
5. **LABEL** the plate with your initials and the toxin you are going to use. **LABEL** the lid above each well with the appropriate treatment group. Use Figure 9 as a guide.
6. **REMOVE** the media from each well using a sterile transfer pipet. **DISPOSE** of the media into a marked waste container.
7. Using a fresh transfer pipet for each condition, **ADD** 900 µL of each dilution into the appropriate well. Remember that each treatment is performed in triplicate.
8. Gently **ROCK** the plate to ensure that the entire surface of each well is covered by media.
9. **INCUBATE** the plate in the chamber for 24 hours.

NOTE:
It is important to maintain sterile technique while mixing and pipetting media. Contamination by bacteria, yeast, or fungus can kill insect cells and ruin the experiment!

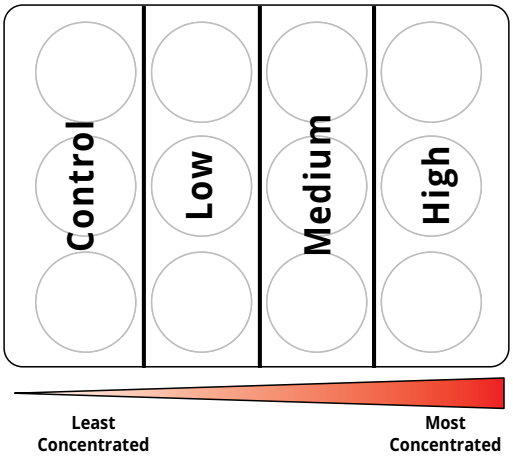
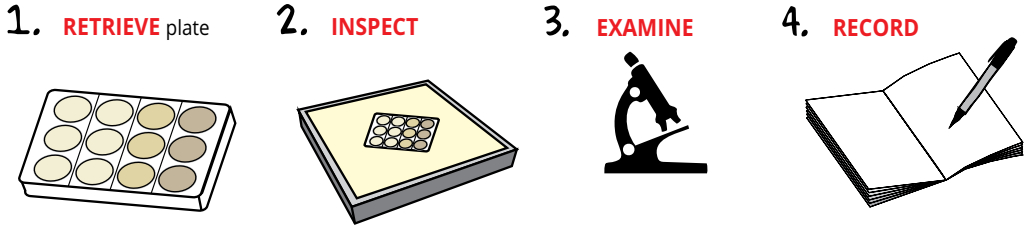


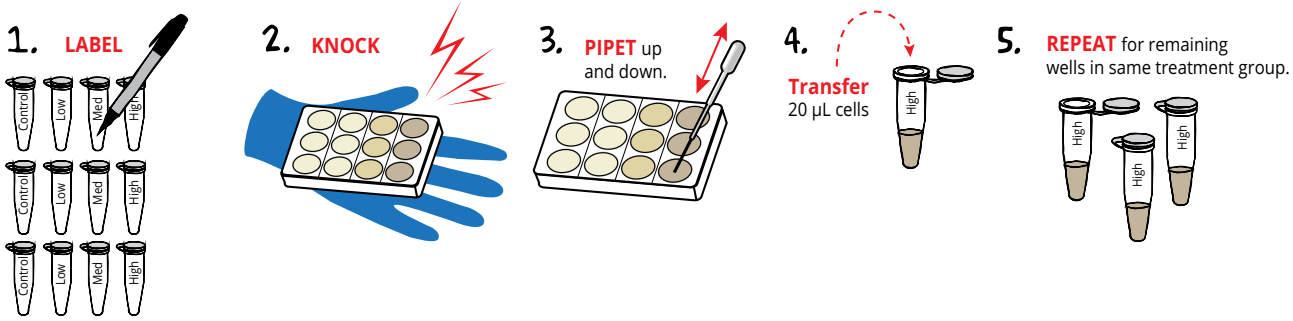
Figure 9: Example setup of experimental plate.

Module V: Cell Viability Assays Using Trypan Blue Staining



A. OBSERVING THE HEALTH OF YOUR CULTURE

1. **RETRIEVE** your cell culture plate from the incubation chamber.
2. **INSPECT** the plate up against a light source. Insect cells should be visible as a pale haze or cluster of cells on the bottom surface of the wells.
3. **EXAMINE** the cells under a microscope, paying attention to your replicates for each treatment group.
4. **RECORD** your observations in the Cell Culture Data Record. **DRAW** a picture of the cell morphology, including the shape of individual cells and the size and distribution of cell clusters. Unhealthy and apoptotic cells will show an increase in small particles (called granules), vacuole formation, cell shrinkage, cell membrane "blebbing" and nucleus fragmentation (Figure 8A). ***NOTE: If possible, take photographs with a digital camera attached to the microscope. Include the digital images or printed photos with your cell culture records***



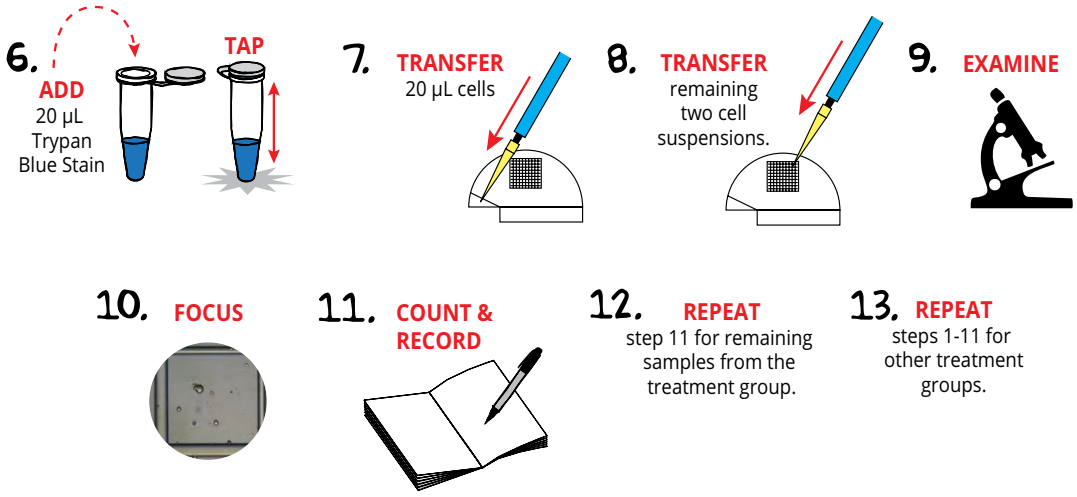
B. PERFORMING A VIABILITY ASSAY

The cell counting chamber, or "hemocytometer", is a device widely used to count the cells in a specific volume of fluid. In this case, the chamber will also be used to differentiate dead from live cells. Live, viable cells exclude Trypan Blue dye whereas dead cells absorb the dye and stain blue.

1. **LABEL** 12 microcentrifuge tubes to correspond to your treatment groups and replicates.
2. **KNOCK** the plate firmly against the palm of your hand to loosen the cells. ***NOTE: Be careful that the media does not spill between wells.***
3. **PIPET** the cells in the first well up and down five times with a fine-tipped transfer pipet to disperse the culture.
4. Using an adjustable volume pipet, **TRANSFER** 20 µL of cell suspension into a microcentrifuge tube.
5. **REPEAT** steps 2-4 for each remaining well in the same treatment group. Do not pipet or remove the cells from the other wells at this time.

NOTE: It is important to only work with one treatment group at a time to limit the length of exposure to Trypan blue.

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



6. ADD 20 µL of Trypan Blue dye to the cells in each tube and gently **MIX** by tapping the tube on the lab bench or pipetting up and down.
7. Slowly **TRANSFER** 20 µL (approximately one drop) of Trypan Blue-stained cell suspension to a notch on the bottom left side of one counting area of the cell counting chamber. The chamber will fill by capillary action.

NOTE: Pipet slowly to minimize the number of bubbles that enter the chamber. If bubbles do enter the chamber press gently on the center of the counting area to displace.

8. Using fresh pipet tips, **TRANSFER** the remaining two Trypan Blue-stained cell suspensions to unused counting areas of the chamber.
9. **EXAMINE** the counting chamber on the microscope using the lowest objective.
10. **FOCUS** on the grid lines in the chamber. Move the slide until you find one of the corners – this might require changing to a higher-powered objective.

NOTE: The counting chamber grid contains 9 clusters, each divided into 9 smaller squares. For this experiment you will count the cells in the 9 small squares that make up three of the four corner regions (Figure 10).

11. **COUNT** the number of living and dead cells within the 9 squares of one corner region. **RECORD** the number of live (clear and bright) and dead (deep blue) cells in the Cell Culture Data Record. Continue to **COUNT** the cells in two of the remaining corner regions, recording the number for each region independently.
12. **REPEAT** step 11 for the remaining samples from the same treatment group.
13. **REPEAT** steps 2 through 11 for the other treatment groups.

NOTE: Do not attempt to clean the used wells of the cell counting chamber.

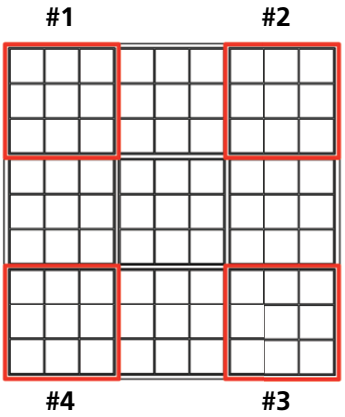


Figure 10: Corner regions of the counting chamber.

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

C. INTERPRETING THE TOXICITY TESTING DATA

Computing Percent Cell Viability

Cell viability calculations give scientists a measurement of the average cell survival after treatment with a substance.

The percentage of viable cells from each well can be calculated using the formula:

$$\text{Percent Cell Viability} = \frac{\text{Number of live cells}}{\text{(Total \# of cells counted)}} \text{ X } 100$$

For Example: You add the cells from the first well of your treatment group to the counting chamber and count a total of 75 cells across the 3 corners. You notice that 70 of the cells are bright and 5 are deep blue.

$$\text{Percent Cell Viability} = \frac{70}{75} \text{ x } 100 = 93.3\% \text{ Viability}$$

1. Using a sheet of graph paper or a computer graphing program, **CREATE** a bar graph of the average percent viability results from your 3 replicates.

   Use this graph to identify any trends or unexpected results. Does the toxicity of your pollutant increase from one concentration to the next? Are there any changes that you did not expect? What could be a possible explanation for these changes?

2. **EXAMINE** the variability between your replicates.

   Toxicity screening experiments will inevitably have some variability due to biological differences between cells. In addition, human error – such as differences in pipetting or counting accuracy – can introduce variability.

   (Optional) **CALCULATE** the standard deviation for each experimental condition and use these values to add error bars to the graphs. Error bars that do not overlap indicate that the difference between two conditions is likely not due to chance. While error bars that do overlap indicate that any difference between the two conditions should be more cautiously interpreted

   The standard deviation of a sample is known as S and is calculated using the formula: $$s = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

   Where x represents each value in the population, $\bar{x}$ is the mean value of the sample, Σ is the summation (or total), and n is the number of values in the sample.

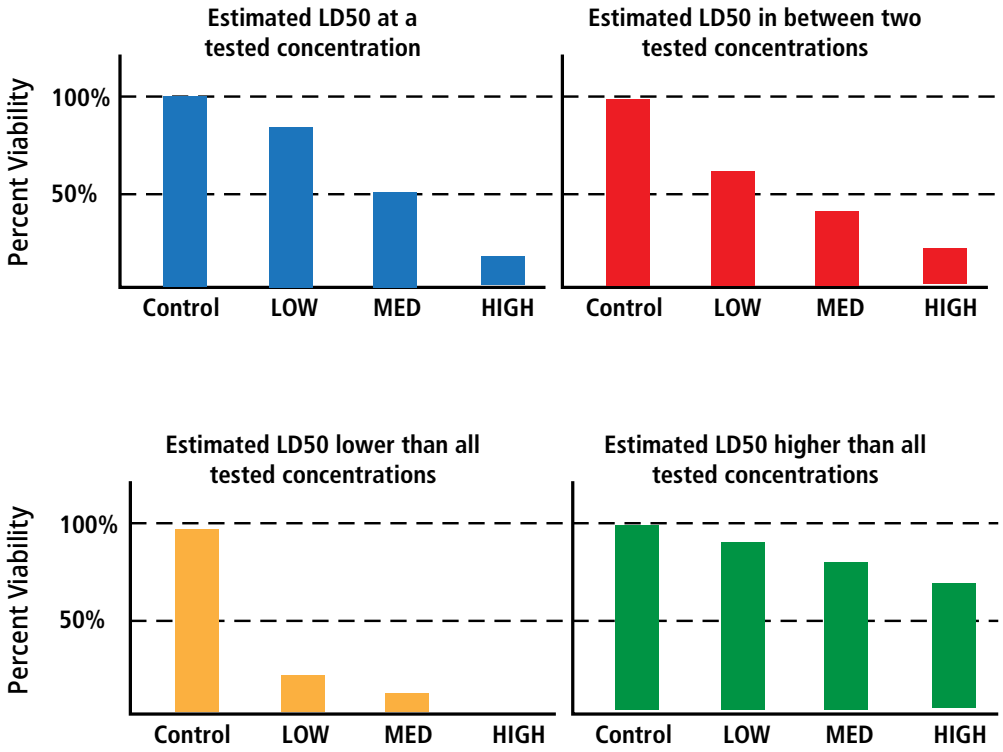
   The standard deviation can be calculated using an appropriate software program or online resource. It can also be calculated by hand in four steps:

   a. Record the average of all the replicates.
   b. Take the first replicate value and subtract the average from step a. Then square the result. Repeat this for all replicates and record the result.
   c. Add the results from step b and then divided by the number of replicates minus one.
   d. Take the square root of the number from step c.

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

3. (Optional) **ESTIMATE** a LD50 value for your pollutant.

   The LD50 value, or median lethal dose, is the concentration of a substance that results in the death of 50% of the tested cells. Use your graph to estimate the concentration that would result in a 50% cell viability in your assay. This value may be at a tested concentration, between two of the tested concentrations, higher than all of the tested concentrations, or lower than all of the tested concentrations. This is illustrated in the example graphs below.



Study Questions

1. Why has cell culture become such an important tool for researchers?

2. What are the advantages of insect cell culture? Why are insect cells useful in toxicology assays?

3. What does the LD_{50} of a substance measure?

4. How do the results of a cell culture toxicity assay apply to the whole organism? How do they relate to different species of organisms?

Instructor's Guide

IMPORTANT - READ ME!!

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

ORGANIZATION AND IMPLEMENTING THE EXPERIMENT

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

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

Approximate Time Requirements

MODULE	PRELAB	EXPERIMENT
I	----	15 min.
II	----	15 min.
III	5 min.	15 min.
IV	20 min.	30 min., overnight incubation
V	20 min.	60-90 min.

Pre-Lab Preparations

ADVANCE PREPARATION:

Preparation for:	What to do:	When?	Time Required:
Establish Cell Culture	Inoculate flask with insect cells	Immediately upon receipt of cells – approx. one week before starting the experiment	15 min., 72 hr. incubation
	Prepare incubation chamber	Anytime before performing the experiment	15 min.
	Prepare cell culture plates	48 hours before performing the experiment	60 min.
Module III: Examination of Insect Cell Cultures	Prepare microscopes	Anytime before performing the experiment	5 min.
Module IV: Exposure of Insect Cells to Toxins	Aliquot and distribute reagents	Anytime before performing the experiment	20 min.
Module V: Cell Viabiity Assays Using Trypan Blue Staining	Aliquot and distribute reagents	Anytime before performing the experiment	20 min.

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

We recommend preparing the equipment and reagents to initiate cell culture and acquiring the incubation chamber before starting the experiment with the students. The reagents for modules IV and V can be prepared as needed once student groups have progressed to those experiments. Have ready a simple compound microscope for analysis of the cells throughout all modules.

Pre-Lab Preparations

ESTABLISHMENT OF INSECT CELL CULTURE

Preparation of Incubation Chamber

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

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

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

Initiation of Insect Cell Culture

The Sf9 insect cells (A) are shipped in a 15 mL conical tube, and should be transferred to a flask as soon as they are received. All 15 mL can be transferred to the flask, or the volume can be split into two flasks. This will provide you with an extra starter flask in case of mistakes, like contamination. In this case, 7.5 mL of cells are transferred to each flask. Make sure to invert the 15 mL conical tube of cells before transferring to flask(s).

1. **WARM** the Insect Cell Media (B) to room temperature.
2. Gently **INVERT** the tube of cells to mix.
3. In a sterile environment and using a sterile transfer pipet or sterile micropipet tip, **TRANSFER** the entire volume of Sf9 insect cells (A) to a sterile cell culture flask. ***NOTE: Do NOT pour the cells into the flask as this greatly increases the risk of contamination.***
4. Using a fresh transfer pipet, **ADD** 15 mL of fresh Insect Cell Media (B) to the flask.
5. **INCUBATE** the cell culture flask in the incubation chamber.
6. After 24 hours, most of the insect cells should have attached to the surface of the flask. **CONFIRM** attachment of the cells under a microscope.
7. **ALLOW** the cells to grow for an additional 24-72 hours, checking health and confluency daily. The cells should be at least 80% confluent before passaging in the following steps.

Preparing Cell Culture Plates

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

1. **WARM** the insect cell media (B) to room temperature.
2. **KNOCK** the side of the flask against the palm of your hand and gently **SCRAPE** the bottom of the flask with a sterile cell scraper. **PIPET** up and down with a sterile 10 mL pipet to loosen the cells. **ADD** the entire flask of cells to the bottle of Insect Cell Media.
3. Gently **SWIRL** to mix the cells.
4. Using a sterile 10 mL pipet, **ADD** 1 mL of cells to each well of the six 12-well cell culture plates.
5. Carefully **PLACE** the student plates into the incubators. The students will perform the experiment once cells have reached 80% confluency.

Pre-Lab Preparations

MODULE III: EXAMINATION OF INSECT CELL CULTURES

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

MODULE IV: EXPOSURE OF INSECT CELLS TO TOXINS

This experiment includes three potential toxin solutions, although additional heavy metals, environmental pollutants, and chemicals can be tested for toxicity. The stock solutions of $CuSO_4$ and $ZnSO_4$ are provided at a concentration of 6 µM in water, while Ethanol is provided as a 50% solution.

1. **SELECT** one toxin per group ($CuSO_4$, $ZnSO_4$, or Ethanol) and aliquot 5 mL into labeled tubes.
2. **DISPENSE** 9.5 mL of Dilution Media (C) into six 15 mL centrifuge conical tubes. **LABEL** tubes as "Dilution Media".

FOR MODULE IV
Each Group Requires:

- Plate of cells
- Three 5 mL tubes
- 6 sterile transfer pipets
- 1 tube of Dilution Media
- 1 tube of toxin

MODULE IV: CELL VIABILITY ASSAYS USING TRYPAN BLUE STAINING

1. **ALIQUOT** individual tubes of 300 µL Trypan Blue (G) for the 6 groups.

FOR MODULE IV
Each Group Requires:

- One aliquot of Trypan Blue
- 12 empty microcentrifuge tubes
- 12 small transfer pipets
- Two counting chambers.

Experiment Results and Analysis

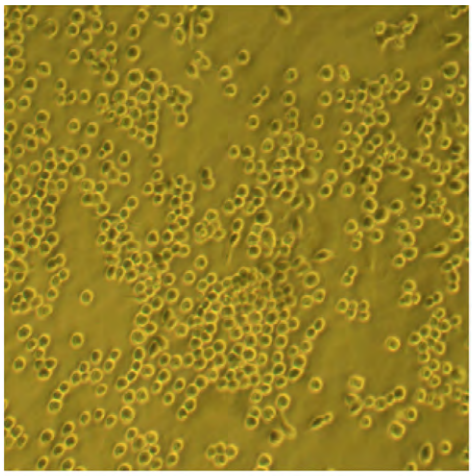
The expected results will vary depending on the growth characteristics of the cells and variations in experimental technique. Cellular viability depends heavily on the conditions in which the cells are grown, including the temperature of the incubator and the type and concentration of the toxin used.

MODULE V: CELL VIABILITY ASSAYS USING TRYPAN BLUE STAINING

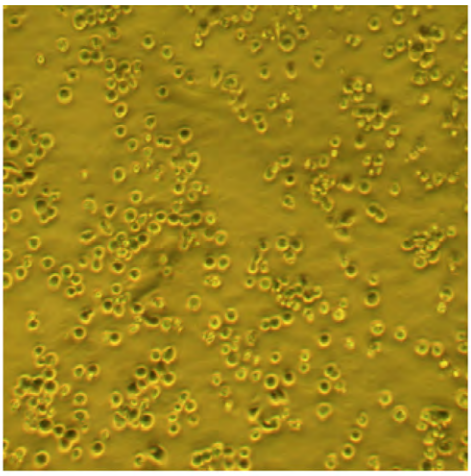
A. Observing the Health of Your Culture

Sample images of Sf9 cells 24 hours after treatment with toxins can be seen below. Healthy cells are rounded with bright cell membranes, while unhealthy cells are shriveled and tend to be darker.

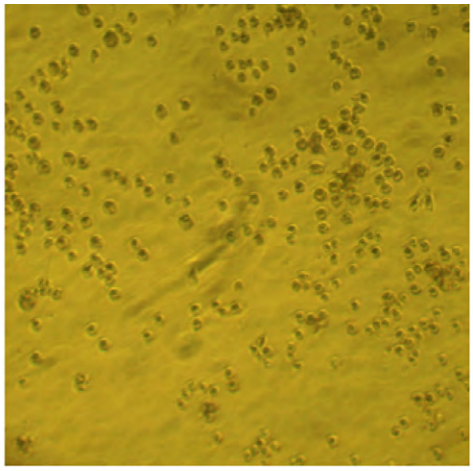
Control



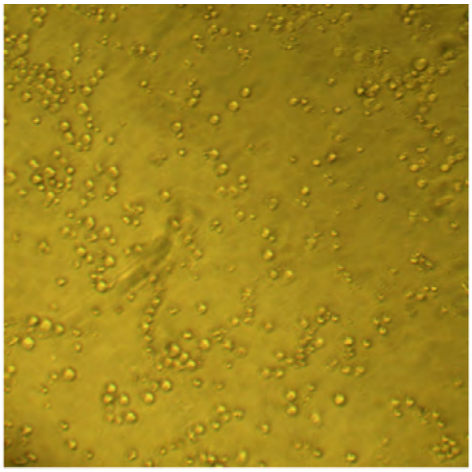
$CuSO_4$ (6 µM)



Alcohol (50%)



$ZnSO_4$ (6 µM)



Experiment Results and Analysis, continued

B. Calculating the Percent Viability of Cells

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

Using Figure 11 for the example:
- Live (bright) cells = 13
- Total cells counted = 17

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

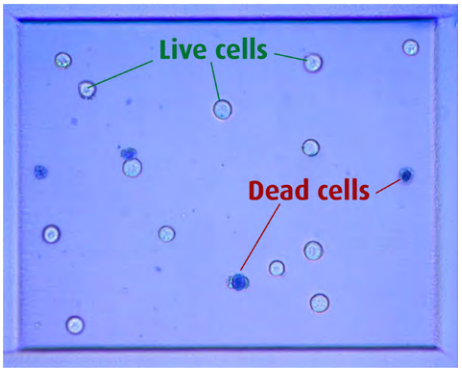


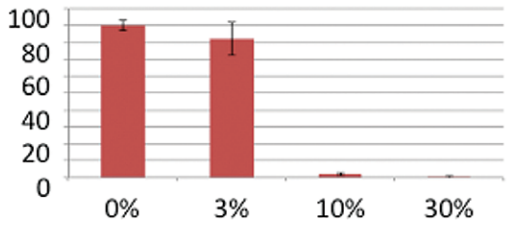
Figure 11: Trypan blue staining of cells

PERFORMING A VIABILITY ASSAY

Representative cell count values from cells treated with the indicated toxins for 24 hours.

Alcohol	1			2			3			Average		
	Live	Dead	Total	Live	Dead	Total	Live	Dead	Total	Live	Dead	Total
Control	180	20	200	120	18	138	70	5	75	**123.33**	**14.33**	**137.67**
Low	27	11	38	37	5	42	100	14	114	**54.67**	**10.00**	**64.67**
Medium	1	59	60	1	97	98	2	59	61	**1.33**	**71.67**	**73.00**
High	0	100	100	0	100	100	1	95	96	**0.33**	**98.33**	**98.67**

% Viability	1	2	3	Average	STDEV
Control	90.00	86.96	93.33	**90.10**	**3.19**
Low	71.05	88.10	87.72	**82.29**	**9.73**
Medium	1.67	1.02	3.28	**1.99**	**1.16**
High	0.00	0.00	1.04	**0.35**	**0.60**

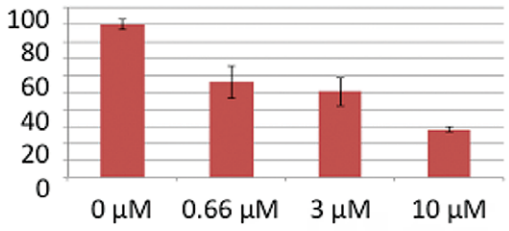


Experiment Results and Analysis, continued

PERFORMING A VIABILITY ASSAY, continued

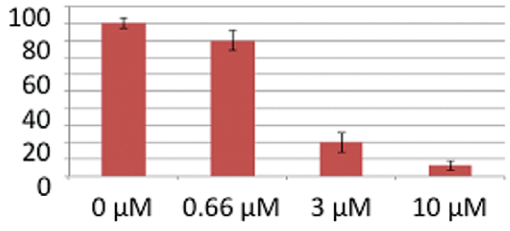
CuSO4	1			2			3			Average		
	Live	Dead	Total	Live	Dead	Total	Live	Dead	Total	Live	Dead	Total
Control	180	20	200	120	18	138	70	5	75	**123.33**	**14.33**	**137.67**
Low	37	38	75	36	33	69	40	20	60	**37.67**	**30.33**	**68.00**
Medium	50	36	86	47	67	114	36	33	69	**44.33**	**45.33**	**89.67**
High	28	77	105	14	34	48	14	34	48	**18.67**	**48.33**	**67.00**

% Viability	1	2	3	Average	STDEV
Control	90.00	86.96	93.33	**90.10**	**3.19**
Low	49.33	52.17	66.67	**56.06**	**9.29**
Medium	58.14	41.23	52.17	**50.51**	**8.58**
High	26.67	29.17	29.17	**28.33**	**1.44**



ZnSO4	1			2			3			Average		
	Live	Dead	Total	Live	Dead	Total	Live	Dead	Total	Live	Dead	Total
Control	180	20	200	120	18	138	70	5	75	**123.33**	**14.33**	**137.67**
Low	120	33	153	125	20	145	105	35	140	**116.67**	**29.33**	**146.00**
Medium	14	80	94	12	34	46	18	80	98	**14.67**	**64.67**	**79.33**
High	7	80	87	6	70	76	3	90	93	**5.33**	**80.00**	**85.33**

% Viability	1	2	3	Average	STDEV
Control	90.00	86.96	93.33	**90.10**	**3.19**
Low	78.43	86.21	75.00	**79.88**	**5.74**
Medium	14.89	26.09	18.37	**19.78**	**5.73**
High	8.05	7.89	3.23	**6.39**	**2.74**



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

CELL CULTURE DATA RECORD

Week of _____

Group # _____ Names: _____

	DATE:			
HEALTH OF CELL CULTURE	Appearance of cells			
	Clarity of media			
	Confluency (Density of cells)			
IMAGE OF CELLS				

CELL CULTURE DATA RECORD

Name: _____ Group ID: _____ Date: _____

Experimental variables

Toxin Tested: _____ Control Tested: _____

	1			2			3			Average		
	Live	Dead	Total	Live	Dead	Total	Live	Dead	Total	Live	Dead	Total
Control µM												
Low µM												
Medium µM												
High µM												