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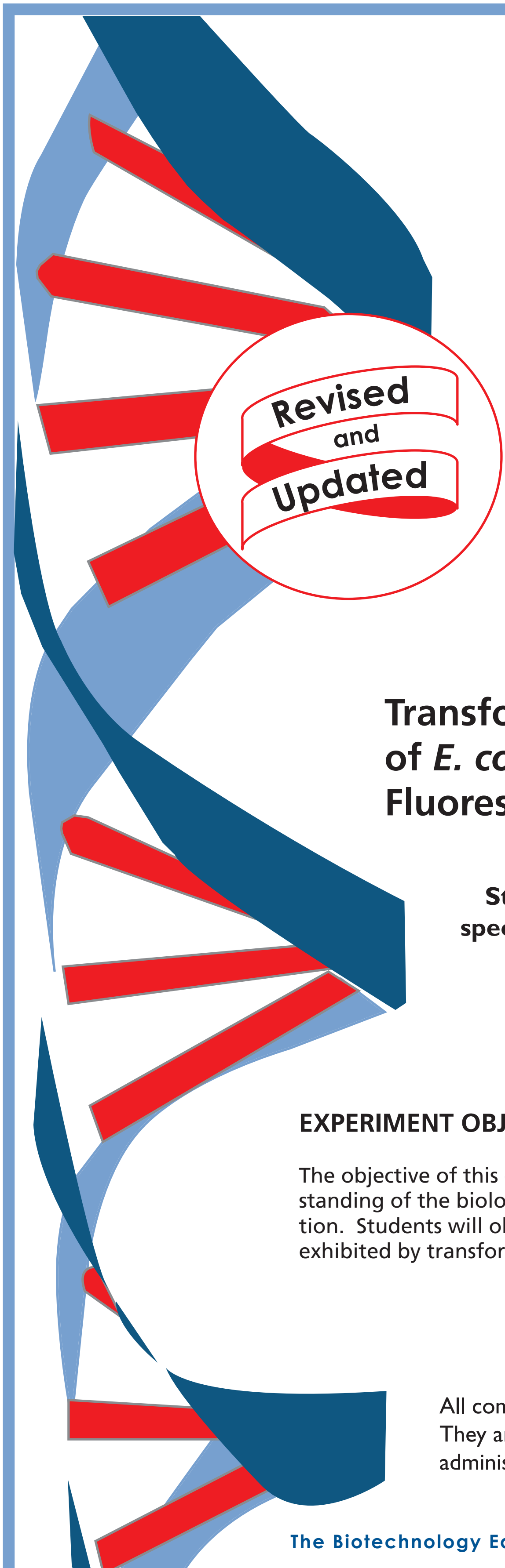

EDVO-Kit #

223

Transformation of of *E. coli* with a Green Fluorescent Protein Plasmid

Storage: See Page 3 for specific storage instructions

EXPERIMENT OBJECTIVE:

The objective of this experiment is to develop an understanding of the biological process of bacterial transformation. Students will observe the acquired fluorescent trait exhibited by transformed bacterial cells.

EVT 005077K

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Important READ ME!

Transformation experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicilllin, ampicillin, kanamycin or tetracycine should not participate in this experiment.

Experiment Components

Component Quantities:

Experiment # 223 is designed for 10 groups.

	Contents	Storage
A	Transformation cell slant (DO NOT FREEZE)	Room temperature
B	Supercoiled pFluoroGreen™	Freezer
C	Ampicillin	Freezer
D	IPTG	Freezer
E	$CaCl_2$	Room temperature
•	Bottle ReadyPour™ Luria Broth Agar, sterile (also referred to as ReadyPour medium)	Room temperature
•	Bottle Luria Broth Medium for Recovery, sterile (also referred to as Luria Recovery Broth)	Room temperature

- Petri plates, small
- Petri plates, large
- Plastic microtipped transfer pipets
- Wrapped 10 ml pipet (sterile)
- Toothpicks (sterile)
- inoculating loops (sterile)
- Microtest tubes with attached lids

All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

None of the experiment components are derived from human sources.

Requirements

- Automatic Micropipet (5-50 µl) and tips
- Two Water baths (37°C and 42°C)
- Thermometer
- Incubation Oven (34°C and 37°C)
- Pipet pumps or bulbs
- Ice
- Marking pens
- Bunsen burner, hot plate or microwave oven
- Hot gloves
- Long wave U.V. light (EDVOTEK cat #969 recommended)

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FAX: (301) 340-0582
web: www.edvotek.com
email: edvotek@aol.com

Please have the following information:

- The experiment number and title
- Kit Lot number on box or tube
- The literature version number (in lower right corner)
- Approximate purchase date

Bacterial Transformation

Bioluminescence of marine microorganisms has been observed by many summer visitors at beaches around the world. Onlookers are always fascinated by the repeated parade of color and light on the sand during the ebb and flow of the tide. This observation pales to the light produced by the bioluminescent jelly fish *Aquorea victoria*, the natural host of the green fluorescent protein (GFP). A bright burst of light is observed when energy is transferred to the green fluorescent protein (GFP) located in specialized photogenic cells in the base of the jellyfish umbrella.

This family of proteins has been known for some time and significant research in this area has been reported. Fluorescent proteins can be expressed both in prokaryotic and eucaryotic cells. These proteins do not require substrates, other gene products, or cofactors. When exposed to long wave U.V. light, they emit a bright green light that is visible in bacteria transformed by plasmids that contain the genes encoding GFP. Likewise, purification of the GFP from crude protein extracts is simplified by their fluorescence.

In cell biology experiments, the GFP protein is often fused to other proteins to study various biochemical processes. There are many examples of chimeric fusion proteins using the GFP protein as a biological tag. Such fusions are either at the N- or C- termini. The chimeric proteins are used as biotechnological tools to study protein localization and trafficking within cells.

The green fluorescent protein (GFP) possesses a molecular weight of approximately 40,000 daltons. Most of the intact protein is required for maintaining fluorescence; only small deletions of a few amino acids are allowed without compromising the integrity of the protein structure. Interestingly, the chromophore responsible for light emission is within the structure of the GFP protein and resides in amino acid residues 65 to 67, a cyclic tripeptide composed of Ser-Tyr-Gly. The importance of protein folding is clearly demonstrated in that the GFP is fluorescent only upon proper conformational folding.

With the 3-D structure of GFP being determined, several other variants of the GFP have been constructed using site-directed mutagenesis (SDM). SDM allows specific (point) mutations to be introduced in a protein to determine the impact of that mutation on the protein structure and function. The GFP protein can also be used as a dramatic tool to visually demonstrate the effect of pivotal amino acid changes on the structure and function of a protein.

Bacterial Transformation

BACTERIAL TRANSFORMATION AND OVEREXPRESSION OF TRANSFORMED GENE

Bacterial transformation is of central importance in molecular biology. Transformation is the process by which a bacterium takes up and expresses exogenous DNA, resulting in a newly acquired genetic trait that is stable and heritable. This exogenous DNA can be recombinant DNA molecules that have been constructed *in vitro*, as well as natural DNA molecules. Transformation is also of historical importance since it led to the discovery by Oswald Avery, in 1944, that DNA was the genetic material. In that historical experiment, Avery and colleagues purified DNA from a lethal strain of *Streptococcus pneumoniae*, removing all protein from the DNA. This DNA was then transformed into a harmless strain of the same organism. Injection of the transformed, formerly harmless, strain into mice resulted in their death.

For transformation to occur, bacterial cells must be in a particular physiological state, referred to as competency, in which the bacterial cell wall is made permeable to macromolecules such as DNA. Competency can occur naturally in certain species of *Haemophilus* and *Bacillus* when the levels of nutrients and oxygen are low. Competent *Haemophilus* cells express a membrane-associated transport complex that binds and transfers certain DNA molecules from the medium into the cell where they are then integrated into the bacterial chromosome and expressed. In nature, the source of the external DNA is from other cells that have died and their cell walls lysed to release their DNA into the surrounding medium.

Much current research in molecular biology involves the transformation of *E. coli*, an organism that does not naturally enter a state of competency. *E. coli* can artificially be made competent when treated with chloride salts of the metal cations calcium, magnesium and rubidium. In addition, abrupt transitioning between heat and cold can induce competency. It is believed that metal ions and temperature changes affect the structure and permeability of the cell wall and membrane, allowing DNA molecules to pass through. Due to their unstable cell walls, competent *E. coli* cells are fragile and therefore must be treated carefully.

The number of cells transformed per 1 microgram (µg) of DNA is known as the transformation efficiency. In practice, much smaller amounts of DNA are used (5 to 100 nanograms, ng) since excessive DNA (>100 ng) inhibits the transformation process. For example, say 10 nanograms (0.01 microgram) of DNA was used to transform cells that were in a final volume of 1 ml. Assume 0.1 ml (100 µg) of these cells were plated on agar medium such that only the cells that acquired the foreign DNA could grow. This procedure is called selection. After incubation (in this

Bacterial Transformation

example) 100 colonies were found on the plate. Realizing that each colony originally grew from **one** transformed cell, the transformation efficiency in this example is 10^5 (outlined in Figure 1). In research laboratories, transformation efficiencies generally range from 1 x 10^5 to 1 x 10^8 cells per microgram of DNA. Special procedures can produce cells having transformation efficiencies approaching 10^{10}.

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol at recovery (ml)}}{\text{vol plated (ml)}} = \text{Number of transformants per } \mu\text{g}$$

Specific example:

$$\frac{100 \text{ transformants}}{0.01\ \mu\text{g}} \times \frac{1 \text{ ml}}{0.1 \text{ ml}} = 100{,}000\ (1 \times 10^5) \text{ transformants per } \mu\text{g}$$

Figure 1:
Bacterial Transformation Efficiency Calculation

Transformation is never 100% efficient. Approximately one in every 10,000 cells successfully incorporates exogenous DNA. However, based on the large number of cells in an average sample (typically 1 x 10^9), only a small number must be transformed to obtain visible colonies on an agar plate.

This concept can be demonstrated by plating the same volume of recovered cells on selective and nonselective agar medium. The nonselective bacterial agar plates will be covered heavily with untransformed cells, forming a "lawn", in contrast to individual colonies obtained on the selective agar plate. Transformed cells will grow on selective medium that contains an antibiotic.

To ferry foreign genes into bacteria, plasmids are usually used. Plasmids are self-replicating extrachromosomal, double-stranded circular DNA molecules found in many strains of bacteria. Many plasmids contain genes that provide resistance to various antibiotics, including tetracycline, kanamycin, and ampicillin (amp). Ampicillin is a derivative of penicillin that inhibits bacterial growth by interfering with the synthesis of bacterial cell walls. The product of the ampicillin resistance gene is the enzyme β-lactamase. This enzyme is secreted by transformed cells into the surrounding medium, where it destroys ampicillin. Due to this extracellular secretion, cells that are not transformed are able to undergo limited growth in the zones surrounding transformed, antibiotic-resistant cells. Colonies consisting of these untransformed cells are called "satellites", since they only appear around larger colonies of transformed cells. Larger plating volumes and longer incubation times increase the number of satellite colonies.

Plasmids naturally exist as supercoiled molecules. The two strands of DNA in the supercoiled molecule wind around each other to produce a condensed, entangled structure when compared to relaxed (non-supercoiled) DNA (Figure 2). Competent *E. coli* cells are sensitive to the

The Experiment

Bacterial Transformation

conformation of the DNA they will accept. Supercoiled DNA gives the highest transformation efficiencies.

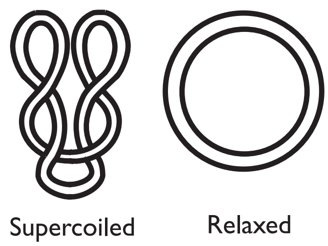


Figure 2: Supercoiled and circular forms of plasmid DNAs

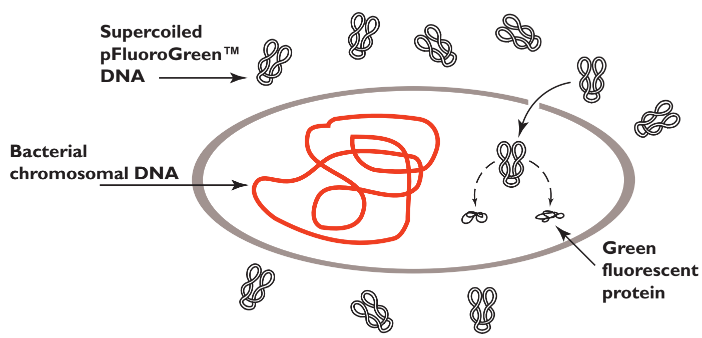


Figure 3:
Overview of transformation and protein expression.

OVERVIEW OF THE GFP EXPRESSION SYSTEM

In this experiment, the goal is to express fluorescent proteins (gfp) in transformed bacterial cells (Figure 3). To begin this process, there must be a means of "turning on" the cloned GFP gene in the recombinant plasmid. In order to have an "off/on" switch for controlling expression, the gene is placed under the control of a DNA sequence known as a "promoter".

A promoter is a sequence of DNA that typically occurs just in front ("upstream") of the DNA coding sequence (the sequence that specifies the amino acid sequence for a protein). The chromosome of the host bacterial strain used in this experiment has been genetically engineered to contain the gene for RNA polymerase, which is under control of the lac promoter, and can be turned on (induced) by the presence of a small molecule called IPTG (*isopropyl-beta-D-thiogalactopyranoside*). IPTG binds to and inactivates an inhibitor protein known as the lac repressor.

The sequence of events required to turn on expression of gfp is as follows:

Bacterial Transformation

1. Cells are grown in the presence of IPTG (to turn on the lac promoter. IPTG binds and releases the lac repressor that binds to the lac promoter (lac) upstream of the RNA polymerase gene. The release of the inhibitor allows the RNA polymerase to be produced from the *E. coli* genome.

2. The RNA polymerase, in turn, recognizes the promoter on the plasmid enabling production of large quantities of the fluorescent protein. In summary, a strong promoter, combined with an active RNA polymerase, allows for very high levels of gfp mRNA (and thus protein expression) in the transformed cells.

Experiment Overview

BEFORE YOU START THE EXPERIMENT

1. Read all instructions before starting the experiment.

2. Write a hypothesis that reflects the experiment and predict experimental outcomes.

EXPERIMENT OBJECTIVE:

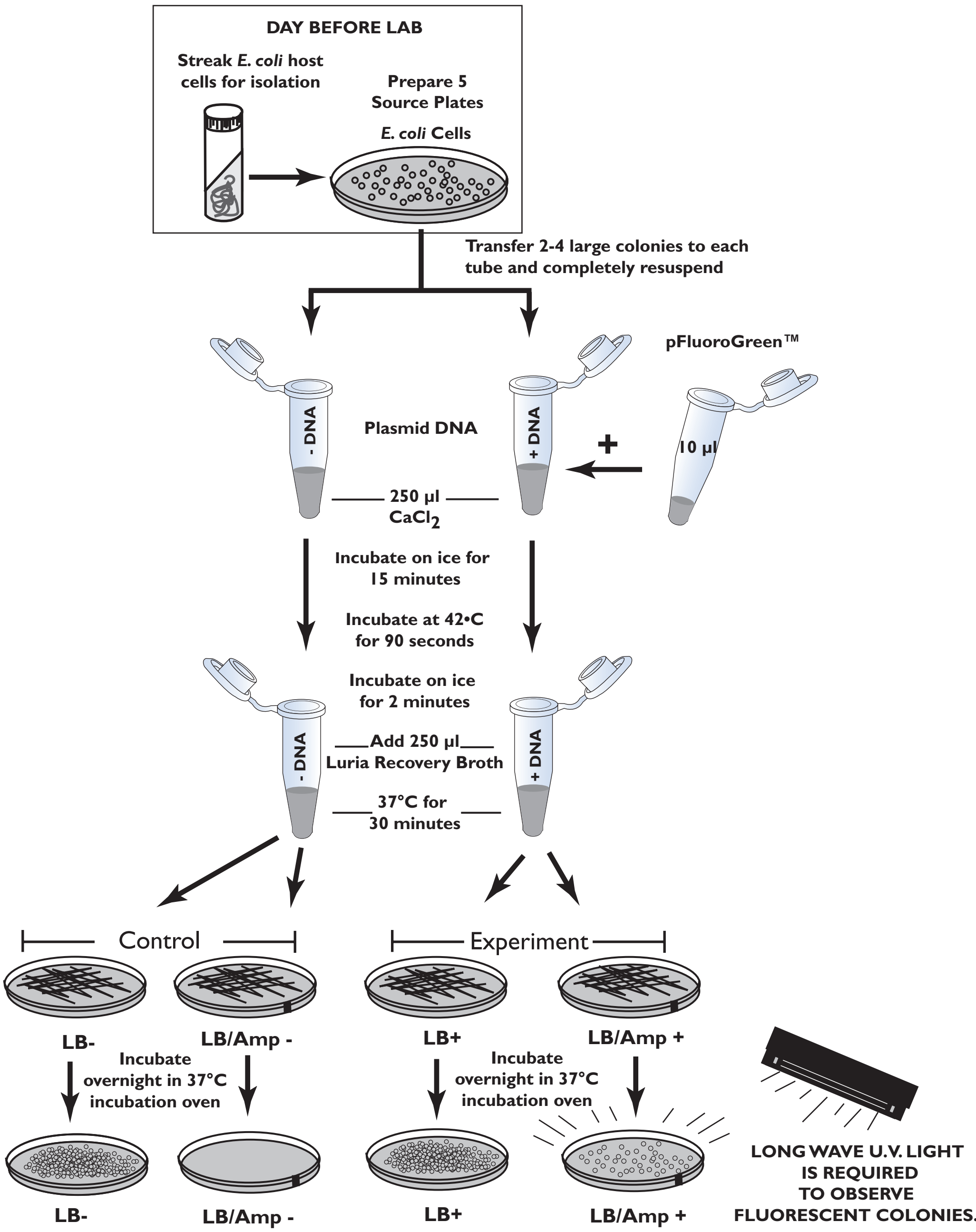
The objective of this experiment module is to develop an understanding of the biological process of bacterial transformation by the pFluoroGreen™ plasmid DNA. This experiment enables the students to observe the acquired phenotypic trait of green fluorescent protein exhibited by transformed bacterial cells.

BRIEF DESCRIPTION OF EXPERIMENT:

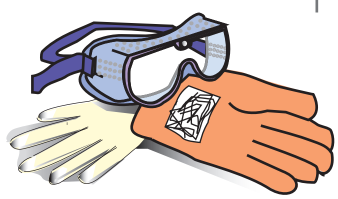
In this experiment, you will transform a strain of competent *E. coli* which has no antibiotic resistance with supercoiled plasmid DNA which has a gene for antibiotic resistance. The plasmid produces the green fluorescent protein, because in addition to the antibiotic resistance gene, it contains the gfp gene known as pFluoroGreen™.

Bacterial cells will be selected for the presence of plasmid by plating them on agar medium containing ampicillin. Only bacterial cells that take up the plasmid will survive selection on ampicillin agar plates and will produce green fluorescent colonies which will be visible under long wave U.V. Light. The transformation efficiency will then be estimated.

Experiment Overview



Laboratory Safety



Important READ ME!

Transformation experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicilllin, ampicillin, kanamycin or tetracycine should not participate in this experiment.

1. Gloves and goggles should be worn routinely as good laboratory practice.

2. Exercise extreme caution when working with equipment which is used in conjunction with the heating and/or melting of reagents.

3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.

4. The *E. coli* bacteria used in this experiment is not considered pathogenic. Although it is rarely associated with any illness in healthy individuals, it is good practice to follow simple safety guidelines in handling and disposal of materials contaminated with bacteria.

5. Properly dispose materials after completing the experiment:

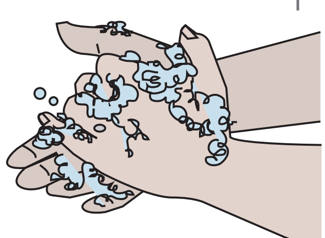
   A. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.

   B. All materials, including petri plates, pipets, transfer pipets, loops and tubes, that come in contact with bacteria should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:

   - Autoclave at 121° C for 20 minutes.
     Tape several petri plates together and close tube caps before disposal. 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 or agar from spilling into the sterilizer chamber.

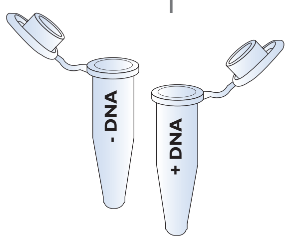
   - Soak in 10% bleach solution.
     Immerse petri plates, open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves and goggles when working with bleach.

6. Wear gloves, and at the end of the experiment, wash hands thoroughly with soap and water.



Transformation of *E.coli*

SETTING UP THE TRANSFORMATION AND CONTROL EXPERIMENT



1. Label one microcentrifuge tube "+ DNA". This will be the transformation tube with pFG plasmid DNA.

2. Label a second microcentrifuge tube "– DNA" . This will be the experimental control tube without pFG plasmid DNA.

3. Using a sterile 1 ml pipet, add 250 µl (0.25 ml) of ice cold $CaCl_2$ solution to each tube.

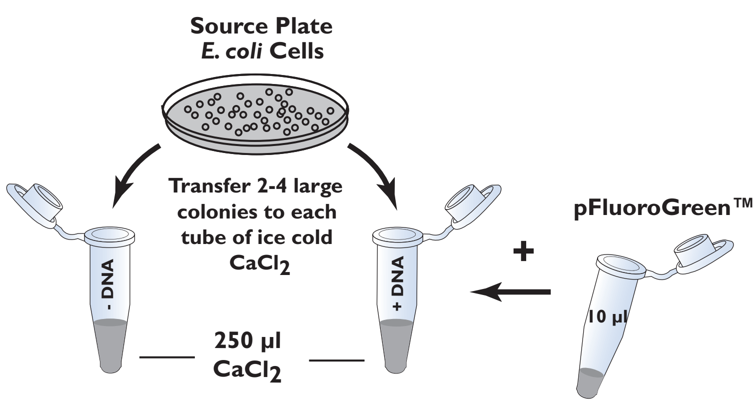
4. As done in research laboratories, pick colonies from the source plate of *E. coli* cells. Use the following method for each of the test tubes labeled "+ DNA" and "– DNA":

   - Use a sterile toothpick to transfer several colonies (2-4 colonies, approximately 2-4 mm in size) from the source plate to the test tube.
   - Between your fingers, twist the toothpick vigorously and up and down in the cold $CaCl_2$ solution to dislodge the cells.

Reminder

"+" Contains plasmid DNA

"-" Does not contain plasmid DNA



5. In both tubes, suspend the cells completely by tapping or vortexing.

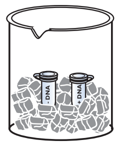
6. To the tube labeled "+ DNA", add the following:

   - 10 µl of pFluoroGreen™ (from tube labeled "pFG")

Avoid scraping up agar when transferring the cells from the source plate to the tubes with calcium chloride solution. It is important that the cells are resuspended in the calcium chloride solution and is not left on the toothpick or on the wall of the tube.

Proper experimental technique is critical - the plasmid DNA (pFluoroGreen™) must be added directly into the cell suspension.

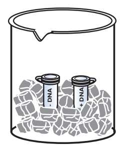
Transformation of *E.coli*



7. Incubate the two tubes on ice for **15 minutes**.

8. Place both transformation tubes at **42°C for 90 seconds**.

   This heat shock step facilitates the entry of DNA in bacterial cells.



9. Return both tubes immediately to the ice bucket and incubate for **two (2) minutes**.

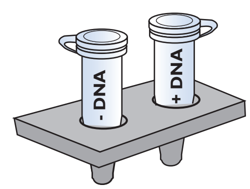
10. Using a sterile pipet, add **250 µl** of Luria Recovery Broth to each tube and mix.

11. Incubate the cells for **30 minutes** in a 37°C waterbath for a recovery period.

12. While the tubes are incubating, label 4 agar plates as indicated below. Write on the bottom or side of the petri plate.

    - Label one unstriped plate: LB -
    - Label one unstriped plate: LB + (pFG plasmid)
    - Label one striped plate: LB / Amp -
    - Label one striped plate: LB / Amp + (pFG plasmid)
    - Put your initials or group number on all the plates.

13. After the recovery period, remove the tubes from the water bath and place them on the lab bench. Proceed to plating the cells for incubation.



Quick Reference:

DNA and competent cells are combined in a suspension. After the cells have incubated with the DNA, growth medium (recovery broth) is added. Bacterial cells continue to grow through the recovery process, during which time the cell wall is repaired. Cells recover and begin to express the antibiotic resistance gene.

Transformation of *E.coli*

Reminder

"+" Contains plasmid DNA

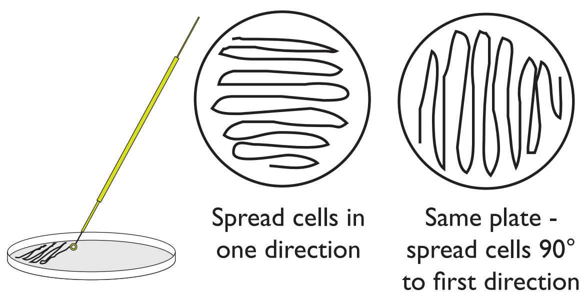
"-" Does not contain plasmid DNA

PLATING THE CELLS

Plating cells from the tube labeled "- DNA" (Control Experiment):

14. Use a sterile 1 ml pipet to transfer recovered cells from the tube labeled " - " to the middle of the following plates:

- 0.25 ml to the plate labeled LB -
- 0.25 ml to the plate labeled LB / AMP -



Spread cells in one direction

Same plate - spread cells 90° to first direction

15. Spread the cells over the entire plater with a sterile inoculating loop (see Figure at left).

16. Cover both plates and allow the liquid to be absorbed.

To avoid contamination when plating, do not set the lid down on the lab bench -- Lift the lid of the plate only enough to allow spreading. Be careful to avoid gouging the loop into the agar.

Plating cells from the tube labeled "+ DNA"

17. Use a sterile 1 ml pipet to transfer recovered cells from the tube labeled "+ DNA" to the middle of the following plates:

- 0.25 ml to the plate labeled LB + (pFG plasmid)
- 0.25 ml to the plate labeled LB / Amp + (pFG plasmid)

18. Spread the cells with a sterile inoculating loop in the same manner as step 15 .

19. Cover the plate and allow the liquid to be absorbed (approximatrly 15-20 minutes).

Reminder: Follow proper procedures for disposal of contaminated materials.

Transformation of *E.coli*

Reminder:
Follow proper procedures for disposal of contaminated materials.

PREPARING PLATES FOR INCUBATION

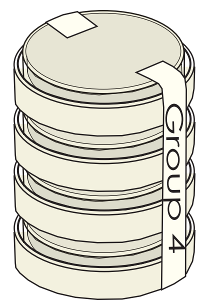
20. Stack your group's set of plates on top of one another and tape them together. Put your initials or group number on the taped set of plates.

    The plates should be left in the upright position to allow the cell suspension to be absorbed by the agar.

21. Place the set of plates in a safe place designated by your instructor.

22. After the cell suspension is absorbed by the agar, you or your instructor will place the plates in the **<u>inverted</u>** position (agar side on top) in a 37°C bacterial incubation oven for overnight incubation (15-20 hours).

    If the cells have not been absorbed into the medium, it is best to incubate the plates upright. The plates are inverted to prevent condensation on the lid, which could drip onto the culture and may interfere with experimental results.



VIEWING PLATES AFTER INCUBATION

23. Darken the room and use a long wave U.V. light to visualize the transformed cells that will glow as green due to the expression of the green fluorescent protein.

    To visualize the fluorescent colonies, the long wave U.V. light (EDVOTEK cat. # 969 recommended) can be held underneath the plates in a darkened room.

24. Proceed to analyzing your results.

Experiment Results and Analysis

LABORATORY NOTEBOOK RECORDINGS:

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

During the Experiment:

- Record (draw) your observations, or photograph the results.

Following the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

ANSWER THESE QUESTIONS BEFORE ANALYZING YOUR RESULTS.

1. On which plate(s) would you expect to find bacteria most like the original non-transformed *E. coli* cells? Explain.
2. On which plate(s) would you find only genetically transformed bacterial cells? Explain.
3. What is the purpose of the control plates? Explain the difference between each and why it is necessary to run each.
4. Why would one compare plates LB/amp- and LB/amp+?

Continued

Experiment Results and Analysis

Data Collection

5. Observe the results you obtained on your transformation and control plates.

   Transformation Plates: + DNA

   - LB +
   - LB/Amp +

   Control Plates: - DNA

   - LB -
   - LB/Amp -

6. Draw and describe what you observe. For each of the plates, record the following:

   - How much bacterial growth do you observe? Determine a count.
   - What color are the bacteria?
   - Why do different members of your class have different transformation efficiency values?
   - If you did not get any results, what factors could be attributed to this fact?

The Experiment

Experiment Results and Analysis

DETERMINATION OF TRANSFORMATION EFFICIENCY

Transformation efficiency is a quantitative determination of how many cells were transformed per 1 µg of plasmid DNA. In essence, it is an indicator of how well the transformation experiment worked.

You will calculate the transformation efficiency from the data you collect from your experiment.

1. Count the number of colonies on the plate with ampicillin that is labeled:

   LB / Amp +

   A convenient method to keep track of counted colonies is to mark the colony with a lab marking pen on the outside of the plate.

2. Determine the transformation efficiency using the formula:

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol at recovery (ml)}}{\text{vol plated (ml)}} = \text{Number of transformants per } \mu\text{g}$$

Example:

Assume you observed 40 colonies:

$$\frac{40 \text{ transformants}}{0.05\ \mu\text{g}} \times \frac{0.5 \text{ ml}}{0.25 \text{ ml}} = 1600\ (1.6 \times 10^3) \text{ transformants per } \mu\text{g}$$

Quick Reference for Expt. 223

50 ng (0.05 mg) of DNA is used.

The final volume at recovery is 0.50 ml.
The volume plated is 0.25 ml.

Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. Exogenous DNA does not passively enter *E. coli* cells that are not competent. What treatment do cells require to be competent?
2. Why did the recovery broth used in this experiment not contain ampicillin?
3. What evidence do you have that transformation was successful?
4. What are some reasons why transformation may not be successful?
5. What is the source of the fluorescence?

Instructor's Guide

Notes to the Instructor:

IMPORTANT READ ME!

Transformation experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicilllin, ampicillin, kanamycin or tetracycine should not participate in this experiment.

ORGANIZING AND IMPLEMENTING THE EXPERIMENT

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students.

The guidelines that are presented in this manual are based on ten laboratory groups consisting of two, or up to four students. The following are implementation guidelines, which can be adapted to fit your specific set of circumstances. If you do not find the answers to your questions in this section, a variety of resources are available at the EDVOTEK web site. In addition, Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call 1-800-EDVOTEK for help from our knowledgeable technical staff.

Day 1: (Prior to the Lab)

- Prepare agar plates
- Prepare *E. coli* Cells (overnight incubation).
- Dispense the DNA and control buffer

Day 2: (Day of Lab Experiment)

- Equilibrate water baths at 37°C and 42°C; incubation oven at 37°C
- Students transform cells and plate for overnight incubation.

Day 3: (Day after Lab Experiment)

- Students observe transformants and controls
- Students calculate transformation efficiency
- Follow clean up and disposal procedures as outlined in the Laboratory Safety section.

Notes to the Instructor:

NATIONAL CONTENT AND SKILL STANDARDS

By performing this experiment, students will develop skills necessary to do scientific inquiry, learn new techniques using several types of biotechnology equipment, and will learn standard procedures used in transformation. Analysis of the experiments will provide students the means to transform an abstract concept into a concrete explanation.

APPROXIMATE TIME REQUIREMENTS

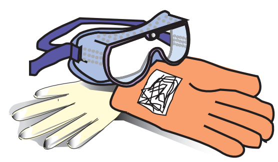
1. Preparation of *E. coli*: plate for individual colonies and incubate at 37°C for 16 to 24 hours before the laboratory (overnight incubation).
2. Preparation of agar plates: plates can be prepared several days in advance and stored inverted (agar side on top) in the refrigerator. Preparation requires approximately 1 hour.
3. Dispensing the DNA and control buffer: This can be done the day before the lab and stored in the refrigerator. Requires approximately 30 minutes.
4. Equilibration of equipment: On the day of the experiment, allow ample time for the equilibration of the water baths at 37°C and 42°C and a bacterial incubation oven at 37°C .
5. Transformation and plating: Each group will perform the transformation experiment and plate four sets of bacterial cells. These procedures require approximately 50 minutes.
6. Overnight incubation: Incubate plates approximately 15-20 hours at 37°C. Additional colonies will also appear between 24 - 48 hours at room temperature.

LABORATORY NOTEBOOKS

It is highly recommended that students maintain a laboratory notebook to formulate hypotheses and to record experimental procedures and results.

- EDVOTEK Cat. # 1401, Laboratory DataBook is recommended.
- Guidelines for keeping a laboratory notebook is available at the EDVOTEK web site.

Pre-Lab Preparations



Wear Hot Gloves and Goggles during all steps involving heating.

POUR AGAR PLATES
(Prior to the Lab experiment)

- For optimal results, prepare plates two days prior to plating and set aside the plates inverted at room temperature.
- If they are poured more than two days before use, they should be stored inverted in the refrigerator. Remove the plates from the refrigerator and store inverted for two days at room temperature before use.

Heat the ReadyPour™ Medium

1. Equilibrate a water bath at 60°C for step 5 below.
2. Loosen, but **do not** remove, the cap on the ReadyPour medium bottle to allow for the venting of steam during heating.

   Caution: Failure to loosen the cap prior to heating or microwaving may cause the ReadyPour medium bottle to break or explode.

3. Squeeze and vigorously shake the plastic bottle to break up the solid agar into chunks
4. Heat the bottle of ReadyPour medium by one of the methods outlined below. When completely melted, the amber-colored solution should appear free of small particles.

   A. Microwave method:

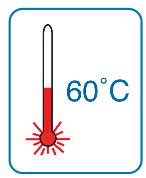
   - Heat the bottle on High for two 30 second intervals.
   - Using a hot glove, swirl and heat on High for an additional 25 seconds, or until all the ReadyPour medium is dissolved.
   - Using a hot glove, occasionally swirl to expedite melting.

   B. Hot plate or burner method:

   - Place the bottle in a beaker partially filled with water.
   - Heat the beaker to boiling over a hot plate or burner.
   - Using a hot glove, occasionally swirl to expedite melting.

5. Allow the melted ReadyPour medium to cool. Placing the bottle in a 60°C water bath will allow the agar to cool, while preventing it from prematurely solidifying.

   When the ReadyPour™ medium reaches approximately 60°C, the bottle will be warm to the touch but not burning hot.



Pre-Lab Preparations

Label ("Stripe") the Plates

6. Use a lab marker to "stripe" the sides of twenty (20) 60 x15 mm petri dishes. This will provide an easy method of differentiating between plates with ampicillin and plates without ampicillin.
   - Open one sleeve of 20 plates and stack the plates neatly.
   - Start the marker at the bottom of the stack and move the marker vertically to the top plate to "stripe" the sides of the 20 plates.
   - These plates will be used for medium with ampicillin.
   - Do not stripe the second sleeve of plates. These will be the control plates.

Pour the Plates

Note: The single bottle of agar medium will be used to make the 5 source plates, 20 control plates and 20 Amp plates.

7. Pour 5 large *E. Coli* source plates

   Use a 10 ml pipet and pipet pump to pour the 5 **large** plates, 10 ml each, with the ReadyPour medium without ampicillin.

8. Add the IPTG to the cooled Ready Pour medium. Recap the bottle and swirl to mix the IPTG.

9. Pour 20 control plates
   (no ampicillin, no-stripe):

   Use a fresh 10 ml pipet (or the same pipet from step 7) and pipet pump to pour the 20 control plates, 5 ml each with ReadyPour medium **without** ampicillin.

Quick Reference: Pouring Agar Plates

- Use a sterile 10 ml pipet with a pipet pump to transfer the designated volume of medium to each petri plate. Pipet carefully to avoid forming bubbles.
- Rock the petri plate back and forth to obtain full coverage.
- If the molten medium contains bubbles, they can be removed by passing a flame across the surface of the medium.
- Cover the petri plate and allow the medium to solidify.

Pre-Lab Preparations

Add reagents to medium which has been cooled. Hot medium will cause reagents, such as ampicillin and IPTG, to rapidly decompose.

10. Add the entire amount of ampicillin powder to the remaining molten ReadyPour medium in the bottle.

11. Recap the bottle and swirl to completely mix the ampicillin.

12. Pour 20 transformation plates (with ampicillin, striped plates):

    Use a fresh 10 ml pipet to pour the twenty (20) striped plates, 5 ml each.

13. Allow the agar to cool and resolidify.

Note: If plates will be used within two days, store in a sealable plastic bag so the plates will not dry out. Store at room temperature, inverted.

If you have extra sterile petri plates on hand, use any remaining medium to pour additional plates for the optional activity described on page 28.

Reminder: Follow proper procedures for disposal of contaminated materials.

Summary of Poured Plates:

5 source plates - large plates:
10 ml each - ReadyPour medium

20 control plates - small no stripe plates: 5 ml each - ReadyPour medium with IPTG (no ampicillin)

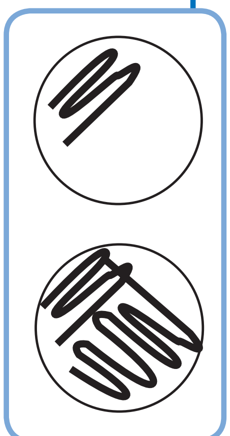
20 transformation plates - small striped plates: 5 ml each - ReadyPour medium with IPTG and ampicillin

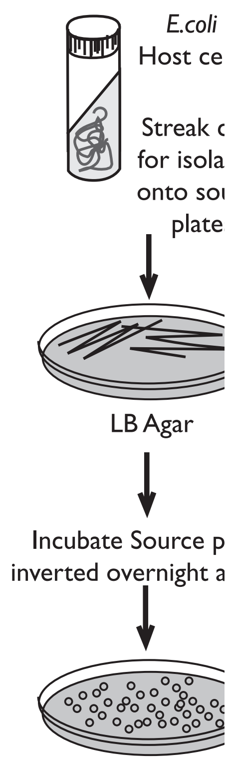
Pre-Lab Preparations

DAY BEFORE THE LAB

This experiment requires preparation of isolated *E.coli* host transformation colonies 16-24 hours before the laboratory experiment, so plan accordingly.

Important: Do not prepare source plates more than 24 hours before the experiment. Old source plates will compromise the success of the transformation experiment.





1. With a sterile loop, streak a small clump of *E.coli* cells from the slant and streak for isolation onto a source plate (see figures at left).

   Do not use plates containing ampicillin.

2. Repeat procedures as described for 4 additional plates. You will have a total of 5 source plates.

3. Incubate plates inverted overnight (16-24 hours) in an incubation oven at 37°C.

Pre-Lab Preparations

DAY OF THE LAB:

1. Dispense 1 ml of $CaCl_2$ into microcentrifuge tubes for each of the 10 groups and place on ice.

2. Dispense 1.5 ml of Luria Broth Medium ("Recovery broth") into tubes for each of the 10 groups and keep at room temperature.

   Alternatively, the Luria Broth Medium bottle can be placed at a class-room pipeting station for students to share.

Preparation of DNA

3. Label 10 tubes "pFG" (pFluoroGreen™).

4. Place the tube of supercoiled pFluoroGreen™ on ice.

5. Before dispensing the DNA, tap the tubes until all the sample is at the tapered bottom of the tube.

6. Using an automatic micropipet, dispense 12 µl of the appropriate supercoiled DNA to each of the microtest tubes labeled "pFG" (pFluoroGreen™).

   Note: Students will use 10 µl for the transformation experiment.

7. Cap the tubes and place them on ice.

Each Group Requires:

- Sharing - one of 5 *E. coli* source plates
- 1 tube (1 ml) $CaCl_2$
- 1 tube pFluoroGreen™ plasmid DNA
- 2 striped plates
- 2 unstriped plates
- 4 sterile 1ml pipets
- 2 sterile inoculating loops
- 1 sterile tube (1.5ml) "Recovery broth"

Classroom Equipment:

- Water bath(s)
- Incubation Oven

Optional Activity:

Do not discard the tubes containing transformed bacteria. After plating an aliquot on selection plates, add an additional 50 µl of recovery broth to the tubes and set them in a rack. Leave on the lab bench overnight. If for some reason, transformants do not grow on the selection plates, the remaining cells can be plated as outlined below:

1. Collect the bacterial cell pellet by centrifugation in a microcentrifuge. If a microcentrifuge is not available, let the bacteria collect by gravity and do not disturb.
2. Remove all except 50 µl of medium (supernatant, top layer).
3. Resuspend the cell pellet in remaining medium.
4. Spread the entire contents of the tube on selection medium.
5. Incubate the plate as before, 16-24 hours in a 37°C incubation oven.
6. Follow proper procedures for disposal of contaminated materials.

Instructor's Guide

Experiment Results and Analysis

ANSWER THESE QUESTIONS BEFORE ANALYZING YOUR RESULTS.

1. **On which plate(s) would you expect to find bacteria most like the original non-transformed *E. coli* cells? Explain.**

   The bacteria on the plate labeled LB- would be identical to the non-transformed starter *E. coli* source plate because they did not have any plasmid added to them, but were re-plated onto an LB plate.

2. **On which plate(s) would you find only genetically transformed bacterial cells? Explain.**

   The bacteria growing on the plate labeled LB/amp+ would be the genetically transformed cells since only those cells that have taken up the plasmid which expresses the ampicillin resistance gene and the fluorescent gene(s) will survive on the plates which contain ampicillin.

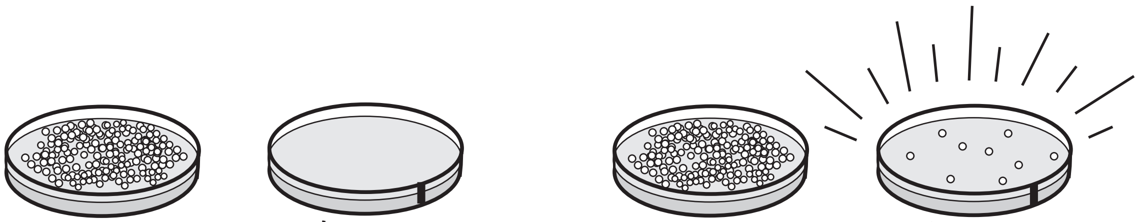
3. **What is the purpose of the control plates? Explain the difference between each and why it is necessary to run each.**

   Control plates help interpret the experimental results. There are three control plates in this experiment. The control plate that is labeled LB/amp- shows that cells without the plasmid which contains the fluorescent gene will not grown in the presence of ampicillin. The control plate labeled LB- shows that the cells without the plasmid are able to grow on agar without ampicillin. The control plate LB+ shows that the cells were not damaged during the transformation process and therefore are able to grow on agar plates that do not contain ampicillin.

4. **Why would one compare plates LB/amp- and LB/amp+?**

   Cells not treated with the plasmid will not grow on the plate with ampicillin (LB/amp-) because they are not expressing the ampicillin resistance gene. However, cells treated with the plasmid will grow on the LB/amp+ plate because they are expressing the ampicillin resistance gene.

Experiment Results and Analysis



LB- plated with control cells (no DNA)	LB/Amp- plated with control cells (no DNA)	LB+ plated with cells pFluoroGreen™	LB/Amp+ plated with cells pFluoroGreen™
Result: No fluorescent cells visible. White colonies. May look like a smeared layer of cells.	Result: No growth	Result: white colonies. May look like a smeared layer of cells.	Result: individual colonies that will fluoresce when exposed to long wave U.V. light.
Demonstrates: Host bacterial cells are viable in the absence of ampicillin.	Demonstrates: Cells are sensitive to ampicillin. Without pFluoroGreen™, they are not ampicillin-resistant.	Demonstrates: Untransformed and transformed cells are viable in the absence of ampicillin. The majority of the growth are the untransformed cells and therefore overshadow the transformed fluorescent cells.	Demonstrates: Transformation of cells resistant to ampicillin due to the uptake of pFluoroGreen™. Host bacterial cells that are not transformed will not grow in the presence of ampicillin.

Study Questions and Answers

1. **Exogenous DNA does not passively enter *E. coli* cells that are not competent. What treatment do cells require to be competent?**

   E. coli can be artificially induced to enter competency when they are treated with the chloride salts of the metal cations calcium, magnesium and rubidium. In addition, sudden cycles of heat and cold help to bring about competency. The metal ions and temperature changes affect the structure and permeability of the cell wall and membrane so that DNA molecules can pass through.

2. **Why did the recovery broth used in this experiment not contain ampicillin?**

   The recovery broth did not contain ampicillin in order to give the cells a chance to repair themselves and to express their newly acquired genes without an immediate challenge.

3. **What evidence do you have that transformation was successful?**

   A successful transformation will show colonies on the plate labeled LB/amp+ and should fluoresce under long UV light.

4. **What are some reasons why transformation may not be successful?**

   Unsuccessful transformations could be the result of many things, including: 1) not adding the plasmid to the host cells in the +DNA tube, or 2) not adding a colony of bacteria to the +DNA tube, and 3) improper timing of the heat shock step.

5. **What is the source of the fluorescence?**

   The source of fluorescence comes from the green fluorescent protein encoded by the plasmid.

Material Safety Data Sheet

EDVOTEK®

May be used to comply with OSHA's Hazard Communication Standard. 29 CFR 1910.1200 Standard must be consulted for specific requirements.

IDENTITY (As Used on Label and List): Ampicillin

Note: Blank spaces are not permitted. If any item is not applicable, or no information is available, the space must be marked to indicate that.

Section I

Manufacturer's Name: **EDVOTEK, Inc.**

Address (Number, Street, City, State, Zip Code): **14676 Rothgeb Drive, Rockville, MD 20850**

Emergency Telephone Number: **(301) 251-5990**

Telephone Number for information: **(301) 251-5990**

Date Prepared: 07/01/03

Signature of Preparer (optional):

Section II - Hazardous Ingredients/Identify Information

Hazardous Components [Specific Chemical Identity; Common Name(s)]	OSHA PEL	ACGIH TLV	Other Limits Recommended	% (Optional)
Ampicillin CAS# 7177-48-2	No data			

Section III - Physical/Chemical Characteristics

Boiling Point	No data	Specific Gravity (H_2O = 1)	No data
Vapor Pressure (mm Hg.)	No data	Melting Point	No data
Vapor Density (AIR = 1)	No data	Evaporation Rate (Butyl Acetate = 1)	No data

Solubility in Water: Slightly soluble

Appearance and Odor: Odorless, white crystaline powder

Section IV - Physical/Chemical Characteristics

N.D. = No data

Flash Point (Method Used)	Flammable Limits	LEL	UEL
No data		N.D.	N.D.

Extinguishing Media: Dry chemical, carbon dioxide, water spray or regular foam

Special Fire Fighting Procedures: Move container from fire area if possible. Do not scatter spilled material with water streams.

Unusual Fire and Explosion Hazards: Avoid breathing vapors.

Section V - Reactivity Data

Stability			Conditions to Avoid
	Unstable		
	Stable	X	Incompatibles

Incompatibility: Strong oxidizers

Hazardous Decomposition or Byproducts: Toxic oxides of carbon, nitrogen and sulfur

Hazardous Polymerization			Conditions to Avoid
	May Occur		
	Will Not Occur	X	Incompacticles

Section VI - Health Hazard Data

Route(s) of Entry: Inhalation? Yes Skin? Yes Ingestion? Yes

Health Hazards (Acute and Chronic): Sensitizers may result in allergic reaction

Carcinogenicity: No data NTP? IARC Monographs? OSHA Regulation?

Signs and Symptoms of Exposure: Repeated exposure may result in sensitization and possible anaphylactic shock.

Medical Conditions Generally Aggravated by Exposure: No data

Emergency First Aid Procedures: Ingestion: Allergic symptoms. Eyes/Skin: Flush with water Inhalation: Move to fresh air

Section VII - Precautions for Safe Handling and Use

Steps to be Taken in case Material is Released for Spilled: Wear suitable protective clothing. Sweep up and place in suitable container for later disposal. Do not flush spilled material down sink.

Waste Disposal Method: Observe all federal, state, and local regulations

Precautions to be Taken in Handling and Storing: Keep away from incompatible substances

Other Precautions: None

Section VIII - Control Measures

Respiratory Protection (Specify Type):

Ventilation				
	Local Exhaust	Yes	Special	None
	Mechanical (General)	No	Other	None

Protective Gloves: Yes | Eye Protection: Splash or dust proof

Other Protective Clothing or Equipment: Eye wash

Work/Hygienic Practices: Wear protective clothing and equipment to prevent contact.

Material Safety Data Sheet

EDVOTEK®

May be used to comply with OSHA's Hazard Communication Standard. 29 CFR 1910.1200 Standard must be consulted for specific requirements.

IDENTITY (As Used on Label and List): IPTG

Note: Blank spaces are not permitted. If any item is not applicable, or no information is available, the space must be marked to indicate that.

Section I

Manufacturer's Name: **EDVOTEK, Inc.**

Address (Number, Street, City, State, Zip Code): **14676 Rothgeb Drive, Rockville, MD 20850**

Emergency Telephone Number: **(301) 251-5990**

Telephone Number for information: **(301) 251-5990**

Date Prepared: 07/01/03

Signature of Preparer (optional):

Section II - Hazardous Ingredients/Identify Information

Hazardous Components [Specific Chemical Identity; Common Name(s)]	OSHA PEL	ACGIH TLV	Other Limits Recommended	% (Optional)
Not applicable				

Section III - Physical/Chemical Characteristics

Boiling Point	None	Specific Gravity (H_2O = 1)	Unknown
Vapor Pressure (mm Hg.)	None	Melting Point	109-110C
Vapor Density (AIR = 1)	None	Evaporation Rate (Butyl Acetate = 1)	None

Solubility in Water: Moderate

Appearance and Odor: White crystals/slight odor thiophenol

Section IV - Physical/Chemical Characteristics

Flash Point (Method Used)	Flammable Limits	LEL	UEL
Unknown			

Extinguishing Media: Water, carbon dioxide, or dry chemical

Special Fire Fighting Procedures: None

Unusual Fire and Explosion Hazards: None

Section V - Reactivity Data

Stability			Conditions to Avoid
	Unstable		
	Stable	X	None

Incompatibility: Strong oxdizing agents

Hazardous Decomposition or Byproducts: Carbon dioxide and sulfur dioxide

Hazardous Polymerization			Conditions to Avoid
	May Occur		
	Will Not Occur	X	

Section VI - Health Hazard Data

Route(s) of Entry: Inhalation? Yes Skin? Not studied Ingestion? Not studied

Health Hazards (Acute and Chronic): Toxcity has not been studied

Carcinogenicity: Unknown NTP? No data IARC Monographs? No data OSHA Regulation? No data

Signs and Symptoms of Exposure: Unknown: avoid dust

Medical Conditions Generally Aggravated by Exposure: Unknown

Emergency First Aid Procedures: External: flush with water Internal: Induce vomiting, consult physician

Section VII - Precautions for Safe Handling and Use

Steps to be Taken in case Material is Released for Spilled: Cover and sweep up with inert carrier

Waste Disposal Method: Dissolve in a combustible solvent and burn in a chemical incinerator with afterburner and scrubber, or sweep up and return inoriginal container.

Precautions to be Taken in Handling and Storing: Avoid dust store cool

Other Precautions: Information CAS #367-93-1

Section VIII - Control Measures

Respiratory Protection (Specify Type): Filter mask

Ventilation				
	Local Exhaust	Yes	Special	None
	Mechanical (General)	Yes	Other	None

Protective Gloves: Rubber or vinyl | Eye Protection: Face mask or goggles

Other Protective Clothing or Equipment: Lab apron

Work/Hygienic Practices: Avoid dust or contact with skin