Chapter 1

Spot-overlay Ames Test of Potential Mutagens

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Introduction

During the genetics section of typical introductory biology courses, students are exposed to topics such as DNA replication, mutations, transcription, and translation. While most students grasp a cursory understanding of these topics quite rapidly, they often experience difficulty understanding how these topics are inter-related or how this group of topics relates to other parts of the course, such as sections on biochemistry and metabolism. Furthermore, students in introductory biology often have difficulty developing experimental protocols and analyzing results. The spot-overlay Ames test of potential mutagens allows us to address all of these concerns in our introductory course.

Before beginning these experiments, students are asked to bring their favorite potential mutagen into class. We encourage students to be creative. They quickly develop a keen interest in the results because of the true unknown aspect of the material with which they will be working.   Furthermore, the nature of these experiments allows students to think about experimental design, including the inclusion of and interpretation of positive and negative controls. Finally, students are required to analyze their preliminary data from the first week and generate a follow-up set of experiments to conduct during the second week of this module.

This series of experiments has been used in the introductory biology course at Davidson College for several years. Students in the course generally are first or second year students. Many, though certainly not all, of the students in this course intend to become biology majors. Laboratory sections are limited to 16 students and students work in groups of four. We spend two weeks conducting the experiments and part of a third week analyzing and discussing the results. We believe this protocol represents a cost-effective means of introducing students to a host of issues related to mutagenesis and biochemistry.

Materials

Equipment

Shaking incubator at 37˚C
Water bath at 47˚C
Refrigerator at 4˚C
Micropipets of 1-20 µL, 20-200 µL, and 200-1000 µL
Sterile pipet tips
UV light box
Plastic wrap
Sun screen of different SPF values
Gloves
Biohazard bags
Autoclave

Reagents

Petri plates of Davis Minimal Agar (DMA), about 25 mL/plate
    14 plates per group of students (six for Week 1, eight for Week 2)
Tubes of top agar
    36 tubes containing 1mL each per group of students (Week 1)
    8 tubes containing 5 mL each per group of students (Week 2)
16-24 hour cultures of *S.typhimurium*, strains grown in tryptic soy broth
    1 mL culture of each strain per group of students (Week 1)
    1 mL culture of appropriate strains per group of students (Week 2)
sodium azide (NaN_3) at 0.1 µg/ µL
    500 µL per group of students (Week 1 and Week 2 if needed)
Potential mutagens prepared in advance
Sterile dH_2O or other solvents

Notes for Instructors

We ask students to bring their potential mutagens to class one week before we begin this exercise. Before the first laboratory session, solid samples are placed in small flasks with about 10 mL of water and autoclaved to sterilize the samples and solubilize them. Ethanol-based solutions and samples solubilized in ethanol are sterilized by filter sterilization through a 0.22 micron filter.

For positive controls, sodium azide and ultraviolet light can be used. Sodium azide, at concentrations of 0.1 to 10 μg per mL of overlay, is a mutagen for strains 100 and 1535. Ultraviolet light is a mutagen for strain 102. The optimal time of exposure of bacteria to UV irradiation varies depending on the intensity of the light source. Generally, exposure times of 10 to 60 seconds provide significant results. Potential mutagens that may yield interesting results include smoked meat, grilled meat, and hair coloring solutions. The maximum volume of potential mutagen that can be added to a spot-overlay is approximately 75 μL. The addition of greater volumes results in an overly diluted overlay mixture.

Because students are working with potentially dangerous bacteria, it is essential that safe laboratory practices are emphasized. We stress the importance of wearing gloves, disposing of materials in biohazard bags, and washing thoroughly before leaving the laboratory. With these precautions, we have experienced no safety problems in our courses.

In addition to discussing DNA replication and mutagenesis during these laboratory exercises, we also use these laboratory sessions to re-address aspects of biochemistry and to emphasize issues of experimental design. Discussions of the his⁻ auxotrophic mutants have proven very useful in helping students grasp issues related to enzyme activity, catabolism, and biosynthetic pathways. Also, these experiments allow the students to gain a better understanding of experimental design. For instance, we emphasize that a compound is considered mutagenic if it results in a mutation rate twice that of the spontaneous mutation rate. We then discuss how we can determine the spontaneous mutation rate accurately. A series of questions can be asked, such as: If we dissolved our potential mutagen in ethanol instead of water, what would an appropriate negative control be? We also address the question of appropriate positive controls. For instance, one can discuss the merits of measuring growth on complete LB agar plates in the absence of a potential mutagen versus growth on minimal plates in the presence of a known mutagen.

Finally, we have found these experiments to be very useful in addressing issues of data interpretation. When students expose bacteria to a time course of UV irradiation, for example, they routinely observe an increase in the number of revertants with increasing exposure times. With excessive UV irradiation, however, no bacterial colonies are observed. A discussion of the possible interpretations of this result often proves quite enlightening for students.

Student Outline

Week 1: Spot-overlay Test of YFPM (your favorite potential mutagen)

[*]Modified from: Dorothy M. Maron and Bruce N. Ames. Revised methods for the *Salmonella* mutagenicity test. *Mutation Research*. Vol. 113:173-215, 1980.

Goals for this Laboratory Session
During this period, you will pour agar plates to be used in next week's laboratory session. You will become familiar with the Ames test, a world-wide standard for testing new compounds to determine if they are mutagenic. The method you will use this week was developed here at Davidson College and allows us to screen more compounds quickly and cheaply. Next week we will use the traditional method to quantify the degree of mutagenicity for selected compounds.

NEWS ITEM: April 25, 1997. Dr. Bruce Ames was awarded the Japan Prize ($210,000 cash) for his lifelong work with carcinogens. "When people ask me if I'm the 'Ames' of the Ames test, I say: 'That was so long ago, that was my father.'" Dr. Ames is professor of biochemistry and molecular biology at UC-Berkeley (for more information about Dr. Ames, see: http://mendel.berkeley.edu/Center.homepage.html) and his research is focusing on the relationship of aging, nutrition, and cancer. You can find 4 papers by his group in the April 1, 1997 issue of *Proceedings of the National Academy of Sciences*.

Background
Our environment is full of potential carcinogens (cancer-causing agents) such as UV light, industrial pollutants, pesticides, food additives, and natural products such as tobacco. These carcinogens can cause cancers because they are mutagens (chemicals that cause mutations); they can change the nucleic acid sequence of DNA. Obviously, it is important to have a rapid and inexpensive assay for testing chemicals we suspect are carcinogenic, including the large number of new synthetic chemicals being produced each year.

It is estimated that 90% of all carcinogens also are mutagens, and with this figure in mind, Bruce Ames and his colleagues developed a test in the 1970s that uses special bacteria that are very sensitive to mutagenic agents. The Food and Drug Administration (FDA) now uses the Ames test to screen many chemicals rapidly and inexpensively. Those few chemicals that appear to be mutagenic by the Ames test are tested further in animals to assess their ability to cause cancer.

Wild-type cultures of the bacterium *Salmonella typhimurium* grow in media without the addition of any amino acids. This growth is possible because they are prototrophic, which means that they have metabolic pathways for making all of their own amino acids. Each amino acid has a separate pathway for its synthesis. For example, Figure 1.1 shows the pathway for histidine

synthesis, which begins with catabolic intermediate **C** and uses nine enzymes (numbered 1 through 9 in Figure 1.1) to convert precursor **C** into histidine.

Prototrophic Bacterial Cell

Energy Source

A
B   1   3   5   7   9
C
D   (a)(b)(c)(d)(e)(f)(g)(h)(i)   **Histidine produced**
E   2   4   6   8   Protein synthesis
F
G   Cell growth

Waste Products

His⁻ Auxotrophic Bacterial Cell

Energy Source

A
B   1   3   5   7   9
C   (a)(b)(c)(d)   **Histidine supplied in medium**
D
E   2   4   6   8   Protein synthesis
F
G   Mutation in enzyme 4   Cell growth
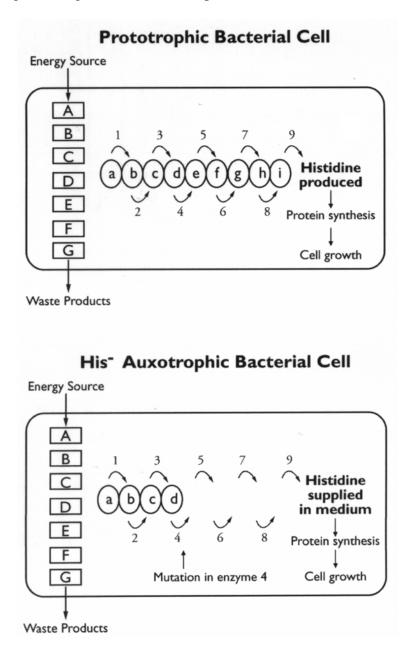
Waste Products

Figure 1.1. Schematic overview of *Salmonella typhimurium* metabolism and the effect of a nonrepaired mutation in the synthesis of histidine. (TOP) Metabolism of a normal (prototrophic) *S. typhimurium*. The catabolism (breakdown) of the food source produces precursor C which is needed for histidine synthesis. The formation of each intermediate (a-i) in the histidine synthesis pathway is catalyzed by a different enzyme (arrows 1-9). Each enzyme is a protein whose synthesis is encoded by a separate gene. The absence of any one of the nine enzymes would prevent the synthesis of histidine. (BOTTOM) Metabolism of an auxotrophic (his⁻) mutant that cannot produce its own histidine. The catabolism of food still produces

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precursor C, but enzyme 4 is not produced due to a point mutation in the encoding DNA. This defect results in the interruption of histidine synthesis because intermediate 'e' cannot be made, which prevents the rest of the enzymes from synthesizing the appropriate products. In order for this auxotroph to grow, then, histidine must be supplied to the medium.

The Ames test uses mutant strains of *Salmonella typhimurium* that cannot grow in the absence of the amino acid histidine because a mutation has occurred in a gene that encodes one of the nine enzymes used in the pathway of histidine synthesis (Figure 1.1). The mutation prevents translation of a functional enzyme, and thus the cell cannot complete the conversion of the catabolic intermediate **C** to histidine. Therefore, the Ames mutants only can grow if histidine is supplied in the growth medium. These **auxotrophic** mutants are called *histidine-dependent* or his ⁻ (pronounced hiss-minus) mutants because they depend on an external source of histidine to grow. Auxotrophs are mutant individuals that cannot make all the metabolic products that wild-type (**prototrophic**) individuals of the same species can make.

There are several different mutant strains of *S. typhimurium* that have different mutations in their DNA. We will use a variety of different mutant strains that have important distinctions that make them suitable for detection of different types of mutagens. Here is a list of the available strains that we can use and what mutation each strain carries:

- **TA 1535** has a base-pair substitution resulting in a **missense mutation** in the gene encoding the first enzyme in the histidine biosynthesis pathway. A –GGG- (proline) substitutes for a –GAG- (leucine) in the wild-type organism.
- **TA 100** contains the same mutation identified in TA 1535. It differs slightly from TA 1535 in other respects and may detect a different range of mutagens.
- **TA 1537** has a +1 frameshift mutation (**insertion of one nucleotide**) in a different gene than is mutated in 1535. A C is inserted in a run of five Cs that exists in the wild-type organism.
- **TA 1538** has a –1 frameshift mutation (**deletion of one nucleotide**) in the same gene that is mutated in TA 1537. In this strain, a C is deleted from a run of Cs that exists in the wild-type organism.
- **TA 98** contains the same mutation identified in TA 1538. It differs slightly from TA 1538 in other respects and may detect a different range of mutagens.
- **TA 102** is significantly different from the others. It has an *ochre* mutation (-TAA-), which means that it has a **nonsense mutation**, in place of the –CAA- present in the wild-type organism. Unlike the other his⁻ strains, this strain has a A:T basepair at the site of reversions. This mutation occurs in the same gene as is mutated in strain TA 1535.

In addition to the mutations listed above, there are two other traits that each of these strains exhibit. First, these mutant strains lack a DNA excision-repair mechanism that exists in wild-type bacteria and normally would repair any new mutations in the DNA that are caused by exposure to mutagens during our experiments. The result of this defect is that DNA errors are not corrected, thus enhancing the strains' sensitivity to mutagens. Second, these strains have a defective lipopolysaccharide layer in their cell wall that allows chemicals to penetrate more easily into the cell than is true with wild-type bacteria.

In summary, we have mutant strains of *Salmonella typhimurium* that cannot synthesize histidine, are very susceptible to additional mutations because they lack the normal repair mechanisms found in bacteria, and are more permeable than wild-type bacteria to external chemicals, including potential mutagens. In order for these cells to survive on a plate that lacks histidine, they must regain the ability to synthesize histidine by undergoing another mutation that corrects the original mutation that prevented the production of the missing enzyme. This type of mutation is known as a back mutation, or **reversion**, because this second mutation returns the mutant to the wild-type (prototrophic) phenotype. This reversion can happen spontaneously due to incorrect DNA replication or as the result of a mutagen. In the Ames test, mutagens are defined as compounds that result in more than double the spontaneous mutation rate. Note that a reversion (either spontaneous or caused by a mutagen) is NOT the same as a random mutation. A reversion is a specific event that happened randomly, but when it happened, we knew exactly which gene was affected.

A brief note about mutations: a mutation is any change in a DNA sequence from the original sequence of nucleic acids, and mutations happen all the time in your cells. Sometimes it is because a mutagen comes from the outside of the cell and in some manner creates changes in the DNA. Often the mutations are just errors that occur during DNA replication when cells divide. In fact, there is an average of nearly one mutation (error) in your DNA every time one cell divides. Our cells have mechanisms to repair the mutated DNA, and they usually do, but if a mistake is overlooked, the change in the DNA is carried on in future replications in the cell. This scenario represents one way that a "spontaneous" mutation can occur, because there was no obvious cause on which to blame the mutation.

To determine the number of **revertants** following exposure to a mutagen, we must have a way to differentiate the mutant strain (his⁻ auxotrophs) and the new mutants we may generate (his⁺ revertants). For this purpose, the Ames test uses a chemically defined medium, that is, a medium in which the amounts of every ingredient are known. If a his⁻ culture were placed on a chemically defined minimal agar lacking histidine, only those cells that have mutated to his⁺ (revertants), would grow and form colonies. In theory, the number of colonies that revert and grow is proportional to the mutagenicity of the test chemical.

The chemically defined medium used for the Ames test actually has a trace (growth-limiting) amount of histidine added only to the soft agar overlay. Trace amounts of histidine in the medium are necessary because some mutagenic agents react preferentially with actively replicating DNA. When his⁻ strains are plated on this medium, they grow until they run out of histidine (only 2-3 cell divisions lasting about one hour), and the result is a faint, nearly invisible lawn of growth within the overlay. Conversely, revertant bacteria should form large colonies because their growth is not limited because they can produce their own histidine. Each large colony represents one revertant bacterium and its offspring. **By definition in the Ames test, a mutagen is any chemical agent that results in more than twice the number of mutants as occur spontaneously,** and thus is potentially carcinogenic for humans.

What you have read is an overview of the theory behind the Ames test that we will use. Many chemicals in nature, however, are not carcinogenic/mutagenic until after they are consumed by an animal. One job of the liver is to detoxify harmful chemicals, but in the process

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some chemicals are converted into very potent mutagens. For this reason, all test chemicals used in the Ames test by the FDA are routinely incubated with rat liver extract in an oxygenated environment so that liver enzymes, such as oxygenases, will "activate" the chemical being screened; the "activated" chemical then is added to the bacteria. Due to the potential hazards associated with this step, we will not use any liver extracts. Instead, we will determine if various agents are mutagenic in their "unactivated" states.

Overview of the Experimental Series with the Ames Test

The ultimate goal of the laboratory series on the Ames test is to design experiments to test an unknown potential mutagen. In the first week, we will use a new variation of the Ames test that was developed at Davidson College during the summer of 1997. This new variation is called the **Spot-Overlay Assay.** It is designed to allow us to screen many different chemicals quickly and cheaply. Each group will use sodium azide as one of its potential mutagens because we know that this chemical is a powerful mutagen (positive control). The second week, we will use the standard overlay assay developed by Dr. Ames to quantitate any potential mutagens detected by the spot-overlay assay. To perform any Ames test successfully, you must be careful to maintain sterile conditions, because you want only *S. typhimurium* in your Petri dish and not other contaminating strains from the air, your fingers, lips ... you get the picture. Furthermore, you must be very careful in this laboratory series:

NOTE: THIS LAB HAS POTENTIAL HAZARDS!

1) Does "*Salmonella*" sound familiar to you? It is the bacterium that turns your stomach inside out after eating bad potato salad.

2) You will be handling potential or known carcinogenic/mutagenic materials.

YOU MUST WEAR GLOVES AT ALL TIMES WHEN HANDLING THE CHEMICALS AND BACTERIA USED IN THIS EXERCISE.

We have faith in your abilities to use your common sense and be careful what you do with test tubes, pipet tips, etc. that have come in contact with the bacteria and chemicals. Enough warnings, now on to the good stuff.

Designing Good Controls for Experiments

In your experimental designs for both weeks, the use of controls will be important, just as it is in every experiment. A positive control is a condition that will test positive in your assay; in this case, that means adding a chemical that should allow the cells to grow and reproduce, thus bacterial colonies will grow in a minimal media lacking histidine. Negative controls are conditions that should not cause anything to happen. For our experiments, this control would be a condition where no mutations should occur above the rate of spontaneous reversions. It is possible that more than one positive and negative control might be needed for each chemical and bacterial strain tested.

Perhaps the best way to think about what controls are needed is to look at the possible results for your "test condition" (i.e. what happens when you add your favorite potential

mutagen?) and make sure that you could explain the results. For example, what would happen if you saw no colonies in any of your spot-overlays? What if there were bazillions of cells in every spot-overlay? Your control condition spot-overlays should be designed so that you can interpret these results.

How will you determine the "spontaneous" mutation rate? Once you understand this question, you can look at the possible outcomes of this experiment. There are only two: either you will see more colonies with a potential mutagen present, or you won't. Take the first possible outcome: if you see more colonies in your spot-overlay with a potential mutagen (due to new mutations) than you see in the spot-overlay with no added chemicals, can you be sure that it was due to the potential mutagen and nothing else? What control(s) do you have to perform to make sure this conclusion is true?

Now the second scenario: Let's say you get few if any colonies with your potential mutagen present. Can you be sure that this chemical is not a mutagen? What other variables could be different, or what could go wrong, to give the result of no colonies? If you were testing bleach and no colonies grew, what could you conclude?

After thinking through the possibilities and discussing them in class, we will have several controls, both positive and negative, to include in our experimental design for this first week.

Safety Precautions
CAUTION! Pay attention to what you are handling. If you are handling a container with bacteria or chemicals, be sure and wear gloves. PLEASE--to conserve the number of gloves needed--have only those people handling these things wear gloves. You can use one pair of gloves for the entire laboratory period.

1) Know where to throw away used gloves, pipet tips, etc. that have come in contact with bacteria and chemicals **BEFORE** you use them. All trash, including tubes with bacteria and pipet tips go in the orange biohazard bags, which will be autoclaved. The metal caps are cleaned and reused.

2) When handling anything that is supposed to be sterile, such as the pipet tips, Petri plates, tubes of distilled water, bacteria, agar, etc., be sure to uncover or uncap the items for as brief a time as possible. Also, be sure to keep the caps of tubes and lids of Petri plates facing down towards the floor when you are holding them to reduce the possibility of contamination.

Pouring Agar Plates
NOTE: You do not need to wear gloves while pouring the agar plates, because no mutagenic chemicals or bacteria are involved in this procedure.

1. Use the work area with a 2.5-3 foot long piece of absorbent lab counter paper. Label the Petri dishes you will use with your initials and your lab time (e.g. Tues. am). I recommend you put your initials on the bottom, along the perimeter.

2. Look in the water bath on your table for a flask labeled DMA. This flask contains Davis Minimal Agar that has been autoclaved to make it sterile, and is being kept at 47°C to keep it liquefied.

3. Think about these important points in pouring a Petri plate before doing it:

   a) You must work quickly, because once the container of minimal agar is removed from the water bath, it will start to harden within a few minutes.
   b) Pour just enough agar into the Petri dishes to fill each dish about half way.
   c) Although you must work fairly quickly, pour the agar gently to minimize the number of bubbles (bubbles look amazingly similar to colonies when the agar hardens).

4. When you are ready to pour the agar:

   a) Pull out the container of DMA and remove the cap.
   b) Open the cover of the Petri dish halfway and pour in enough agar to just cover the bottom of the dish. Try to minimize the introduction of bubbles.
   c) Repeat for all the dishes.
   d) Immediately rinse the flask with warm water to facilitate washing the flask.

5. Let the plates harden 15 minutes before moving them. These plates will be stored upside down until next week's laboratory session.

Spot-Overlay Protocol
   1. Obtain six pre-poured minimal agar Petri plates (these plates contain no histidine at all). Label the plates (where the arrow is in diagram below) with your group name, the date, and what strain of cells will be tested. Divide each plate into 6 equal parts. Label each pie-shaped section with the chemical to be tested in that area. Labeling Petri dishes is tricky: I recommend writing small, and around the perimeter of the bottom.
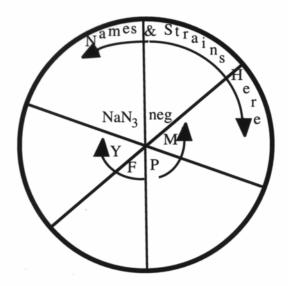
Figure 1.2. Labeling of spot-overlay plate. Each plate is divided into pie-shaped sectors, with each sector corresponding to one of the potential mutagens. Each plate also is labeled to indicate which strain of bacteria is being used.

2. Locate the tubes that contain the soft-agar overlay in the water bath on your table -- each group will have **36** (six plates with six spots on each) of these. These tubes have been autoclaved to sterilize them and put at 47˚C to keep them liquefied.

3. Locate the tubes containing the different strains of *Salmonella* that will be kept at room temperature on the lab bench.

4. If you will be touching tubes, pipettes, or plates that contain bacteria, you must **PUT ON GLOVES.**

5. Locate your chemical to be assayed and any solvents you may be adding to the overlay soft agar tubes.

   NOTE: Now you must work quickly to make sure the soft agar overlay does not harden in the tube. Make sure you understand all aspects of step 6 before proceeding.

6. Sterilely add your test chemical and bacteria to the overlay tube and spot this mixture onto the minimal agar.

   a) Set your pipettor to the appropriate volume of test chemical (to be determined after discussions with your instructor). Open the box of sterile pipet tips, aseptically put one on the micropippetor, and take an aliquot of your test chemical (or solvent used as a control) into the micropipettor.
   b) Remove the cap from the overlay tube, and discharge your aliquot of the test chemical into the overlay tube.

c) Using one strain at a time, swirl the tube containing the bacteria, remove **60 μL of *S. typhimurium* tester strain** and aseptically add it to the overlay tube from step b above.
d) After swirling the tube to mix the bacteria and test chemical, withdraw **200 μL of the mixture**.
e) Lift the top of the Petri plate (open end facing down) and quickly but gently discharge the bacteria/chemical/agar onto the minimal agar - you should create a small puddle.
f) Repeat this step until each strain has been tested with each potential mutagen.
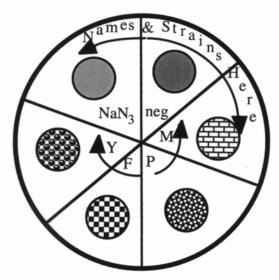g) Invert the plates (i.e., store with the lid on the bottom) and place in a 37˚C incubator for 48-72 hours.

Figure 1.3. Schematic of spot-overlay plate after addition of agar overlays. 200μL of bacteria/chemical/soft agar overlay are added to each section of plate containing Davis minimal agar.

Analysis of Results

After the appropriate incubation period, the plates will be removed from the incubator and stored at 4˚C until next week. During next week's laboratory period, you will count the number of visible colonies in each spot-overlay, including your negative control spot-overlay. Even though the size of the colonies may vary greatly, each colony still arose from only one bacterium that has reverted, thus "all colonies are created equal." A colony consists of a distinct white spot that can be distinguished from a bubble or other similar looking phenomena. Your definition of a colony (anything that shows up vs. only really big ones, etc.) is less important than being consistent in counting colonies from one plate to another. Therefore, it is important that one person counts the colonies in all spot-overlays on a single plate. It should only take you 15 to 30 minutes to count. If there are too many colonies to count, then we will discuss a sampling method that can be used. When you have finished counting the colonies, put the plates in the refrigerator because refrigeration will slow their growth.

NOTE: Be prepared to list the number of colonies in your test conditions and controls for the class to analyze next week. We may look at the plates during the next laboratory period so please do not throw them away yet.

Due before you leave lab
For next week, we will quantify the mutagenicity of any test chemical that your preliminary results suggest are worth further study. You will design the experiments at that time and perform the more traditional method of plate incorporation for the Ames test.

So save any leftovers of YFPM!

Week 2: Quantification of a Mutagen

Goals for This Laboratory Session
This week, you will count the colonies on each spot on each plate from last week. Each group will post its results on the board and all results will be discussed as a group. We will determine which strains and YFPMs suggest that a compound is mutagenic. Then, each group will design a new experiment to quantify the potential mutagen.

Introduction
We will start this week by looking at each group's data from last week. Each group should list their results on the chalkboard. List the number of colonies observed for each strain and test chemical (including the negative control) on the chalkboard. We will discuss these results together and then each group will determine which substance will be analyzed quantitatively today.

Plate Incorporation Protocol
This method is different from last week's, though the principle is the same.

1. Each group will need eight plates that you poured last week. Label them with your group name, and what condition will be tested on it. I recommend writing small, and around the perimeter of the bottom. You will perform two experiments, each experiment will use four plates, with each experiment using one of the four plates for an appropriate control.

2. Locate the tubes of the soft-agar overlay tubes (the volume will be 5 mL instead of last week's volume of 1 mL) in the water bath on your table - each group will have **eight** of these. These tubes have been autoclaved to sterilize them and put at 47°C to keep them liquefied.

3. Locate the tubes containing the appropriate strains of *Salmonella* that will be kept at room temperature on the lab bench. You will test only the one strain that was the most sensitive to your potential mutagen based on your results from last week.

4. If you will be touching tubes, pipettes, or plates that contain bacteria, you must **PUT ON GLOVES.**

5. Locate your chemical to be assayed and any solvents you may be adding to the soft agar overlay tubes.

NOTE: Now you must work quickly to make sure the soft agar overlay does not harden in the tube. Make sure you understand all aspects of step 6 before proceeding.

NOTE: Step 6 is not the same as it was last week.

6. Sterilely add your test chemical to the overlay tube.

   a) Set your pipet to the appropriate volume of test chemical. Open the box of sterile pipet tips, aseptically put one on, and take an aliquot of your test chemical (or solution being used as a control) up into the pipet.
   b) Remove the cap from the overlay tube, and discharge your aliquot of the test chemical into the overlay tube.
   c) Sterilely add **60 μL of the proper strain of *S. typhimurium*** to the appropriate overlay tube. Mix the bacteria, chemical and agar by thumping the tube for a few seconds.
   d) Lift the top of the Petri plate and quickly but gently pour the entire contents of the overlay tube onto the agar surface (try to do it gently and prevent the introduction of bubbles).
   e) Quickly swirl the plate with the soft agar overlay until it completely covers the surface of the minimal agar base layer.
   f) Put the plate down on a flat surface and allow to harden at least 5 minutes before moving the plate again.
   g) Repeat steps a-f until you have tested duplicates of three concentrations of your mutagen and a negative control on a single strain of bacteria.
   h) Invert the plates (i.e. store with the lid on the bottom) and place in a 37°C incubator for 48-72 hours.

Analysis of Results

After the appropriate incubation period, count the number of visible colonies on each plate. Again, an exact size definition of a colony is less important than each group being consistent in counting colonies from one plate to another. You will need to come in on your own time to count the colonies.

Written Report: Full Scientific Paper – Due in one week

Be sure to include results from the control conditions.

Study questions
1. Each group tested different concentrations of a sodium azide. Based on your results, was the mutagenicity of sodium azide proportional to the amount present? Explain your answer.

2. In your experiment testing the mutagenicity of your unknown substance, please explain the ingredients and the purpose of the positive and negative controls.

3. Why did we begin with an auxotrophic strain of bacteria and look for reversion to a prototrophic strain?

4. a) If you added a known mutagen such as sodium azide to the rich medium that contained histidine, what would you expect to see? Explain.
   b) If you knew that 5 μM of sodium azide was highly mutagenic, what would you expect to see if you added 50 mM sodium azide? The exact amount is not the critical issue here. The main point is that you are added a whole lot more.

5. **Fact #1:** Davis Minimal Agar (DMA) has none of the 20 amino acids in it.
   Fact #2: Sodium azide causes base substitution mutations at locations all over the bacterial DNA, not only at the single nucleotide that is wrong in the his- gene of this mutant bacteria.

   Question: What if sodium azide caused a base substitution mutation in a gene coding for an enzyme needed to make a different amino acid, such as leucine, instead of the base substitution for the wrong nucleotide in the gene for the enzyme needed to make histidine? What would you see on your Petri dish of mutant bacteria on DMA and why?

Literature Cited

Ames, B., McCann, J. and Yamasaki, E. 1975. Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian Microsome Mutagenicity Test. *Mutation Research* 31: 347-364.

Ames, B, and Yanofsky, C. 1971. The Detection of Chemical Mutagens with Enteric Bacteria, pp. 267-282. In: *Chemical Mutagens-Principles and Methods for their Detection,* Vol. 1, A. Hollander (Ed.), Plenum Press, N.Y.

Maron, D. R. and Ames, B. N. 1980. Revised methods for the *Salmonella* mutagenicity test. *Mutation Research* 113: 173-215.

Appendix

Ordering Information

Petri plates	Fisher #08-757-14G
Biotin	Sigma #B-4501
DMA (Davis Minimal Agar)	Fisher #DF0544-17-4 (from DIFCO)
L-histidine	Sigma #H-8125
Sodium azide	Sigma #S-2002
Tryptic soy broth	Fisher #DF0370-17-3 (from DIFCO)
Tryptic soy agar	Fisher #DF0369-15-8 (from DIFCO)

Preparation of Media

DMA plates

26.6 g DMA + 980 mL distilled water yields 1 L of solution
Autoclave.
Pour approximately 25 mL per plate.
Allow plates to cool and store them at 4˚C

TSA plates

Follow directions on package.

Tryptic soy broth

Follow directions on package.

Sodium azide

Make 10 mg/mL stock solution in distilled water
Dilute 100 fold to 0.1mg/mL for use.
Store the solution in the dark at room temperature.
Use 0.1 to 0.2μg per plate for a significant mutation rate.
NOTE: Make the sodium azide solution fresh each semester.

Overlay tubes

270 mL distilled water
2.7g DMA
1.5g NaCl
30 mL 10x stock trace solution
Microwave the solution until it is completely melted.
Quickly aliquot 1mL per culture tube and cap the tube with Morton closure.
Autoclave.
Allow the tubes to cool and solidify.
Store the tubes at 4˚C or at 47˚C if they will be used that day.

Trace solution (10x stock)

6.1 mg biotin
5.25 mg histidine

Add distilled water to 100 mL.
Stir while heating to dissolve the components.

Salmonella typhimurium cultures

From ATCC
TA 1537 (catalogue # 29630)
TA 1538 (catalogue # 29631)
TA 1535 (catalogue #29629)
Email: sales@ATCC.org

From Ames Laboratory
TA 98
TA 100
TA 102
Written requests: Ames Laboratory
401 Barker Hall MCB/BMB
University of California at Berkeley
Berkeley, CA 94720
Fax requests: 510 643-7935

Store stock cultures at -70°C. Several days before the laboratory session, transfer a scraping of each strain into a test tube containing 2 mL sterile TSB. Cap and incubate the culture at 37°C with shaking overnight. Store these cultures at 4°C and use these cultures as starter cultures for the exercises. Approximately 18 hours before a laboratory session, transfer 10 µL of starter cultures into separate test tubes containing 2 mL sterile TSB. Incubate at 37°C with shaking until the laboratory period.

For long-term storage of *Salmonella* stocks, combine 700µL of an overnight growth with 300 µL autoclaved glycerol in a screw cap vial. Mix thoroughly by inversion and freeze at -70°C.