Name:________________________________

FOHS Week 9: Spot-Overlay Test of YFPM (Your Favorite Potential Mutagen)

As a student, it is your responsibility to carefully read through the laboratory manual before class. There are new techniques and biological hazards associated with many laboratory exercises, so please come to class prepared and be sure to wear appropriate clothing. If you have any questions about the laboratory activities, then contact Ben prior to the lab.

PRE-LAB: Before the lab, please answer the following questions: ______CHECKED

1. What will serve as the positive control for this experiment?

2. What reagent or reagents will serve as our negative controls?

3. Why are the positive and negative controls important?

4. If you saw no colonies in any of your spot-overlays what could be the explanation?

5. What does the Ames Test measure?

6. How will you determine the “spontaneous” mutation rate?

7. During this laboratory, what potential hazards are there to you?

8. Make a diagram or flow chart of the procedure for preparing an overlay spot on your agar plate.(see Part A, step 6)

Spot-Overlay Test of YFPM (Your Favorite Potential Mutagen)

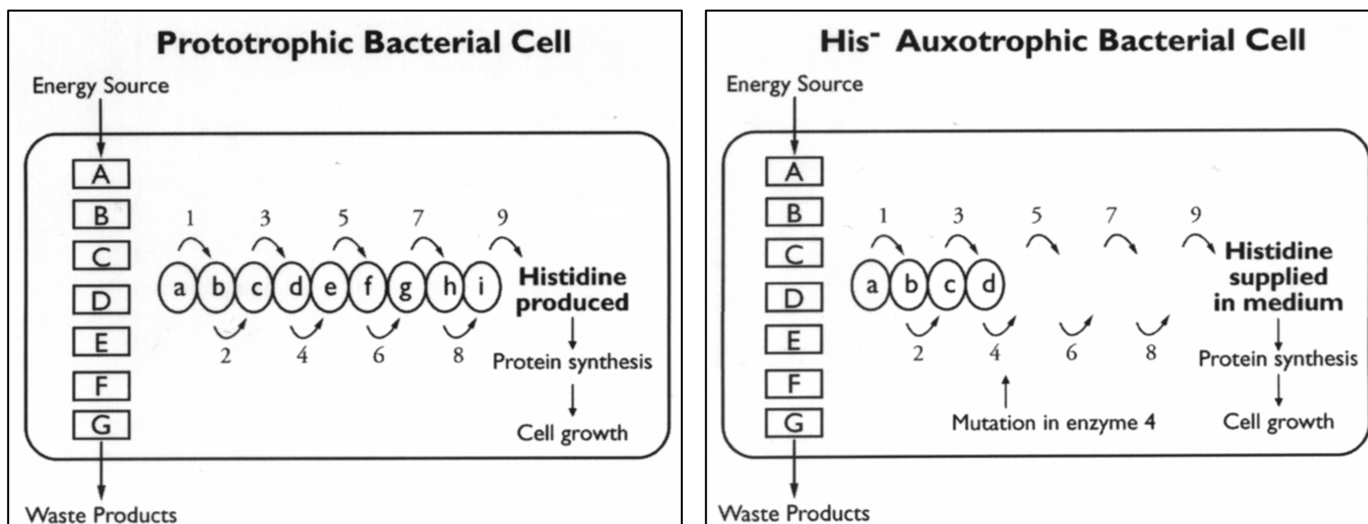
This protocol is modified from: Dorothy M. Maron and Bruce N. Ames. Revised methods for the Salmonella mutagenicity test. Mutation Research. Vol. 113:173-215, 1980. The modified version (Spot-Overlay Assay) and some of the following description is by David R. Wessner, Peggy C. Maiorano, John Kenyon, Ralph Pillsbury, and A. Malcolm Campbell, Department of Biology at Davidson College, Davidson, NC.

The Ames test was developed by Dr. Bruce Ames and is a world-wide standard for testing new compounds to determine if they are mutagenic. The Spot-Overlay method you will use today allows us to screen more compounds quickly and cheaply.

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.

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 the figures below show the pathway for histidine synthesis, which begins with catabolic intermediate **C** and uses nine enzymes (numbered 1 through 9) to convert precursor **C** into histidine.



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. The mutation prevents translation of a functional enzyme, and thus the cell cannot complete the conversion of the catabolic intermediate 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 individuals that cannot make all the metabolic products that wildtype (prototrophic) individuals of the same species can make.
There are several different mutant strains of *S. typhimurium* that have different mutations in their DNA. Here is a list of some strains that are commonly used:

• **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 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 wildtype organism.

• **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, today we will use a mutant strain of *Salmonella typhimurium* that cannot synthesize histidine, is very susceptible to additional mutations because it lacks the normal repair mechanisms found in bacteria, and is 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.

The ultimate goal of the laboratory series on the Ames test is to design experiments to test unknown potential mutagens. To do this, we will use a new variation of the Ames test that was developed at Davidson College. 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. 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.

******** 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 or other contaminated foods.

2) You will be handling potential or known carcinogenic/mutagenic materials. These mutagens have been prepared in ethanol, water or both, depending on their solubility. Before the laboratory session, solid samples were 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 were sterilized by filter sterilization through a 0.22 micron filter.

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

CAUTION! Pay attention to what you are handling. If you are handling a container with bacteria or chemicals, be sure and wear gloves. Be careful not to contaminate items such as your clothes, pens, laboratory papers, backpacks and other items that you will leave with today. THINK ABOUT WHAT YOU HANDLE THROUGHOUT THE ENTIRE EXPERIMENT!

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 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.

Designing Good Controls for Experiments

The use of controls in your experimental designs will be important, just as it is in every experiment. A **positive control** is a condition that will test positive in your assay. **Negative controls** are conditions that should not cause anything to happen. 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.

A. Spot-Overlay Protocol

1. Obtain **four** pre-poured minimal agar Petri plates (these plates contain no histidine at all). Divide each plate into 6 equal parts. Label the plates (where the arrow is in diagram below) with your initals, the date, and the strain of bacteria to be tested. Also label each pie-shaped section appropriately (mutagen code names and controls that will be tested in that area). Write small and near the edge!

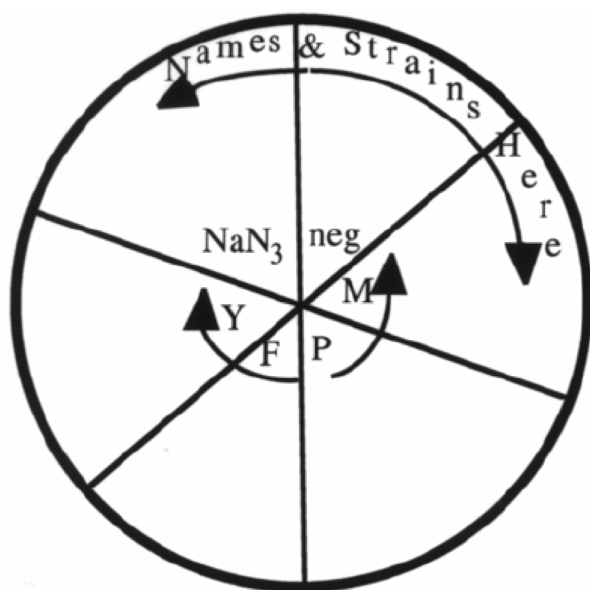


Figure 1. Labeling of spot-overlay plate. Each plate is divided into pie-shaped sectors, with each sector corresponding to one of the potential mutagens or controls.

2. Locate the water bath that contains the soft-agar overlay tubes –
each group will have **24 tubes** (4 plates with six spots on each).. These tubes have been autoclaved to sterilize them and put at 47°C to keep them liquefied.

3. Locate the tubes containing the *Salmonella* strains that will be kept at room temperature.

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

5. Select 4 mutagens from the panel that you would like to test. Locate the positive and negative controls.

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. Aseptically add your one test chemical and one bacterial strain 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 expel your aliquot of the test chemical into the overlay tube.

c) Swirl the tube containing the bacteria by holding the top and flicking the bottom gently. Remove **60 µL** of the *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 (see Fig. 2).

f) Repeat step 6 until each of the four potential mutagens has been tested with each of our tester strains.

7. Let the plates sit for 5-10 minutes before inverting (i.e., store with the lid on the bottom). Place your plate in a 37°C incubator for 48-72 hours.

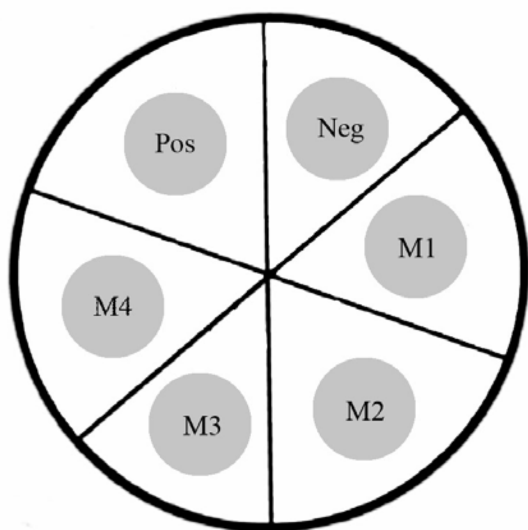


Figure 2. Schematic of spot-overlay plate after addition of agar overlays. 200µL of bacteria/chemical/soft agar overlay are added to each section of plate containing minimal agar. Pos, positive control; Neg, negative control; M1-4, mutagens 1-4.

List the mutagens you used here:

M1	
M2	
M3	
M4	

8. Safely dispose of materials and decontaminate your benches as instructed. Wash your hands and forearms with soapy water immediately after taking off your gloves.

9. Record any important details in your laboratory packet that would allow someone else to repeat your experiment EXACTLY as you did it.

B. Ultraviolet (UV) Light as a Mutagen

Read all of the instructions before proceeding.

1. Obtain and label **one TSA plate** and **one minimal agar plate**

2. Choose one of the tester strains of bacteria to expose to UV light.

3. Using a sterile cotton swab, wet the swab with your bacterial broth and spread the liquid gently and evenly across the entire surface of the agar. (use a new swab for each plate)

4. Locate the UV lamp. **NOTE:** You will need to time this step and wear protective eye wear. (Do not look at the UV lamp, it is damaging to the rods and cones in your retinas).

5. Devise a strategy to expose different parts of the plate surface to different total amounts of UV radiation (ie. shorter or longer time periods). Hint: Cardboard, paper, or the lid of the plate will block UV radiation. **Remember to keep a portion of each plate completely unexposed.**

6. To expose the bacteria to the UV light source for whichever time period you have decided on (probably less than 10 minutes but more than 5 seconds): a) Place the dish under the lamp, turn on the UV, **remove the lid and begin timing**. b) Turn off the UV light and replace the lid. c) Record the time in your lab packet.

7. Repeat this sequence for each section of the plate or each cumulative dose of UV

C. Analysis of Results (Wednesday)

After the appropriate incubation period, the plates will be removed from the incubator and stored at 4°C until next lab meeting. During next 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. If there are too many colonies to count, then we will discuss a sampling method that can be used.

Record your results in the lab packet.

Name: ______________________

LAB REPORT

Name: ______________________
Lab Partners: ______________________
____
____

The following questions are not comprehensive. You should definitely include any other information diagrams or sketches you think are relevant to understanding this experiment.

1. Design a table and record the results from your experiments in the space below. For a given treatment, record the actual number of colonies. If there are too many colonies to count devise a scale to indicate thickness of growth (for example, + is light growth and +++ indicates heavy growth). **Be sure to include which mutagens you used and the results from the control conditions.**

2. Different groups tested different concentrations of a sodium azide. Based on the class results, was the mutagenicity of sodium azide proportional to the amount present? Explain your answer.

Name: ________________________

3. Sketch or describe your results from the UV exposure. Were there any revertants? What happened on the TSA plate?

4. Give some interpretation of your results:
   Which chemicals were mutagenic?
   Were all strains equally susceptible to the different mutagens?
   What types of changes in the DNA were induced by different treatments?

Name: ________________________

5. Did your controls give the expected results? If not, speculate why the results were different.

6. The definition of **auxotrophy** is the inability of an organism to synthesize a particular organic compound required for its growth. Excluding bacteria, name another organism that is an auxotroph.

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

Name: _______________________

8. **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 just 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 minimal agar and why?