Genetics, Bio260
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The Ames Test

The Ames test was developed in the 1970's by Bruce Ames, Professor of Biochemistry at UC-Berkeley, as a fast and sensitive assay of the ability of a chemical compound or mixture to induce mutations in DNA. Because the assay does not use a live animal model, it is inexpensive, easy, and fast. Bruce Ames published his work in a series of papers, including "Identifying Environmental Chemicals Causing Mutations and Cancer" in the journal *Science* (volume 204, 1979). Dr. Ames' work was critical in linking mutations in DNA to carcinogenesis. His work identified many mutagens including pesticides such as DDT, the food additive AF-2 (no longer used), the flame retardant 'tris-BP,' and mutagenic compounds in commercial hair dyes. We will use a version of the Ames test today to test the mutagenicity of a variety of compounds, including materials you brought with you. Next week you will prepare this DNA from mutant bacteria so we can determine what types of mutations were induced.

The Assay: Since DNA is chemically the same in all organisms, any living organism can be used to test for mutagens. Thus, bacteria can be used as a first step in identifying potential human carcinogens without waiting for long-lived mammals to develop cancer. In this assay, mutant strains of the bacteria *Salmonella tymphimurium* (*S. typhimurium*) will be used. These haploid bacteria already contain particular mutations in the gene encoding an enzyme used to synthesize the amino acid histidine; their genotype is given as his^-. Since the bacteria require histidine to make many of their proteins, these mutant bacteria will die unless the media in which they are grown contains histidine. It is known that secondary mutations occur at a low spontaneous rate; these mutants are called revertants because they have reverted to the his^+ genotype and phenotype and can now grow just fine in media lacking histidine. The assay then involves plating his^- *S. typhimurium* onto media with trace amounts histidine and adding chemicals to be tested for mutagenicity. The number of colonies growing on the plate indicates the number of revertants. In a true testing situation, a variety of concentrations of each chemical would be tested to generate a dose-response curve.

A Caveat: The rate of mutagenicity in various organisms can still differ however, due to the rate of chemical absorption by cells and differential metabolism of compounds, in the mammalian liver for example. In the original Ames test, a liver extract is added to the plates as well, simulating how mammalian liver enzymes can modify compounds. In some cases, the liver de-toxifies compounds, but some compounds are actually rendered more toxic after modification. We will be omitting this step of the assay. Note that compounds that test positive in the Ames test would be subjected to further testing in mammalian model systems such as in mice or rats before being labeled as a human carcinogen.

Today's experiment is in two parts: the exposure of *S. typhimurium* strains to potential mutagens (The Ames Test itself) and measurement of the number of bacteria in the cultures used to allow you to quantitatively assess the mutagenicity of the chemicals tested.

Bacterial Strains: We will use two his^- *S. typhimurium* strains in our screen for mutagens. TA1535 contains a T to C missense mutation in the *hisG* gene, leading to a leucine to proline amino acid substitution. TA1538 has a deletion of 1 base pair (C) in the *hisD* gene, which causes a -1 frameshift mutation. This changes two amino acids and brings a stop codon into the reading frame133 amino acids prematurely.

Because there are different his^- mutations in these strains, reversion to the his^+ phenotype will require different molecular lesions (mutations). Since different mutagens can exert their effect on DNA through different mechanisms, using strains containing different mutations allows us to identify mutagens that have differing effects on DNA. Using your knowledge of gene structure and translation, you should try to predict what kinds of mutations could lead to reversion in each case.

You should know that there are additional mutations in the bacterial strains used in the Ames test. Both of the strains have a defect in the lipopolysaccharide cell wall causing them to be more vulnerable to exogenous mutagens. Additionally, both strains have defective DNA excision-repair mechanisms. These mechanisms would normally correct mutations arising during DNA replication or from exogenous mutagens.

A Word about Safety: During this exercise you will be handling strains of *S. typhimurium* and known or suspected mutagens. *S. typhimurium* is a pathogenic organism responsible for certain types of food poisoning, so be sure to follow proper microbiological technique. The mutagens we have chosen for this lab are not very harmful to humans and they are provided in low concentrations. Nonetheless, this is a good time to practice lab safety. Wear gloves when working with the bacteria or mutagens and keep these materials localized in one place on your bench. Known mutagens will only be applied to your plates in one place in the lab. Your instructor will be happy to do this application for you, if you do not wish to do so. Dispose of all gloves/plates/pipette tips in the biohazard containers (not the normal trash). Wash your hands before and after this week's lab.

Part I: Determining the Number of Bacteria in Overnight Cultures

To quantitatively determine the mutagenicity of a compound, one must not only determine the number of mutant bacteria produced, but also the total number of cells exposed to the mutagen to begin with. This is particularly important if one wishes to

compare experiments done on different days or with different bacterial cultures. For example, say a new drug undergoing clinical trials causes allergic reactions in 20 patients. Before we make an assessment of the potential allergic properties of this drug, we need to know how many patients were exposed to the drug: were there 200 or 20,000 patients exposed?

You will do your Ames test with a bacterial culture that was grown overnight. That is, the population of bacteria had been doubling by binary fission for about 10 hours. How many living cells are in that culture, say, per mL? How can we figure this out? We could use a spectrophotometer to determine the cell number per mL, as each cell scatters light when it is in solution. However, overnight cultures are 'saturated,' that is, they are at or near the carrying capacity of the nutrient broth in which they are growing, so some cells are dying but will still scatter light. It would be better to directly measure the *number of living* cells per mL. Each living cell is capable of dividing to produce 2 cells and so on – on a solid surface these aggregate cells will form a colony. We will thus measure colony-forming units (CFU's) in a culture. Overnight cultures of bacteria like *S. typhimurium* exhibit variation in CFU concentration, typically containing $2x10^8$–$2x10^9$ CFU's/mL. The human eye obviously cannot distinguish such a large number of individual colonies, so we will determine the number of CFU's in a given culture by spreading serial dilutions of the overnight cultures on nutrient-rich agar plates and counting the resulting colonies after about 20 hours of growth at 37°C.

The steps involved in calculating CFU concentration are not difficult, but they do require exacting precision when using the pipetters. Think about how a small error at one of the first few dilutions could affect all the subsequent dilutions. How much would a pipetting error of 0.1 mL be magnified after six or seven serial dilutions? With this in mind, take your time and be sure to conduct this experiment with care. Your data will reflect your pipetting skills and plating consistency.

You will want to count 100 or fewer colonies on your plates. You will be plating 100 µl of your dilutions – remember that 1000 µl = 1 mL or, 100µl = 0.1 mL. If you expect your overnight culture to contain $2x10^8$–$2x10^9$ CFU's/mL, how many 1 in 10 or 1:1 serial dilutions should you do? Plan to plate 2 dilutions, one of which is 50% less concentrated than the other. Recall that a 1 in 10 dilution means adding 1 volume of a liquid to 9 volumes of a liquid (as in 1 mL culture + 9 mLs water or 100 µl culture + 900 µl water). Serial dilutions means diluting a stock solution, then diluting the dilution that you just made even further, then diluting that dilution….(see Figure 1). Determine how many serial dilutions you need to make and what the dilution factor is (Figure 1). Lastly, determine how you can make one last dilution that is only half the concentration of the previous dilution. Plan to plate the contents of the last 2 dilutions. Before actually creating the dilutions, check your protocol with the instructor or lab assistant to be sure you have an appropriate plan.

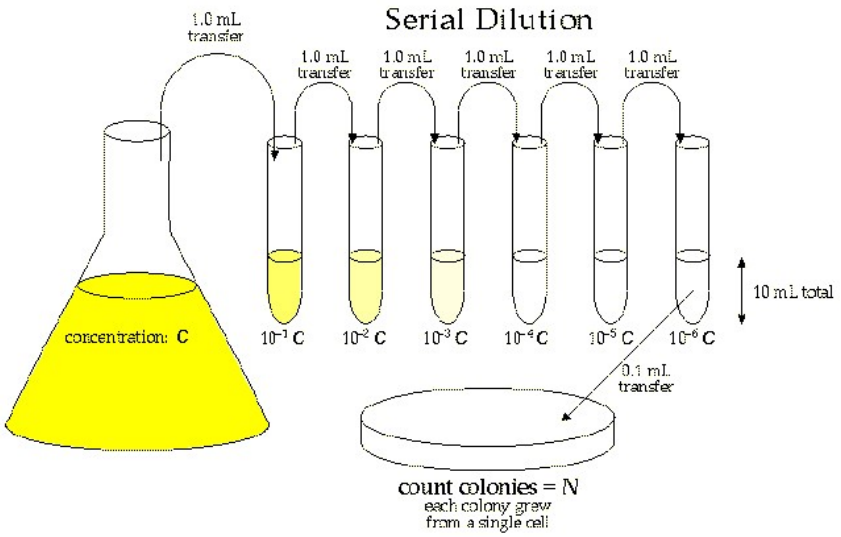

Figure 1: An example of serial dilution. The relative concentration of each dilution (or dilution factor) is shown beneath each tube.

When you count the number of colonies on your plate, you can simply multiply the colony number by the dilution factor to determine how many CFUs were in the 100 µl that you plated. Then multiply by 10 to determine the CFUs/mL of the overnight culture. Will a 10^6 concentration be dilute enough for your experiment?

Creating and Plating Serial Dilutions:

1. Determine the number of serial dilutions that you will perform, and what the final dilution factor of each of your dilutions will be. Write all of these concentrations on labeling tape and apply to the microcentrifuge tubes (include the strain name and your initials).

   Make sure you label all the intermediate dilutions, as it is easy to lose track of which one you are on.

2. Apply Bacdown to your work area and allow it to dry. Your instructor or lab assistant will demonstrate proper microbiological technique and pipetter use at this time.

3. Gently vortex your overnight solution to mix up the sediment at the bottom of the test tube (approximately 5-10 seconds).

4. Pipet approximately 900 µl of your overnight culture into your first empty microcentrifuge tube. Vortex thoroughly for approximately 5 seconds.

5. Pipet 900 µl of the sterile water into every microcentrifuge tube except the ones containing the original overnight culture solution and the final dilution.

6. Pipet 100 µl of your full strength overnight solution into your first serial dilution microcentrifuge tube (should contain 900 µl of sterile water). Vortex thoroughly.

7. Repeat this process for all of your serial dilutions.

8. Label your Petri dishes with the strain name, concentration, group initials, and date.
Write in small letters around the edges of the bottom of the dish. The lids may get mixed up, but the Petri dish will always stay with the agar!

9. Pipet 100 µl of one of your serial dilutions onto the center of the L medium agar plate.
Perform this step carefully, as the liquid tends to bead up and roll around the dish. The desired result is one large drop in the center of the dish. Don't puncture the surface of the agar- this is a surface application.

10. Flame sterilize the hockey stick spreader, briefly touch it to the agar away from the bacteria, and then spread the bacteria over the surface of the agar.

11. Repeat steps 9-10 for one other serial dilution.
Think about why having data from two different dilutions is important.

12. Allow the plates to sit for about 10 minutes, invert and put together with tape, then incubate for 24 hours at 37^{o}C.

13. Count the resulting colonies and record your observations.

14. Using the estimated concentrations of your serial dilutions, determine the CFU/mL content of your original solution and express the standard error as a +/- value.
Find the average of both the dilutions (you will have to adjust for the differences in concentration). Note that you only plated 0.1 mL of the dilutions, not a full mL.

Part II: Ames Test

Experimental Design: Each group of two students will be given materials to prepare an experiment in which a total of 10 plates may be used. Each plate will eventually contain 1 strain of bacteria exposed to one concentration of one chemical, for example. You and your partner should design a carefully controlled experiment using both TA1535 and TA1538. Determine what will work best as positive and negative controls. Two chemicals with known mutagenicity are available to you. **Sodium azide (NaN_3)** is a white solid that is highly soluble in water. Because it easily kills bacteria in high enough concentration, it is used as a preservative in some chemical solutions; its degradation via electric shock is used to inflate car airbags. **4-nitro-o-phenylenediame (4NOP)** is an orange-red powder previously used in hair dyes. In the late 1970's the National Cancer Institute (NCI) noticed that there was an abnormally high incidence of bladder cancer among workers in the dye industry, so they requested a bioassay of 4NOP to determine its mutagenic potential. 4NOP produced positive results in the Ames test and it is considered a potential mutagen.

While choosing your mutagens, you should think about the nature of the organism you are testing. Although the strains of *S. typhimurium* used in the Ames test are his-, most bacteria are not. The incubation conditions used in this experiment are idea for many microorganisms, and many types of fungi and bacteria will readily grow on both top agar and Vogel-Bonner medium. You should be meticulous in your sterile procedure to ensure that no outside contaminants enter your Petri dishes. Furthermore, you should do your best to ensure that there are not microorganisms in the mutagens you are testing. For example, that nasty week-old casserole in your mini-fridge will probably produce many bacterial colonies if you test it as a potential mutagen. This does not necessarily mean your casserole is mutagenic. If you choose a potential mutagen that may have microorganisms in it (e.g. river water), talk to your instructor or lab assistant as soon as possible to see if it is autoclavable. If you suspect your potential mutagen may already have bacteria that can survive on a nutrient-deficient medium, think about how you could test this. Finally, remember that *S. typhimurium* is affected by anti-microbial substances.

Determine what mutagens you will be testing, and discuss your concentrations and application procedure with your professor.

Ensure that your plates are labeled to reflect the mutagens you will test and their concentrations.

Put your potential mutagens into solution at the concentration you discussed with your professor or lab assistant. Typically most mutagens will be suspended in 1 mL of water and placed in an microcentrifuge tubes

Ames Test Basic Procedures:

1. Label each plate with strain name, mutagen+concentration, group initials, and date.

2. Apply Bacdown to the laboratory bench where you will be performing bacterial transfers. Allow the surface to dry. Do not place bacteria near the surfaces that are still wet with Bacdown.

Inoculating Top Agar with *S. typhimurium*:

IMPORTANT NOTE: When working with top agar it is essential that you complete all your transfers quickly. Top agar will begin to set up once you remove it from the hot water bath, so all the following steps should be completed in approximately one minute. So, read through the directions first and figure out who is doing what. Do a "dry run" where you practice each movement as a team first.

3. Briefly vortex the overnight culture of *S. typhimurium* you wish to test (TA1535 & TA1538).

A small pellet of cells often forms at the bottom of test tubes containing overnight bacterial cultures; the goal is a uniform distribution of all cells in the media.

4. Wearing gloves, flame the lip of the overnight culture and remove 100µL of the overnight culture solution. Flame the lip of the test tube before you put the cap back on.

It is often difficult to pipet out of a test tube without contaminating the pipetter itself. If you do touch the edge of the test tube, briefly wipe down the pipetter with Bacdown before using a new bacterial strain to prevent cross-contamination.

5. Remove one tube of top agar from the hot water bath, remove the plastic cap, briefly flame the lip of the tube, and **slowly** add the 100µL of overnight culture solution. Flame the lip again and replace the cap.

Do not forcibly pipette the overnight culture solution into the top agar, as this will introduce bubbles into the agar. Ultimately, these bubbles will be transferred to your agar plate and will make the process of colony counting much more difficult.

5. Briefly (3-5 seconds) vortex the test tube of top agar and bacteria.

6. Gently pour the top agar onto a labeled minimal glucose agar plate.

Again, avoid producing bubbles. Once the agar sets, small bubbles will look almost identical to bacterial colonies.

7. With the lid of the Petri dish on, gently distribute the top agar over the surface of the plate. A rocking figure eight motion typically works best. The top agar will start to harden in 15-20 seconds, so be quick.

8. Wait ten minutes before moving any plates with top agar on them.

Exposing *S. typhimurium* to Different Mutagens:

While allowing the top agar to harden completely, you may prepare your potential mutagens for use. Several potential mutagens will be supplied for you and you will have brought some of your own as well. Solid substances can be blended with sterile water as aqueous substances will diffuse in the agar and contact the maximum number of bacteria. Waxy materials might be easily melted in the microwave and then applied to filters. We find that placing mutagen solutions on sterile filter paper disks produces consistent results. If your mutagen is difficult to put into solution, direct application is a viable option.

The following steps are for application of a mutagen in solution:

9. Flame sterilize your forceps after dipping them in 95% ethanol and allow the forceps to cool for a few seconds.

10. Using the lid of the glass Petri dish as a protective cover, remove one sterile filter paper disk with your forceps.

11. Ensure that the cover for your microcentrifuge tube is secured and lightly flick the tube to evenly distribute your mutagen in the solution.
This is especially important with 4NOP because it is highly insoluble in water. Repeat this step every time you use a mutagen.

12. Pipette 10 µL of the test solution directly onto the center of the filter paper.

13. Using the lid of the Petri dish as a protective cover, place the filter paper (mutagen-side down) onto the top agar.

14. Repeat this process for each plate.

15. Tape your plates together, label them with the date and your group initials, invert the plates (to prevent condensation) and incubate for approximately 48 hours at 37^{o}C

Counting the Revertant Colonies on Your Plates:

1. Remove nutrient-rich plates (CFU concentration experiment) after 18-24 hours of incubation.

2. Make notes on colony characteristics such as size, shape, and color.

3. Count each colony on your plates.

Make sure to be consistent in your definition of a colony. All colonies come from one revertant bacterium, so all colonies should be counted the same, regardless of size. If there are an excessive number of revertant colonies on a plate (over four-hundred), you may want to count only ¼ of the plate and then multiply your final result by four. This is known as the quadrant counting method, and it is less accurate than counting all the colonies on a given plate.

4. Remove nutrient-deficient plates (Ames test) after about 48 hours.

5. Repeat steps 2+3 for these plates.

6. Calculate reversion frequency as # of revertant colonies per mL/total # of CFU's per mL of appropriate overnight culture.

7. Select one plate which contains revertant colonies you wish to test for molecular lesions. Label one of the provided large test tubes (this should contain approximately 5mL of L. medium) with the same information that is on your selected plate. Place your plate in the 4^{o}C refrigerator and replace the test tube in the appropriate area.

Your lab assistant will prepare one colony from your selected plate in an overnight culture solution before the next laboratory period.

Ames Test Week I: Materials Needed in Lab

Materials for each group (groups of 2):

10 mL overnight L medium broth culture of each of the following strains:
TA1538, TA1535

12- L medium plates (L. medium+1.5% bacto agar)- 12 plates per lab group
10 Vogel-Bonner medium E plates
11 tubes of 3 mL bio-his top agar, liquified (autoclaved) in hot water bath (57^{o}C)
1 test tube rack (for large test tubes)
1 test tube containing 5ml of sterile L. medium
1 p1000
1 p200
1 p20
1 pipetman rack
1 box p200 tips
1 box p1000 tips (can probably share between groups- only need 20 or so)
1 pipette tip bucket
1 Bunsen burner
1 bottle Bacdown
1 Miracloth
1 jar 95% ethanol
1 Plating hockey stick
1 vortex mixer
1 bottle sterile ddH_20
Labeling Tape

Materials for Lab:

1 mL of sodium azide @ 0.05 mg/mL
1 mL of 4-nitro-o-phenylenediamine @ 0.5 mg/mL
3 large hot water baths (set to 57^{o}C)
1 Box each size glove
1 biohazard trash bag
3 bottles of sterile H20
3 sterile forceps
2-3 Petri dishes of sterilized filter paper disks
1 jar 95% ethanol
3 blenders
5 microfuge racks
1 beaker sterile microfuge tubes
1 square bottle sterile H20
1 area of Benchkote for very hazardous chemicals
2 beakers of sterile 1.5 ml microcentrifuge tubes
Forceps & alcohol & matches near mutagens (or move near gas jet)

Bio260, Genetics **Reversion: Phenotype to DNA**
E. De Stasio

Introduction:

During last week's lab you exposed his^- mutant strains of *S. typhimurium* to suspected mutagens. Some of your his^- bacteria reverted to a his^+ phenotype, but how do you suppose this happened? As you remember, different mutagens affect DNA in different ways. However, even though a specific mutagen will typically cause one type of mutation, this is not necessarily the only mutation that particular mutagen can induce. Furthermore, we know that TA1535 and TA1538 have contain different mutations, so perhaps different secondary mutations can lead to the his^+ phenotype. This week we will prepare DNA to identify the molecular changes that confer the revertant phenotype.

Isolation of Genomic DNA from *S. typhimurium*:

Before you can amplify the most probable areas of mutation, you must isolate the genomic DNA from your revertants. An overnight culture of the revertant bacteria you selected will be provided and it is your job to extract genomic DNA from these bacteria. We will use DNeasy™ mini-prep spin columns to isolate genomic DNA away from cell membranes, proteins, and RNA. This kit can be used to isolate DNA from a variety of sources, including blood, tissue samples, plants, and bacteria. To obtain genomic DNA, you will first lyse the bacteria with a buffered Proteinase K solution and a high temperature incubation. Then you will pipet the lysed bacteria onto the spin column membrane and centrifuge the sample. The bacterial genomic DNA will adhere to the membrane, but all contaminants (proteins, salts, divalent cations, etc.) will flow through the membrane and be discarded. You will wash the spin column membrane several times to ensure the purity of your DNA, and then you will dry the membrane. Finally, you will elute the DNA into a microcentrifuge tube to use as a template for PCR.

Please be careful with your pipetting and check off each step of the protocol as you complete it.

Protocol for Bacterial Genomic DNA Extraction (Qiagen mini-prep spin column):

1. Vortex your overnight culture solution for approximately 15 seconds.

2. Using the P1000, transfer approximately $2x10^8$ cells (1.5 mls) from your solution into a small labeled microcentrifuge tube. *Use your calculations from the CFU concentration experiment to determine how many µL this will be. This is a separate overnight solution, but the number should be relatively close.*

3. Centrifuge the solution for 10 minutes at 7500 rpm. *Check to ensure that a small pellet has formed after this step. If no pellet forms, repeat centrifugation.*

4. Remove the supernatant with a P1000 and discard. *Be careful not to disturb the pellet.*

5. Resuspend the pellet in 180 µL of Buffer ATL. *You may need to perform multiple up and down pipettings to ensure uniform suspension.*

6. Add 20 µL of proteinase K solution and mix by vortexing for at least 10 seconds.

7. Incubate bacteria for approximately one hour at 56°C in a hot water bath.
Vortex twice during the incubation period to ensure complete lysis of the cells.

8. After incubation is complete, pulse vortex your sample for 15 seconds.

9. Add 400 µL of Buffer AL/ethanol solution and vortex immediately for approximately 15 seconds. *You should have a homogenous solution at this point. Vortex the sample again if there is an excessive amount of precipitate.*

10. Pipette this mixture into the Qiagen DNeasy™ spin column (the column should come pre-packaged in a 2 mL collection tube). Centrifuge for 1 minute at 8000 rpm.

11. Discard the flow-through in the appropriate waste disposal area and place the spin column in a new collection tube.

12. Pipette 500 µL of Buffer AW1 into the spin column and spin for 1 min. at 8000 rpm.

13. Discard the flow-through and place the spin column in a new collection tube.

14. Pipet 500 µL of Buffer AW2 into the spin column and centrifuge for **3 minutes at 14000 rpm**.
This will dry the spin column membrane, so be sure that the column is empty after this step. Do not allow the flow-through to touch the spin column.

15. Discard the flow-through and place the spin column back in the collection tube and respin for 1 minute. Discard the flow-through and the collection tube. Place the spin column into a clean (labeled) 1.5 ml eppendorf tube.
*This tube will contain your DNA prep, so be clearly label it. Additionally, place an uppercase P on the side of the tube to indicate that it is a DNA **P**rep.*

16. Pipette 200 µL of Buffer AE into the spin column (directly onto the membrane). Allow the spin column to sit at room temperature for approximately one minute.

17. Centrifuge the spin column at 8000 rpm to elute.

18. Place your DNA prep on ice and turn it in to your lab assistant.

Amplifying the *hisG* or *hisD* Gene via PCR:

You should now have an isolated prep of genomic DNA from your revertant colony. However, this DNA is from the entire genome of your bacteria, and we are only interested in determining what causes the his^+ phenotype, most likely due to changes in the coding regions of the His operon. Since we know where the mutations are that render TA1535 and TA1538 his^-, and since the bacteria will have to somehow overcome these mutations, we will be amplifying these specific regions of the *hisG* and *hisD* genes.

We will use a polymerase chain reaction (PCR) to amplify portions of the *hisG* (mutant in TA1535) and *hisD* (mutant in TA1538) genes surrounding the original mutations which confer the his^- phenotype (Figure 2). The final amplified regions will be approximately 500 base pairs long.

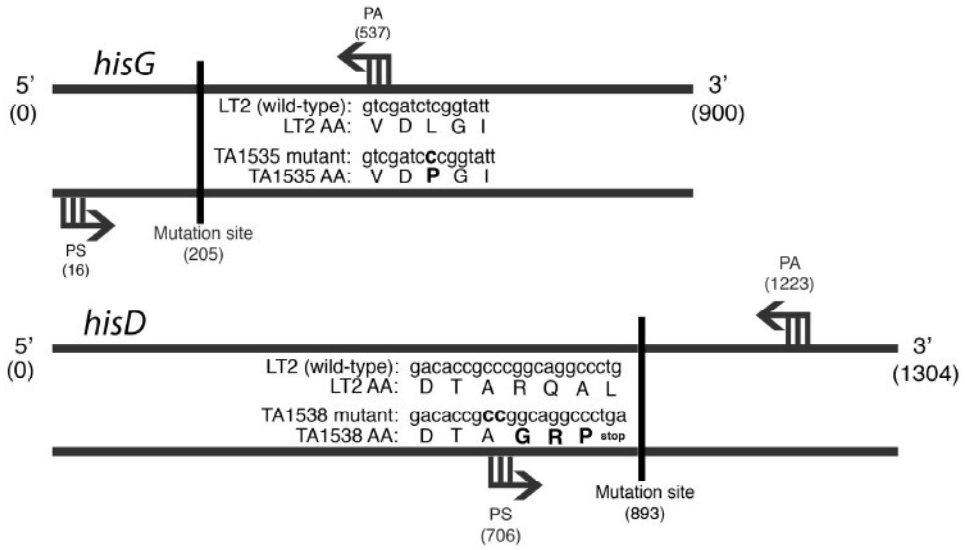

Figure 1. The *hisG* and *hisD* genes of *S. typhimurium* and the location of the mutation sites found in TA1535 and TA1538, respectively. Amino acid (AA) sequences are shown under the base sequences. Actual mutations are shown in bold and are surrounded by bases/AA's on both sides to indicate the effect of the mutation. PS and PA denote the binding site for sense and antisense primers. Mutation site locations indicate the first mutated base. All base locations are relative to the first base of the respective gene in the 5' to 3' direction. Image created by Nathan Goodson-Gregg.

The two primer sets have different optimum annealing temperatures and will require separate thermocycling. The primer sets are:

hisG primer set (optimum annealing temperature of 38°C):
hisG 919 PS: CGCTTACGCATAGCT
hisG 1440 PA: AGCTTCAAGCGTCGC
hisD primer set (optimum annealing temperature of 44°C):
hisD 2611 PS: CCGTCTGAAGTACTG
hisD 3114 PA: TCAATGGTTGATGCC

Protocol for PCR Amplification:

Important note: To ensure that you obtain good results from your PCR, you do not want contaminants getting into your sterile 0.2 mL PCR tube; be sure to use fresh pipette tips every time you transfer a new solution. Keep reagents on ice at all times.

1. Obtain a 0.2 mL thin-walled PCR tube and label the top with your group initials.

2. Determine which primer set you will use and obtain the appropriate set of primers.

3. Pipette 10.5 µL of nuclease-free water into your reaction tube.

4. Pipet 0.5 µL of each desired primer into the PCR tube (6.25 pmole each primer)
   You should have two primers in your reaction, so there will be 1 µL added to your total reaction volume in this step.

5. Add 1 µL of your bacterial genomic DNA prep to the PCR tube.
   Try to pipette the DNA directly into the reaction mixture.

6. Add 12.5 µL of the Promega Master Mix to the reaction tube.
   This contains the Taq polymerase, MgCl2, reaction buffer, and dNTP mixture.

7. Gently vortex the tube to mix all the components and briefly centrifuge to collect the reagents at the bottom of the PCR tube.

8. Place the PCR tube in a thermocycler.
   Your PCR products will be removed after the reaction is complete and stored at –20°C until they are needed.

Technical Information about PCR:

PCR master mix contains the following ingredients:
- 3mM MgCl2
- 400uM dNTP's (each dATP, dGTP, dCTP, dTTP)
- 50units/mL

The thermocycler program is:
- 92°C- 5 minutes (denatures DNA)
- 30 cycles of:
  - 94°C- 2 minutes (separation)
  - 38°C/44°C (*hisD* primers/*hisD* primers)- 30 seconds (annealing)
  - 72°C- 2 minutes (extension)
- 72°C- 20 minutes (elongation)
- 4°C- hold temperature

Purifying PCR Products for Sequencing:
Estimated time: 30 minutes total

We will employ Sanger DNA sequencing to look for mutations associated with reversion to the his^+ phenotype. Recall that Sanger sequencing is basically DNA replication from a single primer hybridized to one of the two strands of template DNA. Because the PCR reaction includes many extra copies of two different primers, the amplified DNA fragment must be purified away from the primers. We will use another spin column to purify the DNA fragment (PCR product) away from the primers, dNTPs, and TAQ polymerase. Once you have purified your DNA fragment, you will add a portion of it, along with just one of the PCR primers, to a clean tube. We will send these samples to a DNA sequencing facility and you will get a data file of the finished sequence. Be sure to obtain a sample number from your instructor so that your sample can be identified when the sequences are returned. You will then use software and a sequence file you can find on Moodle to compare the original TA1535 or TA1538 sequence to the sequence of your revertant.

Protocol for Purifying PCR Products:

1. Add 125 µL of Buffer PBI (should have a yellow color) to your PCR product and pipette to mix.

    Check to make sure that the mixture is still yellow. If the mixture has a violet hue you will need to adjust the pH with 3M sodium acetate (pH 5.0) until the color is yellow. Obtaining the appropriate pH is essential for efficient purification of your PCR products.

2. Apply this mixture to the spin column and centrifuge for one minute at 14,000 rpm to bind the DNA.

3. Discard the flow-through and place the spin column back in the same collection tube. Add 650 µL of Buffer PE to the column and centrifuge for 60 seconds at 14,000 rpm.

4. Discard the flow-through and place the column back in the same collection tube.

5. Centrifuge for an additional minute at 14,000 rpm to completely dry the column.

6. Discard the flow-through and place the column in a clean labeled 1.5 mL microcentrifuge tube. *This tube contains your final purified PCR product, so be sure it is clearly labeled.*

7. Add 50 µL of Buffer EB and centrifuge for one minute at 14,000 rpm.

Verifying Results:

Typically one would run a gel of the final purified PCR products to determine DNA concentration and confirm the success of PCR amplification. Although you will not be performing this step, here is what one of these gels would look like:

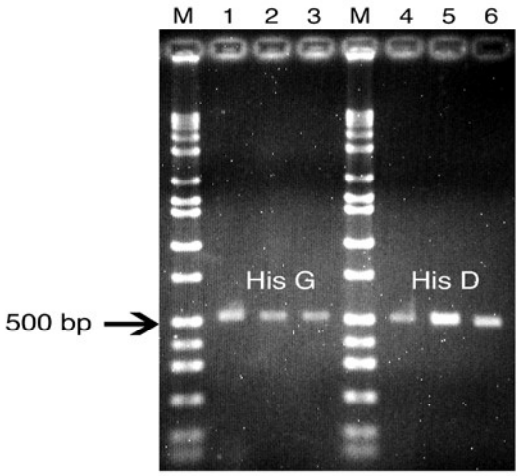

Figure 2. Agarose gel of purified PCR products. 1-3 amplified with *hisG* primer set, and 4-6 amplified with *hisD* primer set. Image created by Nathan Goodson-Gregg.

Preparing your Sample to Send off for Sequencing:

1. Obtain a sterile 0.5 mL PCR tube, close it, and label it with a unique number obtained from your instructor. Fill in the small form that details which strain you began with and the mutagen used to create your revertant. Record your number so you can identify your sequence.

2. Add 8.7 µL of nuclease free water to your microcentrifuge tube.

3. Add 0.3 µL of one sense primer (12.5 pm/L) to your microcentrifuge tube.
   You only need to use one primer, so you should use either His G 919 PS, ***or*** *His D 2611 PS, same as one primer from the primer set you used for PCR.*

4. Add 3.0 µL of your purified PCR product to your microcentrifuge tube.

5. Close the lid tightly, invert to ensure that it is sealed, and give your tube to your instructor or lab assistant.

6. Enter your purified PCR product into the PCR product library.

Materials Needed in the Lab, Ames Test parts 2 and 3

Material for each group:

- 1 p1000
- 1 p200
- 1 p20
- 1 pipetman rack
- 1 pipette tip bucket
- 1 box p200 tips
- 1 labeling marker
- 1 ice bucket
- 1 microcentrifuge tube block

Materials for lab:

- 2 thermocyclers
- 2 tubes of Promega Master Mix (clear)
- 6 tubes of nuclease free water (for PCR)
- 50uL each of HisG919, HisG1440, HisD2611, and HisD3114 primers (primers should be at 12.5pm/μL)
- 4 microcentrifuges (more if they are available)
- 4 small hot water baths (for microcentrifuge tubes)
- 2 square bottles sterile H20
- 1 box each size glove
- 1 jar labeled **Salmonella Waste** (to be autoclaved)
- 1 biohazard trash bag
- 2 boxes p1000 tips
- 2 boxes p2 tips
- 2 beakers of sterile 1.5ml microcentrifuge tubes
- 1 beaker sterile PCR tubes (thin pink ones)
- 2 Qiagen DNeasy Blood+Tissue DNA prep kits (50 tubes per kit)
- 2 10ml containers of 1:1 Buffer AL (Qiagen product)/100% ethanol
- 2 Qiagen Qiaquick PCR purification kits (50 tubes/kit)
- 1 roll of parafilm
- 1 beaker sterile 1.5 ml microcentrifuge tube
- Two primers from last lab-HisG919, HisD2611 (@ 12.5pm/μL)
- 1 ice bucket (for the primers)
- 4 tubes of nuclease free water

Bio260 Genetics
E. De Stasio **Ames Test: Data Analysis**

Part I: Reversion Frequencies
Compute both CFU/per ml and the reversion frequencies for controls and each potential mutagen tested. Be sure these are reported in visual and prose form in your manuscript.

Part II: Sequence Analysis
Download FASTA DNA sequence files that are available on Moodle. These are stored in .txt format, so use whatever text editor program you have to open and manipulate these.

To align sequence files easily (to compare them), use CLUSTALW – a free on-line program at: http://www.ebi.ac.uk/Tools/msa/clustalw2/

At step one, use the pull-down menu to select 'DNA' rather than 'Protein.' You do not need to change any of the parameters, simply copy in each sequence, hitting return between each one that you enter. Do include the line at the top that identifies the sequence; it will look like: >HisD blah blah…Then press the "Submit" button. The output will be the best alignment possible. Be sure you can tell which sequence is which by the first label in the name (the first line that begins with > is the label). It is this line that makes the text file a FASTA file. Wherever the sequences are all identical, there will be an asterisk under the sequence alignment. Look for the differences between mutant and wild type first.

To 'conceptually translate' a DNA sequence into amino acid sequence, there are many programs available. An easy one to use is:
http://www.vivo.colostate.edu/molkit/translate/

Input your sequence via copy/paste (leave off that first labeling line this time), then hit translate. All 6 reading frames will be displayed. One should be an open reading frame that starts on the first ATG start codon. Using your alignment, you should be able to find the location of the mutation. You may then copy and save these protein sequences into a text file and do an alignment in ClustalW, this time using 'Protein' rather than 'DNA.'

Be sure to indicate how the DNA sequence and the protein sequence correlates with the histindine phenotype for each bacterial strain you analyze. You must analyze the wild type sequence, the original his- mutant sequence, and at least two different revertant sequences for *each* of the TA1538 and TA1535 strains.

Bio260 Genetics
E. De Stasio **Ames Test – Mini-Manuscript**

Each lab group may submit one report. You should share data from your entire lab table, but each report may be written by no more than 3 students.

Limit of 3 pages of prose, 5 pages total

Use the following section headings:

Abstract: A brief summary of the goals and results of this experiment.

Introduction: Describe the assay system and the research goals and your hypotheses.

Methods: Describe which substances you tested, at what concentrations or amounts, and how long it took for the bacteria to grow to give you quantifiable results. You may refer to the lab handout for basic methods, do not re-write the handout here.

Results:
Figures/Tables: Use whatever form of presentation you think best serves the data you collected. Data describing reversion frequencies should be quantitative and in scientific units. Figures or tables should be clear, well labeled, and contain proper headings and prose legends. Be sure to make use of your controls! You might wish to compare data quantitatively as well. Qualitative data should be presented visually as well. What mutations did you find? How is the protein changed?

Prose: Descriptions of your results should be in well-constructed paragraphs begun with a topic sentence (a statement of a result). Descriptions should be qualitative and quantitative and go beyond that which is presented in your figures and tables. Figures & Tables are referred to parenthetically.

Discussion: Interpret the data you present. What does it mean? What was the spontaneous mutation rate compared to rates of induced mutation? Is there a difference in the rates and types of mutations induced by a particular chemical? Why might that be? Why do we care?

Acknowledgements: Acknowledge the input of data from lab mates as well as any editorial assistance or helpful discussions with anyone other than a co-author. This is proper scientific ethics and etiquette.

Honor Code: of course.