STREAM

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Educator Version

Student Lab Report

Introduction

EBPI's synthetic Muta-Lab™ kit is a convenient approach that simulates a widely used test for the mutagenicity of chemicals. This procedure is able to simulate a reverse mutation in bacteria after exposure to different compounds. EBPI designed these kits to give reproducible results that are similar to the bacterial based experiment.

Purpose

With this simple lab activity, students gain practical knowledge by conducting a real-world procedure by simulating a test of different samples and watch as the bacteria in the prepared samples perform a reverse mutation. Students will also learn how different chemicals and compounds can cause various types of mutations, the importance of metabolic activation, as well as how bioengineered bacteria are utilized in a battery of biological testing techniques. This activity is designed for any classroom environment and requires no specialized equipment or reagents.

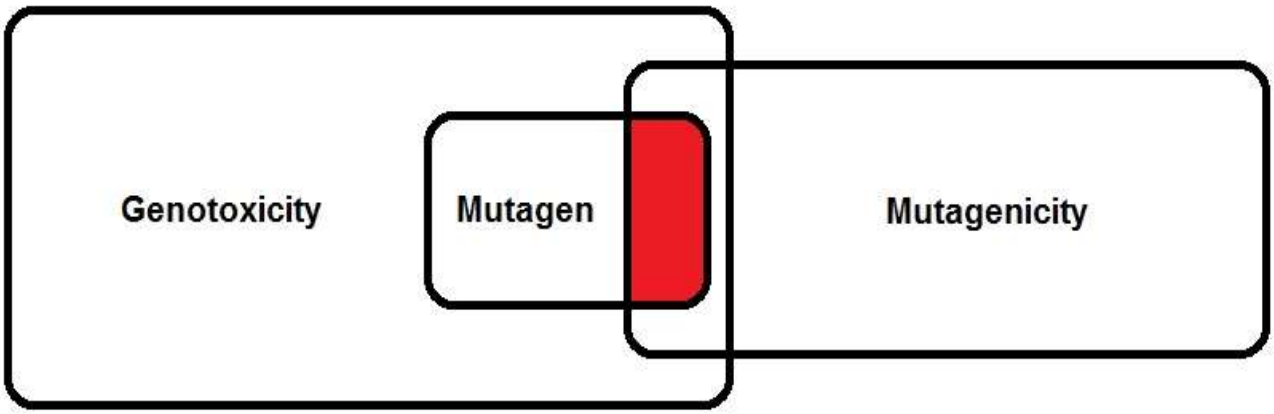
WARRANTY

EBPI warrants that, at the time of shipment, the synthetic Muta-Lab™ kit is free of defects in material and workmanship, and complies with the company specifications. Since actual experimental conditions prevailing at user's laboratory are beyond the control of EBPI or its representatives, EBPI makes no other warranty, express or implied, with respect to the product. Notification of any breach of warranty must be made within 120 days of delivery. The sole and exclusive remedy of the customer for any liability of EBPI of any kind, including liability based upon warranty (express or implied, whether contained herein or elsewhere) is limited to the replacement of the product or the refund of the invoice price of the product.

Concept: Genotoxicity and Mutagenicity

Genotoxicity and Mutagenicity

Genotoxicity and mutagenicity are closely related terms. A genotoxic agent interacts with DNA and/or cellular apparatus that regulate the fidelity of the genome. Any such interaction can result in mutations and possibly lead to the development of cancer. While the concepts of genotoxicity and mutagenicity are related, it is important to note that all mutagens are genotoxic, however, not all genotoxins are mutagenic. Simply put, mutagenicity is a particular case of genotoxicity.



Mutagen and Mutagenicity

Mutagenicity refers to the induction of permanent transmissible changes in the structure of the genetic material of cells or organisms. Changes may involve a single gene or a block of genes. A mutagen is an agent, such as a chemical, radiation, or ultraviolet light that is capable of inducing a mutation or increasing the rate of mutations in an organism. Mutagenicity is strongly correlated with carcinogenicity. Many mutagens are known carcinogens. Mutagenic materials may be hazardous due to their ability to alter the structure and function of DNA. Mutations are often the initiating factor inducing cancerous transformations of normal cells.

Mutagenicity Testing

During the 1950s, bacteria strains with specifically engineered mutations were developed which allowed for relatively simple testing of the mutagenic nature of a substance. Testing for mutagenicity has since become a standard process in the risk assessment of cosmetics, pesticides, pharmaceuticals, and medical use products. The tester strains of bacteria were mutated from their natural phenotype to be unable to produce histidine, in the case of *Salmonella*, or tryptophan, in the case of *E. coli*. When grown in a medium that contains little or no histidine (or tryptophan) the bacteria are unable to grow. However, in the presence of a mutagen, the bacteria are mutated back to their natural phenotype and survive. The growth of the surviving bacteria, called revertants as these bacteria have undergone a reverse mutation back to the natural phenotype, can be measured and correlated with mutagenicity. The strains have also been developed to be sensitive to a range of types of mutagenic agents.

The synthetic Muta-Lab™ kit is based on the most popular and validated bacterial reverse-mutation test, known as the `Ames Test'. Traditionally, reverse-mutation tests have used agar plates, known as `pour plate', `plate-incorporation' or `agar-overlay' assays. An alternate method performed entirely in liquid culture is the `Fluctuation test', originally devised by Luria and Delbruck (1943) and modified by Hubbard et al. (1984).

The Reaction

Bacterial strains used in the ‘Ames test’ carry a specific mutation in a biosynthesis or metabolism pathway. As a result, Ames strains are dependent on the supplementation of the nutrient normally processed by this pathway and will not survive in normal growth media. However, specific mutations caused by toxicant interactions at sites in the bacterial DNA can produce reversions back to the wild type genome, a state in which the bacteria regain the ability to synthesize histidine.

This experiment involves a simulation of that test that includes four samples (A to D) using two simulated Salmonella mutant strains. The tests will be carried out in the presence or absence of the S9 enzyme. The results will determine which type of mutation, if any, each of the chemicals caused.

Expectations

The synthetic Muta-Lab™ kit is a realistic simulation of a reverse mutation in bacteria after exposure to different compounds. Students will be performing a simulation of a test that includes 4 samples using 2 simulated strains. The tests will be carried out in the presence or absence of the S9 enzyme. Students will prepare their microplate according to their respective microplate assignment and dispense the different concentrations of samples or backgrounds into the four quadrants of each microplate. See the handout "*Microplate Setup Table - Synthetic Muta-Lab*™" for further details. Once your class completes the experiment and the colour change occurs, the results can be recorded to determine toxicity levels and each group will complete a lab report for evaluation.

Contents of the Synthetic Muta-Lab™ Kit

Each kit contains sufficient components for 12x 96-well microplates. The bottles and vials in the synthetic Muta-Lab™ are labelled with clear, bold letters.

Each kit should contain the following:

- 12x 96-well Microplates
- Sample A 24 mL (1 unit)
- Sample B 24 mL (1 unit)
- Sample C 24 mL (1 unit)
- Sample D 24 mL (1 unit)
- Diluent Buffer 100 mL (1 unit)
- Chromogen 10 mL (8 units)
- Reaction Mixture 10 mL (6 units)
- Bacteria 20 mL (2 units)
- Positive Control 1 mL (3 units)
- S9 mix 10 mL (1 unit)

Further materials required, but not included in the kit (contact EBPI if you require these items):

- 20x units of 200 uL micropipettes & tips
- 48x units of Test Tubes

Collecting the Samples and Preparation of the Samples

No samples or advance preparation is required.

Student Station Preparation

Below is the suggested distribution of the reagents needed for the experiment based on 12 lab groups.

STUDENT LAB STATIONS	FRONT OF THE CLASSROOM
Groups 1 to 12 should have 1x 96-well Microplate, approximately 8 mL of Diluent Buffer, 4 Test Tubes, 2 to 3 micropipettes with tips. Groups 1 to 4 will NOT receive any of the unknown Samples A to D. Groups 5 and 6 should have Sample A (12 mL per group) Groups 7 and 8 should have Sample B (12 mL per group) Groups 9 and 10 should have Sample C (12 mL per group) Groups 11 and 12 should have Sample D (12 mL per group)	2 Units of Bacteria 6 Units of Reaction Mixture 3 Units of Positive Control 1 Unit of S9 Mixture

Lab Safety Protocols – Review this with your class before beginning the experiment

Storage:

- All kit components can be stored at room temperature.
- Store the Reaction Mixture in a dark place as the purple dye used is light sensitive.
- Store the 96-well microplates in a dry, dark location at room temperature to ensure optimum test performance.
- Improper storage may result in an instantaneous colour change as opposed to a gradual colour change.

Handling:

- The simulated reaction mixture contains a purple dye, bromocresol purple, which may cause slight staining of skin as well as clothing. All other reagents are a sodium bicarbonate buffer solution.
- It is important to use the Dilution Buffer provided, as it is a buffer solution and cannot be replaced by distilled or tap water.

Microplate Setup Table
Synthetic Muta-Lab™

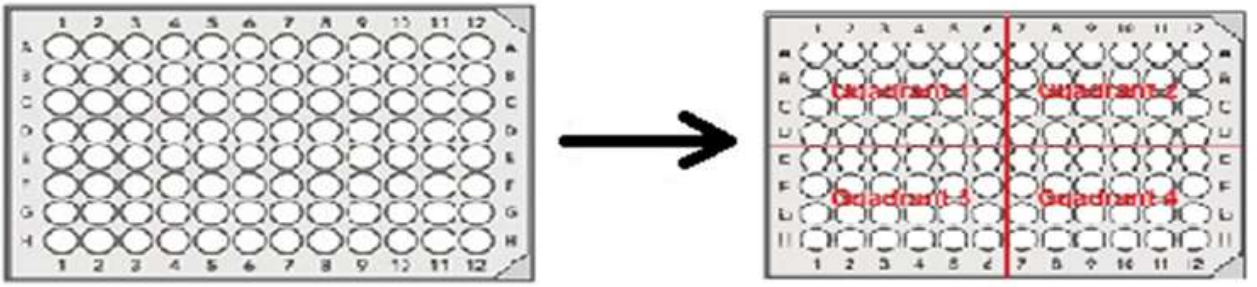
To Educators: Please ensure that each student group have the appropriate items and volumes before conducting the experiment.

Plate	Quadrant	Treatment	Standard	Sample (mL)	dH_20 (mL)	Reaction Mixture (mL)	S9 (mL)
1	1	Blank			4	1	
	2	Background TA100 (S9)			4	1	1 Drop
	3	Background TA100 (S9)			4	1	1 Drop
	4	NaN_3 (TA100)	1 Drop		4	1	
2	1	Blank			4	1	
	2	Background TA98 (S9)			4	1	1 Drop
	3	Background TA98 (S9)			4	1	1 Drop
	4	2-NF TA 98	1 Drop		4	1	
3	1	Blank (S9)			4	1	1 Drop
	2	Background TA 100			4	1	
	3	NaN_3 (TA100)	1 Drop		4	1	
	4	2-NF (TA98)	1 Drop		4	1	
4	1	Blank (S9)			4	1	1 Drop
	2	Background TA 98			4	1	
	3	2-AA (TA100) (S9)	1 Drop		4	1	1 Drop
	4	2-AA (TA98) (S9)	1 Drop		4	1	1 Drop
5	1	100% Sample A TA100		3	1	1	
	2	50% Sample A TA 100		1.5	2.5	1	
	3	100% Sample A TA100 (S9)		3	1	1	1 Drop
	4	50% Sample A TA100 (S9)		1.5	2.5	1	1 Drop
6	1	100% Sample A TA98		3	1	1	
	2	50% Sample A TA98		1.5	2.5	1	
	3	100% Sample A TA98 (S9)		3	1	1	1 Drop
	4	50% Sample A TA98 (S9)		1.5	2.5	1	1 Drop
7	1	100% Sample C TA100		3	1	1	
	2	50% Sample C TA 100		1.5	2.5	1	
	3	100% Sample C TA100 (S9)		3	1	1	1 Drop
	4	50% Sample C TA100 (S9)		1.5	2.5	1	1 Drop
8	1	100% Sample D TA100		3	1	1	
	2	50% Sample D TA 100		1.5	2.5	1	
	3	100% Sample D TA100 (S9)		3	1	1	1 Drop
	4	50% Sample D TA100 (S9)		1.5	2.5	1	1 Drop
9	1	100% Sample B TA100		3	1	1	
	2	50% Sample B TA 100		1.5	2.5	1	
	3	100% Sample B TA100 (S9)		3	1	1	1 Drop
	4	50% Sample B TA100 (S9)		1.5	2.5	1	1 Drop
10	1	100% Sample B TA98		3	1	1	
	2	50% Sample B TA98		1.5	2.5	1	
	3	100% Sample B TA98 (S9)		3	1	1	1 Drop
	4	50% Sample B TA98 (S9)		1.5	2.5	1	1 Drop
11	1	100% Sample C TA98		3	1	1	
	2	50% Sample C TA98		1.5	2.5	1	
	3	100% Sample C TA98 (S9)		3	1	1	1 Drop
	4	50% Sample C TA98 (S9)		1.5	2.5	1	1 Drop
12	1	100% Sample D TA98		3	1	1	
	2	50% Sample D TA98		1.5	2.5	1	
	3	100% Sample D TA98 (S9)		3	1	1	1 Drop
	4	50% Sample D TA98 (S9)		1.5	2.5	1	1 Drop

Procedure – Synthetic Muta-Lab™

1. Each Microplate was **labelled with a number** on the top left which corresponds with the treatment set up as described in the '**Microplate Setup Table**.'

Divide your plate into **quadrants** as shown below and **label the microplate** cover with the appropriate mixtures being added.

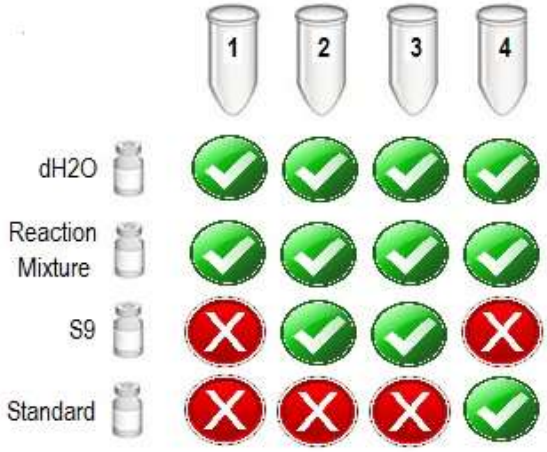


Example - Microplate 1	
- Quadrant 1: 4 mL dH2O, 1 mL Reaction Mixture	Blank Treatment
- Quadrant 2: 4 mL dH2O, 1 mL Reaction Mixture, 1 Drop S9	Background TA100 with S9 Treatment
- Quadrant 3: 4 mL dH2O, 1 mL Reaction Mixture, 1 Drop S9	Background TA100 with S9 Treatment
- Quadrant 4: 4 mL dH2O, 1 mL Reaction Mixture, 1 Drop Standard	Sodium Azide NaN3 Treatment

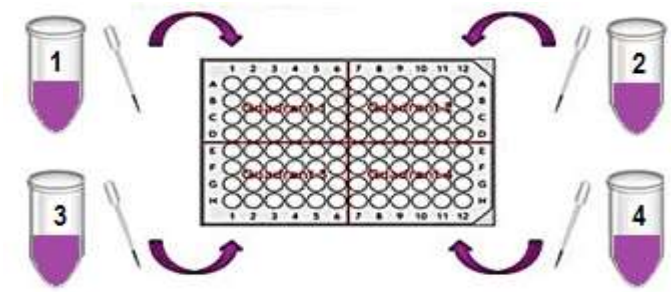
2. Label four test tubes 1 to 4 to correspond for each quadrant number on the microplate.

Once the test tubes are labelled, dispense the proper reagents according to the '**Microplate Setup Table**.' In the end, each test tube should contain approximately 5 mL once all the reagents have been added. Ensure the test tubes are well mixed and a uniform purple colour.

The following setup is for Microplate 1.

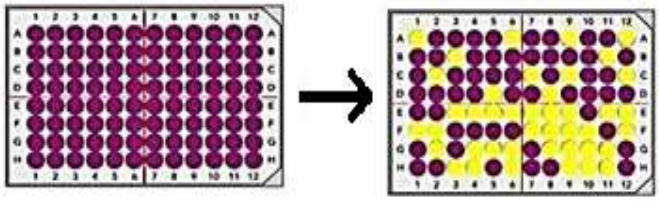


3. Using a transfer pipette, add 2 drops or 100 uL of the contents of each respective test tube to the corresponding quadrant (i.e. Test Tube 1 to Quadrant 1), and so on until all of the wells in the microplate are filled.



4. Cover the microplate and wait for 15 to 20 minutes.

An observed colour change (yellow) represents the growth of bacteria.



5. Clean Up. All materials are can be disposed of through regular solid waste disposal.

To Educators: Please review the information as a visual guideline of the experiment results, recording the results, further analysis, and the answer sheet for the questions.

Recording the Results: After the experiment, students can record the results from their microplates. If a sample results in a number of positive wells greater than the background, then the '*Significance Values Table'* (the Table below) is used in order to determine the level of certainty that the sample is mutagenic. During this time, you can assess and evaluate their results to note accuracy and any possible errors. Students should be encouraged to record their errors in their lab reports.

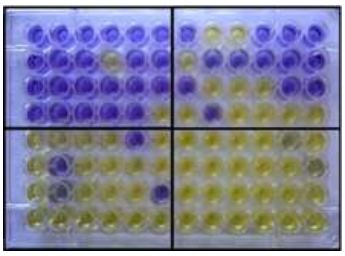
Guidelines

Counting the Microplates:

All yellow, partially yellow or turbid wells are positives. All purple wells are negative. The `Blank' (i.e. no test material added) microplates will show the level of spontaneous or background mutation of the assay organism. Students will record the number of positive wells for each plate using the provided table of the results significance handout. It is important to determine if the negative (blank) and positive (known mutagen) controls worked properly. The blank does not contain any bacteria and no yellow wells should appear in the blank quadrants. Each positive control is a known mutagen and contains strong mutagenic properties which should result in all or nearly all of the wells turning yellow in that quadrant. After the experiment, students will record the results for each of the plates using the '*Significance Values Table*' provided in the next page, analyze which samples are mutagenic, and complete their lab report for evaluation.

Sample of the Synthetic Lab Results:

An example of the Synthetic Muta-Lab™ Results



Quadrant 1: Background
(2 Positive Results =
2 Wells Turned Yellow)

Quadrant 2: Mild Mutagen
(11 Positive Results)

Quadrant 3: Strong mutagen
(21 Positive Results)

Quadrant 4: Positive Control
(24 Positive Results)

Significance Values Table:

# positive wells in the background quadrant	# positive wells in the treatment quadrant			# positive wells in the background quadrant	# positive wells in the treatment quadrant		
----------	95%	99%	99.9%	----------	95%	99%	99.9%
0	3	5	9	13	19	21	23
1	5	8	11	14	20	22	24
2	7	9	12	15	21	22	24
3	9	11	14	16	21	23	
4	10	12	15	17	22	24	
5	11	13	16	18	23	24	
6	12	15	17	19	23	24	
7	13	16	18	20	24		
8	14	17	19	21			
9	15	18	20	22			
10	16	19	21	23			
11	17	19	22	24			
12	18	20	22				

Interpreting the Results:

Once the microplate experiment is done and the results from the controls are verified, students can evaluate the mutagenicity of the samples by comparing sample quadrants to respective background quadrants (i.e. compare the response of TA 100 in Sample A, to the background response of TA 100). Have your student lab groups complete the following:

1. Use the '**Significance Values Table**' to determine the level of significance of the difference between the background and the sample response to classify compounds are either mutagenic or non-mutagenic.

2. Complete the '**Microplate Results Table**' by placing the significance of the difference between the sample and the background (e.g. 95%, 99%, or 99.9%) by using the 'Significance Values Table.'

3. Based on the response of the different strains to Samples A, B, C, and D, and the genotype of each of the bacterial strains, determine which type of mutation (if any) is caused by each of the samples. Justify this with a brief explanation based on the concepts of mutagenicity and genotoxicity learned in class.

Further Analysis:

To further analyze the results, your students can pick further analysis questions based on your preference and the project stream's overall objectives.

Questions and Answers

1. Explain all components of the reaction mixture for the negative and positive control. Why are these controls needed?

   Negative Control contains all reagents A-F but no sample or positive mutagenic compound, as well as bacteria. This control serves to show the spontaneous mutation rate of the bacteria, if it is not exposed to any possible mutagens. Also important to note is that you may want to include the solvent used to dissolve your samples in the negative control in order to make sure the solvent is not mutagenic or toxic to the bacteria.

   Positive Control contains all reagents A-F as well as the mutagenic standard. This control serves to show what the response of the bacteria will be if it is, in fact, exposed to a mutagen. It is expected that the response in this preparation will be very high (with 24/24 wells (or close) turning yellow after the incubation period).

2. If your sample were to have a mutation rate less than that of the background, what would that mean? How would you adjust the protocol in order to determine whether your sample were in fact mutagenic or not?

   If your sample has a lower mutation rate than the spontaneous background, it could mean that your sample is toxic to the bacteria, limiting growth and potential for mutation. If this is the case, you should perform dilutions of your sample in order to determine if there is a limit of toxicity, and if it becomes non-toxic, does the response rise above the background to prove mutagenic, or not.

3. If you knew that adding 5 µM of positive control was highly mutagenic (all 24-wells turn yellow after 3 days), what would you expect to see if you kept increasing the concentration?

   It would be expected that after a certain amount of positive control was added, it would prove toxic, decreasing the number of positive responses, eventually reaching 0 positive wells as all the bacteria would be killed outright. Although not always the case, this is a reasonable assumption.

Student Lab Report

Group #	Group Student Names	Role

Checkmark the appropriate Stream below:

Water 	Chemical 	Soil 	Synthetic 

Note: At the beginning of class, your teacher will have the lab stations ready. Remember to check your lab station, ensure you have the appropriate supplies, and read over the safety protocols.

Pre-Lab Notes	

Instructions: Read over the preparation and the procedure for the specific biotechnology kit that you will be working on. Complete the lab and record the results.

Preparation
Synthetic Muta-Lab™

To Students: Please follow the instructions and information below to help prepare for the lab experiment.

Expectations

The synthetic Muta-LabTM kit is a realistic simulation of a reverse mutation in bacteria after exposure to different compounds. Your group will be performing a simulation of a test that includes 4 samples using 2 simulated strains (named TA 100 and TA 98). The tests will be carried out in the presence or absence of the S9 enzyme. There are four unknown samples being tested, called A, B, C, and D. Once your group apply the mixtures and the colour change occurs, record the results and one of the group members will note the results for the class. Later, your group will determine which type of mutation, if any, each of the chemicals caused. Ensure that your group prepares the microplate according to your group and microplate number assignment.

Lab Station Preparation

Note: Your educator will assign your group a microplate number which will be your group number. This number is important because it will correspond to the mixture that your group will need to prepare. Your educator will let you know which mixture you need to prepare on the day of the experiment.

At the beginning of the lab, you should have the following available in the front of the classroom and lab station:

FRONT OF THE CLASSROOM	GROUP LAB STATION	
2 Units of Bacteria 6 Units of Reaction Mixture 3 Units of Positive Control 1 Unit of S9 Mixture	- 1x 96-well Microplate - 4x Test Tubes - Micropipettes	- ~ 8 mL of Diluent Buffer - ½ bottle of Reaction Mixture

Before starting an experiment it is important to ensure all of the materials, supplies and equipment needed are available and ready. Be sure to speak to your educator if there is anything missing.

Ensure that you review the Lab Safety Protocols before beginning the experiment

Storage:

- All kit components can be stored at room temperature.
- Store the Reaction Mixture in a dark place as the purple dye used is light sensitive.
- Store the 96-well microplates in a dry, dark location at room temperature to ensure optimum test performance
- Improper storage may result in an instantaneous colour change as opposed to a gradual colour change.

Handling:

- The simulated reaction mixture contains a purple dye, bromocresol purple, which may cause slight staining of skin as well as clothing. All other reagents are a sodium bicarbonate buffer solution.
- It is important to use the Dilution Buffer provided, as it is a buffer solution and cannot be replaced by distilled or tap water.

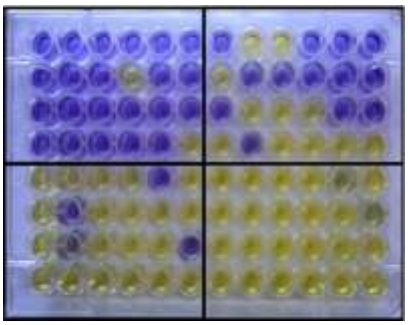
Results & Analysis
Synthetic Muta-Lab™

Instructions: Once you obtained the results, complete the following steps below. Read over the guidelines before recording your results.

If you have any errors from the experiment, please describe them here and the reasons why it occurred:

Observing the Results

Visual Guidelines: The diagram below is a general guideline to compare your results, ensure accuracy, and complete your analysis of the results.



Quadrant 1: Background
(2 Positive Results = 2 Wells Turned Yellow)

Quadrant 2: Mild Mutagen
(11 Positive Results)

Quadrant 3: Strong mutagen
(21 Positive Results)

Quadrant 4: Positive Control
(24 Positive Results)

Criteria for a Valid Test: It is important to determine if the negative (blank) and positive (known mutagen) controls worked properly. The blank does not contain any bacteria and therefore no yellow wells should appear in the blank quadrants. Each positive control is a known mutagen and will contain strong mutagenic properties which should result in all or nearly all of the wells turning yellow in that quadrant.

Significance Values Table:

Significance Values				Significance Values			
# positive wells in the background quadrant	# positive wells in the treatment quadrant			# positive wells in the background quadrant	# positive wells in the treatment quadrant		
----------	95%	99%	99.9%	----------	95%	99%	99.9%
0	3	5	9	13	19	21	23
1	5	8	11	14	20	22	24
2	7	9	12	15	21	22	24
3	9	11	14	16	21	23	
4	10	12	15	17	22	24	
5	11	13	16	18	23	24	
6	12	15	17	19	23	24	
7	13	16	18	20	24		
8	14	17	19	21			
9	15	18	20	22			
10	16	19	21	23			
11	17	19	22	24			
12	18	20	22				

Recording the Results

Once the microplate experiment is done and the results from the controls are verified, your group can evaluate the mutagenicity of the samples by comparing sample quadrants to respective background quadrants.

Use the **'Significance Values Table'** to determine the level of significance of the difference between the background and the sample response to classify compounds are either mutagenic or non-mutagenic. You can place the significance of the difference between the sample and the background (e.g. 95%, 99%, or 99.9%) by using the **'Significance Values Table'** (see the previous page).

Quadrant		**Microplate Description**	**Number of Positive Wells**	**Significance (%)**
Plate ___	1			
	2			
	3			
	4			

Once you are done recording your own results, record the results of the other plates that were done in the table below:

Micro Plate	Quadrant	Treatment	# of Yellow Wells	% Significance	Micro Plate	Quadrant	Treatment	# of Yellow Wells	% Significance

Interpreting the Results

Based on the response of the different strains to Samples A, B, C, and D, and the genotype of each of the bacterial strains, determine which type of mutation (if any) is caused by each of the samples. Justify this with a brief explanation based on the concepts of mutagenicity and genotoxicity from your research.

Further Analysis

Explain all components of the reaction mixture for the negative and positive control. Why are these controls needed?

If your sample were to have a mutation rate less than that of the background, what would that mean? How would you adjust the protocol in order to determine whether your sample were in fact mutagenic or not?

If you knew that adding 5 μM of positive control was highly mutagenic (all 24-wells turn yellow after 3 days), what would you expect to see if you kept increasing the concentration?