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Tobacco Induced Mutation: Teacher Instructions

Summary: Students expose bacteria to 4 different concentrations of tobacco extract and observe the mutagenic effect on the bacteria. (The bacteria change from red to white when they mutate). Mutations are an important precursor to cancer.

Objectives: Students will learn how to plate bacteria.
Students will learn how to collect & analyze data.
Students will observe the mutagenic properties of tobacco.
Students will observe a dose/response relationship.

Time: Preparation - Make Agar Petri Plates: 2 hours
Make Stock Plates: 10 minutes
Make Tobacco Solution:30 minutes

Experiment - Perform the Experiment: 1 hour
Incubate Bacteria: 24 -48 hours (doesn't require class time)
Data Collection & Analysis: 1-3 hours (depending on extent of analysis)

Materials:

Material	Vendor and Catalog Number
Petri Dishes (1-2 Plates per student)	VWR 100mm sterile 25384-071Case of 500 $96.30
Wire Loops (one for each student)	
Tryptone	Difco, 211703, 100 g
Yeast Extract	Difco, 212740, 100g
Cigarettes (2 per group)	
NaCl (Sodium Chloride)	
UV lamp	
Beaker (one per group)	
Stir sticks	
Hot plate or microwave	

Test tube racks which fit 14 ml tubes (1 per group)	
Cheese Cloth or other Filter Paper (1 per group)	
3 500 ml Bottles	
Bunsen Burners (3-4)	
Graduated Cylinder	
1 ml Size Plastic Transfer Pipettes (4 per group)	VWR 14670-345 500 for $24.78
14 ml Plastic Test Tubes (5 per group)	VWR 60819-761, case of 500, 111.37
Agar	Difco, 214010, 454g
Autoclave or pressure cooker	
Serratia Marcescens	
Distilled Water	
Marking Pens (1 per student)	

Methods:

TEACHER PREPARATION

Make the Agar Petri (LB) Plates and liquid bacterial media (food); allow minimum two days to complete the preparations

- LB- Plates:

Mix the following ingredients with 1L of distilled water (20ml media per plate, makes about 35 plates):

10g NaCl
10g tryptone
5g yeast extract
20g agar

Autoclave the solution (121 ° C, 30 minutes); wait for the solution to cool (should be cool enough to handle but not cold) and pour carefully into the Petri dishes avoiding bubbles as much as possible. Allow gel to solidify at room temperature overnight (O/N). LB-Plates can be stored in a refrigerator for up to 2 months or at room temperature for a couple of days.

Tips:

1) use a 2 L glass Erlenmeyer flask for the mixture to prevent bubbling over of the mixture during heating, cover the top of the Erlenmeyer with foil before autoclaving.

2) add all ingredients except agar initially; once all the other ingredients are in solution, add the agar. The agar will not go into solution at room temperature but this is okay as the agar will go into solution during autoclaving.
3) Whether inoculated with bacteria or not, store the plates upside down to avoid condensation buildup on the surface of the agar.

- Liquid LB-Media:

Mix the following ingredients with 1L of distilled water:

10g NaCl
10g tryptone
5g yeast extract

Transfer solution to 2 - 500 ml glass bottles and autoclave (121 ° C, 30 minutes). This solution should be stored in the bottles and can be stored at room temperature indefinitely.

<u>Prepare bacteria and tobacco solution for the experiment</u>

- Serratia Marcescens: *S. Marcescens* is the strain of bacteria utilized in this activity and is non-infectious to humans. *S. Marcescens* produces a red pigment (i.e. colonies are red) when grown at 30 ° C. When grown at cooler or warmer temperatures, this red pigment is not produced and colonies are white. Mutations in the bacteria will produce white colonies at 30 ° C. Thus, it is important to grow the bacteria at 30 ° C when testing for the ability of the cigarette extract to produce mutations.

- Generate Stock Plates: After obtaining *S. Marcescens*, the teacher should make some stock plates which will serve as a reservoir of bacteria for activities with students. Stock plates can be generated by:
    1) inoculating 3-4 LB-plates with S. *Marcescens*
    2) incubating the plates overnight at 30 ° C. These plates remain viable and can be stored up to several months in the refrigerator.

Generate 3-4 "student stock plates" from stock plates of Serratia marcescens for the students to use in order to prevent contamination of the refrigerated stock plates: Prior to any activity with students, 3-4 student stock plates should be generated by:

1) flaming wire loop
2) removing a small amount of bacteria from the refrigerated stock LB-plates using the wire loop.
3) Inoculating 3-4 new LB plates with the bacteria on the wire loop, incubate 24-48 hr. at 30 °C.

- *Make Tobacco Solution:
  1) Remove the tobacco from 2 cigarettes and place in 200 ml of distilled water in a beaker.
  2) Heat the water for 10-15 minutes with constant stirring .
  3) Filter the leaves out of the extract using cheese cloth or other filter paper.
  4) Pour into a bottle for storage

* This step can be performed by the teacher or by the students depending on time constraints.

DAY 1. PERFORMING THE EXPERIMENT: 1 hour

- Place the 3-4 "student stock plates" around the room.
- Divide the students into groups of 4-5 and distribute 6 LB media plates to each group and a wire loop to each student.
- Have students label 5 tubes: 1:1, 1:100, 1:1000, 1:10,000, bacteria
- Have students make dilutions of the cigarette solution using graduated pipettes according to the scheme below.

1:1	- no dilution, place 0.1 ml of cigarette solution into the 14ml tube labeled 1:1
1:100	- place 0.1ml cigarette solution + 10 ml liquid LB media into the 14ml tube labeled 1:100, shake solution to mix
1:1000	- using a new pipet, place 0.1 ml of the 1:100 ml cigarette solution + 10 ml LB media into the 14ml tube labeled 1:1000, shake solution to mix
1:10,000	- using a new pipet place 0.1 ml of the 1:1000 cigarette solution + 10 ml LB media into the 14ml tube labeled 1:1000, shake solution to mix
Bacteria	- The students should save this tube for later. Further instructions for this tube will be given later in this protocol.

- Have one student in each group prepare an LB-plate with one of the above cigarette solutions. The teacher may want to point out the position of 0.1 ml mark on the graduated pipet. To prepare the plates, have students:

  1) label the bottom of the plate with their name, date, and NT (no treatment), UV (UV light Positive control), 1:100, 1:1000, or 10,000
  2) add 0.1 ml of dilution solution to properly labeled LB-plate
  3) bend wire loop into an L shape
  4) flame loop

5) carefully spread 0.1 ml of dilution solution around the agar with the wire loop bent in the shape of an L.
6) Flame the loop again to sterilize for the next step.

Make sure the students remember to use a different pipet for each of the different dilution solutions to prevent contamination!!

- Let the plates sit right-side up (large lid on top) at room temperature to allow the cigarette solutions to sink into the surface of the Agar Petri Plates (approximately 20-30 minutes, can be left overnight due to time contraints if need be).

Treatment Conditions

Tobacco Solution	Controls
1:1	No treatment = NT (negative)
1:100	UV light (positive)
1:1000	
1:10,000	

1) While the cigarette solutions are allowed to sink into the LB-plates students should:

1) Using a graduated cylinder, place 10 ml liquid LB-media in the 14 ml plastic tube previously labeled - bacteria.
2) flame a wire loop
3) once the loop has cooled, remove a very small amount of bacteria from the "student stock plates" situated around the room.
4) The bacteria should then be placed in the 10 ml of LB liquid media in the 14 ml plastic tube to dilute the bacteria. The students should "stir" the loop around in the LB media and watch to make sure the "chunk" of bacteria comes off the loop.
5) Students should then **GENTLY TURN THE TUBE UPSIDE DOWN 4-5** times to mix the bacteria.

- Once the cigarette solutions have been allowed to sink into the surface of the agar each student should inoculate the labeled plates with bacteria.

1) unbend wire loop
2) flame loop and wait for the loop to cool

3) dip the wire loop into the tube labeled Bacteria
4) Use the wire loop to plate the bacteria on the appropriate LB-plate using the plating pattern included in the "Student Guide to Aseptic Technique". Reflame the loop to sterilize for storage.

Students should make only 1 pass with their loop, while plating, otherwise, bacteria end up all over the plate and no colonies will be seen!

5) The UV light positive control plate and NT negative control plates should be plated identically to the treatment plates but without cigarette solution.
6) The teacher should expose the UV light plate to UV light by:
   - removing the Petri dish lid
   - placing the open plate in front of a UV light for 60 seconds.
7) Incubate plates at 30 °C for 48 hr.

Note: Plating bacteria should be done utilizing aseptic (sterile) technique. A description of plating bacteria utilizing aseptic technique is included in the "Guide to Plating Bacteria Using Sterile Technique". The instructor may want to set aside a class period to allow the students to practice working with bacteria.

Day 2: BACTERIA INCUBATING

Day 3-4: DATA COLLECTION AND ANALYSIS : 2-3 hr.

Collecting the data:

- Each student should obtain his/her plate and begin to count the white colonies.
- The number of white colonies for each dilution should then be recorded in a laboratory notebook or on the data sheet provided.
- If no colonies are seen on the students plate and the bacteria grow in a lawn, data can be collected as an estimation of the percentage of white bacteria on the plate. Percentage can be easily determined by utilizing the grid included in this packet.
  1) Students should place their previously inncoulated petri plate down onto the grid, lining up the outline of the circle with the outline of the bottom of their plate.
  2) The students should then estimate the percentage of white bacteria, red bacteria, or empty of each of the 32 small squares

and record the percentages in the appropriate column next to the grid circle.

3) The total percentages of each color can then be calculated by adding up the percentages from all 32 small squares and dividing the total by 32.

Analyzing the Data : Discussion Points

- Did the students see any trends in their group? (e.g. The number of mutations increase as the tobacco solution concentration increases)
- Why are mutations important?
- Did any groups have mutations at the 1:10,000 dilution? If so, discuss how small that amount is.

Statistical Analysis (optional): The instructor may collect the data for the whole class and post it such that each student can analyze all the data. An overhead for the class data sheet is included in this packet.

- Once all of the data has been collected the average and standard deviation for the number of white colonies for each treatment condition can be calculated.
- T-tests can then be performed to determine statistical significance of the results. The following comparisons might prove interesting:
  - NT X 1:1 (no treatment negative control compared to 1:1 dilution)
  - NT X 1:100
  - NT X 1:1000
  - NT X 1:10,000
  - NT X UV light
- Additionally, the results can be graphed as number of colonies vs. dilution of cigarette solution on graph paper such as that included in this packet.

Tobacco Induced Mutations: Student Instructions

Materials:

6 Petri Dishes
Wire Loops (one for each group member)
Cigarettes (2)
Beaker
Stir stick
Hot plate or microwave
LB-Plates (bacteria food)
LB liquid media (bacteria food)
Test tube racks which fit 14 ml tubes Cheese cloth or other filter paper
1 500 ml bottle
Bunsen Burner
Graduated Cylinder
4 1ml size plastic pipettes
5 14 ml plastic test tubes
Serratia marcescens
Distilled Water
Marking Pens

Methods:

Day 1: TREAT THE BACTERIA

Make Tobacco Solution:

- Remove the tobacco from 2 cigarettes and place in 200 ml of distilled water in a beaker.
- Heat the water for 10-15 minutes with constant stirring .
- Filter the leaves out of the extract using cheese cloth or other filter paper.

Performing the experiment:

- Obtain 6 LB media plates and 5 wire loops for your group.
- Label 5 tubes: 1:1, 1:100, 1:1000, 1:10,000, bacteria
- Make dilutions of the cigarette solution using graduated pipettes according to the scheme below. Make a check mark in the box beside the directions for each dilution as you make them.

☐ 1:1 - no dilution, place 0.1 ml of cigarette solution into the 14ml tube labeled 1:1

☐ 1:100 - place 0.1ml cigarette solution + 10 ml liquid LB media into the 14ml tube labeled 1:100, shake solution to mix

☐ 1:1000 - using a new pipet, place 0.1 ml of the 1:100 cigarette solution + 10 ml LB media into the 14ml tube labeled 1:1000, shake the solution to mix.

☐ 1:10,000 - using a new pipet, place 0.1 ml of the 1:1000 ml cigarette solution + 10 ml LB media into the 14ml tube labeled 1:1000, shake solution to mix

☐ Bacteria - Save this tube for later. Further instructions for this tube will be given later in this protocol.

Treatment Conditions

Cigarette Solution	Controls
1:1	No treatment = NT (negative)
1:100	UV light (positive)
1:1000	
1:10,000	

Each student in your group should prepare an LB-plate with one of the above cigarette solutions. To prepare the plate with cigarette solution:

1) label the bottom of each plate with your name & date
2) label one plate with: NT (no treatment), UV (UV light positive control), 1:1, 1:100, 1:1000, or 1:10,000.
3) add 0.1 ml of diluted cigarette solution to the properly labeled LB-plate using a pipette
4) bend wire loop into an L shape
5) flame loop
6) carefully spread 0.1 ml of dilution solution around the agar with the wire loop.

Remember to use a different pipet for each of the different cigarette solutions to prevent contamination!!

- Let the plates sit right-side up (large lid on top) at room temperature to allow the cigarette solutions to sink into the surface of the LB-Plates (approximately 15-20 minutes to overnight).

While the cigarette solutions are sinking into the LB-plates:

1) Get the 14 ml tube labeled "bacteria."
2) Using a graduated cylinder, place 10 ml liquid LB-media tube.
3) Flame a wire loop
4) Once the loop is cool, remove a very small amount of bacteria from the "student stock plates" situated around the room.
5) Place bacteria in the 14 ml plastic tube to dilute the bacteria. "Stir" the loop around in the LB media and watch to make sure the "chunk" of bacteria comes off the loop.
6) **GENTLY TURN THE TUBE UPSIDE DOWN 4-5** times to mix the bacteria.

Inoculate all 6 plates with bacteria:

1) unbend wire loop
2) flame loop and wait for the loop to cool
3) dip the wire loop into the tube labeled "bacteria"
4) Use the wire loop to plate the bacteria onto the appropriate LB-plate using the plating pattern included in this packet. Reflame loop after each plating.

Make only 1 pass with your loop, while plating, otherwise, bacteria end up all over the plate and no colonies will be seen!

5) Take your UV light plate to the teacher for exposure to UV light.

Note: Plating bacteria should be done utilizing aseptic (sterile) technique. A description of plating bacteria utilizing aseptic technique is included in the "Guide to Plating Bacteria".

Day 2: BACTERIA INCUBATING

Day 3: DATA COLLECTION AND ANALYSIS

Collecting the data:

- Obtain your plate and count the white colonies.
- Record the number of white colonies for each dilution on the data sheet provided.

Analyzing the Data:

- Share your data with the teacher and collect the data from the other groups from the teacher.

- Calculate the average and standard deviation for the number of white colonies for each dilution of cigarette solution from the class data sheet.
- Perform T-tests for the following comparisons:

    NT X 1:1 (no treatment negative control compared to 1:1 dilution)
    NT X 1:100
    NT X 1:1000
    NT X 1:10,000
    NT X UV light

- Graph number of colonies vs. dilution of cigarette solution.
    - Label Axis
    - Graph averages from the class data for each dilution solution
- Paste the results of the analysis in your laboratory notebook.

Tobacco Induced Mutations: Student Data Sheet

Names of Group Members: _____

Date: _____

	Treatment Conditions					
	Controls		Dilution of Cigarette Solution			
	No Treatment (NT - negative control)	UV light (positive control)	1:1	1:100	1:1000	1:10000
White (# of colonies)						

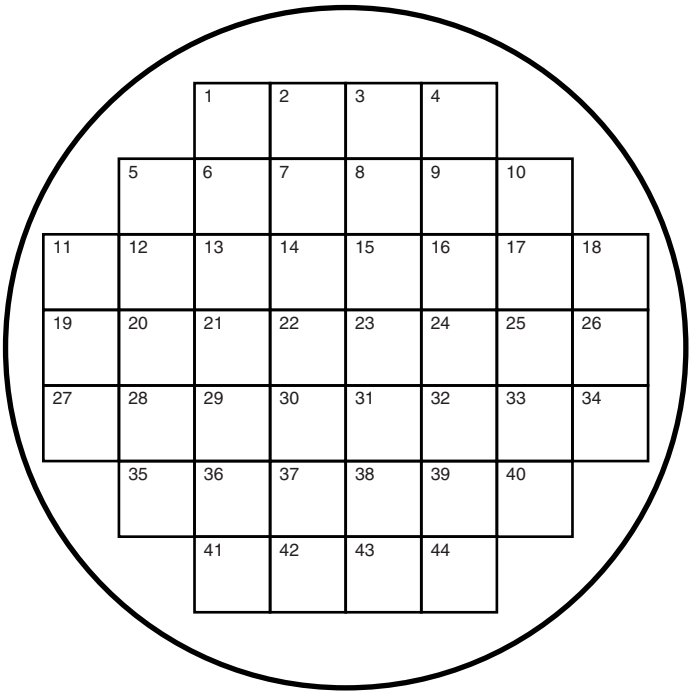
Tobacco Induced Mutations: Class Data Sheet

	Treatment Conditions					
	Controls		Dilution of Cigarette Solution			
	No Treatment (NT - negative control)	UV light (positive control)	1:1	1:100	1:1000	1:10000
Group 1						
Group 2						
Group 3						
Group 4						
Group 5						
Group 6						
Group 7						
Group 8						
Group 9						
Group 10						
Group 11						
Group 12						
Average						
Standard Deviation						

Tobacco Induced Mutations:

Statistical Analysis

	Comparisons				
	NT X UV	NT X 1:1	NT X 1:100	NT X 1:1000	NT X 1:10000
P value					
Statistically Significant ? Y/N					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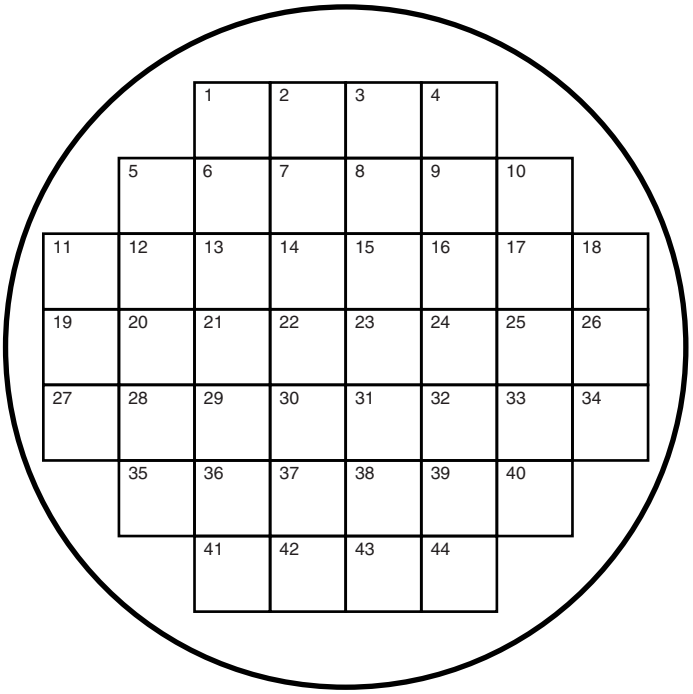
5 6 7 8 9 10
11 12 13 14 15 16 17 18
19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34
35 36 37 38 39 40
41 42 43 44


1 2 3 4
5 6 7 8 9 10
11 12 13 14 15 16 17 18
19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34
35 36 37 38 39 40
41 42 43 44

NAME: Tobacco Induced Mutations %

Sample: _____

% of square covered	
White	Red
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9
10	10
11	11
12	12
13	13
14	14
15	15
16	16
17	17
18	18
19	19
20	20

White	Red
21	21
22	22
23	23
24	24
25	25
26	26
27	27
28	28
29	29
30	30
31	31
32	32
33	33
34	34
35	35
36	36
37	37
38	38
39	39
40	40

	White	Red
	41	41
	42	42
	43	43
	44	44
Total	/44	/44
	%	%
	White	Red

Sample: _____

% of square covered	
White	Red
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9
10	10
11	11
12	12
13	13
14	14
15	15
16	16
17	17
18	18
19	19
20	20

White	Red
21	21
22	22
23	23
24	24
25	25
26	26
27	27
28	28
29	29
30	30
31	31
32	32
33	33
34	34
35	35
36	36
37	37
38	38
39	39
40	40

	White	Red
	41	41
	42	42
	43	43
	44	44
Total	/44	/44
	%	%
	White	Red