Upper Division Biology class - (AP, IB or independent electives)

Introduction:

The study of evolution is arguably one of the more difficult subjects for students to understand and appreciate in the field of biology. There are many reasons for this difficulty, from experimental limitations in the classroom to varied interpretations of the data and phenomena. One potential barrier of student exploration of evolutionary theory is that it is relatively easy to gain a superficial understanding of the process and outcomes. Superficial understanding opens the door for misunderstanding, and the concept of evolution seems particularly susceptible to misconceptions by students. One of the main goals of this project is to guide students towards a deeper understanding of evolution as a process and the many dynamics involved. Using a harmless bacteria P.fluorescens, and a series of transfers and dilutions, students will begin to see morphological changes in less than 7 days. This process is meant to facilitate discussions in class and between students to steps taken in the procedure and the results observed.

Microorganisms are ideal study subjects for this experiment because you can obtain many generations of organisms quickly and easily. The experimental evolution described here involves multiple populations founded by a single clone of a harmless bacterium. Pseudomonas fluorescens SBW25 is a plant-associated microbe that grows on plant leaves and roots and serves as a probiotic by controlling various fungal pathogens. Changes in colony morphology represent evidence of a beneficial mutations within the population.

This experimental evolution model is simply a series of daily transfers of a bead from one culture tube to the next, which preferentially selects for cells that attach to the bead, and selects against free-swimming planktonic cells. Only the cells that attach to the bead are transferred to the new test tube. The major product of this selection is production of a biofilm, which is a complex structure of aggregated cells on a surface (think dental plaque) that can cause pathogenic or beneficial interactions with their host or environment. In the labs students use microbiology lab procedures to investigate the process of bacteria reproduction and track how offspring of a single clone origin can diversify in morphology, quantity, and genetic makeup in a short period of time. This lab has direct connections to **NGSS HS-LS 4 (biological evolution: unity and diversity)** as well as connections to other standards depending on what direction the teacher and students want to pursue.

Included in this packet of information:

1) Learning targets
    a) Lesson plan outlines, with links to resources
    b) Teacher notes for specific concerns in lessons
2) Student pre-assessment tool (link is embedded on Lesson Day 1)
    a) google form that can be shared with students online
    b) an evaluation of answers to use as a metric (*separate document*)
3) Next Generation Science Standards connections outlined
4) Background sources for student and teacher
    a) Background readings (are linked in lesson plan outline)
    b) Lab packet and protocol for student information
    c) Teacher answer key for lab packet

5) Graphics to explain process and outcomes; daily process and results
    a) Teacher graphics for teacher prep
6) Lab protocol, safety procedures; (anti fire protocol/ what to do if)
    a) short videos about sterile protocol are linked in the NSTA presentation
    b) suggestions for teacher about how to approach this work with students
    c) Powerpoint to drive while students work through the protocol

1) Teacher notes:Upper level Biology class Lesson outline: (AP, IB or other upper division biology classes)

Prior to the start of this lab, students should have a background in the basics of genetics, cell reproduction (mitosis and meiosis), as well as a basic understanding of evolution. If students have a basic understanding of RNA/DNA and protein synthesis processes, that is great for some extension discussions. This unit includes a pretest, meant to identify and quantify the students' current ideas about Evolution and Natural selection prior to the activities. The pretest can be given online or hard copy, a rubric is provided to help categorize answers by students, so that misconceptions can be addressed during the activities. It is recommended the test be given afterward also, so students can compare and evaluate their own changes in the learning process.

Student Learning objectives:

1) Based on a set of given problem statements, students will choose one to focus and refer to during the experiment. This will be the problem statement that they use to focus their lab report, individual research and analysis process.
2) As a result of the lab protocol, students will use the key concepts to make connections to evolution trends and the natural selection process in biofilm building bacteria in other aspects of life/Earth. In a lab report students will explain how the protocol created a selection process to increase and highlight the proliferation of wrinkly spreaders vs smooth round morphology and how those variables related to the outcome.
3) Students will practice microbiology lab techniques, and effectively record lab procedures, observations, and results during the given lab protocol using Pflu as a model for evolution. Discussion questions will be used to highlight and confirm understanding of the process and how the results link to the NGSS HS-LS4-2.
4) Using the data from the experiment, students will create a spreadsheet/ graphs to illustrate the class results and explain how those results connect to their lab team's problem statement. Students will analyze this data and how it correlates to another larger evolution topic, which will be communicated in a formal lab report.
5) By the end of the experimental protocol students will develop 3 hypothesis statements about the genetic variation between the original ancestor and any new morphologies present. ***

Day of lesson	topic	resources	Follow up/assessment
1) **Introduction**	Introduction - outline of history of evolution; misconception probe, Class discussion/ history outline overview	online test (google form or AAAS/hard copy test option Students copy of lab protocol Intro to prokaryotic origin/bacteria background reading	Build a question board for any and all ?? on evolution (engage) Give students NGSS outline, learning objectives and assessment goals

2) **Background and practice**	Background reading/ and intention of the lab previewed; Practice lab skills: pipetting; sterilizing tubes; dilution series; plating techniques ***	Pre-read Lab journal set up w/ protocol for lab (explore) Outline/ Choose problem statement for class	Lab Journal set up: Problem statement; materials; generic procedure (overview); Vocab list on separate sheet;
3) **experiment begins**	**Day - 1:** transfer colonies to grow overnight into LacZ+ and LacZ- tubes w/ bead, for each lab station	Plates grown 48+ hours prior; sterile glassware and media for each lab station	Discussion questions and lab journal time. (explore)
4	**Day 0** - bead and create Evo tube #1; series dilution to 10^2; plate to confirm and get baseline data of SR colonies	Sterile glassware; media; plates; vortex, etc	Lab journal work: procedure outlined; observations for the day; any anomalies in procedure; Hypothesis re: plates when checked Day 2
5	**Day 1** - bead transfer; Conversation re: Natural selection vs artificial selection; context in real world of biofilm species Plates note ***	Sterile glassware; media	updates/ observations; notes about their role in the selection process and why they are performing this task based on intended outcome related to hypothesis
6 (optional discussion day)	Class conversation re: connections of lab to research/ evolution, etc) (explain)	Read plates from Day 0	Wrinkly spreader reading
7	**Day 2** - bead transfer, dilutions and plating;		
8	**Day 3** - bead transfer		Natural selection reading
9	**Day 4** - option to finish or continue (if finishing, dilution series and plating)	Read plates from Day 2	
10 discussion day	data analysis re: problem statements	Day 5 bead transfer option	DNA mutation background ideas/ research info
11	Read day 4 plates Class data sharing/ analysis expectations		Day 6 final day option
12 wrap up day	review procedure,	Post test re: Evolution and	Lab report rubric

	results, and final lab report expectations	natural Selection evaluate Discussion re: new understandings as a result of the activity	(elaborate)

Blue highlights in the chart reflect alignment with the 5E's strategies

Teacher notes:
This series of lesson plans and activities are designed for upper division biology classes, which could include IB, AP or other science elective courses that have time to cover the topic of Evolution with a lab activity and extended resource/ research components.

Day 1 lesson:
****Pre-test designed** along the lines of the ACORNS research (Opfer et al, June 2012; Cognitive foundations for science assessment design) See the google form which allows for individual analysis and class analysis. Evaluation of learning at the end of the project can be part of a discussion re: how student perceptions or answers have changed. Scoring of pre-assessment rubric

Students should **pre-read the background Prokaryotic origin** to have an overall view of prokaryotic cells, Pseudomonas fluorescens (P.flu), evolution and adaptation, and bacteriological practices. Background topics include evolution of single celled organisms, difference between a colony and individual organisms, and some facts about the strain of bacteria they are about to start working with in the lab.

As the lab progresses, there are other supplemental readings that can be supplied to the students to inform them about the process of morphological changes, DNA changes, and how these specifically relate to early evolution of organisms on Earth.

Day 1:
Resource links: history of Evolution outline; Pre-test online; prokaryotic origin reading

1) Give students a chance to write down all of their questions re: evolution, on poster paper that will be posted around the room. Continue to use these as a referral point as it comes up in class discussions.
2) Handout lab packet to students which includes learning objectives, protocol, and assessment goals

Day 2 lesson:
***** Pre- lab work -**
Students will need to practice aseptic methods with test tubes of practice media, including safety with mini bunsen burners, and how to sterilize tubes, etc. It will also be important to practice other lab protocols: changing polystyrene ball from one culture media to another; practice in dilution techniques; and practice plating samples using spreader bars and use of pipetting tools. It is suggested that students have an opportunity to practice these skills prior to the lab when working with the actual bacteria. Set up lab stations which have ***Plain water in media tubes, plain agar plates, alcohol beakers for the forceps and plating bar, mini bunsen burners, etc***. Practicing aseptic protocol prior to the lab sequence could potentially yield better results, which allows for better quality observations, discussion and opportunities for the students to build ideas regarding evolutionary changes in these bacteria colonies.

Students should pre-read the Lab journal set up and procedures for the lab prior to the practice session, so that they have an overview of the process and intentions. Be sure that students have an understanding about the learning objectives and assessment outcomes for this project.

Day 2:

1) Class discussion re: prokaryotic origin reading, and how it relates to P.flu experiment.
2) Have students set up their lab journals with heading, date, and Problem statement of their choice.
3) Show students the lab protocol practice procedures - they should practice them, and highlight quantities in the note packet, and other steps to focus on in the protocol.
    a) Why sterilization is important - in microbiology sterilization is a concern for both the validity of results (to prevent contamination from other sources than what you are testing) and safety of the lab technicians, depending on the organism they are working with in their research.
    b) Use of pipette tools and how to load/ clear and release tips these are precision instruments, it is important to use them correctly in order to improve quality of results
    c) Bead transfer w/ sterilization procedures have student suggest ideas of the role of bead transfer in this activity.
    d) Dilution series and why/ how from bead liquid in small tube, to 10^{-2}, 10^{-4}, 10^{-5}
    e) If time practice plating and spreading liquid w/ spreader bar

Safety briefing: be sure to note any steps that need extra caution due to open flame and use of alcohol for sterilization practices. Also note how to deal with a fire emergency should one occur. Other concerns include labeling of tubes and plates to maintain quality data throughout the protocol. The most common mistake is taking the forceps or spreader bar from the flame and back to the alcohol beaker. This will cause the beaker to start on fire. (precaution - put only enough alcohol in the beaker to cover the bottom of the bar and tips of the forceps.)

Day 3 lesson:

Experiment begins, this is a good time to make sure students have lab journals set up and are ready to record lab data, observations, etc.

Day 5 lesson:

Day 1 experiment - Teacher Note:

Remove the Day 0 agar plates from the incubator and examine. It is normal, to not have quantifiable data until 48 hours after plating. Students should have data charts that will account for the number of blue and white colonies *(ideally the ratio should be approximately 1:1 on Day 0 given equal competition for the media. Longer incubation times for day -1 might change that ratio)*. If plates have developed they can be stored in the refrigerator for later observations and comparisons. It is a good plan to keep all of the plates until the students have finished reading all of them from Day 0, 2 and 4. They may need to refer back to previous plates as they get better at observations of colony formations.

*warning - plates get odorous by the end of the experiment, sealing them in a container might help the smell from spreading

Day 6 lesson:

This is a good time to have a discussion with the class to make sure they understand what is going on. It is also a good day to re-look at the questions they set up on Day 1 - can they answer any yet? This is a way to also check on how their research is going. Within the context of this protocol there are long procedural days and short days (bead transfer), if you find the protocol is interfering with the learning process, it is ok to leave the

tubes to incubate an extra day in order to have students catch up with the learning/ discussion points. Once students get proficient with bead transfer, there will be time to work in their lab journals, or have discussions to clarify points of interest.

See the answer key for lab packet questions.

Other discussion points that may come up during the lab:

1) **Reproductive rates - for Pflu** - Pseudomonas in general have a range of reproductive rates depending on the species and growing conditions. There is a diversity of range in the genus from 9.8 min to significantly longer. For P.flu specifically, references list anywhere from 51 min to to 200 min depending on conditions. Have students find resources to compare the rates of different bacteria, and suggest why different organisms have different reproductive rates - connection to the current lab variables is also useful.
    a) http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1392930/
    b) http://textbookofbacteriology.net/growth_3.html
    c) http://www.cabdirect.org/abstracts/20093116814.html;jsessionid=27C5525007A3A49537FAAC9530FF482B

2) **Biofilm connections w / mutations** evidenced - what is the role of WS in other contexts - Cystic fibrosis; stromatolites; what others? Is this mutation beneficial or not? From the perspective of the bacteria organism, how does this mutation create other complications for survival?
3) Emphasize how random mutations occur all the time during both meiosis and mitosis, as well as protein synthesis (freq rate 1 in 10^{7}). Consider that there are many checks in each of these process to prevent a mutation from being fatal to the organism. In spite of those checks in the process, mutations still happen and ultimately lead to changes in organism. Have students explain how this fits in with both evolution, natural selection and maintaining a population.

Potential end of experiment on Day 4 - at this point the students have evidence that can be analyzed, and they can suggest connections from the background resources to suggest why they are seeing these results. Stopping here also allows the teacher to cover other related topics depending on which standard or strand of curriculum needs to be emphasized, re: difference/ benefits between organism vs colony formations, which can lead to multicellularity topics; the differences of phenotypes, and exploration of genotypic information. Which could include DNA comparisons of each observed phenotypic model; cellular mechanisms that result in the morphological changes, ie: protein synthesis, mutations at the genome level; evolution trends with more conversation about natural selection and other factors that contribute to changes at the organism level or population level; and so many more depending on the class interest.

This is a good time to have students reflect back on the early prompts connected with the background information to assess understanding of the lab process itself and how it connects to bigger themes in biology.

If there is time and interest, this protocol can be extend 7 days, which would include one more transfer day and one more plating day using the same protocol from day 3, and day 2 respectively. The last day of the experiment is simply a dilution and plating day with no further bead transfers(day 4 here). To continue this procedure for one more round, offers that many more possibilities for students to see a variety of wrinkly spreader morphology, and have more conversation about competition, modifications, etc. absolutely worth it if you have time.

Further extension can be used with students include: isolating DNA; having students review DNA information and analyze where mutations have occurred with known databases; using class samples of isolated DNA, then using a restriction enzyme, and running gel electrophoresis to look at different samples banding size.

3) NGSS Connections chart - Adv Biology level

Standard: HS LS4-2 Biological Evolution, Unity and Diversity

Performance Expectations: Students will use lab techniques and technology to observe morphological changes in a species over many generations; students will collect data on specimen reproductive rates; observe mutations which lead to morphological changes; suggest ideas re: competition/mutation processes that lead to class results.

HS LS4- 2 Construct an explanation based on evidence that the process of evolution primarily results from four factors: (1) the potential for a species to increase in number, (2) the heritable genetic variation of individuals in a species due to mutation and sexual reproduction, (3) competition for limited resources, and (4) the proliferation of those organisms that are better able to survive and reproduce in the environment. *[Clarification Statement: Emphasis is on using evidence to explain the influence each of the four factors has on number of organisms, behaviors, morphology, or physiology in terms of ability to compete for limited resources and subsequent survival of individuals and adaptation of species*. [Assessment Boundary: Assessment does not include other mechanisms of evolution, such as genetic drift, gene flow through migration, and co-evolution.]

Dimension	**NGSS name/citation**	**Connections to activity**	**Other resources**
Science and Engineering Practices	**- Obtaining, Evaluating, and Communicating Information**: progresses to evaluating the validity and reliability of the claims, methods, and designs. Communicate scientific information (e.g., about phenomena and/or the process of development and the design and performance of a proposed process or system) in	Students will use a standard lab protocol, individually choose a problem statement as an avenue to focus their analysis and research; they will document and evaluate the results on a daily basis relative to their choice of topic; they will then present their data graphically, and textually in a formal lab report, using the	Students will need to research at least 2 outside sources for reference to either evolution or biofilm bacteria and how this information connects to their

	multiple formats (including orally, graphically, textually, and mathematically).	activity information recorded in their lab journal.	findings for their final presentation.
Disciplinary Core ideas	**LS4.B: Natural Selection** - The traits that positively affect survival are more likely to be reproduced, and thus are more common in the population. (HS-LS4-3) **LS4.C: Adaptation** Evolution is a consequence of the interaction of four factors: (1) the potential for a species to increase in number, (2) the genetic variation of individuals in a species due to mutation and sexual reproduction, (3) competition for an environment's limited supply of the resources that individuals need in order to survive and reproduce, and (4) the ensuing proliferation of those organisms that are better able to survive and reproduce in that environment. (HS-LS4-2)	Students will be able to observe/ document reproduction ratio's of both the lacZ- and lacZ+ strains of Pseudomonas fluorescens; students will also observe/document morphological structures/ changes from the ancestor strain to the final offspring plates, as a result of selection processes. Students will make hypothesis and suggestions re: the 4 adaptation factors and how they are at work in the lab protocol.	Documentation of plate results by each student lab group in their lab journal of the colony numbers and type from even day lab protocol (day 0, 2, 4, etc)
Crosscutting concepts	**Patterns** Different patterns may be observed at each of the scales at which a system is studied and can provide evidence for causality in explanations of phenomena.	As students record evidence from plating results of P.flu lacZ- and lacZ+, discussions in class will develop ideas of causality, which may include topics of competition, resource use, benefits, etc ; Observation of morphological changes/ patterns will lead to discussions in which students will be asked to suggest ideas re: DNA mutations. Based on supplemental readings, students will be asked to predict causality for this particular species, and other biofilm species.	Supplemental reading on morphology of wrinkly spreaders and natural selection Optional use of other species data patterns to compare/ contrast Student observations of patterns will be reported in both their lab journal

			and a formal lab report.
Connections to Nature of Science	**Scientific Knowledge Assumes an Order and Consistency in Natural Systems** Scientific knowledge is based on the assumption that natural laws operate today as they did in the past and they will continue to do so in the future	Students will use evidence to support scientific laws, related to both data for this lab, and extrapolate how this works in the world past and present with reference to other organisms with morphological changes. Class discussions of other biofilms ie: stromatolites, cystic fibrosis, etc or larger organisms connections.	Research papers - found on own, those supplied with the lab, and the historical outline of evolution ideas as a references

Recommended student Assessments for this unit:

1) keep a lab journal of process and findings, including the questions and writing prompts presented within this protocol and the student lab sheets. Journal will be graded at the end of the experimental process.
2) Ask lab partners to present their specific claims and findings to the class. At this time it is important to emphasize salient points in a focused, coherent manner with relevant evidence, sound valid reasoning, and well-chosen details. Additionally students can be evaluated on the following public speaking skills: use appropriate eye contact, adequate volume, and clear pronunciation (refer to Common core public speaking rubric for more ideas)
3) Students individually present their findings in a written lab report format with research citations, charts and diagrams, etc. (see rubric suggestions) Use of an online grading program is useful for students to receive feedback on both their writing skills and scientific presentation on technical lab reports. (Turnitin.com)

4 b.) Student lab guide for Pseudomonas fluorescens: Name _____

Lesson #2

As a result of reading the background material, please be prepared to discuss the following questions in class:

- What is the difference between a colony and an individual multicelled organism? Are there benefits to each form?
- What type of bacteria are we using and why is it a good choice for this experiment?
- In which organisms can we observe Evolution in action today both in the laboratory and in the field?
- What evidence needs to be documented when studying changes in organisms to support the idea that evolution has occurred?
- Why is it hard to use experimental models of larger more complex organisms when we want to observe changes?
- In what ways does Natural Selection play a role in the evolution of bacteria? Does artificial selection have a role in the evolution of bacteria?

Lab activity Objectives: the purpose of this lab is to 1) model evolution and morphological changes of single cell organisms, *Pseudomonas fluorescens (P.flu),* through a series of generations while purposefully selecting biofilm strains that attach to a bead. 2) You will practice bacteriological lab skills, in the process of growing generations of harmless bacteria. 3) As the selected population continues to grow through a process of bead transfers, you will be able to make observations of phenotypic changes, as well as make predictions regarding future colony growth and morphological changes as the laboratory proceeds. 4) This lab activity will culminate in a lab report that will be "published" by the student which will offer connections to past and present trends in evolution. This report will include data analysis from the experiment and offer suggestions as to how it does or does not relate to their research findings of another experimental specimen.

Student Lab Journal guide:

As a student of science we are going to pursue this experiment as a research scientist would, be sure to document all important information, quantitative and qualitative data, as well as procedural events that might affect the outcome. Include questions that come to mind, any anomalies that occur, as well as any other key information that would be necessary to publish your findings.

1) **Develop a problem statement:** here are some suggestions*, you and your lab partners need to choose one and put it in your lab journals in a complete form.*

- To observe Evolution in action, Pseudomonas fluorescens is an ideal model because _____
  - *Be sure to include conversation about what documentation would need to be considered to be "significant" and therefore a good model of the process of evolution?*
- The key variables that prompt evolutionary change at the level of the organism level are_____
  - *be sure to include those related to laboratory experiments and in nature*
- The variables that affect Natural selection in single celled organisms include _____
  - *in the long run you will need to consider how could you determine if selective advantage had occurred?*
- There are benefits/ costs for a single cell organisms to mutate/ change morphologically, leading to either colony formation or multicellularity, for example _____

2) Research: Once you and your team have chosen a problem statement, you will need to find at least 2 research sources of information, related to either evolution or biofilm bacteria specifically. ***This step should be completed in the next two days, remember to take notes on the articles as well as citations for future reference.***

Based on your background readings, and class discussions:
3) Create a Hypothesis: choose one of the discussion points related to evolution of single celled organisms (prokaryotic cells) and build a hypothesis statement which must include variable manipulation and outcomes expected.

4) List all possible variables that could be involved: based on your reading of the lab protocol, please identify the variables, be sure to identify which are controlled, the independent variable, dependent variable, and confounding variables. Explain how the protocol addresses these to justify your identification.

- As a class agree which protocols are necessary and essential, which all students will use to improve validity of class results.

5) Lab protocol prep: make sure you have already read the protocol from Day - 1 to Day 1; using the prepared materials, your teacher will demonstrate each of the following processes. As a lab team you will then practice each process and make sure you have a thorough understanding in how to perform the skills and be safe in the process. Practicing these aseptic laboratory protocols could potentially yield better class results, which allows for more discussion and opportunities to build ideas regarding evolutionary changes in bacteria colonies. You will be using ***Plain water in tubes, flame sterilization, plain agar plates etc***. before using the actual bacteria for the experiment.

Materials: 1 set each per lab station: 1 large test tubes with water and bead, caps on; 1 small test tube w/ water/ cap on; 1 forcep/tweezer; 1 beaker w/ small amount of ETOH 75%; mini bunsen burners; vortex; 2 large test tubes w/ water and caps on for dilution series; 50 µl pipette and tips; practice plates have plain agar; parafilm

A. Students practice bead transfer and sterilization protocol using the actual tubes and beads, flame, with water. *(large test tubes with water and bead, caps on; 1 small test tube w/ water/ cap on; 1 forcep/tweezer; 1 beaker w/ small amount of ETOH 75%; Bunsen burner/ mini; vortex)*
B. Students practice dilution series protocol with tubes and pipettes from small tube, to create a 10^{-2} and 10^{-4} series; *(mini bunsen burners; vortex; 1 small tube w/ cap and water; 2 large test tubes w/ water and caps on for dilution series; 50 µl pipette and tips)*
    a. students document how the math works on the dilution series, and why this is an important step before plating bacteria from the evolution tubes.
C. Students pipette 50 µl water onto a plate and use sterile spreader bar to practice plating; students also learn how to seal petri dishes with parafilm.

Document all of these things in your lab journal; sketch set ups, and explain why it is important to follow this procedure and sterilization process.

Lesson 3:

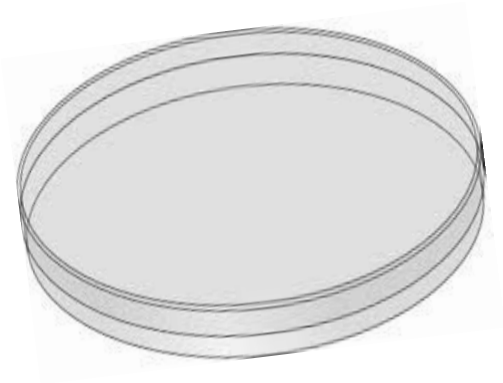
Discussion prompts for students:

- Which variables do you think are key to the evolution in early organisms?
- What conditions are necessary to culture bacteria in a lab setting?
- Are there any new terms that you need to clarify from your research?
- Identify the variables involved in the process for single celled organism reproduction.
- What factors would be necessary for a single celled organism to move to multicellular organism.

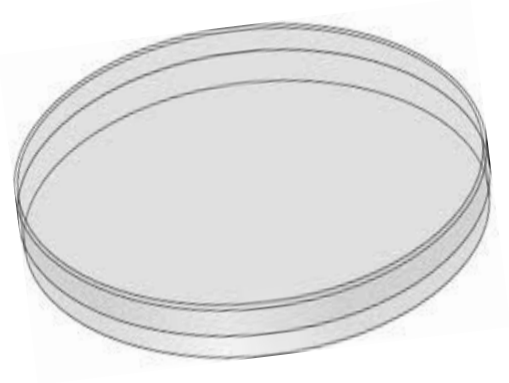
Day -1 Protocol and observations: Today's task is to transfer original colonies from plates to incubation tubes day. Once they are incubated, we'll begin our observations of morphological changes to the original ancestor strain, as well as quantifying the LacZ+ and LacZ- colonies.

GOALS for Day -1: please two goals of today's procedure in your lab journal.

1) Before starting the procedure, observe and sketch what you see in the original *P.flu* LacZ - and *P.flu* LacZ+ plates: *be prepared to explain the difference between isolated colonies and overgrowth sites.*



P.flu LacZ - colonies



P.flu LacZ+ colonies

observational notes: record your observations including color, size and location of colonies, patterns, etc

- be sure to look under the dissecting scope for more observations of the colonies on the original plate.

Experimental Day - 1 - preparing the *P.flu* for our lab observations:

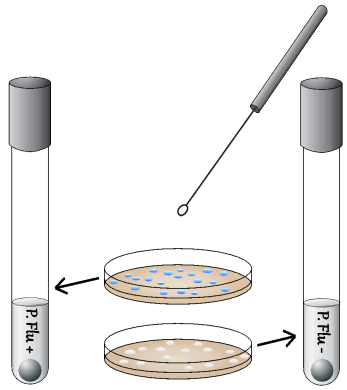
Before the bacteria got to our classroom, they had been stored in a cryo solution and frozen at -80°Celsius (-112° Fahrenheit). They were extracted from the cryo tubes, and plated. The colonies you see on the plate should be color coded blue and white due to a specific color treatment added to the agar to help differentiate them in this procedure. ***This is not a correlation to the bead colors*** *used in the experiment. the strain of P.flu that is LacZ+ will show up on the plates as blue colonies, and the strain that is P.flu LacZ- will appear as white colonies. This color coding allows you to easily identify colony types and quantify results quickly.*

When selecting a colony to move from the original plate to the test tube, choose one that is self contained, and by itself about the size of a punctuation mark rather than a large clump or sheet of bacteria. Initial colony selection is one variable related to the type of results you see further you go in the experiment.

Day -1 Materials

- Metal inoculation loop, in beaker of 75% ethanol
- gloves
- mini bunsen burners/ lab station
- vortex
- parafilm to reseal plates at finish
- *Pseudomonas fluorescens* SBW25 LacZ+ colonies *(these colonies should be blue, due to agar + Xgal)*
- *Pseudomonas fluorescens* SBW25 LacZ– colonies *(these colonies will be white, due to agar + Xgal)*
- (2) Large glass culture tubes per team containing:
  - 4 mL King's B Medium (KB)
  - 1 mL T-Soy Broth
  - 1 blue polystyrene bead

Figure 1: Use a sterile inoculation loop to inoculate *P. flu* + culture tubes with blue colonies, and *P. flu* – tubes with white colonies.



Day -1 Procedure:

1. You will need Two culture tubes (one for your white colony selection, and one for your blue colony selection. Be sure to label the side of the tubes using a permanent marker, with either "*P. flu +"* and your team name or "*P. flu –*" and your team name. There should be a blue bead in each tube as the Day -1 tube.
2. One team member will flame sterilize an inoculating loop until it becomes "red-hot". (use the same technique demonstrated in class) Remember to dip the loop in 70% ETOH before flaming the loop, let it cool.
3. Once cooled, use the loop to transfer a **single** isolated *Pseudomonas fluorescens* Lac+ (blue**)** colony from an agar plate to the "*P. flu* +" culture tube. Sterilize the vial and lid before returning the test tube to your lab tray. Then vortex the test tube to mix the cultures into the media.
4. Flame sterilize the inoculating loop and repeat the same process, this time transferring a single *P. fluorescens* Lac– (white) colony to the "*P. flu –*" culture tube. Remember to sterilize the vial and lid before using the vortex to mix the cultures into the media.
5. Your lab group should now have a single test tube for each strain of ancestor P.flu.
6. Incubate the culture tubes at 28°C for 24 hours.

Teacher note regarding timeline: there may be a bottleneck on this day as each lab group selects their colonies from the plate, then moves to the vortex.

****Once you have completed the protocol, make notes in your lab journal re: observations, anomalies and any other information that will be useful later for your lab report. Using your lab journal is a key skill in this process.

On some of the following days it may be important to designate lab partner tasks, so that observations and results are documented, and protocol is completed. Taking pictures with your smart phones is a great tool, but not the only way to record results.

In your lab journal leave a page to record questions that come up during the protocol that we might not have time to answer at that specific time. These questions or ideas will add to our discussions as we proceed, and will also be good information to add to your research outline to be used in your introduction or conclusion of the lab report.

Lesson # 4

Even days are longer protocol days, please use your time wisely, be safe and methodical in your procedure.

Your bacteria have been incubating at 28°C (82.4°C) for the past 24 hours. Today, the polystyrene bead will be removed from the overnight culture and vortexed to remove all of the biofilm that has formed on the bead. ***An equal volume of the LacZ- white and LacZ+ blue P. fluorescens culture will be added to a single evolution test tube using a pipette****, in which they will compete for media (nutrition) both in the test tube and on the petri dishes. space on the bead and test tube.*

a. *Make a prediction about the competition factor for media by each type of P.fluorescens at this stage of the experiment. What would you expect to see in your results if there is a competition for resources?*
b. *Why are we only selecting the bacteria that has been growing on the bead?*
c. *Is it possible that there is a competition for resources in other areas of the test tube? Choose 1 to explain.*
d. *Why are we only selecting the bacteria that has been growing on the bead? Is there another location that might also yield biofilm bacteria? Defend your answer.*
e. *Suggest one reason why that is a necessary step before putting a sample of the new combinations on an agar plate.*

Lab Protocol Day 0: Bead transfer, dilution series, plating day

End products for Day 0:

- 1 Evolution tube per team which contains both LacZ+ and LacZ- colonies
- 1 agar plate made from a 10^{-4} dilution per team

Confirm that your lab stations have the following Day 0 Materials:

- Metal forceps, in a beaker of 75% ethanol
- (2) Small glass tubes containing: 1 mL KB/ team (for bead transfer)
- Vortex
- A p200 and p1000 pipette and sterile tips
- (1) Large glass evolution tube containing: 4 mL KB and 1 white polystyrene bead/ team
- (2) Large glass tubes containing: 5 mL Phosphate Buffered Saline (PBS)/ team
- (1) ½ Strength Tsoy-Agar plate containing X-gal/ team
- 75% ethanol spray bottles
- Glass rod (for spread plates), in a beaker of 75% ethanol
- parafilm for sealing plates, permanent marker for labelling
- Gloves
- Mini bunsen burner

Day 0 Procedure:

SAFETY: remember to tie back long hair, no loose clothing when working around flames, and gloves are required. If there are any accidents or mishaps, please report those immediately to your teacher. Be especially careful when working with an open flame and ETOH (which is flammable). There are a number of sequence steps in today's protocol, be methodical as you proceed.

1. You will need to label the small KB tubes *P. flu* + and *P. flu* –; also label the large evolution tubes and agar plates with your team name, replicate dilution, and the day of the series and date; and the large PBS tubes have been labeled 10^{-2} and 10^{-4} with team names. *See the examples as a model for how to label. Do not open any of the media tubes or plates until you are ready to use them, this will help maintain sterile conditions inside each item.*

2. Using sterile techniques, Pour the contents of the "*P. flu* + " overnight culture into its plastic cap, then use sterile forceps to transfer ONLY the bead to the corresponding small KB tube. (*Flame sterilize the forceps and allow them cool for 30 seconds. Make sure that once you have flamed the forceps, they do not touch anything else! If they touch something else after they have been flame sterilized, they are no longer considered sterile!*) **It is possible that you may hear a sizzle; this is normal and just means that the forceps are still hot from sterilization. Allow them to cool until you no longer hear a sizzle before you touch the polystyrene bead.** Flame the forceps and repeat the same process with "*P. flu* – " overnight culture (Figure 2) into it's small KB tube. *You now have two small KB tubes.*

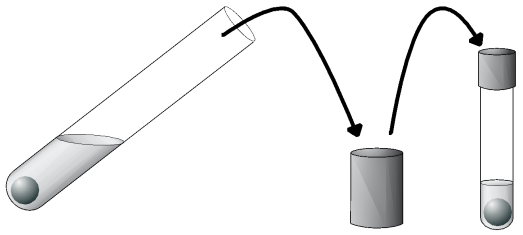


Figure 2: The overnight culture (left) is poured into its cap, and then sterile forceps are used to move only the bead to the small KB tube (right).

3. Vortex both (+ and -) small KB tubes for at least 45 seconds to remove biofilm from the bead.

4. Once your small KB tubes have been vortexed, it is time to transfer a portion of this solution to the Day 0 evolution tube. Briefly vortex one of the small tubes for about three seconds, and then use a **p1000 pipette** set to transfer 500 µl of this vortexed "*P. flu* +" liquid media from the small KB tube to the large evolution tube labeled with your team name and the Day#. Repeat with the vortexed "*P. flu* – " liquid, adding it to the same large evolution tube (Figure 3). **Your end result will now be 1 evolution tube w/ white bead for each team.**

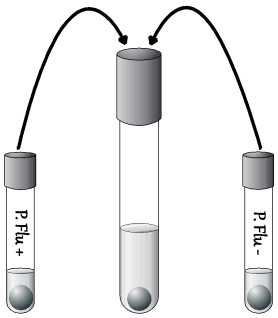


Figure 3: The evolution tube (center) is inoculated with vortexed liquid media from *P. flu* + and *P. flu* – cultures.

5. Briefly vortex your team evolution tube , then use a **p200 pipette** set to move 50 µl from the large evolution tube to the PBS tube marked 10^{-2} . **Set the evolution tube in your team test tube tray to incubate overnight.

6. Briefly vortex the 10^{-2} A PBS tube, then pipette 50 µl from it into the PBS tube marked *10^{-4}* A. (Figure 4). Be sure to label which tube is dilution 10^{-4} (NOTE: *this is a dilution process - discussion point w/ students before and after. Be prepared to show the math procedure for quantification of the dilution process in your lab journal)*

7. Briefly vortex the 10^{-4} A tube, then from this tube, pipette 50 µl onto the agar plate and spread the culture by using the sterilized glass spreader bar. Once you have finished spreading the culture, use parafilm to seal the plate.
8. The end product for today will be 1 Evo tube and 1 agar plates of 10^{-4} dilution per team. *When done be sure plate is labeled with team name, date, and dilution #.*
9. Incubate the evolution tubes at 28°C for 24 hours. Incubate the plate, **agar side up**, at 28°C for 48 hours.
    a. *why are plates stored agar side up? In your lab note section suggest some reasons why this is a standard microbiology protocol.*

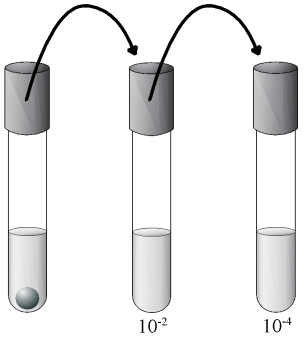


Figure 4: Serial dilution from the evolution tube (left) into PBS.

10. CLEAN UP Procedure:

- **a.** in the back of the classroom there is a bucket of 15% Lysol solution. Please pour leftover media (strain out the beads) into the bucket. Once empty place test tubes in the cleaning tub.
- **b.** The cleaning tub has 15% Lysol for the used test tubes and caps to be soak for 10 min. Once they have soaked, all tubes and lids will need washing/ scrubbing w/ brush.
- **c.** once scrubbed, these tubes and caps go to a 2nd rinse tub of 25% bleach and water to sit for 10 min. Finish with final rinse under the faucet before setting to air dry upside down in a rack.
- **d.** after mixing with a 15% lysol bath, all solutions can be disposed of down the drain,
    - **i.** Pflu is a harmless to humans bacteria, but can be persistent and contaminate other laboratory cultures. Clean up is an important step for students to understand and take seriously.
- e. Disposable materials include:
    - i. pipette tips, disposable vials if used, gloves,

<u>Lab Questions to reflect on today's protocol:</u>*answer these questions in your lab notes*

- *To make sure we have things straight, describe the contents of the evolution tube we made today.*
- *What do you believe is the purpose of the dilution we performed today?*
- *The types of media and solutions are a key component to this protocol. Knowing that bacteria need to grow and reproduce, which of the media types would you expect to find nutrients in? KB solution, PBS solution, T-soy agar? What kinds of nutrients would you expect to find in those you selected? Explain your rationale based on the steps of today's protocol.*

Lesson #5
DAY 1: Normal bead transfer

Over the course of 24 hours, the millions of cells that you added to your tube will grow to become billions. It doesn't take long before the bacteria consume the food and nutrients provided by the media inside of the test tube. In order to make sure that the bacteria continue to survive, we have to transfer a small number each day into a new tube. In the case of our experiment, we transfer only the bacteria that are good at forming biofilm and have thus successfully stuck to the bead.

Pre-lab Discussion: *Please answer these questions in your lab journal*

- *what role are you playing by transferring the bead? Suggest why that is a key part of this protocol. What if there were not a bead? What section would we want to transfer? How would that change our results?*
- *Identify which variables we are managing in today's procedure. Which do you think are simply basic protocol ie: controlled variables, and which might make a difference in our results, especially wanting to select for biofilm mutations. Suggest at least one variable that might make a significant difference in our daily data.*
- *How could we calculate how many generations of P.fluorescens have formed? remember that we are talking about an organism that reproduces asexually. Make a hypothesis about how many generations are growing in a 24 hour incubation period.*

Student lab materials:

- Metal forceps in beaker of 75% ETOH
- mini bunsen burners/ lab station
- (1) Large glass evolution tube containing 5ml KB and 1 **blue** polystyrene bead/ team

SAFETY: remember to tie back long hair, no loose clothing when working around flames, and gloves are required. If there are any accidents or mishaps, please report those immediately to your teacher. Be especially careful when working with an open flame and ETOH (which is flammable). There are a number of sequence steps in today's protocol, be methodical as you proceed, record your actions, any anomalies and questions in your lab journal for today's work.

Day 1, Bead transfer procedure:

1. Using sterile procedures - Pour the contents of the overnight culture into its plastic cap, and then use sterile forceps to transfer the **white bead** to the new corresponding evolution tube with fresh media and a **blue bead**. (*use the clean up protocol from Day 0 to dispose of old liquid, glassware and beads)*

2. Incubate the evolution tube at 28°C for 24 hours.

3. Remove the Day 0 agar plates from the incubator*(if used)* and allow them to sit on the bench for an additional 24 hours to fully develop. Students may make brief observational notes about what they currently see in their plates. Are they able to make observations to quantify and quality on the current colony growth. This is a good time to consider vocabulary of what they see now and will see at the end of 48 hours.

Today's summary Questions:

- *Why are we transferring only the bead, and not the liquid contents into the new evolution tube? Refer to the wrinkly spreader background reading, and offer a hypothesis about the results you would expect to see if we plated colonies from the solution in the test tube compared to those that form on the bead?*
- *Decide with your lab group, how best to record your plate data tomorrow. What important information will need to be included, to evaluate our predictions from Day 0? (competition for resources, how to identify the "wild type" original strains of P.flu compared to any genetic variants; how to count single colonies, etc) are there any other qualities we should consider when recording data from our first plate?*

Refer back to your problem statement and research - are there any other questions or observations you and your lab team need to make as you proceed with this experiment?What data do you need from other groups in the class to support your hypothesis?

Lesson # 7

DAY 2: Bead transfer, Dilution series and plating

Since you last transferred your bacterial culture 24 hours ago, you may have noticed that the appearance of your test tube has changed. Your test tube may have an increased amount of biofilm on its sides and your culture may have a neon-yellow tint and chunks of biofilm in it. Believe it or not, this is normal! It is also normal, however, that you may not observe these changes.

*Your incubated test tubes now contain both a white (W_0) and a blue bead (B_1). Today, you are transferring your 24-hour **blue** bead to a new tube containing fresh media and a white bead (W_2). Over the course of 24 hours, some of the bacteria from the blue bead will detach and re-adhere to the surface of the white bead. In addition to this, we want to verify the bacteria inside of the test tube on an agar plate. In order to do this, we will plate the bacterial cultures from each of our even experimental days (ie: Day 0, Day 2 and Day 4).*

Pre-lab discussion:

Before starting today, make a prediction about the competition factor between your P.flu + and - colonies, on Day 0 they were combined in the same media, then plated after a dilution. If your group wanted to find out about competition between the two strains, what would you expect to see in this first set of results. What would you be looking for in future plates? (list evidence that would help you determine the effect or lack of competition)? Use observational predictions and quantitative predictions.

If your lab team is looking for morphological changes, what should we see on the initial plating from Day 0, and what would you predict should happen in future plating results?

Support your ideas with at least 1 scientific principle used in this lab procedure; give 1 idea of what evidence we will need to see to prove your hypothesis and prediction of the process in action.

This is the point that you will notice that the procedure repeats the process from Day 0 - bead transfer, dilution series (with an additional dilution to 10-5) and plating of both 10-4 and 10 - 5 dilutions. The addition of plate reading from the previous series will need to also take place, be prepared to quantify your results, and make observations under the microscope.

Day 2 lab group materials:

- Metal forceps/ 70% ETOH beaker
- Vortex
- A p200 and p1000 pipette and sterile tips
- (1) Large glass evolution tube containing: 5 mL QB and 1 **white** polystyrene bead/ team
- (1) Small glass tubes containing: 1 mL Phosphate Buffered Saline (PBS)/ team
- (2) Large glass tubes containing: 5 mL Phosphate Buffered Saline (PBS)/ team
- (1) Large glass tubes containing: 4.5 mL Phosphate Buffered Saline (PBS)/ team
- (2) ½ Strength Tsoy-Agar plate containing X-gal/ team
- glass spreader bar in beaker of 70% ETOH
- mini bunsen burners/ team
- gloves
- parafilm to seal plates/ permanent markers
- dissecting microscopes for plate analysis from Day0

SAFETY: remember to tie back long hair, no loose clothing when working around flames, and gloves are required. If there are any accidents or mishaps, please report those immediately to your teacher. Be especially careful when working with an open flame and ETOH (which is flammable). There are a number of sequence steps in today's protocol, be methodical as you proceed, record your actions, any anomalies and questions in your lab journal for today's work.

Procedure:

1. Using sterile processes, for both the test tubes and forceps, Pour the contents of the overnight evolution culture into its metal cap, and then use sterile forceps to transfer the **blue bead** (B_1) to the new evolution tube with fresh KB media and a **white bead (** W_2).

DO NOT POUR THE CONTENTS OF THE OVERNIGHT CULTURE BACK INTO THE TUBE YET!

2. Use sterile forceps to transfer the **white bead (W_0)** to the corresponding labeled 1 mL PBS tube.
    a. Vortex the small PBS tubes for at least 45 seconds to remove cells from the bead.
3. Before proceeding, be sure to label all tubes with your team name, the day # and the dilution amount.
4. Then Use a P200 pipette to move **50 µl from** the small PBS tube to the large PBS tube marked 10^{-2} .
5. Briefly vortex the 10^{-2} A PBS tube, then pipette **50 µl** from it into the PBS tube marked 10^{-4} .
6. Briefly vortex the 10^{-4} A PBS tube, then pipette **500 µl** from it into the PBS tube marked 10^{-5}. (10^{-5} tubes w/ 4.5 ml PBS)

7. Briefly vortex the 10^{-4} PBS tube. From this tube, **pipette 50 µl onto the agar plate labeled 10^{-4},** and spread plate with a sterilized glass spreader bar. Be sure plate is labeled with team name, date, and dilution #. When done seal with parafilm.

8. Repeat step 6, but instead of using the 10^{-4} PBS tube, use the 10^{-5} PBS tube. From this tube, **pipette 50 µl onto the agar plate labeled 10^{-5},** and spread plate with a sterilized glass spreader bar. Be sure plate is labeled with team name, date, and dilution #. When done seal with parafilm.

Incubate the plate, agar side up, at 28°C for 48 hours. This can be done on the lab bench.
Incubate the evolution tube at 28°C for 24 hours.
Use the clean up procedure from Day 0 to clean up your lab supplies.

Record any key details in your lab journal. Set up your data charts for reading the Day 0 plates. You will also need to use the microscope to verify shape, consistency, etc.

Plating results protocol:

1) *Count the number of blue and white colonies on each plate. Record the number of each in the data table in your lab journal. Calculate the ratio of these colonies?_____.*

What strategy did you use to count your colonies. Have your lab partner count also - did you both come up with the same amount? Explain.

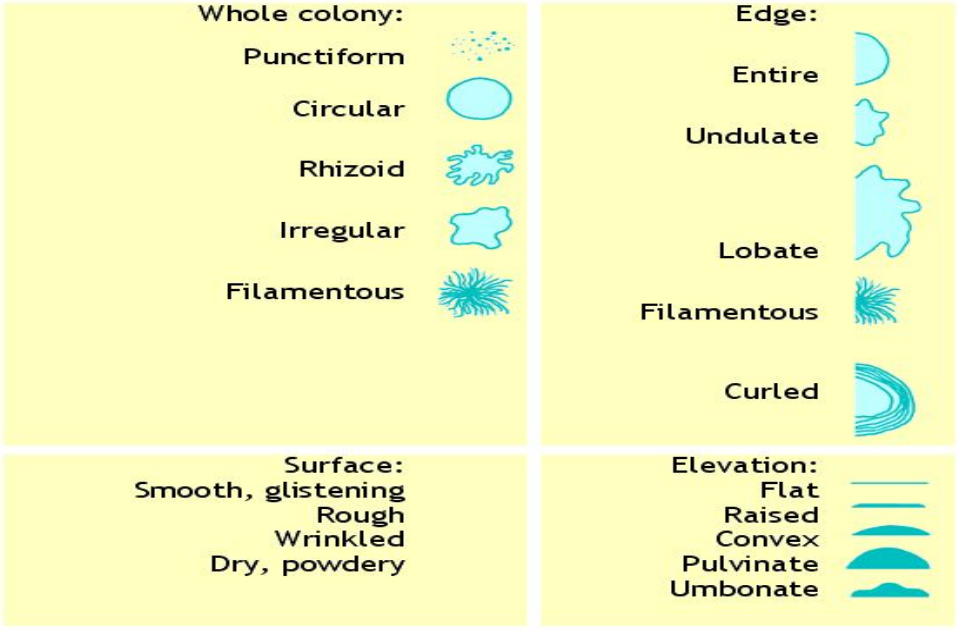
Be prepared to share with the class how you counted, and how accurate your process was when compared to your partners.
What other methods could be used to count colonies that would improve the accuracy?

2) Record observations for colony morphology, including: color; size; shape using the vocabulary from the chart below. These observations can best be done under a dissecting scope once they have a colony count and drawing.

3) ***As the lab continues, notice if the ratio of color or type stays the same? Or if there are other quantifiable observations that change.***

- *Does the ratio of irregular remain consistent?*
- *Make a prediction based on the variables that we are manipulating, for our ending plate results - for both blue to white colony ratio; and irregular to "regular" colony ratio.*
- *How does the data relate back to your choice of problem statement?*

Use this reference guide for describing your colonies as we continue to observe plates. This is universal science vocabulary you should become more familiar with in the process of observing your colonies.



Data for this section can include, sketches, pictures, charts, etc - initial observations will be key for noting changes as the lab continues. It may also be a good idea to keep the plates until the end of the protocol for comparison, in case not enough data was gathered the first time. Ask your teacher about how this will work.

Analysis of data:

1) For each plate - make ½ page sketch of the entire plate as it appears to the naked eye; include observations; note colony shape, size; and any interesting features you found.

- **NOTE:** For any and all sketches, you are encouraged to use labels & arrows to identify important features on your sketches.

2) Now, for each plate, choose a few colony shapes to make a ½ page sketch from the microscope view. Choose the microscope power and lighting conditions that best allow you to see colony morphology. Be sure to note differences in size and shape in your sketches. Additionally, pictures taken with your smart phones would be nice, but should not be the only pics in your lab journal.

3) If there are any anomalies, be sure to note what they are and why you think they happened. (Are you seeing a few different "types" of colony morphologies, or in your view, are all the colonies the same?… different?) Lastly, you should include a statement about how your data compares to the rest of the class and one other lab group.

Plating data charts Day 0:

lab group	10^{-4} White total	10^{-4} blue total	10^{-4} white irregular	10^{-4} blue irregular	Ratio of blue:white	comments/ other notes

Analysis of data: include observations; sketches of your plates, and colony shape, size; and any interesting features you found on your plates. Choose a few colony shapes to sketch from the microscope view, be sure to note differences in size and shape in your sketches. Addition of pictures taken with your smart phones can be a good addition, but should not be the only pics in your lab journal. If there are any anomalies, be sure to note what they are and why you think they happened. Lastly, You should include a statement about how your data compares to the rest of the class and one other lab group.

Teacher/ Student option: This is a good time to take a break in the protocol to have class discussion related to the student problem statements, research and any other questions.

Lesson # 8
Day 3: Normal Bead transfer:
Day 3 procedure is the same as Day 1, please refer to that procedure.

Questions to consider:

1) Why is this step necessary to repeat? What variable are we most focused on with this step?
2) Which generation is this from the ancestors?
3) Remember each new evolution tube has new media for the bacteria to consume. Review your competition predictions and decide if they need revision based on your initial set of plate data.
4) What other types of colonies might be living in the rest of the solution we dispose of? (*additional reading from morphology paper*)
5) What is the benefit of different colonization types in each evolution tube? What are the variables that would drive diversification?
6) How could we test to confirm that indeed diversification is happening to these different colonies? What information/ evidence would help confirm your hypothesis?

Lesson # 9
Day 4 - possible end of experiment, create dilution series and final plating day

- If you choose, the experiment can continue, which would mean using lab protocol from Day 2.

Today's goal is to wrap up the process and create one more plate of cultures for Day 4.

1) Use sterile forceps to transfer the **white bead** to the corresponding labeled 1 mL PBS tube. **this is the generation we are going to dilute and plate today.**

a. Vortex the small PBS tubes for at least 45 seconds to remove cells from the bead. *This step is important to remove the cells from the bead before we create a dilution series to plate both the 10-4 dilution and the 10 -5 dilution.*

2) Dilution steps: Use a P200 pipette to move **50 µl from** the small PBS A tube to the large PBS tube marked 10^{-2} .

a. Briefly vortex the 10^{-2} A PBS tube, then pipette **50 µl** from it into the PBS tube marked 10^{-4} .
b. Briefly vortex the 10^{-4} A PBS tube, then pipette 500 µl from it into the PBS tube marked 10^{-5} A. (**10-5 tubes w/ 4.5 ml PBS**)

3) **Plating the dilutions**: Briefly vortex the 10^{-4} PBS tube. From this tube, **pipette 50 µl onto the agar plate labeled 10^{-4},** and spread plate with a sterilized glass spreader bar. When done be sure plate is labeled with team name, date, and dilution #. *Lastly, seal with parafilm, use proper sterile protocol when plating*.

a. repeat the process for the 10^{-5} dilution. be sure to label the plate. These plates will need to incubate for about 48 hours before results can be documented.

Results from the Day 2 plates: these plates have now incubated for 48 hours. As you record your results remember which counting strategy seemed to work best from our last plate reading day. This time you will have 2 plates on at dilution 10-4, and one at dilution 10-5.

- What differences do you anticipate between the two plates?

(Again, keep in mind to be methodical in recording your results, and documenting as you go. **Remember to leave your plates sealed throughout this process.)**

1) **Count** the number of blue and white colonies: record your totals for each color in your data chart. What is the ratio?_____. Before adding your data to the class data sheet, have your lab partner count also - did you both come up with the same amount? if not, how can you resolve your different counts, so the data can be shared with the class. Explain what you decided.

_____

Based on our discussion in class last time, do you think your accuracy in counting colonies has improved? explain which process you believe yields the best results in this process.

2) Sketch and explain the colony morphology you are seeing as you collect data and look in the microscope, Be sure to share with the class any unusual, new and exciting colonies found in your plates. refer to the charts for terms, or let's agree as a class on any new findings and terms we will use, so that we can compare data.

Refer back to the data from our 1st set of plates, does the ratio of color or type stays the same? How can you explain this observation? _____

- Does the ratio found on these plate correlate to the prediction you made on Day 2 from our first set of data? as you explain our results, do not forget to include connections to the variables that we are manipulating, and correlations to color and shape of colonies.

Plating data charts for Day 2 plates:

lab group	10^{-4} White total	10^{-4} blue total	10^{-4} white irregular	10^{-4} blue irregular	ratio wht/blue	comments/ other notes

lab group	10^{-5} White total	10^{-5} blue total	10^{-5} white irregular	10^{-5} blue irregular	ratio wht/blue	comments/ other notes

Analysis of data: include observations; sketches of your plates, and colony shape, size; and any interesting features you found on your plates. Choose a few colony shapes to sketch from the microscope view, be sure to note differences in size and shape in your sketches. Addition of pictures taken with your smart phones can be a good addition, but should not be the only pics in your lab journal. If there are any anomalies, be sure to note what they are and why you think they happened. Lastly, You should include a statement about how your data compares to the rest of the class and one other lab group.

Graph 4 sets of class data. Based on this data write 3-4 analysis statements pertaining to what you think this data indicates at this point in the process. Attach your graph in your lab notebook along with any pictures. Which data will better show a casual observer our findings? How can you best graph the data and summarize our findings to have your graph support your current understanding of colony morphology changes over a series of generations. Which dilution series is a better set of data to graph? Be prepared to defend why you chose that series as a reference.

Lesson # 11

Final plate reading- Day 4 plates (48 hours after)

Use the same protocol we outlined in Day 4 for the reading of Day 2 plates. Once you have all of the data compiled for the class, reflect back on your initial predictions/ hypothesis and whether the data we found supports your predictions.

Be sure to include in your lab journal - the data charts, graphs, and pictures from your results. Once those pieces have been recorded, it is time to reflect on what the take home message of this activity has been.

- What do we now know and understand as a result of our observations, readings and discussions from this past week. (If this were a lab report, this reflection and connection piece would be called a conclusion :))
- What other questions arose during this process? Did you and your classmates find answers that helps to improve your understanding of the diversification process that leads to evolution changes in organisms?
- What other questions do you still have that this lab did not address? Can these questions be solved by research? If not, how could you design an experiment that would help resolve your particular question?

4c.) Teacher's answer key to Student lab journal questions:

Use misconception pre-assessment to continue to highlight information related to natural selection, the lab protocol, and the key concepts related to Evolution and natural Selection. Referring back to the key concepts will assist in students scaffolding of information and development in the topic.

Lesson #2 Pre lab work discussions,

As a result of reading the background material, please be prepared to discuss the following questions in class:

- What is the difference between a colony and an individual multicelled organism? Are there benefits to each form? A colony is composed of cells that originate from one individual cell. Accept most answers students offer for benefits of a colony, for later discussion and clarification.
- What type of bacteria are we using and why is it a good choice for this experiment? Pseudomonas fluorescens, it is a harmless bacteria that has a symbiotic relationship with beets.
- In which organisms can we observe Evolution in action today both in the laboratory and in the field? Accept most reasonable answers
- What evidence needs to be documented when studying changes in organisms to support the idea that evolution has occurred? Morphological changes, DNA changes, improved success in given environment, survival to reproductive status, adaptability, long term changes,
- Why is it hard to use experimental models of larger more complex organisms when we want to observe changes? Gestational time of organisms, time it takes for DNA mutations to result in changes at a population level
- In what ways does Natural Selection play a role in the evolution of bacteria? Does artificial selection have a role in the evolution of bacteria? Natural selection is the mechanism of change at the organism level, so as an individual changes that will lead to colony changes; Artificial selection is the process we are using in this lab; additionally allow conversation about antibiotic resistant bacteria strains, humans use of antibiotics have played a role in those strains, as well as other selection processes.

Lab Day -1 Protocol and observations:

Discussion prompts for students:

- Which variables do you think are key to the evolution in early organisms? Number of generations, selection process, environmental/ nutritional conditions, competition with other species, time, temperature,
- What conditions are necessary to culture bacteria in a lab setting? Nutrition - in this case carbon based, temperature 25-30 C, pH of solution, oxygen,
- Are there any new terms that you need to clarify from your research? Make a list in lab journals and discuss as they come up
- Identify the variables involved in the process for single celled organism reproduction. Single cell organism ie: bacteria reproduce by way of binary fission or sexual reproduction - reproductive rate variables are the same as above. However, it is worth clarifying reproduction strategies and resulting differences in DNA as a result.
- What factors would be necessary for a single celled organism to move to multicellular organism. Multicellular organisms have to have a division of labor to meet the demands of complexity; there is a need to organize sharing of nutrients/ oxygen to meet the needs of the whole organism, reproduction strategies that consistently pass on genetic information to the offspring; and for some movement in order to better compete in their environment.

Lab Protocol Day 0: Bead transfer, dilution series, plating day

Pre-lab questions for discussion and journal notes

a. *Make a prediction about the competition factor for media by each type of P.fluorescens at this stage of the experiment. What would you expect to see in your results if there is a competition for resources? This is a good time to have a discussion about random ratios of colonies vs obvious differences in colony ratio on plates. It might also include conversations about what variables in this experiment might contribute to a difference in ratios.*

b. *Why are we only selecting the bacteria that has been growing on the bead? We are looking for bacteria that are modifying into biofilm builders; what other area might be worth selecting from and plating?*

c. *Is it possible that there is a competition for resources in other areas of the test tube? Choose 1 to explain. Refer to the wrinkly spreader reading: Air to liquid interface, mid level tube liquid, bead level, sides of test tube. Make sure variables are listed that connect to the layer identified.*

d. *Why are we only selecting the bacteria that has been growing on the bead? Is there another location that might also yield biofilm bacteria? Defend your answer. Refer to discussion in letter b, and defend your answer.*

Lab Questions to reflect on today's protocol: *answer these questions in your lab notes*

- *To make sure we have things straight, describe the contents of the evolution tube we made today. In the evo tube today there is a bead, two cultures of Pflu, LacZ- and LacZ+, media for nutrition for the bacteria*
- *What do you believe is the purpose of the dilution we performed today? Bacteria reproduce rapidly, if we did not dilute there would be too many colonies to count and observe on the plate*
- *The types of media and solutions are a key component to this protocol. Knowing that bacteria need to grow and reproduce, which of the media types would you expect to find nutrients in? KB solution, PBS solution, T-soy agar? KB (Kings B contains a number of carbohydrate nutrients for the bacteria), PBS is simply a neutral "salt" solution, T-soy agar has a number of carbohydrate nutrients for the bacteria to reproduce on the agar. What kinds of nutrients would you expect to find in those you selected? Explain your rationale based on the steps of today's protocol.*

Lab Protocol Day 1:

Pre-lab Discussion: *Please answer these questions in your lab journal*

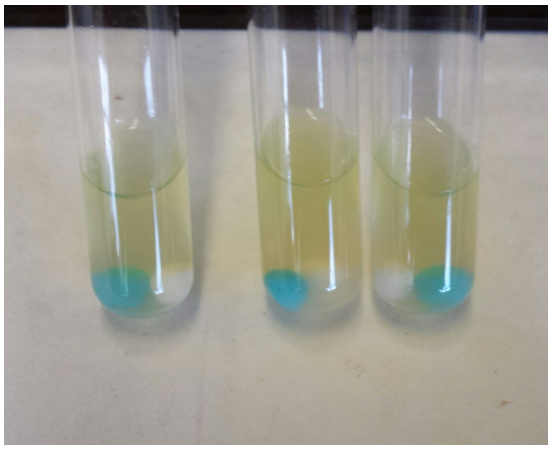
- *what role are we playing by transferring the bead? Selection for biofilm mutations Suggest why that is a key part of this protocol. What if there were not a bead? We could select from the air - liquid interface What section would we want to transfer? How would that change our results? Different levels of solution will yield different types of bacteria mutations*
- *Identify which variables we are managing in today's procedure. Which do you think are simply basic protocol ie: controlled variables, and which might make a difference in our results, especially wanting to select for biofilm mutations. Suggest at least one variable that might make a significant difference in our daily data. Variables - selection of bead, vortex, new media/nutrients, time between selections,*
- *How could we calculate how many generations of P.fluorescens have formed? remember that we are talking about an organism that reproduces asexually. Make a hypothesis about how many generations are growing in a 24 hour incubation period. Pseudomonas genera range from 9.8 min to 100 min, Pflu has an average reproductive rate of 51 min (O'Mahony, 2006).*

Lab Protocol Day 1 summary Questions:

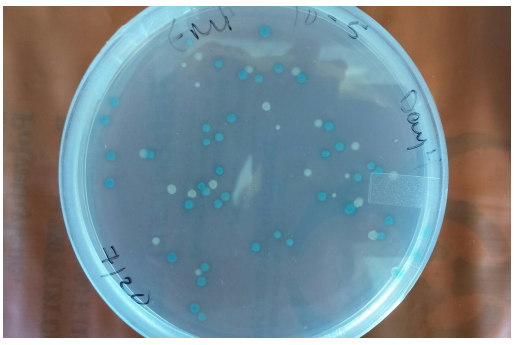
- *Why are we transferring only the bead, and not the liquid contents into the new evolution tube? Refer to the wrinkly spreader background reading, and offer a hypothesis about the results you would expect to see if we plated colonies from the solution in the test tube compared to those that form on the bead?*
- *Decide with your lab group, how best to record your plate data tomorrow. What important information will need to be included, to evaluate our predictions from Day 0? (competition for resources - # of blue and white colonies, growth patterns on the plate, how to identify the "wild type" original strains of P.flu compared to any genetic variants- smooth round is the ancestor strain, any other options re: edges, transparency/color, elevation appearance, etc; how to count single colonies, etc) are there any other qualities we should consider when recording data from our first plate? Day 0 plates should yield an approximate 1:1 ratio from total plate counts, ratios may vary on individual plates; also note size of colonies - large, medium, small;*
    - *Have each lab partner count, using two different methods - suggestions, count only white, then only blue; draw a grid on the plate to count, draw a pie chart w/ slices or quarters to count, etc. Students should suggest which way works best for their team, then agree as a class for future plate counts. Day 0 plates will be the easiest 10 -4 plates to read, there will be more growth on the plates in the following days.*

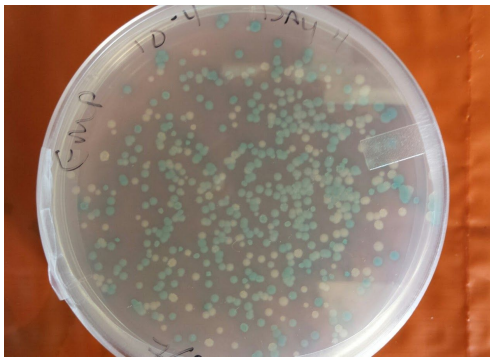
Lab Protocol Day 2 - what to expect from plates and tubes images

Day 2 test tubes after 24 hours, with two beads:



Plating data to anticipate:

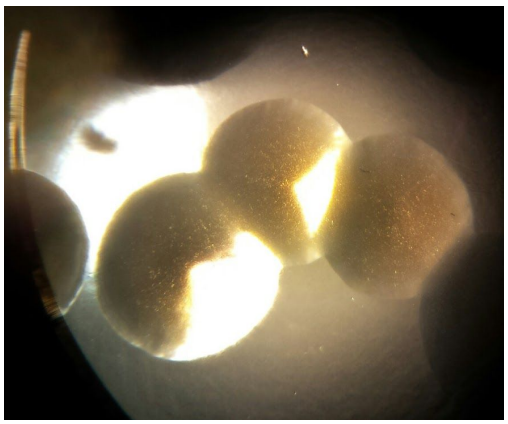




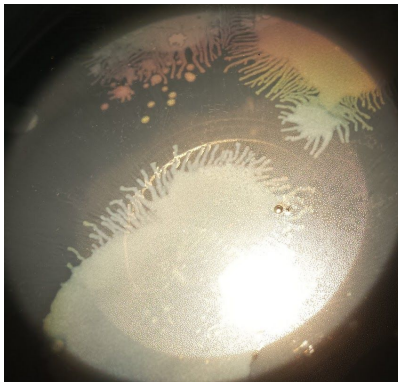
10 -4 dilution; Day 4 plate

Day 4 plate 10-5

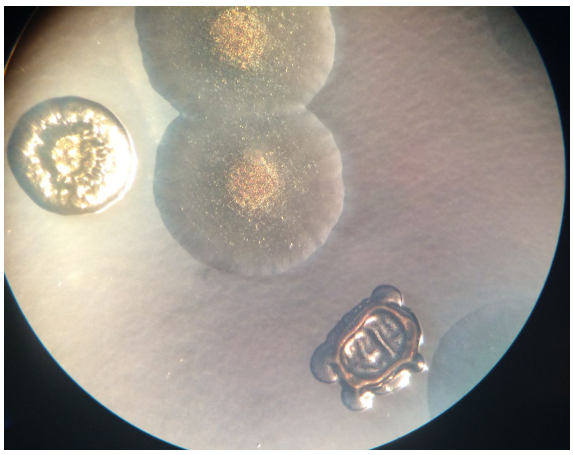
Colony morphology expectations - be looking for any changes

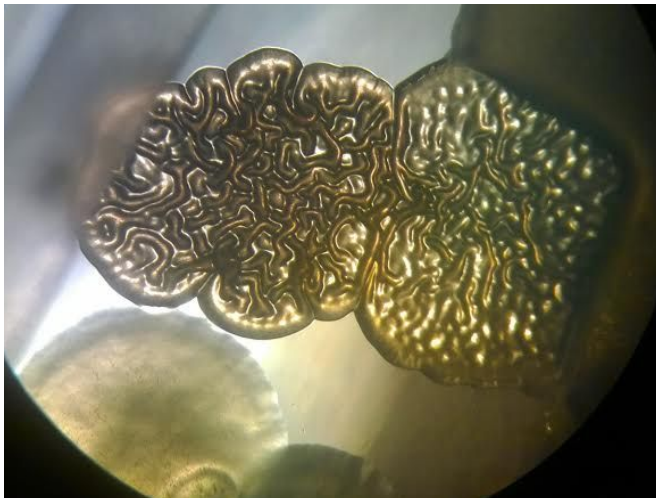


circular, convex colonies



filamentous colony formation





Have students describe these forms

Lab protocol Day 3 - Bead transfer

Questions to consider:

1) Why is this step necessary to repeat? What variable are we most focused on with this step? Bead transfer is this protocol's selection process for collecting wrinkly spreader mutations; if a lab group would like to try removing and plating other locations in the tube, this might be a good day to suggest some groups consider that for thier Day 4 plating process in addition to the normal plating; if a group is

going to plate a different location in the tube that should be done carefully before bead transfer on Day 4, then follow the rest of the protocol as written.

2) Which generation is this from the ancestors? This question is going to require some math - P.flu reproductive rate is 51 min approx, under ideal conditions- have students show their work as they make the prediction.
3) Remember each new evolution tube has new media for the bacteria to consume. Review your competition predictions and decide if they need revision based on your initial set of plate data. Suggested conversation w/ students to increase ideas as well as research options - if the media in the tube and on the plate is always fresh and full of nutrients, where/ why would you expect to see competition? There are conditions that wrinkly spreaders have more advantage than the smooth round, where would that location in the tube be? Is there a ratio difference between the LacZ+ and Lac Z- (if there have been weekends or longer incubation times between plating, you might begin to see these differences);
4) What other types of colonies might be living in the rest of the solution we dispose of? (*additional reading from morphology paper as well as the morphology chart options*) Accept all reasonable ideas
5) What is the benefit of different colonization types in each evolution tube? The test tube is like its own ecosystem, there are a number of niches which some forms of Pflu will be best fit. What are the variables that would drive diversification? Amount of nutrients/ oxygen, surfaces to form on, temperature differences, space, predation, random mutation rates, which types were selected from the previous bead transfer day in the solution that was transferred,
6) How could we test to confirm that indeed diversification is happening to these different colonies? Plating offers morphological changes visually, in addition you could select samples and isolate their DNA to see what if any mutations are occurring, even if morphology has not yet shown evidence of change. What information/ evidence would help confirm your hypothesis? Morphological changes are evidence, DNA changes are evidence.

Lab Protocol Day 4 - possible end of experimental protocol -this depends on your class time, and how the plating results are looking. If you have the time, you could put it up to student vote. As the lab progresses and more wrinkly spreader diverse forms show up on the plates, it becomes very exciting for the students.

Reading Day 2 plates, same protocol as before, but there may be (more) wrinkly spreader colonies in the data

- If this is the case, show everyone what they look like, and then send them back for careful observations in the scopes. Students do not tend to see the nuances of change at first, ie: edges changing appearance, transparency differences, etc. Though some will be very obvious, other changes will be more subtle - the lobate edges, umbonate elevation, texture, etc.
- If time, have students look at Day 0 and Day 2 plates alongside of the Day 4 plates and make comparisons under the microscope. There are some colony vocabulary observations they may not have noticed on the first two plates.
- You may begin to have morphology other other than a composite wrinkly spreader, ie: lobate, larger less compact colonies, swarming, etc all are worth conversation about how this is different than the ancestor plates and what may be happening that would cause these results. Refer back to the variables, lab techniques, any anomalies that might have occurred. This is a great discussion to have with students about “real science” is not always a perfect canned lab. Many students do not understand when asked to suggest improvements at the end of a lab report. This is an opportunity to have that conversation, so they can analyze their own work and procedures to see if they could improve on the experiment, or have other suggestions they

would like to try to see if they could improve the results based on the selection process, media types, etc.

Recommendation:
While waiting for the Day 4 plates to develop, it is a good time to go back to questions students asked at the beginning of the lab and see if they have been covered. If not, they can be used for class discussion, or assigned to groups that have a similar topic for their problem statements, and used for research topics. If this is the case be sure to have students report back with what they have found, so other groups have the information.

Safety protocol: Be safe.

Pseudomonas fluorescencs SWB25 is a harmless bacteria appropriate for use in high school laboratory settings. Students will be asked to use good bacteriological protocols and maintain integrity of growing this particular strain vs other random microbes in the classroom activities.

Sterilization protocol involves flames - either of bunsen burners or alcohol burners. Long hair should be tied back, loose clothing should be managed. Lab surfaces can be sprayed down with 70% ETOH to both prepare the surface before work and clean the surface after work.

- when moving tools (forceps, spreader bar, etc) from ETOH beaker to flame sterilization, only go from ETOH to flame. Do not move hot metal or glassware to the ETOH beaker. EtOH is highly flammable.
- Students should know how to handle fire issues in the classroom when dealing with flammables.

Solutions used in this lab should not be ingested. Students should use good hand washing protocol, before, during, and after handling all solutions, cultures and petri dishes with colonies. During this protocol students should wear gloves, vinyl or nitrile gloves are recommended to avoid allergy issues with latex. If you are using glassware, remind students of safe glass handling, and how to deal with clean up if breakage happens.

Disposal of media, cultures in test tubes can be cleaned up in the classroom and disposed down the drain (see protocol for clean up action). Petri dishes should be kept closed, then can be wrapped in a trash bag and disposed of in the classroom trash. These cultures can get stinky - if disposal is not soon after result gathering, they should be stored in a refrigerator. A more specific clean up protocol is included in the student lab sheets, and in the teacher media preparation.

As always when working with biology labs, there should be no food or drink at the work areas while students are handling media, cultures, etc in this lab activity.