Name:_____ February 2017

Winnacunnet High School

Evolution in Action

Block ____

Bench 1	Bench 2	Bench 3
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Bench 4	Bench 5	Bench 6
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PRE-LAB QUESTIONS: DAY -1

1. Describe the purpose of the "pre-conditioning" step that will be carried out on Day -1 of your experiment? How many colonies are used to inoculate one test tube containing media and a white bead?

2. Suzie has just used a sterilized inoculating loop to obtain a single isolated colony of bacteria, which she then transferred into her test tube containing fresh media and a white bead. After she has put the cap of her newly inoculated test tube back on, she grabs the petri dish and goes to grab another colony. Before she can touch the inoculating loop to the petri dish, Larry stops her and tells her that she is doing it wrong. Which student is correct in this case, and why?

EVOLUTION-IN-ACTION

Pseudomonas fluorescens Experimental Evolution Protocol

You are about to embark on a journey through a world that you might be unfamiliar with; one filled with odd instruments that you will use to study oddly shaped slimy bacterial colonies and neon yellow biofilm-coated test tubes. Over the course of the next week you will be taking care of bacterial cultures, and your ordinary looking colonies will evolve to produce distinct mutants that have adapted to inhabit different parts of a test tube.

ALWAYS REMEMBER

Proper **aseptic technique** is a very important part of microbiology! All tubes, beads, and media have been sterilized in an **autoclave** prior to use in these experiments. When tubes were prepared, media was always distributed using sterile pipettes, and sterile beads were added using forceps that have been heated over a flame until *"red-hot"* to prevent contamination.

USEFUL TERMS

- **Aseptic Technique** – a sterile set of practices and procedures performed to minimize contamination by other bacteria.
- **Autoclave** – a strong, heated container that reaches high temperature and pressure to sterilize equipment and media.

DAY -1: PRE-CONDITIONING YOUR BACTERIAL CULTURE

The season for your favorite sport has just begun. It has been a long off-season, and it is clear that it is going to take some time for everyone to get situated and back in shape before the big season opener, when your Blue Devils take on the long-time rivaled Great White Sharks. Lucky for you, your coach has prepared some different conditioning exercises and workouts that help everyone brush up on their skills and get acclimated before the big day!

Before the bacteria got to your classroom, they had been stored in a freezer for a long period of time at -80° Celsius (-112° Fahrenheit); somewhat similar to a long off-season. Before we can continue with our experiment, we want to ensure that the bacteria are used to being out of the freezer so they are performing at their prime. In order to do so, we give them a day to get acclimated to their new environmental conditions – this day is known as our "Preconditioning Day".

NECESSARY MATERIALS

- Metal inoculation loop
- *Pseudomonas fluorescens* SBW25 LacZ+ colonies (on agar plate)
- *Pseudomonas fluorescens* SBW25 LacZ– colonies (on agar plate)
- (4) Large glass culture tubes containing:
  - 4 mL Queen's B Medium (QB)
  - 1 mL Tsoy Broth
  - 1 white polystyrene bead

PROCEDURE:

1. Two culture tubes for each replicate evolution being performed have been prepared for you. The glass side of the tubes have been labeled either "*P. flu* +" A-B or "*P. flu* –" A-B using a permanent marker.

2. As shown in the demonstrational video, flame sterilize an inoculating loop until it becomes "red-hot".

3. Once cooled, use the loop to transfer a **single** isolated *Pseudomonas fluorescens* Lac+ (blue) colony from an agar plate to the "*P. flu* + A" culture tube by swirling it in the media. Make sure that all of the bacteria transfers from the loop 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* – A" culture tube (Figure 1).

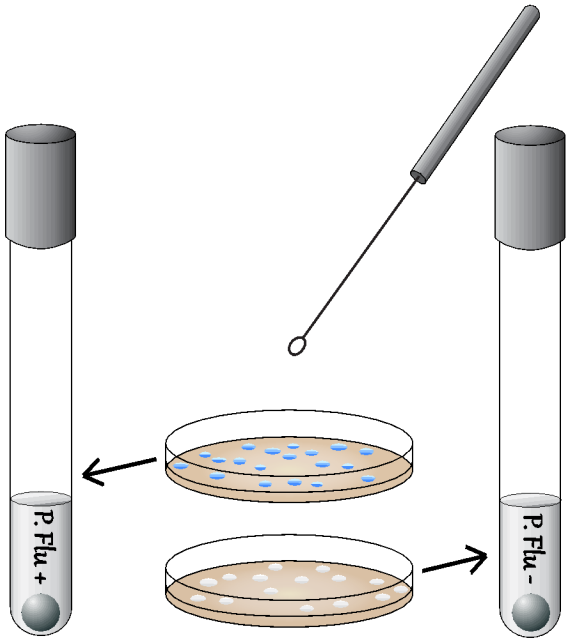

Figure 1: Use a sterile inoculation loop to inoculate *P. flu* + culture tubes with blue colonies, and *P. flu* – tubes with white colonies.

5. Repeat steps 3 and 4 until you have inoculated both a *P. flu* + and *P. flu* – tube for the B replicate evolution. For example, because we are running two replicates, four total cultures should be inoculated, 2 *P. flu* + (A-B) and 2 *P. flu* – (A-B).

BE SURE TO USE A NEW COLONY TO INOCULATE EACH TUBE!

6. Incubate the culture tubes on a roller at 28°C for 24 hours.

POST-LAB QUESTIONS: DAY -1

1. Describe the characteristics of bacteria that make them advantageous when studying evolution?

2. Summarize the different stages that occur throughout the biofilm lifecycle. How does this relate to the bead transfer model that is used in the experiments?

PRE-LAB QUESTIONS: DAY 0

1. Once the bead has been transferred from the large glass evolution tube to the INTER glass tube containing 1 mL of Queen's B media, how long should this tube be vortexed for? What is the purpose of vortexing?

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2. Draw the series of steps that are required to complete a serial dilution on Day 0. Include the amount of liquid that is being transferred, the amount of liquid that is in the dilution tube, and the dilution that is achieved with each step. Circle the dilution(s) that will be plated on Day 0.

3. Draw and briefly summarize the process of plating a bacterial culture. What is achieved by plating, and why is it incredibly important to ensure that you are plating on the agar side of the plate?

DAY 0: CREATION OF COMPETITION CULTURES & PLATING

You and your team have been hard at work, removing all the cobwebs from the long off-season. Tonight is the night when you get to see if all of your hard work from pre-season paid off. You proudly wear your blue uniform that has the Blue Devils mascot on left chest of your jersey, as you walk out onto the field that is lit under the bright lights. The Great White Sharks are in their stark white uniforms and they too are ready for the game to begin. Everyone is in their rightful positions, and the whistle blows. The game is underway and the competition has begun, but who will come out on top?

Your bacteria have been on the roller drum, incubating at 28°C (82.4°C) for the past 24 hours. Now that they have had a chance to get used to a lifestyle in liquid medium, they too are ready for primetime. Your 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 your Lac- white and Lac+ blue P. fluorescens culture will be added to the same test tube using a pipette, in which they will compete for media, and space on the bead and test tube.

NECESSARY MATERIALS:

- Metal forceps
- (4) Large glass tubes labeled "INTER": 1 mL QB
- Vortex
- A p200 and p1000 pipette
- (2) Large glass evolution tube containing: 4 mL QB and 1 white polystyrene bead
- (4) Large glass tubes containing: 5 mL Phosphate Buffered Saline (PBS)
- (2) ½ Strength Tsoy-Agar plate containing X-gal

PROCEDURE:

1. All tubes have once again been prepared for you. QB tubes labeled INTER have been labeled *P. flu* + A-B and *P. flu* – A-B; the large evolution tubes and agar plates have been labeled using an identifiable manor (class section, bench number, replicate letter); and the large PBS tubes have been labeled 10^{-2} A-B and 10^{-4} A-B.

2. 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!

3. Pour the contents of the "*P. flu* + A" overnight culture into its metal cap, then use sterile forceps to transfer ONLY the bead to the corresponding INTER QB tube. **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 with "*P. flu* – A" overnight culture (Figure 2). Repeat for replicate B.

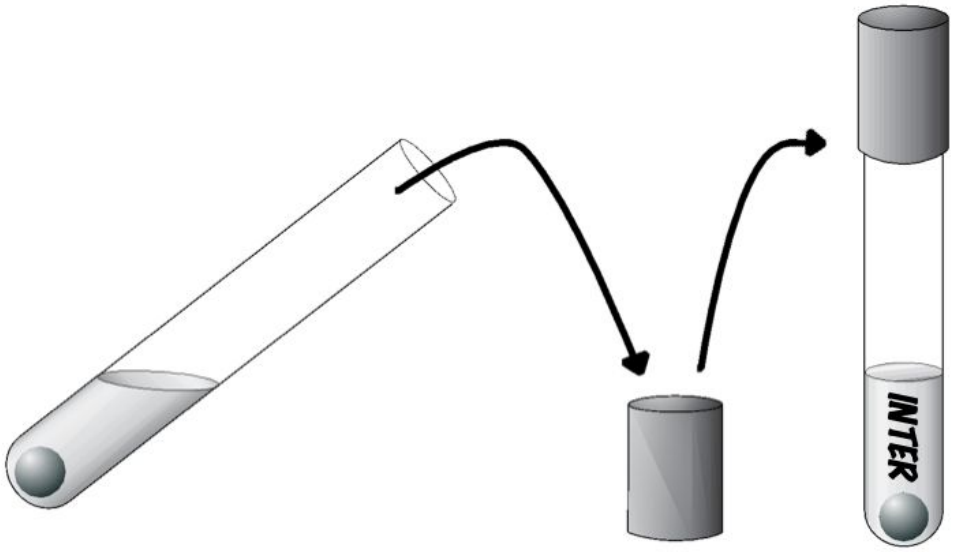


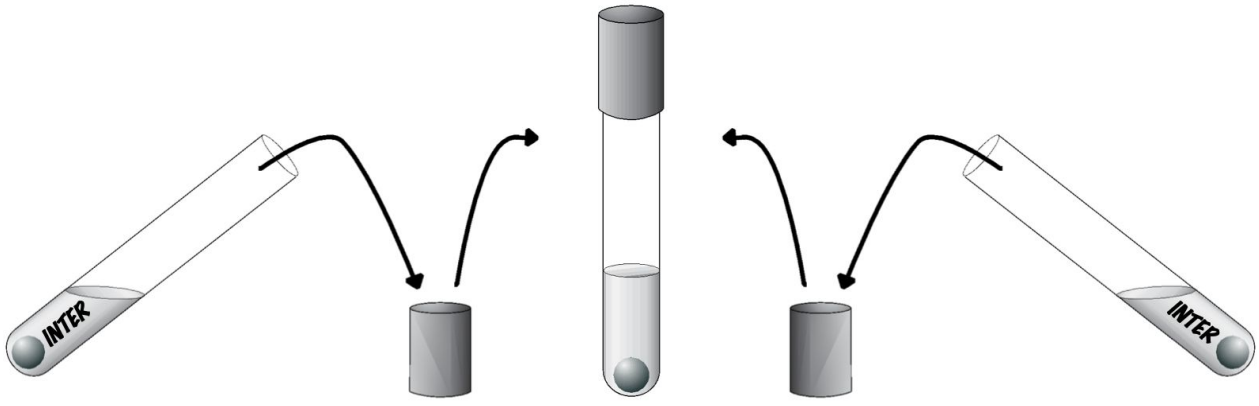
Figure 2: The overnight culture (left) is poured into its cap, and then sterile forceps are used to move only the bead to the INTER QB tube (right).

4. Vortex both INTER QB tubes for at least 45 seconds to remove biofilm from the bead.

5. Once all of your INTER QB tubes have been vortexed, briefly vortex one of the INTER tubes for three seconds, and then pour the contents of the tube into its cap.

6. Use a **p1000 pipette** to transfer 500 µl of this vortexed "*P. flu* +A" liquid media from the cap to the large evolution tube labeled "A". Repeat with the vortexed "*P. flu* – A" liquid, adding it to the same large evolution tube (Figure 3). Repeat with replicate B.

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



7. Briefly vortex evolution tube A, then use a **p200 pipette** to move 50 µl from the large evolution tube to the PBS tube marked 10^{-2} A.

8. Briefly vortex the 10^{-2} A PBS tube, then pipette 50 µl from it into the PBS tube marked *10^{-4}* A. (Figure 4). Once again, make sure that this is done for both replicates.

9. Briefly vortex the 10^{-4} A tube, and from this tube, pipette 100 µl onto the corresponding agar plate and spread plate by shaking the plate with the agar side down using the glass beads. Repeat for replicate B. Remove the glass beads from your agar plate as sterilely as possible.

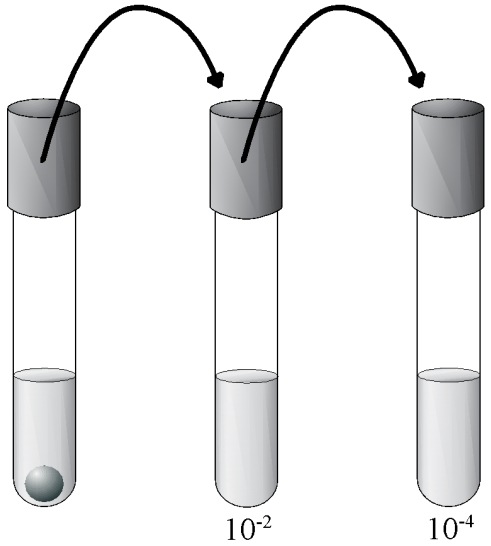


Figure 4: Serial dilution from the evolution tube (left) into PBS.

10. Incubate the evolution tubes on a roller drum at 28°C for 24 hours. Incubate the plate, **agar side up**, at 28°C for 24 hours.

POST-LAB QUESTIONS: DAY 0

1. Why is it important to transfer the bacteria every 24 hours? Draw a graph that illustrates the growth of a bacterial culture. Make sure to label your axes!

A point of Darwin's Theory of Evolution by Natural Selection: _____

2. Provide a detailed hypothesis that describes what you think might occur in your test tube over the next 24 hours when your bacteria from inside the test tube are adhering to the new bead. Try to use the following vocabulary in your predictions: planktonic, biofilm, and overproduction and polystyrene bead.

PRE-LAB QUESTIONS: DAY 1

1. What is the color of the old bead that is being transferred from the 24-hour large glass evolution tube? What is the color of the new bead that is in the new large glass evolution tube? Create a diagram showing the bead transfer for Day 1.

2. Why is it important to disrupt the bead as little as possible during your daily bead transfer? Explain three different ways you might disrupt the bead.

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.

NECESSARY MATERIALS:

- Metal forceps
- (2) Large glass evolution tube containing 5ml QB and 1 **black** polystyrene bead

PROCEDURE:

1. Flame sterilize forceps and let cool.

2. Pour the contents of the overnight culture into its metal cap, and then use sterile forceps to transfer the **white bead** to the new corresponding evolution tube with fresh media and a **black bead**.

3. Incubate the evolution tubes on a roller drum at 28°C for 24 hours.

4. Remove the Day 0 agar plates from the incubator and allow them to sit on the bench for an additional 24 hours to fully develop.

POST-LAB QUESTIONS: DAY 1

1. Describe in detail the three different types of mutations that can occur and the possible effect of that given type of mutation. Which two types of mutations are generally more common, and which is the least likely to occur?

2. What is being accomplished by transferring only the bacteria that have successfully attached to the bead? What is this type of selection called and explain why?

PRE-LAB QUESTIONS: DAY 2

1. Create a diagram that shows the bead transfer that you will carry out on Day 2. Be sure to include bead color in your diagram, and label all of the materials that you will use.

2. We will be viewing our plates from Day 0. What do you expect to see in terms of blue and white colonies? What do you think the shape of each colony will be and why?

DAY 2: NORMAL BEAD TRANSFER

Necessary Materials:

- Metal forceps
- (2) Large glass evolution tube containing 5 mL QB and 1 **white** polystyrene bead

Procedure:

1. Flame sterilize forceps and let cool.

2. Pour the contents of the overnight evolution culture into its metal cap, and then use sterile forceps to transfer the **black bead** to the new corresponding evolution tube with fresh media and a **white bead**.

3. Incubate the evolution tubes on a roller drum or shaking surface at 28°C for 24 hours.

4. Plate analysis of Day 0.

POST-LAB QUESTIONS: DAY 2

1. We already know that bacteria grow at an incredibly fast rate and can potentially overproduce, causing them to produce more bacteria inside the test tube than can survive. This overproduction leads to another phenomenon, which is another point of Darwin's Theory of Evolution by Natural Selection. Explain how this is occurring inside of the test tube and how it relates to overproduction.

   Another point of Darwin's Theory of Evolution by Natural Selection: _____

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PRE-LAB QUESTIONS: DAY 3

1. Draw the series of steps that are required to complete a serial dilution on Day 3. Include the amount of liquid that is being transferred, the amount of liquid that is in the dilution tube, and the dilution that is achieved with each step. Circle the dilution(s) that will be plated on Day 3.

2. Provide a detailed hypothesis as to why you believe it is necessary to dilute one step further on Day 3 than on Day 0.

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DAY 3: BEAD TRANSFER & PLATING DAY

Since you last plated your bacterial culture, 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. Based on your knowledge of evolution, can you provide an explanation as to why this might be true?

You may have noticed that your incubated test tubes now contain both a white and a black bead. Today, you are transferring your 24-hour white bead to a new tube containing fresh media and a black bead. Over the course of 24 hours, some of the bacteria from the white bead will detach and re-adhere to the surface of the black bead. In addition to this, we want to visualize the bacteria inside of the test tube on an agar plate. In order to do so, we want to plate the bacterial culture responsible for these changes. This can be done by plating in a similar fashion to when you last plated on Day 0.

Necessary Materials:

- Metal forceps
- Vortex
- A p200 and p1000 pipette
- (2) Large glass evolution tube containing: 5 mL QB and 1 **black** polystyrene bead
- (2) Large glass tubes labeled "INTER" containing: 1 mL Phosphate Buffered Saline (PBS)
- (4) Large glass tubes containing: 5 mL Phosphate Buffered Saline (PBS)
- (2) Large glass tubes containing: 4.5 mL Phosphate Buffered Saline (PBS)
- (4) ½ Strength Tsoy-Agar plate containing X-gal

Procedure:

1. Flame sterilize forceps and let cool.

2. Pour the contents of the overnight evolution culture into its metal cap, and then use sterile forceps to transfer the **white bead** to the new evolution tube with fresh media and a **black bead**.

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

3. Flame sterilize forceps and let cool.

4. Use sterile forceps to transfer the **black bead** to the corresponding INTER 1 mL PBS tube. Repeat for both replicates.

5. Vortex the INTER PBS tubes for at least 45 seconds to remove cells from the bead.

6. Briefly vortex the INTER PBS tube, and pour the contents of the tube into its metal cap.

7. Use a P200 pipette to move 50 µl from the cap containing INTER PBS A to the PBS tube

marked 10^{-2} A.

8. Briefly vortex the 10^{-2} A PBS tube, then pipette 50 µl from it into the PBS tube marked 10^{-4} A.

9. Briefly vortex the 10^{-4} A PBS tube, then pipette 500 µl from it into the PBS tube marked 10^{-5} A.

10. Repeat steps 6-8 for replicate B.

11. Briefly vortex the 10^{-4} PBS tube. From this tube, pipette 100 µl onto the agar plate labeled 10^{-4}, and spread plate by shaking the plate with the agar side down using the glass beads. Repeat for replicate B.

12. Repeat step 10, but instead of using the 10^{-4} PBS tube, use the 10^{-5} PBS tube.

13. Incubate the plates, agar side up, at 28°C for 24 hours.

14. Incubate the evolution tubes on a roller drum or shaking surface at 28°C for 24 hours.

POST-LAB QUESTIONS: DAY 3

1. It is possible that when you removed your tubes today, only one of them has significantly more biofilm on the sides of the tubes and has a neon culture. As we discussed, this is a possible indication that you have a beneficial mutation in your population. Can you provide an explanation for why only one of your two replicates looks like this if you started with identical bacteria at the beginning of your experiment?

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DAY 4: NORMAL BEAD TRANSFER

Necessary Materials:

- Metal forceps
- (2) Large glass evolution tube containing 5 mL QB and 1 **white** polystyrene bead

Procedure:

1. Flame sterilize forceps and let cool.

2. Pour the contents of the overnight evolution culture into its metal cap, and then use sterile forceps to transfer the **black bead** to the new corresponding evolution tube with fresh media and a **white bead**.

3. Incubate the evolution tubes on a roller drum or shaking surface at 28°C for 24 hours.

4. Remove plates from incubator. Allow them to sit on bench for 24 hours.

DAY 5: NORMAL BEAD TRANSFER

Necessary Materials:

- Metal forceps
- (2) Large glass evolution tube containing 5 mL QB and 1 **black** polystyrene bead

Procedure:

1. Flame sterilize forceps and let cool.

2. Pour the contents of the overnight evolution culture into its metal cap, and then use sterile forceps to transfer the **white bead** to the new corresponding evolution tube with fresh media and a **black bead**.

3. Incubate the evolution tubes on a roller drum or shaking surface at 28°C for 24 hours.

4. Wrap plates with parafilm and refrigerate.

PRE-LAB QUESTIONS: DAY 6

1. Predict what your tubes will look like when you view them in lab. Also, how do you think your plates will be different from the Day 0 plates? Consider blue to white ratio, colony morphology and other possible differences?

2. What is the color of the old bead that is being transferred from the 24-hour large glass evolution tube? What is the color of the new bead that is in the new large glass evolution tube?

DAY 6: NORMAL BEAD TRANSFER

Necessary Materials:

- Metal forceps
- (2) Large glass evolution tube containing 5 mL QB and 1 **white** polystyrene bead

Procedure:

1. Flame sterilize forceps and let cool.

2. Pour the contents of the overnight evolution culture into its metal cap, and then use sterile forceps to transfer the **black bead** to the new corresponding evolution tube with fresh media and a **white bead**.

3. Incubate the evolution tubes on a roller drum or shaking surface at 28°C for 24 hours.

4. Plate analysis.

POST-LAB QUESTIONS: DAY 6

1. Explain how two distinctly different phenotypic and genotypic mutants can inhabit the same test tube simultaneously. Be sure to incorporate the importance of an ecological niche in your answer.

2. What is the newest point that you learned that is a requirement of Darwin's Theory of Evolution by Natural Selection? Explain what it is and why this newest point is essential for evolution by natural selection to take place.

3. When you removed your tube from the incubator on Day 3, it had not appeared to have changed greatly from when you started your experiment. When you removed your tube from the incubator on Day 6, the culture was neon yellow. You are sure that when you plate tomorrow, you will definitely have mutants on your plate. Your group member also states that it is possible that you have mutants on your Day 3 plates. Is he/she correct? Discuss the potential results that may be observed.

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PRE-LAB QUESTIONS: DAY 7

1. What is the color of the bead that is going to be plated? Why isn't it necessary to transfer the other bead to a new evolution tube containing fresh media and an oppositely marked bead?

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2. Draw the series of steps that are required to complete a serial dilution on Day 7. Include the amount of liquid that is being transferred, the amount of liquid that is in the dilution tube, and the dilution that is achieved with each step. Circle the dilution(s) that will be plated on Day 7.

DAY 7: FINAL PLATING DAY

Necessary Materials:

- Metal forceps
- Vortex
- A p200 and p1000 pipette
- (2) Large glass tubes labeled "INTER" containing: 1 mL Phosphate Buffered Saline (PBS)
- (4) Large glass tubes containing: 5 mL Phosphate Buffered Saline (PBS)
- (2) Large glass tubes containing: 4.5 mL Phosphate Buffered Saline (PBS)
- (4) ½ Strength Tsoy-Agar plate containing X-gal

Procedure:

1. Flame sterilize forceps and let cool.

2. Pour the contents of the overnight evolution culture into its metal cap, and then use sterile forceps to transfer the **white bead** to the corresponding labeled 1 mL PBS tube. Repeat for both replicates.

3. Vortex the INTER PBS tubes for at least 45 seconds to remove cells from the bead.

4. Briefly vortex the INTER PBS tube, and pour the contents of the tube into its metal cap.

5. Use a P200 pipette to move 50 µl from the cap containing INTER PBSA to the PBS tube marked 10^{-2} A.

6. Briefly vortex the 10^{-2} A PBS tube, then pipette 50 µl from it into the PBS tube marked 10^{-4} A.

7. Briefly vortex the 10^{-4} A PBS tube, then pipette 500 µl from it into the PBS tube marked 10^{-5} A.

8. Repeat steps 6-8 for replicate B.

9. Briefly vortex the 10^{-4} PBS tube. From this tube, pipette 100 µl onto the agar plate labeled 10^{-4}, and spread plate by shaking the plate with the agar side down using the glass beads. Repeat for replicate B.

10. Repeat step 10, but instead of using the 10^{-4} PBS tube, use the 10^{-5} PBS tube.

11. Incubate the plate, agar side up, at 28°C for 24 hours.

12. Incubate the evolution tubes on a roller drum or shaking surface at 28°C for 24 hours.

POST-LAB QUESTIONS: DAY 7

1. What is the newest point that you learned that is a requirement of Darwin's Theory of Evolution by Natural Selection? Use this new point to provide a possible explanation as to why the number of ancestral colonies that you are seeing on your plate (and thus bacterial cells in your test tube) may have decreased since Day 0 when you started your experiment.

2. Explain the difference between evolution and adaptation.