Protocol: Developing Survival Curve for Escherichia *coli* PRS413

**Abstract:** Calorie restriction (CR) is an effective method for lifespan extension in eukaryotes. TOR mediated nutrient sensing pathway and mitochondria play key roles in the lifespan extension effect of CR in eukaryotes. E. coli is a bacterium without mitochondria, and no ortholog for TOR has been found in E. coli. Here, we investigated whether CR can extend the lifespan of E. coli cells. Our study can address whether TOR-independent pathways can also play a role in lifespan extension effect of CR, and whether mitochondria is indeed an essential factor of CR.

**AIM:** This protocol is a detailed summary for the development and analysis of a survival curve for E. *coli* PRS413 under conditions favoring caloric restriction.

**Overview:** PRS413 is being grown in two separate conditions. The first condition for growth is in M9 media with .4% glucose concentration and the second condition is in M9 with .1% glucose concentration. The .1% concentration provides the conditions for caloric restriction, as glucose availability is limited. Previous research shows that the replicative lifespan of E. *coli* is approximately 4 to 5 days. Over a span of 5 days serial dilution done in 10x folds will be conducted and an average number will be take twice a day. A plot will be developed to determine the survival curve of E. *coli* PRS413 when grown in these conditions. If caloric restriction does have an effect, the bacteria grown in M9 with .1% glucose concentration will have a higher survival rate than the bacteria grown in M9 with .4% glucose concentration. This increase in the .1% glucose concentration is due to an increase in the lifespan of the cell due to caloric restriction.

**Methods**

**Day One: Growth of PRS413 in YPD media**

1. **Pour 5mL of YPD media in a tube**
2. **Flame a loop and take a colony from the PRS413 plate found in the 4oC fridge located under the lab bench**
3. **Parafilm the PRS413 plate and place it back in the 4oC fridge**
4. **Label the tube: Maite, Date, PRS413, Time i.e 03:00**
5. **Place the tube in the shaker at 37 oC overnight for growth \*\*Be sure the thermometer reads 37 oC and that the hours is greater than 70hrs.**

**Day Two: Caloric Restriction and Dilution at Time T=0**

1. **Label 12 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(1) tube: 1% OC (original culture)**
   2. **(1) tube: .4% OC**
   3. **(5) tubes: .1% 102, 103,104,105,106 respectively**
   4. **(5) tubes: .4% 102, 103,104,105,106 respectively**
2. **Label 6 LB plates as follow – These plates can be found in the cold room in the hall by the classrooms**
   1. **(3) plates: Maite/Date/T0/Time/PRS413/.1%/106**
   2. **(3) plates: Maite/Date/T0/Time/PRS413/.4%/106**
3. **Create the following solutions**
   1. **990uL M9, MgSO4, CaCl2 Solution – x2**
      1. **20uL MgSO4**
      2. **2uL CaCl2**
      3. **979uL M9**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **900uL M9, MgSO4, CaCl2 Solution – x8**
      1. **80uL MgSO4**
      2. **8uL CaCl2**
      3. **7.2mL M9**
   4. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **(4) tubes: .1% 103,104,105,106 respectively**
      2. **(4) tubes: .4% 103,104,105,106 respectively**
4. **Harvest the overnight culture cells**
   1. **Take 1000uL of the PRS413 grown overnight in the YPD media and place it in the 1.5mL ependorf tube labeled (1) tube: .1% OC (original culture)**
   2. **Take 1000uL of the PRS413 grown overnight in the YPD media and place it in the 1.5mL ependorf tube labeled (1) tube: .4% OC**
   3. **Centrifuge the .1% and .4% OC ependorf tubes at 7 for 4mins**
   4. **Empty the excess fluid from the ependorf tube and lightly tap the ependorf tube on a paper towel to ensure the YPD fluid is gone**
   5. **Repeat steps a-d until all of the overnight culture has been collected and all that is left in the ependorf tubes is the harvested cells**
   6. **Wash the cells in M9 and centrifuge twice**
   7. **after the last centrifuge cycle empty the excess fluid from the ependorf tube and lightly tap the ependorf tube on a paper towel to ensure the M9 fluid is gone**
5. **Creation of Caloric restriction**
   1. **In the tube .1% OC place the following solution**
      1. **100uL 20%glucose**
      2. **10uL MgSO4**
      3. **1uL CaCl2**
      4. **889uL M9**
   2. **In the tube .4% OC place the following solution**
      1. **400uL 20%glucose**
      2. **10uL MgSO4**
      3. **1uL CaCl2**
      4. **589uL M9**
   3. **Vortex the tubes**
6. **Dilution and Plating of .1% OC**
   1. **Take 10uL from the tube labeled .1% OC and place it in the tube labeled .1% 102**
   2. **Vortex the tube labeled .1% 102**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Vortex the tube labeled .1% 103**
   5. **Take 100uL from the tube labeled .1% 103 and place it in the tube labeled .1% 104**
   6. **Vortex the tube labeled .1% 104**
   7. **Take 100uL from the tube labeled .1% 104 and place it in the tube labeled .1% 105**
   8. **Vortex the tube labeled .1% 105**
   9. **Take 100uL from the tube labeled .1% 105 and place it in the tube labeled .1% 106**
   10. **Vortex the tube labeled .1% 106**
   11. **Take 100uL from the tube .1% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .1% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
7. **Dilution and Plating of .4% OC**
   1. **Take 10uL from the tube labeled .4% OC and place it in the tube labeled .4% 102**
   2. **Vortex the tube labeled .4% 102**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Vortex the tube labeled .4% 103**
   5. **Take 100uL from the tube labeled .4% 103 and place it in the tube labeled .4% 104**
   6. **Vortex the tube labeled .4% 104**
   7. **Take 100uL from the tube labeled .4% 104 and place it in the tube labeled .4% 105**
   8. **Vortex the tube labeled .4% 105**
   9. **Take 100uL from the tube labeled .4% 105 and place it in the tube labeled .4% 106**
   10. **Vortex the tube labeled .4% 106**
   11. **Take 100uL from the tube .4% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .4% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
8. **Place the .4 and .1% OC tubes in a blue cap tube and place in the shaker at 37 oC**
9. **Place the plates in a bag and place upside down (Label up) in the inoculator located in the autoclave room at 37 oC overnight**

**Day Three: Caloric Restriction and Dilution at Time T=1 and T=2, Plate Counting**

1. **Label 10 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(5) tubes: .1% 102, 103,104,105,106 respectively**
   2. **(5) tubes: .4% 102, 103,104,105,106 respectively**
2. **Label 6 LB plates as follow – These plates can be found in the cold room in the hall by the classrooms**
   1. **(3) plates: Maite/Date/T1/Time/PRS413/.1%/106**
   2. **(3) plates: Maite/Date/T1/Time/PRS413/.4%/106**
3. **Create the following solutions**
   1. **990uL M9, MgSO4, CaCl2 Solution – x2**
      1. **20uL MgSO4**
      2. **2uL CaCl2**
      3. **979uL M9**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **900uL M9, MgSO4, CaCl2 Solution – x8**
      1. **80uL MgSO4**
      2. **8uL CaCl2**
      3. **7.2mL M9**
   4. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **(4) tubes: .1% 103,104,105,106 respectively**
      2. **(4) tubes: .4% 103,104,105,106 respectively**
4. **Dilution and Plating of .1% OC**
   1. **Take 10uL from the tube labeled .1% OC and place it in the tube labeled .1% 102**
   2. **Vortex the tube labeled .1% 102**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Vortex the tube labeled .1% 103**
   5. **Take 100uL from the tube labeled .1% 103 and place it in the tube labeled .1% 104**
   6. **Vortex the tube labeled .1% 104**
   7. **Take 100uL from the tube labeled .1% 104 and place it in the tube labeled .1% 105**
   8. **Vortex the tube labeled .1% 105**
   9. **Take 100uL from the tube labeled .1% 105 and place it in the tube labeled .1% 106**
   10. **Vortex the tube labeled .1% 106**
   11. **Take 100uL from the tube .1% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .1% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
5. **Dilution and Plating of .4% OC**
   1. **Take 10uL from the tube labeled .4% OC and place it in the tube labeled .4% 102**
   2. **Vortex the tube labeled .4% 102**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Vortex the tube labeled .4% 103**
   5. **Take 100uL from the tube labeled .4% 103 and place it in the tube labeled .4% 104**
   6. **Vortex the tube labeled .4% 104**
   7. **Take 100uL from the tube labeled .4% 104 and place it in the tube labeled .4% 105**
   8. **Vortex the tube labeled .4% 105**
   9. **Take 100uL from the tube labeled .4% 105 and place it in the tube labeled .4% 106**
   10. **Vortex the tube labeled .4% 106**
   11. **Take 100uL from the tube .4% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .4% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
6. **Place the .4 and .1% OC tubes in a blue cap tube and place in the shaker at 37 oC**
7. **Place the plates in a bag and place upside down (Label up) in the inoculator located in the autoclave room at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates labeled:**
      1. **(3) plates: Maite/Date/T0/Time/PRS413/.1%/106**
      2. **(3) plates: Maite/Date/T0/Time/PRS413/.4%/106**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room located in the hall by the classrooms**
      1. **\*\*there is a box by the door with the plates place in the box**
9. **COMPLETE STEPS 1-7 AGAIN PRIOR TO LEAVING LAB, THIS WILL BE T=2**

**Day Four: Caloric Restriction and Dilution at Time T=3 and T=4, Plate Counting**

1. **Label 10 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(5) tubes: .1% 102, 103,104,105,106 respectively**
   2. **(5) tubes: .4% 102, 103,104,105,106 respectively**
2. **Label 6 LB plates as follow – These plates can be found in the cold room in the hall by the classrooms**
   1. **(3) plates: Maite/Date/T3/Time/PRS413/.1%/106**
   2. **(3) plates: Maite/Date/T3/Time/PRS413/.4%/106**
3. **Create the following solutions**
   1. **990uL M9, MgSO4, CaCl2 Solution – x2**
      1. **20uL MgSO4**
      2. **2uL CaCl2**
      3. **979uL M9**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **900uL M9, MgSO4, CaCl2 Solution – x8**
      1. **80uL MgSO4**
      2. **8uL CaCl2**
      3. **7.2mL M9**
   4. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **(4) tubes: .1% 103,104,105,106 respectively**
      2. **(4) tubes: .4% 103,104,105,106 respectively**
4. **Dilution and Plating of .1% OC**
   1. **Take 10uL from the tube labeled .1% OC and place it in the tube labeled .1% 102**
   2. **Vortex the tube labeled .1% 102**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Vortex the tube labeled .1% 103**
   5. **Take 100uL from the tube labeled .1% 103 and place it in the tube labeled .1% 104**
   6. **Vortex the tube labeled .1% 104**
   7. **Take 100uL from the tube labeled .1% 104 and place it in the tube labeled .1% 105**
   8. **Vortex the tube labeled .1% 105**
   9. **Take 100uL from the tube labeled .1% 105 and place it in the tube labeled .1% 106**
   10. **Vortex the tube labeled .1% 106**
   11. **Take 100uL from the tube .1% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .1% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
5. **Dilution and Plating of .4% OC**
   1. **Take 10uL from the tube labeled .4% OC and place it in the tube labeled .4% 102**
   2. **Vortex the tube labeled .4% 102**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Vortex the tube labeled .4% 103**
   5. **Take 100uL from the tube labeled .4% 103 and place it in the tube labeled .4% 104**
   6. **Vortex the tube labeled .4% 104**
   7. **Take 100uL from the tube labeled .4% 104 and place it in the tube labeled .4% 105**
   8. **Vortex the tube labeled .4% 105**
   9. **Take 100uL from the tube labeled .4% 105 and place it in the tube labeled .4% 106**
   10. **Vortex the tube labeled .4% 106**
   11. **Take 100uL from the tube .4% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .4% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
6. **Place the .4 and .1% OC tubes in a blue cap tube and place in the shaker at 37 oC**
7. **Place the plates in a bag and place upside down (Label up) in the inoculator located in the autoclave room at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates labeled:**
      1. **(3) plates: Maite/Date/T1/Time/PRS413/.1%/106**
      2. **(3) plates: Maite/Date/T1/Time/PRS413/.4%/106**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room located in the hall by the classrooms**
      1. **\*\*there is a box by the door with the plates place in the box**
9. **COMPLETE STEPS 1-7 AGAIN PRIOR TO LEAVING LAB THIS WILL BE T=4**
10. **EVENING PLATE COUNTING**
    1. **Remove the 6 plates labeled:**
       1. **(3) plates: Maite/Date/T2/Time/PRS413/.1%/106**
       2. **(3) plates: Maite/Date/T2/Time/PRS413/.4%/106**
    2. **Count the plates and place in the google docs excel sheet**
    3. **After done counting place the plates in the cold room located in the hall by the classrooms**
    4. **\*\*there is a box by the door with the plates place in the box**

**Day FIVE: Caloric Restriction and Dilution at Time T=5 and T=6, Plate Counting**

1. **Label 10 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(5) tubes: .1% 102, 103,104,105,106 respectively**
   2. **(5) tubes: .4% 102, 103,104,105,106 respectively**
2. **Label 6 LB plates as follow – These plates can be found in the cold room in the hall by the classrooms**
   1. **(3) plates: Maite/Date/T5/Time/PRS413/.1%/106**
   2. **(3) plates: Maite/Date/T5/Time/PRS413/.4%/106**
3. **Create the following solutions**
   1. **990uL M9, MgSO4, CaCl2 Solution – x2**
      1. **20uL MgSO4**
      2. **2uL CaCl2**
      3. **979uL M9**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **900uL M9, MgSO4, CaCl2 Solution – x8**
      1. **80uL MgSO4**
      2. **8uL CaCl2**
      3. **7.2mL M9**
   4. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **(4) tubes: .1% 103,104,105,106 respectively**
      2. **(4) tubes: .4% 103,104,105,106 respectively**
4. **Dilution and Plating of .1% OC**
   1. **Take 10uL from the tube labeled .1% OC and place it in the tube labeled .1% 102**
   2. **Vortex the tube labeled .1% 102**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Vortex the tube labeled .1% 103**
   5. **Take 100uL from the tube labeled .1% 103 and place it in the tube labeled .1% 104**
   6. **Vortex the tube labeled .1% 104**
   7. **Take 100uL from the tube labeled .1% 104 and place it in the tube labeled .1% 105**
   8. **Vortex the tube labeled .1% 105**
   9. **Take 100uL from the tube labeled .1% 105 and place it in the tube labeled .1% 106**
   10. **Vortex the tube labeled .1% 106**
   11. **Take 100uL from the tube .1% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .1% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
5. **Dilution and Plating of .4% OC**
   1. **Take 10uL from the tube labeled .4% OC and place it in the tube labeled .4% 102**
   2. **Vortex the tube labeled .4% 102**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Vortex the tube labeled .4% 103**
   5. **Take 100uL from the tube labeled .4% 103 and place it in the tube labeled .4% 104**
   6. **Vortex the tube labeled .4% 104**
   7. **Take 100uL from the tube labeled .4% 104 and place it in the tube labeled .4% 105**
   8. **Vortex the tube labeled .4% 105**
   9. **Take 100uL from the tube labeled .4% 105 and place it in the tube labeled .4% 106**
   10. **Vortex the tube labeled .4% 106**
   11. **Take 100uL from the tube .4% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .4% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
6. **Place the .4 and .1% OC tubes in a blue cap tube and place in the shaker at 37 oC**
7. **Place the plates in a bag and place upside down (Label up) in the inoculator located in the autoclave room at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates labeled:**
      1. **(3) plates: Maite/Date/T3/Time/PRS413/.1%/106**
      2. **(3) plates: Maite/Date/T3/Time/PRS413/.4%/106**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room located in the hall by the classrooms**
      1. **\*\*there is a box by the door with the plates place in the box**
9. **COMPLETE STEPS 1-7 AGAIN PRIOR TO LEAVING LAB THIS WILL BE T=6**
10. **EVENING PLATE COUNTING**
    1. **Remove the 6 plates labeled:**
       1. **(3) plates: Maite/Date/T4/Time/PRS413/.1%/106**
       2. **(3) plates: Maite/Date/T4/Time/PRS413/.4%/106**
    2. **Count the plates and place in the google docs excel sheet**
    3. **After done counting place the plates in the cold room located in the hall by the classrooms**
    4. **\*\*there is a box by the door with the plates place in the box**

**Day SIX: Caloric Restriction and Dilution at Time T=7 and T=8, Plate Counting**

1. **Label 10 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(5) tubes: .1% 102, 103,104,105,106 respectively**
   2. **(5) tubes: .4% 102, 103,104,105,106 respectively**
2. **Label 6 LB plates as follow – These plates can be found in the cold room in the hall by the classrooms**
   1. **(3) plates: Maite/Date/T7/Time/PRS413/.1%/106**
   2. **(3) plates: Maite/Date/T7/Time/PRS413/.4%/106**
3. **Create the following solutions**
   1. **990uL M9, MgSO4, CaCl2 Solution – x2**
      1. **20uL MgSO4**
      2. **2uL CaCl2**
      3. **979uL M9**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **900uL M9, MgSO4, CaCl2 Solution – x8**
      1. **80uL MgSO4**
      2. **8uL CaCl2**
      3. **7.2mL M9**
   4. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **(4) tubes: .1% 103,104,105,106 respectively**
      2. **(4) tubes: .4% 103,104,105,106 respectively**
4. **Dilution and Plating of .1% OC**
   1. **Take 10uL from the tube labeled .1% OC and place it in the tube labeled .1% 102**
   2. **Vortex the tube labeled .1% 102**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Vortex the tube labeled .1% 103**
   5. **Take 100uL from the tube labeled .1% 103 and place it in the tube labeled .1% 104**
   6. **Vortex the tube labeled .1% 104**
   7. **Take 100uL from the tube labeled .1% 104 and place it in the tube labeled .1% 105**
   8. **Vortex the tube labeled .1% 105**
   9. **Take 100uL from the tube labeled .1% 105 and place it in the tube labeled .1% 106**
   10. **Vortex the tube labeled .1% 106**
   11. **Take 100uL from the tube .1% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .1% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
5. **Dilution and Plating of .4% OC**
   1. **Take 10uL from the tube labeled .4% OC and place it in the tube labeled .4% 102**
   2. **Vortex the tube labeled .4% 102**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Vortex the tube labeled .4% 103**
   5. **Take 100uL from the tube labeled .4% 103 and place it in the tube labeled .4% 104**
   6. **Vortex the tube labeled .4% 104**
   7. **Take 100uL from the tube labeled .4% 104 and place it in the tube labeled .4% 105**
   8. **Vortex the tube labeled .4% 105**
   9. **Take 100uL from the tube labeled .4% 105 and place it in the tube labeled .4% 106**
   10. **Vortex the tube labeled .4% 106**
   11. **Take 100uL from the tube .4% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .4% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
6. **Place the .4 and .1% OC tubes in a blue cap tube and place in the shaker at 37 oC**
7. **Place the plates in a bag and place upside down (Label up) in the inoculator located in the autoclave room at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates labeled from:**
      1. **(3) plates: Maite/Date/T5/Time/PRS413/.1%/106**
      2. **(3) plates: Maite/Date/T5/Time/PRS413/.4%/106**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room located in the hall by the classrooms**
      1. **\*\*there is a box by the door with the plates place in the box**
9. **COMPLETE STEPS 1-7 AGAIN PRIOR TO LEAVING LAB THIS WILL BE T=8**
10. **EVENING PLATE COUNTING**
    1. **Remove the 6 plates labeled:**
       1. **(3) plates: Maite/Date/T6/Time/PRS413/.1%/106**
       2. **(3) plates: Maite/Date/T6/Time/PRS413/.4%/106**
    2. **Count the plates and place in the google docs excel sheet**
    3. **After done counting place the plates in the cold room located in the hall by the classrooms**
    4. **\*\*there is a box by the door with the plates place in the box**

**Day Seven: Caloric Restriction and Dilution at Time T=9 and T=10, Plate Counting**

1. **Label 10 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(5) tubes: .1% 102, 103,104,105,106 respectively**
   2. **(5) tubes: .4% 102, 103,104,105,106 respectively**
2. **Label 6 LB plates as follow – These plates can be found in the cold room in the hall by the classrooms**
   1. **(3) plates: Maite/Date/T7/Time/PRS413/.1%/106**
   2. **(3) plates: Maite/Date/T7/Time/PRS413/.4%/106**
3. **Create the following solutions**
   1. **990uL M9, MgSO4, CaCl2 Solution – x2**
      1. **20uL MgSO4**
      2. **2uL CaCl2**
      3. **979uL M9**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **900uL M9, MgSO4, CaCl2 Solution – x8**
      1. **80uL MgSO4**
      2. **8uL CaCl2**
      3. **7.2mL M9**
   4. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **(4) tubes: .1% 103,104,105,106 respectively**
      2. **(4) tubes: .4% 103,104,105,106 respectively**
4. **Dilution and Plating of .1% OC**
   1. **Take 10uL from the tube labeled .1% OC and place it in the tube labeled .1% 102**
   2. **Vortex the tube labeled .1% 102**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Vortex the tube labeled .1% 103**
   5. **Take 100uL from the tube labeled .1% 103 and place it in the tube labeled .1% 104**
   6. **Vortex the tube labeled .1% 104**
   7. **Take 100uL from the tube labeled .1% 104 and place it in the tube labeled .1% 105**
   8. **Vortex the tube labeled .1% 105**
   9. **Take 100uL from the tube labeled .1% 105 and place it in the tube labeled .1% 106**
   10. **Vortex the tube labeled .1% 106**
   11. **Take 100uL from the tube .1% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .1% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
5. **Dilution and Plating of .4% OC**
   1. **Take 10uL from the tube labeled .4% OC and place it in the tube labeled .4% 102**
   2. **Vortex the tube labeled .4% 102**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Vortex the tube labeled .4% 103**
   5. **Take 100uL from the tube labeled .4% 103 and place it in the tube labeled .4% 104**
   6. **Vortex the tube labeled .4% 104**
   7. **Take 100uL from the tube labeled .4% 104 and place it in the tube labeled .4% 105**
   8. **Vortex the tube labeled .4% 105**
   9. **Take 100uL from the tube labeled .4% 105 and place it in the tube labeled .4% 106**
   10. **Vortex the tube labeled .4% 106**
   11. **Take 100uL from the tube .4% 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .4% 106.**
   12. **Using glass beads evenly spread the 100uL sample on the plate**
6. **Place the .4 and .1% OC tubes in a blue cap tube and place in the shaker at 37 oC**
7. **Place the plates in a bag and place upside down (Label up) in the inoculator located in the autoclave room at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates labeled from:**
      1. **(3) plates: Maite/Date/T7/Time/PRS413/.1%/106**
      2. **(3) plates: Maite/Date/T7/Time/PRS413/.4%/106**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room located in the hall by the classrooms**
      1. **\*\*there is a box by the door with the plates place in the box**
9. **COMPLETE STEPS 1-7 AGAIN PRIOR TO LEAVING LAB THIS WILL BE T=8**
10. **EVENING PLATE COUNTING**
    1. **Remove the 6 plates labeled:**
       1. **(3) plates: Maite/Date/T8/Time/PRS413/.1%/106**
       2. **(3) plates: Maite/Date/T8/Time/PRS413/.4%/106**
    2. **Count the plates and place in the google docs excel sheet**
    3. **After done counting place the plates in the cold room located in the hall by the classrooms**
    4. **\*\*there is a box by the door with the plates place in the box**

**Day Eight: Plate Counting**

1. **Plate Counting**
   1. **Remove the 6 plates labeled from:**
      1. **(3) plates: Maite/Date/T9/Time/PRS413/.1%/106**
      2. **(3) plates: Maite/Date/T9/Time/PRS413/.4%/106**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room located in the hall by the classrooms**
      1. **\*\*there is a box by the door with the plates place in the box**
2. **EVENING PLATE COUNTING**
   1. **Remove the 6 plates labeled:**
      1. **(3) plates: Maite/Date/T10/Time/PRS413/.1%/106**
      2. **(3) plates: Maite/Date/T10/Time/PRS413/.4%/106**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room located in the hall by the classrooms**
   4. **\*\*there is a box by the door with the plates place in the box**