Protocol: Developing a Survival Curve for Escherichia *coli* PRS413 factoring in Osmolarity

**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 four 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. In a parallel study the bacteria is grown in a .3M NaCl concentration of M9 and a .1M M9 concentrated with NaCl. 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 factoring in osmolarity. 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 LB media**

1. **Pour 5mL of LB media in a tube- x2**
   1. **One tube is for Study A (Caloric Restriction) and the other for Study B (NaCl2 Osmolarity)**
2. **Flame a loop and take a colony from the E. *coli* (PRS413) plate found in the 4oC fridge**
3. **Label each tube: Initials/ Date/ Strain/ Media/ Time**
   1. **Maite/12\_03\_19/PRS413/LB/15:00**
4. **Place the tube in the shaker at 37 oC overnight for growth**

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

**Study A: Caloric Restriction**

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 (3 for each concentration, .4% and .1%) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1%/T=0/15:00**
3. **Make the following solutions**
   1. **9.2mL of 1x M9 Buffer (1090uL 10xM9, 8090uL dH20)**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **tubes: .1% 103,104,105,106 respectively**
      2. **tubes: .4% 103,104,105,106 respectively**
4. **Harvest the overnight culture cells (SPIN DOWN)**
   1. **Take 1000uL of the PRS413 grown overnight in the LB media tube labeled study A and place it in the 1.5mL ependorf tube labeled: .1% OC (original culture)**
   2. **Take 1000uL of the PRS413 grown overnight in the LB media tube labeled study A and place it in the 1.5mL ependorf 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 LB 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 1x M9 Buffer (900uL dH20, 100uL 10x M9) and centrifuge- X2**
   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. **892.9uL dH2O, 100uLM9, 2uLMgSO4, .1uL CaCl2, 5mL 20% glucose**
   2. **In the tube .4% OC place the following solution**
      1. **877.9uL dH2O, 100uLM9, 2uLMgSO4, .1uL CaCl2, 20mL 20% glucose**
   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**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Complete a serial dilution ending with tube .1% 106**
   5. **Vortex the tube labeled .1% 106**
   6. **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.**
   7. **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**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Complete the serial dilution ending with tube .4% 106.**
   5. **Vortex the tube labeled .4% 106**
   6. **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.**
   7. **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 at 37 oC overnight**

**Study B: NaCl Osmolarity**

1. **Label 12 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(5) tubes: .MM 102, 103,104,105,106 respectively**
   2. **(5) tubes: .4M 102m 103,104,105,106 respectively**
2. **Label 6 LB plates (3 for each molar concentration) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1M/10^6/T=0/15:00**
3. **Create the following solutions**
   1. **9.2L M9,MgSO 4M CaCl2 stock solution**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1M 102**
      2. **.3M 102**
   3. **Place 900uL of this solution into each of the following 1.5m ependorf tubes:**
      1. **tubes: .1M 103,104,105,106 respectively**
      2. **tubes: .3M 103,104,105,106 respectively**
4. **Harvest the overnight culture cells (SPIN DOWN)**
   1. **Take 1000uL of the PRS413 grown overnight in the LB media tube labeled study B and place it in the 1.5mL ependorf tube labeled: .1M OC (original culture)**
   2. **Take 1000uL of the PRS413 grown overnight in the LB media tube labeled study B and place it in the 1.5mL ependorf tube: .3M OC**
   3. **Centrifuge the .1M and .3M 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 LB 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 1x M9 Buffer (900uL dH20, 100uL 10x M9) and centrifuge- X2**
   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**

**14. Creation of Caloric restriction**

* 1. **In the tube .1M OC place the following solution**
     1. **.099uL NaCl, 100uL 10X M9M 2uL MgSO4, .1uL CaCl2, 893.3 uL dH2O**
  2. **In the tube .3M OC place the following solution**
     1. **.299uL NaCl, 100uL 10X M9M 2uL MgSO4, .1uL CaCl2, 897.6 uL dH2O**
  3. **Vortex the tubes**

1. **Dilution and Plating of .1M OC**
   1. **Take 10uL from the tube labeled .1M OC and place it in the tube labeled .1M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .1M 102 and place it in the tube labeled .1M 103**
   4. **Complete a serial dilution ending with tube .1M 106**
   5. **Vortex the tube labeled Osmolarity Conditions from the tube .1M 106 and place it on the labeled 93 3late, 3 plates should be made ea. Contai.099uL NaCl the tube .1M 106.**
   6. **Evenly spread the 100uL sample on the plate**
2. **Dilution and Plating of .3M OC**
   1. **Take 10uL from the tube labeled .3M OC and place it in the tube labeled .3M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .3M 102 and place it in the tube labeled .3M 103**
   4. **Complete the serial dilution ending with tube .3M 106.**
   5. **Vortex the tube labeled .3M 106**
   6. **Take 100uL from the tube .3M 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .3M 106.**
   7. **Evenly spread the 100uL sample on the plate**
3. **Place the .3 and .1M OC tubes in a blue cap tube and place in the shaker at 37 oC**
4. **Place the plates in a bag and place upside down (Label up) in the inoculator at 37 oC overnight**

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

**Study A: Caloric Restriction**

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 (3 for each concentration) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1%/10^6/T=0/15:00**
3. **Create the following solutions**
   1. **9.2L M9,MgSO4, CaCl2 stock solution**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **tubes: .1% 103,104,105,106 respectively**
      2. **tubes: .4% 103,104,105,106 respectively**
4. **Harvest the overnight culture cells**
   1. **Centrifuge the .1% and .4% OC ependorf tubes at 7 for 4mins**
   2. **Empty the excess fluid from the ependorf tube and lightly tap the ependorf tube on a paper towel to ensure the M9 glucose fluid is gone**
   3. **Wash the cells in M9 and centrifuge- X2**
   4. **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 Growth Conditions**
   1. **In the tubes .1 and .4% add 887.9uL dH2O, 100uL 10x M9, 2uL MgSO4, .1uL CaCl2**
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**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Complete a serial dilution ending with tube .1% 106**
   5. **Vortex the tube labeled .1% 106**
   6. **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.**
   7. **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**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Complete the serial dilution ending with tube .4% 106.**
   5. **Vortex the tube labeled .4% 106**
   6. **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.**
   7. **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 at 37 oC overnight**
10. **Plate Counting**
    1. **Remove the 6 plates from the evening prior**
    2. **Count the plates and place in the google docs excel sheet**
    3. **After done counting place the plates in the cold room \*\*there is a box by the door with the plates place in the box**
11. **COMPLETE STEPS 1-7 EXCLUDING STEPS 4 AND 5 PRIOR TO LEAVING LAB, THIS WILL BE another time count**

**Study B: NaCl Osmolarity**

1. **1. Label 10 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(5) tubes: .1M 102, 103,104,105,106 respectively**
   2. **(5) tubes: .3M 102, 103,104,105,106 respectively**
2. **Label 6 LB plates (3 for each molar concentration) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1M/10^6/T=0/15:00**
3. **Create the following solutions**
   1. **9.2L M9,MgSO4, CaCl2 stock solution**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1M 102**

**.3M 102**

* 1. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
     1. **tubes: .1M 103,104,105,106 respectively**
     2. **tubes: .3M 103,104,105,106 respectively**

1. **Harvest the overnight culture cells**
   1. **Centrifuge the .1M and .3M OC ependorf tubes at 7 for 4mins**
   2. **Empty the excess fluid from the ependorf tube and lightly tap the ependorf tube on a paper towel to ensure the M9 NaCl2 fluid is gone**
   3. **Wash the cells in M9 and centrifuge- X2**
   4. **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**
2. **Creation of Growth Conditions**
   1. **In the tubes .1 and .3M add 887.9uL dH2O, 100uL 10x M9, 2uL MgSO4, .1uL CaCl2**
3. **Dilution and Plating of .1M OC**
   1. **Take 10uL from the tube labeled .1M OC and place it in the tube labeled .1M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .1M 102 and place it in the tube labeled .1M 103**
   4. **Complete a serial dilution ending with tube .1M 106**
   5. **Vortex the tube labeled .1M 106**
   6. **Take 100uL from the tube .1M 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .1M 106.**
   7. **Evenly spread the 100uL sample on the plate**
4. **Dilution and Plating of .3M OC**
   1. **Take 10uL from the tube labeled .3M OC and place it in the tube labeled .3M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .3M 102 and place it in the tube labeled .3M 103**
   4. **Complete the serial dilution ending with tube .3M 106.**
   5. **Vortex the tube labeled .3M 106**
   6. **Take 100uL from the tube .3M 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .3M 106.**
   7. **Evenly spread the 100uL sample on the plate**
5. **Place the .4 and .1M OC tubes in a blue cap tube and place in the shaker at 37 oC**
6. **Place the plates in a bag and place upside down (Label up) in the inoculator at 37 oC overnight**
7. **Plate Counting**
   1. **Remove the 6 plates from the evening prior**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room \*\*there is a box by the door with the plates place in the box**
8. **COMPLETE STEPS 1-7 AGAIN PRIOR TO LEAVING LAB, THIS WILL BE another time count**

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

**Study A: Caloric Restriction**

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 (3 for each concentration) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1%/10^6/T=0/15:00**
3. **Create the following solutions**
   1. **9.2L M9,MgSO4, CaCl2 stock solution**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **tubes: .1% 103,104,105,106 respectively**
      2. **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**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Complete a serial dilution ending with tube .1% 106**
   5. **Vortex the tube labeled .1% 106**
   6. **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.**
   7. **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**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Complete the serial dilution ending with tube .4% 106.**
   5. **Vortex the tube labeled .4% 106**
   6. **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.**
   7. **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 at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates from the evening prior**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room \*\*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 another time count**

**Study B: NaCl2 Osmolarity**

1. **1. Label 10 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(5) tubes: .1M 102, 103,104,105,106 respectively**
   2. **(5) tubes: .3M 102, 103,104,105,106 respectively**
2. **Label 6 LB plates (3 for each molar concentration) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1M/10^6/T=0/15:00**
3. **Create the following solutions**
   1. **9.2L M9,MgSO4, CaCl2 stock solution**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1M 102**
      2. **.3M 102**
   3. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **tubes: .1M 103,104,105,106 respectively**
      2. **tubes: .3M 103,104,105,106 respectively**
4. **Dilution and Plating of .1M OC**
   1. **Take 10uL from the tube labeled .1M OC and place it in the tube labeled .1M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .1M 102 and place it in the tube labeled .1M 103**
   4. **Complete a serial dilution ending with tube .1M 106**
   5. **Vortex the tube labeled .1M 106**
   6. **Take 100uL from the tube .1M 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .1M 106.**
   7. **Evenly spread the 100uL sample on the plate**
5. **Dilution and Plating of .3M OC**
   1. **Take 10uL from the tube labeled .3M OC and place it in the tube labeled .3M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .3M 102 and place it in the tube labeled .3M 103**
   4. **Complete the serial dilution ending with tube .3M 106.**
   5. **Vortex the tube labeled .3M 106**
   6. **Take 100uL from the tube .3M 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .3M 106.**
   7. **Evenly spread the 100uL sample on the plate**
6. **Place the .3 and .1M 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 at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates from the evening prior**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room \*\*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 another time count**

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

**Study A: Caloric Restriction**

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 (3 for each concentration) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1%/10^6/T=0/15:00**
3. **Create the following solutions**
   1. **9.2L M9,MgSO4, CaCl2 stock solution**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **tubes: .1% 103,104,105,106 respectively**
      2. **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**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Complete a serial dilution ending with tube .1% 106**
   5. **Vortex the tube labeled .1% 106**
   6. **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.**
   7. **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**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Complete the serial dilution ending with tube .4% 106.**
   5. **Vortex the tube labeled .4% 106**
   6. **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.**
   7. **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 at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates from the evening prior**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room \*\*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 another time count**

**Study B: NaCl2 Osmolarity**

1. **1. Label 10 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(5) tubes: .1M 102, 103,104,105,106 respectively**
   2. **(5) tubes: .3M 102, 103,104,105,106 respectively**
2. **Label 6 LB plates (3 for each molar concentration) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1M/10^6/T=0/15:00**
3. **Create the following solutions**
   1. **9.2L M9,MgSO4, CaCl2 stock solution**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1 102**
      2. **.4% 102**
   3. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **tubes: .1% 103,104,105,106 respectively**
      2. **tubes: .4% 103,104,105,106 respectively**
4. **Dilution and Plating of .1% OC**
   1. **Take 10uL from the tube labeled .1M OC and place it in the tube labeled .1M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .1M 102 and place it in the tube labeled .1M 103**
   4. **Complete a serial dilution ending with tube .1M 106**
   5. **Vortex the tube labeled .1M 106**
   6. **Take 100uL from the tube .1M 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .1M 106.**
   7. **Evenly spread the 100uL sample on the plate**
5. **Dilution and Plating of .4M OC**
   1. **Take 10uL from the tube labeled .4M OC and place it in the tube labeled .4M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .4M 102 and place it in the tube labeled .4M 103**
   4. **Complete the serial dilution ending with tube .4M 106.**
   5. **Vortex the tube labeled .4M 106**
   6. **Take 100uL from the tube .4M 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .4M 106.**
   7. **Evenly spread the 100uL sample on the plate**
6. **Place the .4 and .1M 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 at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates from the evening prior**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room \*\*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 another time count**

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

**Study A: Caloric Restriction**

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 (3 for each concentration) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1%/10^6/T=0/15:00**
3. **Create the following solutions**
   1. **9.2L M9,MgSO4, CaCl2 stock solution**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **tubes: .1% 103,104,105,106 respectively**
      2. **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**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Complete a serial dilution ending with tube .1% 106**
   5. **Vortex the tube labeled .1% 106**
   6. **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.**
   7. **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**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Complete the serial dilution ending with tube .4% 106.**
   5. **Vortex the tube labeled .4% 106**
   6. **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.**
   7. **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 at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates from the evening prior**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room \*\*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 another time count**

**Study B: NaCl2 Osmolarity**

1. **1. Label 10 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(5) tubes: .1M 102, 103,104,105,106 respectively**
   2. **(5) tubes: .4M 102, 103,104,105,106 respectively**
2. **Label 6 LB plates (3 for each molar concentration) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1M/10^6/T=0/15:00**
3. **Create the following solutions**
   1. **9.2L M9,MgSO4, CaCl2 stock solution**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **tubes: .1% 103,104,105,106 respectively**
      2. **tubes: .4% 103,104,105,106 respectively**
4. **Dilution and Plating of .1% OC**
   1. **Take 10uL from the tube labeled .1M OC and place it in the tube labeled .1M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .1M 102 and place it in the tube labeled .1M 103**
   4. **Complete a serial dilution ending with tube .1M 106**
   5. **Vortex the tube labeled .1M 106**
   6. **Take 100uL from the tube .1M 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .1M 106.**
   7. **Evenly spread the 100uL sample on the plate**
5. **Dilution and Plating of .4M OC**
   1. **Take 10uL from the tube labeled .4M OC and place it in the tube labeled .4M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .4M 102 and place it in the tube labeled .4M 103**
   4. **Complete the serial dilution ending with tube .4M 106.**
   5. **Vortex the tube labeled .4M 106**
   6. **Take 100uL from the tube .4M 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .4M 106.**
   7. **Evenly spread the 100uL sample on the plate**
6. **Place the .4 and .1M 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 at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates from the evening prior**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room \*\*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 another time count**

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

**Study A: Caloric Restriction**

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 (3 for each concentration) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1%/10^6/T=0/15:00**
3. **Create the following solutions**
   1. **9.2L M9,MgSO4, CaCl2 stock solution**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **tubes: .1% 103,104,105,106 respectively**
      2. **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**
   3. **Take 100uL from the tube labeled .1% 102 and place it in the tube labeled .1% 103**
   4. **Complete a serial dilution ending with tube .1% 106**
   5. **Vortex the tube labeled .1% 106**
   6. **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.**
   7. **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**
   3. **Take 100uL from the tube labeled .4% 102 and place it in the tube labeled .4% 103**
   4. **Complete the serial dilution ending with tube .4% 106.**
   5. **Vortex the tube labeled .4% 106**
   6. **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.**
   7. **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 at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates from the evening prior**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room \*\*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 another time count**

**Study B: NaCl2 Osmolarity**

1. **1. Label 10 autoclaved 1.5mL ependorf tubes as follows:** 
   1. **(5) tubes: .1M 102, 103,104,105,106 respectively**
   2. **(5) tubes: .4M 102, 103,104,105,106 respectively**
2. **Label 6 LB plates (3 for each molar concentration) as follows: Initials/Date/Strain/Concentration/Dilution/Timepoint/Time**
   1. **MM/12\_03\_19/PRS413/.1M/10^6/T=0/15:00**
3. **Create the following solutions**
   1. **9.2L M9,MgSO4, CaCl2 stock solution**
   2. **Place 990uL of this solution into the each of following 1.5mL ependorf tubes:**
      1. **.1% 102**
      2. **.4% 102**
   3. **Place 900uL of this solution into each of the following 1.5mL ependorf tubes:**
      1. **tubes: .1% 103,104,105,106 respectively**
      2. **tubes: .4% 103,104,105,106 respectively**
4. **Dilution and Plating of .1% OC**
   1. **Take 10uL from the tube labeled .1M OC and place it in the tube labeled .1M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .1M 102 and place it in the tube labeled .1M 103**
   4. **Complete a serial dilution ending with tube .1M 106**
   5. **Vortex the tube labeled .1M 106**
   6. **Take 100uL from the tube .1M 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .1M 106.**
   7. **Evenly spread the 100uL sample on the plate**
5. **Dilution and Plating of .4M OC**
   1. **Take 10uL from the tube labeled .4M OC and place it in the tube labeled .4M 102**
   2. **Vortex the tube**
   3. **Take 100uL from the tube labeled .4M 102 and place it in the tube labeled .4M 103**
   4. **Complete the serial dilution ending with tube .4M 106.**
   5. **Vortex the tube labeled .4M 106**
   6. **Take 100uL from the tube .4M 106 and place it on the labeled LB plate, 3 plates should be made ea. Containing 100uL from the tube .4M 106.**
   7. **Evenly spread the 100uL sample on the plate**
6. **Place the .4 and .1M 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 at 37 oC overnight**
8. **Plate Counting**
   1. **Remove the 6 plates from the evening prior**
   2. **Count the plates and place in the google docs excel sheet**
   3. **After done counting place the plates in the cold room \*\*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 another time count**

**Day Eight: Plate Counting**

1. **Plate Counting**
   1. **Remove the 6 plates from the morning prior**
   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 from the evening prior**
   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**