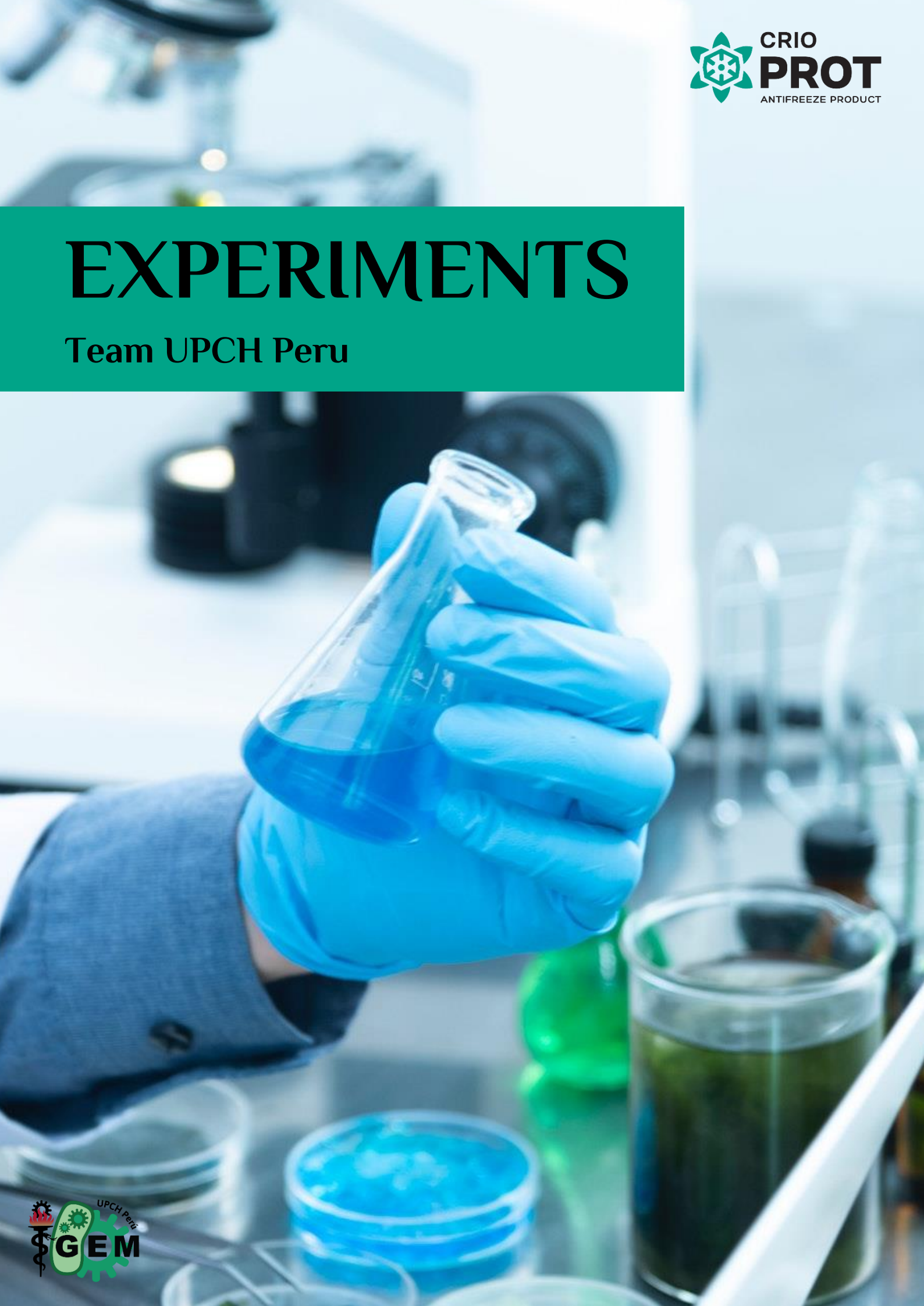
CRIO PROT
ANTIFREEZE PRODUCT

EXPERIMENTS

Team UPCH Peru



UPCH Perú
GEM

Contents

1.1 PCR of AFP

Introduction:

This protocol is based on the **PCR Protocol for Phusion® High-Fidelity DNA Polymerase (M0530)** by *New England BioLabs Inc.*

Materials (for a 50 µL reaction) :

Component	Volume
5X HF Phusion buffer	10 µL
10 mM dNTPs	1 µL
10 uM of Fw Primer (T7)	2.5 µL
10 uM of Rv Primer (T7)	2.5 µL
Approximately 10 ng of template DNA	1 µL
Pol HF Phusion	0.5 µL (1 U)
Molecular-grade water	up to 50 µL
DMSO 1g/mL (optional)	1.5 µL

Procedure:

1. Mix all the components in one tube for the thermocycler. Put first the buffer and the water, and lastly the Pol HF Phusion.
2. The termocycling conditions are as follows:

Stage	Temperature Time
Initial denaturation	98°C for 30 seconds
25 cycles of elongation	98°C for 10 seconds 62°C° for 30 seconds 72°C for 13 seconds
Final extension	72°C for 10 minutes
End	4°C for infinite

1.2 PCR products purification

Introduction:

This procedure is based on the GeneJet PCR Purification Kit. We perform it after obtaining the products of PCR and double digestion of our vector or insert DNA

Materials (for a 50 μL reaction) :

1. Binding, Elution and Wash Buffer
2. Purification columns and tubes for centrifuge
3. Centrifuge

Procedure:

1. Add Binding Buffer to the PCR mixture volume in a 1:1 relation. If it turns yellow, is good for binding
2. Pour up to 800 μL of the mix in step 1 to the purification column. Centrifuge at 13500 rpm for 60 s. Discard the flow through
3. Add 700 μL of Wash Buffer to the column. Centrifuge as in step 2. Discard the flow through
4. Centrifuge the empty column as in step 3. Discard any residual flow
5. Transfer the column to a new tube. Add 30 uL of the elution buffer and heat it at 50°C. The centrifuge as in step 4.

1.3 Double restriction digestion of AFP and expression Vector

Introduction:

This procedure details the steps to double digest our AFP g-block and the expression vector *(pET28)*. Is based on the **Double Digest Protocol with Standard Restriction Enzymes** from New England BioLabs Inc. (NEB).

Materials (for a 100 µL reaction) :

Component	Volume
10X NEBuffer 2.1	10 µL
Approximately 1 µg of DNA	1 µL
NcoI	1 µL
HindIII	0.2 µL
Molecular-grade water	up to 100 µL

Procedure:

1. Mix components in a reaction tube. End with the NcoI and HindIII
1. Incubate at 37°C for at least 1 hour

1.4 Ligation of digested products (g-block and vector)

Introduction:

For ligation of our digested products, we based these next steps from the **Thermo Scientific T4 DNA Ligase protocol for DNA Insert Ligation into Vector DNA**.

Materials (for a 50 µL reaction) :

Component	Volume
10X T4 DNA Ligase buffer	2 µL
Linearized vector	Depends on the concentration of the product. Has to lie between 20-100 ng
DNA insert	Proportion 5:1 over vector* in molarity
T4 DNA Ligase (5 U/uL)	1 µL
Molecular grade-water	up to 20 µL

Note*: For example, if the vector is 47. 1 ng/uL and insert DNA is 3 ng/uL, we could use: 1 uL of vector and 3.14 uL of insert DNA

Procedure:

1. Mix all components in a tube and leave the reaction overnight at 22°C.

1.5 Bacterial transformation by electroporation

Introduction:

For the insertion of our ligated product we used electroporation for transformation into *Escherichia coli* BLR(DE3).

Materials (for a 50 µL reaction) :

Componets

1. Ligation product (vector + insert)
2. 50 µL of electroporation *E. coli* BLR (DE3)
3. SOC media
4. LB agar plate with kanamycin

Equipment

1. Electroporation cuvette
2. Electroporation pulser

Procedure:

1. 1 µL of ligation product was added to a tube with 50 uL of electrocompetent *E. coli*
2. This mix was put in the electroporation cuvette, which was previously cooled in ice.
3. The cuvette was placed in the cuvette and received 1,8 kV
4. 1 mL of SOC media was added in the cuvette after the pulse, to collect the bacteria in another tube. Warning: Be quick and gentile, this step is very important, you can't take too much time here
5. The bacteria in SOC media were placed at 37°C for 1 hr
6. The tube was centrifuged at 6000 rpm during 8 minutes
7. The pellet was resuspended in SOC media
8. Plated in LB agar and kanamycin and left overnight for growth of transformant colonies

1.6 Colony PCR introduction

Introduction:

We made a Colony PCR to evaluate the presence of the insert DNA in the bacteria carrying our expression vector.

Materials:

Component	Volume
5X HF Phusion buffer	4 µL
10 mM dNTPs	0.4 µL
10 uM of Fw Primer (T7)	1 µL
10 uM of Rv Primer (T7)	1 µL
Suspension template	variable (we used 0.4 µL)
Pol HF Phusion	0.2 µL (1 U)
Molecular-grade water	up to 20 µL
DMSO 1g/mL (optional)	0.6 µL

Procedure:

1. Pick the colonies and put each one in tubes with 10 µL of molecule grade-water. Heat them at 95°C for 10 minutes
2. Mix all the components in one tube for the thermocycler. Put first the buffer and the water, and lastly the Poll HF Phusion.
3. The thermocycling conditions are as follow:

Stage	Temperature Time
Initial denaturation	98°C for 30 seconds
25 cycles of elongation	98°C for 10 seconds 62°C° for 30 seconds 72°C for 13 seconds
Final extension	72°C for 10 minutes
End	4°C for infinite

1.7 Protein production

Introduction:

This procedure details the steps to induce protein production in *E. coli* (BLR) DE3. There are two sections: for evaluation by temperature and evaluation by time. Same materials are used for both sections.

Materials:

1. 500 mL of LB media with kanamycin
2. 5 flasks.
3. Incubator shaker
4. Spectrophotometer
5. A pre inocμLum of 10 mL of transformed bacteria with our ligated product, prepared 1 day before procedure
6. Lysis buffer
7. Lysis beads

Procedure:

Section 1 (by temperature)

1. Put 100 µL of the pre inocμLum in a 500 mL flask with 50 mL of LB with kanamycin
2. Put in a shaker at 37°C and 220 rpm. Measure constantly and wait until OD=0.6, at 600 nm
3. Add IPTG for a final concentration of 0.5 mM. Put it in a shaker at 16°C at 220 rpm
4. Put each one at different temperatures as follows. Leave them overnight (16 h approximately):

Conditions	F1	F2	F3	F4
IPTG (+/-)	+	+	+	-
Temperature (°C)	37	30	16	30

5. Next day, centrifuge the content of each flask at 13500 rpm for 5 minutes and suspend the pellet in 1 mL of PBS. Centrifuge again, and resuspend in 1 mL of PBS for a last centrifuge.
6. Resuspend the pellet in 500 µL of lysis buffer and put it in a microtube with beads. Centrifuge a 3000 rpm for 20 s, so that beads go at the bottom.
7. Form the lysates take a sample of 15 µL, and other of 5 µL. Dilute the latter in 10 µL of molecμLar-grade water
8. Heat the samples at 85°C for 5 minutes
9. Run the samples in a polyacrylamide gel 15% for 1h50 at 150 V

Section 2 (by time since induction)

1. Put 1 mL of the pre inocμLum in a 1 L flask with 250 mL of LB with kanamycin
2. Put in a shaker at 37°C. Measure constantly and wait until OD=0.6, at 600 nm
3. Pour 50 mL of the flask in 4 smaller flasks (of 0.5 L) and add IPTG for a final concentration of 0.5 mM, except in one.
4. Take one sample of 5 mL for each of the following hours: 0h(right after induction),4h, 6h and 16 h (overnight).
5. Centrifuge the content of each flask at 13500 rpm for 5 minutes and refrigerate the samples at -20°C for later use
6. Suspend the pellet in 1 mL of PBS. Centrifuge again, and resuspend in 1 mL of PBS for a last centrifuge.
7. Resuspen the pellet in 500 µL of lysis buffer and put it in a microtube with 4 µL of lysozyme. Vortex for 20 minutes.
8. Take 400 µL from the lysates and centrifuge for 13500 rpm for 5 minutes. Separate the supernatant and resuspend the pellet in 400 µL of PBS. Supernatant is the insoluble fraction, and the resuspenden is the soluble
9. Take a sample of 20 µL of each fraction
10. Run the samples in a polyacrylamide gel 15% for 2h at 150 V
11. Stain with Coomassie Blue

2.1 Heterogeneous nucleation experiments

Introduction:

The change of state from liquid water to ice involves two processes: nucleation and crystal growth. The first stage, nucleation, can be of two types: homogeneous and heterogeneous. The homogeneous one has elevated activation energy and occurs at temperatures below -40 °C. On the other hand, heterogeneous implies the formation of an ice core around the surface of a particle present in water (soluble or insoluble). The latter allows the freezing of liquid water to occur at higher temperatures and close to the theoretical freezing point (0 °C). For understanding the importance of nucleating agents in ice formation, this protocol details the steps to evaluate the nucleating ability of different substances/materials: plating beads, ground oregano, and bond paper.

Materials :

Equipments

- PolyScience Circulator + accessories
- Electronic balance
- Photographic camera

Materials

- 2 racks
- Mercury thermometer
- 10 mL graduated pipette
- Pipet bulb
- 24 Test tubes
- Parafilm
- 2 sheets of bond paper
- Ziploc bag
- Aluminum foil
- Perforator
- Ground oregano
- Plating beads
- Spatula
- Chronometer

Reagents

- Distilled water
- Ethanol:Water 60%:40% mixture

Procedure:

Turning ON the equipment

1. NOTE: Always use biosecurity implements such as a mask and gloves.
2. Turn on the equipment and select a temperature of -5 °C, having previously added the mixture of Ethanol: Water 60%: 40% (see Protocol "Manipulation of the recirculating bath"). This should be done first, as it may take time for the equipment to reach the temperature of interest.

Sample preparation of distilled water with nucleating agents

3. Wash all test tubes with bleach, water, and finally distilled water. This must be done to eliminate interference such as nucleators or antifreeze agents.
4. Add 5 mL of distilled water to each of the test tubes.
5. Take a pinch of ground oregano, weigh it on the scale and record the mass. Add this amount of oregano to one of the test tubes with distilled water.
6. Weigh the same amount of ground oregano five more times and add it to 5 test tubes with distilled water.
7. Take two sheets of bond paper and, with the help of a hole punch, obtain circular-shaped pieces of paper (discs) and store them in a Ziploc bag.
8. Take the necessary bond paper discs so

that it has a weight similar to that of the pinch of oregano. Add the discs to a test tube with distilled water.

9. Add the same amount of bond paper discs in another 5 test tubes.
10. Add one plating bead to 6 test tubes with distilled water.

Freezing experiments

11. Immerse the racks with all test tubes in the recirculation bath previously set at -5 ° C.
12. After 20 min, remove 3 test tubes for each group (only distilled water, plating beads, ground oregano, bond paper), shake each one, and note if there is a change in the physical appearance of the tube content. Take photos and videos and re-immerse in the recirculation bath.
13. Remove the tubes that have not been shaken. Note if there is a change in the physical appearance of the tube contents. Take photos and videos and re-immerse in the recirculation bath.

2.2 Manipulation of recirculation bath

Introduction:

A low-temperature medium (less than or equal to 0 °C) is required to study the transition from liquid water to ice. One of the equipment that allows us to work in these conditions is a circulator. It makes a liquid or mixture circulating constantly at a certain temperature. This protocol details the steps to properly operate a PolyScience brand circulator and perform experiments at temperatures less than or equal to 0 °C.

Materials :

Equipments

- PolyScience Circulator

Materials

- Hose (approx. 1 m long)
- 2 Clamps
- Screwdriver
- 5 L closed container
- 1 and 2 L graduated cylinders
- Thermometer

Reagents

- Distilled water
- Ethanol 70%

Procedure:

Preparation of the refrigerant mixture: Ethanol 60%: Water 40%

1. Measure 3 L of 70% ethanol with the 1 and 2 L graduated cylinders.
2. Transfer the 3 L of ethanol to the 5 L container.
3. Measure 430 mL of 70% ethanol in the 1 L graduated cylinder. Add distilled water in this same cylinder until the liter is completed.
4. Transfer the contents of the 1 L cylinder to the 5 L container.
5. Cover the container and shake to homogenize the mixture. With this, you have 4 L of an Ethanol: Water 60%: 40% solution.

Turning on the equipment and selecting the working temperature

6. Verify that the circulator is in a cold environment (approx. 4 °C). This is done so that the cooling process of the recirculating mixture takes as little time as possible.
7. Connect the hose to both outlet pipes at the rear of the equipment. Fasten the hose on both sides using the clamps and a screwdriver. This is done to avoid dripping of the recirculating mixture.
8. Select “PUMP SPEED LOW”.
9. Uncover the equipment and add the Ethanol: Water 60%: 40% mixture. Replace the lid.
10. Connect the equipment to a 110 V source (“IN THE OPPOSITE CASE, USE A TRANSFORMER”).
11. On the front digital display of the equipment, press the “ON” button.
12. To select a working temperature, press the “SET / MENU” button and choose the desired temperature using the up and down arrows to the left of the “SET / MENU” button. The temperature value is inserted digit by digit. When you have already edited a digit and want to change the next one, press “SET / MENU”. When you finish editing all the digits, press the “SET / MENU” button one last time.
13. Wait for the fluid temperature (“FLUID”) to reach the chosen temperature (“SET”).
14. Uncover the equipment and measure the temperature of the circulating mixture when the temperature of the fluid has reached the desired temperature. This is done to check that the temperature indicated by the equipment is correct.

Equipment shutdown and refrigerant mixture storage

15. When you have finished using the equipment, press the “OFF” button. The equipment will shut down, and the liquid will stop recirculating.
16. Disconnect the equipment from the 110 V source.
17. Using a screwdriver, loosen the clamp located on the right (looking at the rear of the equipment from the front). Carefully remove the hose and bring it closer to the 5 L container where the refrigerant mixture is stored. Tilt the equipment to extract as much liquid as possible. Use a syringe or clean small containers to remove the liquid remaining in the equipment. Cover the 5 L container and store it in a cold room at 4 °C.
18. Reconnect the hose and tighten it with the clamp and the screwdriver.

2.3 Freezing experiments in the presence of a nucleating agent and chemical antifreeze agents

Introduction:

The freezing of liquid water is promoted in the presence of nucleating agents by the phenomenon of heterogeneous nucleation (Protocol "Heterogeneous nucleation experiments"). To show how antifreeze agents compete with nucleating agents, this protocol details the steps to evaluate the antifreeze activity of NaCl and glycerol in the presence of a nucleating agent such as a plating bead.

Materials :

Equipments

- **PolyScience Circulator + accessories**
- **Photographic camera**

Materials

- 10 Test tubes
- Spatula
- 10 mL graduated pipette
- Pipet bulb
- 1000 µL micropipette + tips
- Rack
- 10 Plating beads

Reagents

- NaCl 3 Osmol/L stock solution
- Glycerol 3 Osmol/L stock solution
- Distilled water
- Ethanol:Water 60%: 40% mixture

Procedure:

Turning on the equipment

1. NOTE: Always use biosecurity implements such as a mask and gloves.
2. Turn on the equipment and select a temperature of -5 °C, having previously added the mixture of Ethanol: Water 60%: 40% (see Protocol "Manipulation of the recirculating bath"). This should be done first, as it may take time for the equipment to reach the temperature of interest.

Materials' cleaning

3. Wash all test tubes with bleach, water, and finally distilled water. This must be done to eliminate interference such as nucleators or antifreeze agents.

Preparation of dilute solutions

4. Label the 10 test tubes appropriately.
5. 5. From the NaCl 3 Osmol/L stock, prepare by dilution in 2 test tubes two solutions of concentration 0.3 Osmol/L. Similarly, from the 3 Osmol/L glycerol stock, prepare by dilution in 2 test tubes two solutions of concentration 0.3 Osmol / L. To do this, take the respective aliquots, transfer them to the tubes, and complete with the necessary volume of distilled water to reach 5 mL of solution (see Table 1 in next page).
6. Add one plating bead to each tube, which will act as a nucleating agent.

Preparation of dilute solutions

7. Immerse the rack with the 10 test tubes in the recirculation bath previously set at -5 ° C.
8. After 30 min, remove test tubes 1, 3, 5, 7, and 9 (see Table 1), shake each one, and note if there is a change in the physical appearance of the tube contents. Take photographs and videos and re-immerse the tubes in the recirculation bath for a further 30 min. Remove the remaining test tubes, record photos, and videos, and immerse them in the recirculation bath for a further 30 min.
9. After the new 30 min, remove all the test tubes, note how each one looks (frozen or unfrozen water), and take photos and videos.

Table 1. Preparation of solutions and distribution of test tubes

Tube	Solute	Stock Volume (uL)	Distilled water volume (uL)	Final volume (mL)	Solute concentration (Osmol/L)
1	NaCl	5000	0	5	3
2	NaCl	5000	0	5	3
3	NaCl	500	4500	5	0.3
4	NaCl	500	4500	5	0.3
5	Glycerol	5000	0	5	3
6	Glycerol	5000	0	5	3
7	Glycerol	500	4500	5	0.3
8	Glycerol	500	4500	5	0.3
9	-		5000	5	0
10	-		5000	5	0

2.4 Preparation of antifreeze agents aqueous solutions

Introduction:

Antifreeze agents are substances that lower the freezing point of a liquid. Before studying how antifreeze proteins (AFPs) prevent liquid water from freezing into ice, it is relevant to look at how other compounds serve the same goal and how their effectiveness depends on their concentration. Two examples of chemical antifreeze agents are sodium chloride, NaCl, and glycerol, $CH_2(OH)CH(OH)CH_2(OH)$.

Materials:

Equipments
- Electronic balance

Materials
- Spatula
- 2 glass bottles of 150 mL
- 100 µL micropipette + Tips
- 100 mL graduated cylinder

Reagents
- Sodium chloride
- Glycerol
- Distilled water

Procedure:

Material's cleaning
1. Wash all glassware with bleach, water, and distilled water.

Preparation of NaCl 3 Osmol/L stock solution
2. Weigh out 8.775 g of NaCl.
3. Add them to a 150 mL glass bottle.
4. Add to the bottle approx. 70 mL of distilled water.
5. Cap the bottle and shake until all the NaCl dissolves.
6. Transfer the solution to a 100 mL cylinder. Add distilled water to make up 100 mL.
7. Pour the mixture from the test tube into the previously used bottle and shake. With this, you have 100 mL of NaCl 3 Osmol/L.

Preparation of Glycerol 3 Osmol/L stock solution
8. Measure 22 mL of glycerol with a 100 mL graduated cylinder.
9. Using the 100 µL micropipette, add 120 µL of glycerol to the 100 mL cylinder.
10. Add distilled water to make up 100 mL.
11. Pour the mixture from the cylinder into a 150 mL bottle, cap the bottle and shake until a single phase is formed. With this, you have 100 mL of glycerol 3 Osmol/L.

2.5 Manipulation of the recirculating bath

Introduction:

A low-temperature medium (less than or equal to 0 °C) is required to study the transition from liquid water to ice. One of the equipment that allows us to work in these conditions is a circulator. It makes a liquid or mixture circulating constantly at a certain temperature. This protocol details the steps to properly operate a PolyScience brand circulator and perform experiments at temperatures less than or equal to 0 °C.

Materials:

Equipments
- o PolyScience Circulator

Materials
- o Hose (approx. 1 m long)
- o 2 Clamps
- o Screwdriver
- o 5 L closed container
- o 1 and 2 L graduated cylinders
- o Thermometer

Reagents
- **o Distilled water**
- **o Ethanol 70%**

Procedure:

Preparation of the refrigerant mixture: Ethanol 60%: Water 40%

1. Measure 3 L of 70% ethanol with the 1 and 2 L graduated cylinders.
2. Transfer the 3 L of ethanol to the 5 L container.
3. Measure 430 mL of 70% ethanol in the 1 L graduated cylinder. Add distilled water in this same cylinder until the liter is completed.
4. Transfer the contents of the 1 L cylinder to the 5 L container.
5. Cover the container and shake to homogenize the mixture. With this, you have 4 L of an Ethanol: Water 60%: 40% solution.

Turning on the equipment and selecting the working temperature

6. Verify that the circulator is in a cold environment (approx. 4 °C). This is done so that the cooling process of the recirculating mixture takes as little time as possible.
7. Connect the hose to both outlet pipes at the rear of the equipment. Fasten the hose on both sides using the clamps and a screwdriver. This is done to avoid dripping of the recirculating mixture.
8. Select “PUMP SPEED LOW”.
9. Uncover the equipment and add the Ethanol: Water 60%: 40% mixture. Replace the lid.
10. Connect the equipment to a 110 V source (“IN THE OPPOSITE CASE, USE A TRANSFORMER”).
11. On the front digital display of the equipment, press the “ON” button.
12. To select a working temperature, press the “SET / MENU” button and choose the desired temperature using the up and down arrows to the left of the “SET / MENU” button. The temperature value is inserted digit by digit. When you have already edited a digit and want to change the next one, press “SET / MENU”. When you finish editing all the digits, press the “SET / MENU” button one last time.
13. Wait for the fluid temperature (“FLUID”) to reach the chosen temperature (“SET”).
14. Uncover the equipment and measure the temperature of the circulating mixture when the temperature of the fluid has reached the desired temperature. This is done to check that the temperature indicated by the equipment is correct.

Equipment shutdown and refrigerant mixture storage

15. When you have finished using the equipment, press the “OFF” button. The equipment will shut down, and the liquid will stop recirculating.
16. Disconnect the equipment from the 110 V source.
17. Using a screwdriver, loosen the clamp located on the right (looking at the rear of the equipment from the front). Carefully remove the hose and bring it closer to the 5 L container where the refrigerant mixture is stored. Tilt the equipment to extract as much liquid as possible. Use a syringe or clean small containers to remove the liquid remaining in the equipment. Cover the 5 L container and store it in a cold room at 4 °C.
18. Reconnect the hose and tighten it with the clamp and the screwdriver.

2.6 Freezing experiments with culture media

Introduction:

This protocol details the steps to evaluate the nucleating or antifreeze ability of the culture media used in microbiological experiments: a salty medium for P. bathycetes (PB) and Luria-Bertani medium (LB) with and without *E. coli*.

Materials:

Equipments

- PolyScience Circulator + accessories
- Photographic camera

Materials

- 6 Test tubes
- 10 mL graduated pipette
- Pipet bubl
- Rack

Reagents

- Salty medium P. bathycetes (PB)
- Medium Luria-Bertani (LB)
- *E. coli* in medium Luria Bertani (LB + *E. coli*)
- Ethanol:Water 60%: 40% mixture

Procedure:

Turning on the equipment

1. NOTE: Always use biosecurity implements such as a mask and gloves.
2. Turn on the equipment and select a temperature of -5 °C, having previously added the mixture of Ethanol: Water 60%: 40% (see Protocol "Manipulation of the recirculating bath"). This should be done first, as it may take time for the equipment to reach the temperature of interest

Materials' cleaning

3. Wash all test tubes with bleach, water, and finally distilled water. This must be done to eliminate interference such as nucleators or antifreeze agents.

Samples preparation

4. Label and prepare the six test tubes by taking 5 mL aliquots, in duplicate, of the three samples: the salted medium for P. bathycetes (PB), Luria-Bertani medium (LB), and *E. coli* in Luria Bertani medium (LB + *E. coli*) (see **Table 1**).

Tube	Sample	Volume (uL)
1	PB	5
2	PB	5
3	LB	5
4	LB	5
5	LB + *E. Coli*	5
6	LB + *E. Coli*	5

Freezing experiments

5. Immerse the rack with the 10 test tubes in the recirculation bath previously set at -5 ° C.
6. After 30 min, remove test tubes 1, 3, and 5 (see Table 1), shake each one, and note if there is a change in the physical appearance of the tube contents. Take photographs and videos and immerse the tubes in the recirculation bath for a further 20 min. Remove the remaining test tubes, take photos and videos, and re-immerse them in the recirculation bath for an additional 20 min.
7. After 20 min, remove all test tubes, note how each one looks (frozen or unfrozen sample), and take photos and videos.

2.7 Manipulation of the recirculating bath

Introduction:

A low-temperature medium (less than or equal to 0 °C) is required to study the transition from liquid water to ice. One of the equipment that allows us to work in these conditions is a circulator. It makes a liquid or mixture circulating constantly at a certain temperature. This protocol details the steps to properly operate a PolyScience brand circulator and perform experiments at temperatures less than or equal to 0 °C..

Materials:

Equipments
- o PolyScience Circulator

Materials
- o Hose (approx. 1 m long)
- o 2 Clamps
- o Screwdriver
- o 5 L closed container
- o 1 and 2 L graduated cylinders
- o Thermometer

Reagents
- o Distilled water
- o Ethanol 70%

Procedure:

Preparation of the refrigerant mixture: Ethanol 60%: Water 40%

1. Measure 3 L of 70% ethanol with the 1 and 2 L graduated cylinders.
2. Transfer the 3 L of ethanol to the 5 L container.
3. Measure 430 mL of 70% ethanol in the 1 L graduated cylinder. Add distilled water in this same cylinder until the liter is completed.
4. Transfer the contents of the 1 L cylinder to the 5 L container.
5. Cover the container and shake to homogenize the mixture. With this, you have 4 L of an Ethanol: Water 60%: 40% solution.

Turning on the equipment and selecting the working temperature

6. Verify that the circulator is in a cold environment (approx. 4 °C). This is done so that the cooling process of the recirculating mixture takes as little time as possible.
7. Connect the hose to both outlet pipes at the rear of the equipment. Fasten the hose on both sides using the clamps and a screwdriver. This is done to avoid dripping of the recirculating mixture.
8. Select “PUMP SPEED LOW”.
9. Uncover the equipment and add the Ethanol: Water 60%: 40% mixture. Replace the lid.
10. Connect the equipment to a 110 V source (“IN THE OPPOSITE CASE, USE A TRANSFORMER”).
11. On the front digital display of the equipment, press the “ON” button.
12. To select a working temperature, press the “SET / MENU” button and choose the desired temperature using the up and down arrows to the left of the “SET / MENU” button. The temperature value is inserted digit by digit. When you have already edited a digit and want to change the next one, press “SET / MENU”. When you finish editing all the digits, press the “SET / MENU” button one last time.
13. Wait for the fluid temperature (“FLUID”) to reach the chosen temperature (“SET”).
14. Uncover the equipment and measure the temperature of the circulating mixture when the temperature of the fluid has reached the desired temperature. This is done to check that the temperature indicated by the equipment is correct.

Equipment shutdown and refrigerant mixture storage

15. When you have finished using the equipment, press the “OFF” button. The equipment will shut down, and the liquid will stop recirculating.
16. Disconnect the equipment from the 110 V source.
17. Using a screwdriver, loosen the clamp located on the right (looking at the rear of the equipment from the front). Carefully remove the hose and bring it closer to the 5 L container where the refrigerant mixture is stored. Tilt the equipment to extract as much liquid as possible. Use a syringe or clean small containers to remove the liquid remaining in the equipment. Cover the 5 L container and store it in a cold room at 4 °C.
18. Reconnect the hose and tighten it with the clamp and the screwdriver.

Marine broth adapted Medium

Introduction:

The present protocol details the steps to be followed to prepare the adaptation of the Marine broth 2216 medium, produced by Difco®. For that, we first calculated the total ion concentrations for all the salts in the medium, obtaining at the end this table of components:

Components	Quantities (g) for a liter	Components in our lab	Quantities for 250 ml
Peptone	5.0 g	Peptone	1.25 g
Yeast Extract	1.0 g	Yeast Extract	0.25 g
Ferric Citrate	0.1 g	$FeCl_3 \cdot 6H_2O$ (stock)**	10.132 ml
NaCl	19.45 g	NaCl	4.485 g
$MgCl_2$	8.8 g	$MgCl_2$ 1M*	23.1 ml
Na_2SO_4	3.24 g	Na_2SO_4	0.81 g
CaCl2	1.8 g	$CaCL_2$ 1M*	4.06 ml
KCl	0.55 g	KCl	0.1375 g
$NaHCO_3$	0.16 g	$NaHCO_3$ (stock)	1 ml
KBr	0.08 g	KBr (stock)	0.5 ml
$SrCl_2$	34.0 mg	SrCl2 (stock)	0.5 ml
H3BO3	22.0 mg	H_3BO_3	0.5 ml
Na_2SiO_3	4.0 ,g	Na2SiO3	0.5 ml
NaF	2.4 mg	NaF	0.5 ml
NH_4NO_3	1.6 mg	NH_4NO_3	5 ml
Na_2HPO_4	8.0	Na_2HPO_4	0.5 ml
		Sodium citrate (stock)**	0.877 ml
Agar	15 g	Agar*	3.75 g

*: Some salts were already prepared as stocks before we did the experiment. **: Since we could not find Ferric Citrate, we used $FeCl_3 \cdot 6H_2O$ and sodium citrate instead. When the medium is being prepared, we will have the same concentrations of both ions as the original medium. Is important to denote that a lower amount of NaCl was added to the medium since we were including Na+ and Cl- ions from the former salts.

In order to add the salts exact quantities, for smaller concentrations, we prepared new stocks. Then, from these stocks we transferred the equivalent amount to the dis-

tilled water used to prepare the medium. The order in which the salts were added were regarding the possibility of reaction between both salts, for that we started transferring those with higher solubility until the ones with lower solubility. The possibility of precipitation was always checked after adding the salts. Then we autoclaved for approximately an hour at 121°C. In the case of preparing Marine agar, after autoclave, cool to 45-50°C, mix well and dispense in Petri dishes.

Peptone and yeast extract precipitation test:

Due to the precipitation observed in the recreated Marine Broth, we decided to test if the reason for it was the presence of an old peptone or the yeast extract. Therefore in four 50ml Falcon® tubes we setted 4 conditions:

- 1st tube: Old peptone dissolved in distilled water
- 2nd tube: New medium(containing new peptone and yeast extract)
- 3rd tube: New peptone dissolved in distilled water
- 4rth tube: Yeast extract dissolved in distilled water

The same amount of elements were added according to the components table for the Marine Broth. Then, everything was stirred very well.

Bacterium activation protocol in solid and liquid medium

This protocol was based on the manual called:
ATCC® Bacterial Culture Guide: tips and techniques for culturing bacteria and bacteriophages. (Pages 2 - 5).

Firstly we rehydrated the bacteria with 0.5ml of Marine Broth as the way it is detailed in the manual. For that it is important to resuspend the pellet slowly in the medium. Once the pellet was entirely homogenised, we transferred aliquots to 2 test tubes filled with 10ml of Marine Broth and to 2 Marine agar plates, spreading all over the plate in the latter case. After that, we incubate with shaking both tubes and plates at 25°C overnight and kept at 4°C.

Cryopreservation of *Pseudoalteromonas nigrifaciens* strains

Equipment: Centrifuge

Materials: *Pseudomonas bathycetes*(PB) medium, 1.5ml cryotubes, sterile Glycerol 20%

Procedure:

1. Sow a colony of *P. nigrifaciens* in 10ml of PB broth.
2. Incubate at 25 ° C with shaking overnight
3. Centrifuge at 5000 rpm for 20 min at 4 ° C
4. Discard the supernatant and for a final volume of 1ml, add 200µL of 20% glycerol and 800µL of marine broth.
5. Homogenize and transfer to sterile microcentrifuge tubes or cryotubes. Store at -80 ° C.

Streaking method

Equipment: Incubator

Materials: loop handle, Petri dish with PB agar medium(see the **PB medium preparation)**, Bunsen lighter, stored active bacteria(in this case: *P. nigrifaciens*).

Procedure:

1. Defrost completely the bacteria stored in the cryotubes in ice. Once defrosted, invert the tube twice to homogenise the bacteria in the medium.
2. Sterilise the loop handle with the lit Bunsen lighter until the wire is red. Let it cool for 1 min, near the lit Bunsen lighter.
3. Always keeping the closed plate, the loop handle and the cryotubes near the lighter to avoid contamination, take the loop handle and open the lid of the tube containing the bacterium to be cultured.
4. Immerse the handle and put the lid

again. Then, open the plate lid with one hand and culture the bacteria by streaking method over the agar, without making pressure.
5. Close the plate lid and sterilise the handle again as expressed in the first step.
6. Incubate the plate overnight at 25°C and later keep at 4°C.

Pseudomonas bathycetes (PB) adapted Medium

The present protocol details the steps to be followed to prepare the *Pseudomonas bathycetes* (PB) medium, used for the growth of *P.nigrifaciens*. The preparation was based on the book called: "Handbook of Media for Environmental Microbiology". It contains 1% (wt/vol) proteose peptone, 0.3% (wt/vol) yeast extract, 2.4% (wt/vol) NaCl, 0.07% (wt/vol) KCl, 0.53% (wt/vol) MgCl2 and 0.7% (wt/vol) MgSO4 · 7H2O. When prepared, we adapted the elements to the composition of the LB medium and added the missing salts.

For 1L of PB adapted Medium:

Composition	Quantity (g/L)
LB Medium (10g NaCl, 10g Peptone and 5g Yeast extract)	20
NaCl	19
KCl	19
$MgSO_4 * (H_2O)$	0.7
$MgCl_2 * (H_2O)$	11.3
Agar (Optional)	15

For the preparation of this medium, all the components were added to distilled or deionised water and adjusted the volume to 1.0 L. We mixed thoroughly and distributed in different flasks. Then we autoclaved for approximately an hour at 121°C. In the case of adding bacteriologic agar, after autoclave, cool to 45-50°C, mix well and dispense in Petri dishes. After culturing the bacteria, plate incubation was made at 25°C overnight. The plates then were kept at 4°C.

References: Atlas R M.(2005) Handbook of Media for Environmental Microbiology. 2nd ed. CRC Press. p 656.

Medium selectivity

To ensure PB medium selectivity, we cultured different types of bacteria: *E. coli*, *Salmonella spp.* and *Pseudomonas spp*, besides *P. nigrifaciens*.

Equipment: Incubator

Materials: loop handle, Petri dish with PB agar medium(see the **PB medium preparation**), Bunsen lighter, stored active bacteria(in this case: *P. nigrifaciens, E. coli, Salmonella spp., and Pseudomonas spp.*).

Procedure:
1. Defrost completely the bacteria stored in the cryotubes in ice. Once defrosted, invert the tube to homogenise the bacteria in the medium.
2. Sterilise the loop handle with the lit Bunsen lighter until the wire is red. Let it cool for 1 min, near the lit Bunsen lighter.
3. Always keeping the material near to the lighter to avoid contamination, take the loop handle and open the lid of the tube containing the bacterium to be cultured.
4. Immerse the handle and put the lid again. Then, open the plate lid with one hand and draw a line over the agar medium, without making pressure.
5. Close the plate lid and sterilise the handle again as explained in the first step.
6. Repeat this process as needed.
7. Incubate the plate overnight at 25°C and later keep at 4°C.

P. nigrifaciens antibiotic sensitivity and growth curve characterization

To compare the antibiotic sensitivity of *P. nigrifaciens*, we used different antibio-

tics such as ampicillin, chloramphenicol, gentamicin, kanamycin, tetracycline and spectinomycin. All the growth curves were compared to the one obtained without any inhibitor.

Equipment: Incubator

Materials: loop handle, Petri dish with PB agar medium(see the **PB medium preparation)**, Bunsen lighter, stored active bacteria(in this case: *P. nigrifaciens*).

Procedure:

1. A day before 5ml of liquid medium were inoculated with an isolated UFC of P. nigrifaciens previously cultured in a plate. Then the tube containing the inoculum was incubated overnight at 25°C.
2. The following day, defrost completely the antibiotics stored in ice. Once defrosted, invert the tube twice to homogenise the antibiotic concentration in the medium.
3. Always keeping the material near the lighter to avoid contamination, add 800ul of inoculum for the antibiotic treatment and 800ul of PB medium for the control, in the plate titer. Each treatment was done in quadruplicate. The conditions were distributed in the plate as the following table:

Blank PB medium only	**Control** Inoculum only **(Characterise the growth curve)**	**Amp** 800µl inoculum + 50µg/ml Amp	**Chlo** 800µl inoculum + 34µg/ml Chlo	**Gen** 800µl inoculum + 50µg/ml Gen	**Kan** 800µl inoculum + 10µg/ml Kan	**Tet** 800µl inoculum + 5µg/ml Tet	**Spe** 800µl inoculum + 100µg/ml Spe
Blank	Control	Amp	Chlo	Gen	Kan	Tet	Spe
Blank	Control	Amp	Chlo	Gen	Kan	Tet	Spe
Blank	Control	Amp	Chlo	Gen	Kan	Tet	Spe

4. The volume of the antibiotics was added based on their initial concentration and the final volume.
5. Incubate the plate in a spectrophotometer at 600nm overnight or 10h approximately, at room temperature.

Morphological characterization: Gram staining method

The morphological characterization was made by the regular procedure for Gram staining. Using a sterile loop, we took a small portion of the liquid bacterial culture and made the smear on a clean slide. Then, we fixed the smear with gentle heat, using a bunsen burner, and covered the slide with an aqueous crystal violet solution for 1 minute. We washed the preparation under running water and covered it with lugol solution for 1 minute, as well. Subsequently, we washed the slide, decolorized the preparation with 96% ethanol, and washed it again immediately. We covered the smear with safranin solution for 30 seconds, washed the preparation, and dried it using the bunsen burner. Finally, we observed the slide under the microscope from lowest to highest magnification, using immersion oil when appropriate.

CRIOPROT

https://2020.igem.org/Team:UPCH_Peru

@igem.upch.peru

@igem.upch.peru

@igem.upch.peru