CRYOPRESERVATION OF MICROALGAE GUIDE

Cryopreservation stabilizes genomic integrity, preserves culture quality, minimizes maintenance costs and reduces the risk of catastrophic loss. This guide provides a foundation for the long-term cryopreservation of microalgal strains using various cryoprotective agents and stored in the vapor-phase of liquid nitrogen (LN_2).

ITEMS REQUIRED FOR CRYOPRESERVATION

- Liquid nitrogen (LN_2) storage dewar equipped with racks for maintaining cryo storage boxes.
- A Nalgene 1°C · min^{-1} freezing container with isopropanol as specified by the manufacturer.
- A -80°C freezer (no cryopreserved samples are stored permanently in a -80 °C freezer)
- Square cryo storage boxes that hold a quantity of 81, 2-mL cryovials.
- Sterile 2-mL cryovials (specific type not critical)
- Sterile work area if cultures are to be maintained axenically
- Clinical centrifuge with a rotor adapted to hold 2-mL cryovials
- A permeating or nonpermeating cryoprotective agent (CPA) that is known to result in good viability for the specific algal strain (if unknown, it may be desirable to test several different ones simultaneously).

  ! *Please note for this protocol, methanol (MeOH) will be used as the example CPA but may not work for all strains.*

PROCEDURE FOR STRAINS THAT GROW PREFERENTIALLY ON AGAR SLANTS

Cryopreserving agar strain

1. A nutrient agar slant of a composition known to support growth of the alga of interest is prepared inside of a 2-mL cryovial. The vial should contain approximately 1.0 mL of nutrient agar. After the slant solidifies, it is inoculated with the alga of interest and then placed under normal growth conditions. The culture is ready for cryopreservation when a good lawn of algae forms on the agar surface. It should be cryopreserved before the lawn begins to decline.
2. Prepare the following materials in advance:
   - Cryoprotective solution: CPA diluted in culture medium, e.g., methanol (MeOH) diluted to 10 % *(v/v)*

- Cold Nalgene 1°C · min^{-1} freezing container placed into a 4 °C refrigerator at least a day before use
- Place the square cryo storage box placed into a cryo rack and stored in a liquid nitrogen dewar for at least several hours before use

3. To minimize disturbing the lawn, gently add 0.9 mL of the 10% methanolic culture medium at room temperature to the cryovial.
4. Add an additional 0.9 mL of regular culture medium so the total volume of material in the cryovial is 1.8 mL with a 5% MeOH *(v/v)* final concentration. Most of the algal lawn should remain on the agar surface.

   ! ***CAUTION:*** *Algal cultures should be kept in subdued light any time they are mixed with the cryoprotective solution.*
5. Remove the pre-chilled freezing container from the refrigerator and place the cryovial into one of the vial holder locations. Replace the lid back onto the container and place into a -80°C freezer.
6. After at least 1.5 hours in the -80 °C freezer, but not as long as overnight, remove the freezing container from the freezer.
7. The cryo storage box is immediately removed from the rack in the liquid nitrogen dewar and the cryovial is quickly transferred from the container to the cryo storage box to avoid excessive warming.
8. Return the cryo storage box back to the rack and place it into the LN_2 storage dewar (vapor phase) for short-term or long-term storage.

Thawing a cryopreserved agar strain

CAUTION: The next steps should be performed under subdued lighting until the CPA has been removed and the vials placed under normal growth conditions.

1. Prewarm 400-mL of water in a beaker to approximately 37°C.
2. Retrieve the cryo storage rack from the dewar and quickly remove the cryovial and insert it into a cryovial float in the 37°C water bath.
3. Gently agitate the cryovial while thawing and leave in the water bath until all ice has just melted (generally under 2 minutes).
4. If a significant amount of algae has remained adhered to the agar, then it may be possible to remove the cryoprotective solution from above the agar with a disposable pipette without disturbing the algae on the surface of the slant.

! ***NOTE:*** *If the algae do not remain adhered to the agar surface when first thawed, it may be necessary to subject the cryovial to centrifugation before decanting the liquid in each wash. The room-temperature centrifugation should be performed as gently as possible to avoid damaging the fragile cells.*

5. When the liquid has been removed, slowly add fresh culture medium to fill the cryovial.
6. Leave the cryovial undisturbed for several minutes, then remove the liquid gently with a disposable pipette and fill once again with fresh culture medium.
7. After letting the cryovial sit undisturbed for several minutes, remove the liquid and place the cryovial under normal growth conditions. A successfully cryopreserved culture will produce a fresh lawn on the agar surface within a few weeks and may then be transferred to a fresh slant when desired.

PROCEDURE FOR STRAINS THAT GROW PREFERENTIALLY IN LIQUID MEDIUM

Cryopreserving liquid strain

1. A liquid culture should be grown in a medium that supports active growth. The culture should be cryopreserved while it remains in exponential growth. Actively growing cultures generally survive cryopreservation with higher viability than those in stationary or declining phase, or those growing under stressful conditions.
2. Prepare the following materials in advance:
   - Cryoprotective solution: CPA diluted in culture medium, e.g., methanol (MeOH) diluted to 10 % *(v/v)*
   - Cold Nalgene 1°C · min^{-1} freezing container placed into a 4 °C refrigerator at least a day before use
   - Place the square cryo storage box placed into a cryo rack and stored in a liquid nitrogen dewar for at least several hours before use
3. Add 0.9 mL of the liquid culture to the cryovial.
4. Add 0.9 mL of the cryoprotective solution and quickly, but gently, mix the cryo vial so the total volume of material is 1.8 mL with a 5% MeOH *(v/v)* final concentration.

   ! ***CAUTION:*** *Algal cultures should be kept in subdued light any time they are mixed with the cryoprotective solution.*
5. Remove the pre-chilled freezing container from the refrigerator and place the cryovial into one of the vial holder locations. Replace the lid back onto the container and place into a -80°C freezer.

6. After at least 1.5 hours in the -80 °C freezer, but not as long as overnight, remove the freezing container from the freezer.
7. The cryo storage box is immediately removed from the rack in the liquid nitrogen dewar and the cryovial is quickly transferred from the container to the cryo storage box to avoid excessive warming.
8. Return the cryo storage box back to the rack and place it into the LN_2 storage dewar (vapor phase) for short-term or long-term storage.

Thawing a cryopreserved liquid strain

CAUTION: The next steps should be performed under subdued lighting until the CPA has been removed and the vials placed under normal growth conditions.

1. Prewarm 400-mL of water in a beaker to approximately 37°C.
2. Retrieve the cryo storage rack from the dewar and quickly remove the cryovial and insert it into a cryovial float in the 37°C water bath.
3. Gently agitate the cryovial while thawing and leave in the water bath until all ice has just melted (generally under 2 minutes).
4. Centrifuge the cryovial at room-temperature as gently as possible to pellet the algae.
5. Gently decant the liquid from the vial and fill with fresh culture medium.
6. Leave it undisturbed for several minutes, then gently centrifuge to pellet and decant the liquid.
7. Add fresh culture medium to suspend the algae then transfer to a larger volume of fresh culture medium and grow under normal culturing conditions.

IMPORTANT:

The storage dewar must never run out of liquid nitrogen, even briefly, and the cryo storage box must only be removed from the dewar for brief periods of time (preferably less than 3 minutes) to prevent the contents of the cryovials to rise above a temperature of -130°C.