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 liquid nitrogen (LN_2).

PRINCIPLE ITEMS REQUIRED FOR CRYOPRESERVATION

- A liquid nitrogen storage dewar equipped with racks for maintaining cryo storage boxes
- A Nalgene 1 °C Freezing Container (often affectionately called "Mr. Frosty" and herein called a freezing canister, sold by many general laboratory suppliers)
- A -80 °C freezer (conveniently located; no cryopreserved samples are stored permanently in a -80 °C freezer)
- Square cryo storage boxes that hold a quantity of 81, 2-mL cryovials
- 2-mL cryovials (specific type not critical, providing storage is in the liquid nitrogen vapor phase)
- Sterile work area (if cultures are to be maintained axenically)
- A clinical centrifuge with a rotor adapted to hold 2-mL cryovials. We have designed custom Plexiglass sleeves that fit into a clinical centrifuge for that purpose.

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:
    - Culture medium diluted in methanol (MeOH) to 5 % (v/v)
    - Cold Nalgene 1 °C freezing container (canister) that contains isopropanol as specified by the manufacturer, placed into a 4 °C refrigerator at least a day before it is used for cryopreservation
    - An 81-position square cryo storage box designed to hold 2-mL cryovials is placed into a rack and stored in a liquid nitrogen dewar for at least several hours before it is used to store cryovials
3. The 5 % methanolic culture medium at room temperature is added gently to the agar slant in the cryovial until the total volume of material in the vial reaches 1.5 - 1.8 mL. (**CAUTION:** Algal cultures should be kept in subdued light any time they are directly exposed to a methanolic solution). Most of the algal lawn should remain on the agar surface after the solution has been added to the vial.
4. The pre-chilled freezing canister is removed from the refrigerator, the cryovial is placed into one of the vial holder locations in the canister, and the lid is placed back onto the canister. The canister is then placed into a -80 °C freezer.

5. After at least 1.5 hours, but not as long as overnight, in the -80 °C freezer, the freezing canister is removed. The cryo storage box is immediately removed from the rack in the liquid nitrogen dewar and the cryovial is transferred from the canister to the box. The cryo storage box is then placed back into the rack, which is then placed into the LN_2 storage dewar for short-term or long-term storage.

IMPORTANT: The storage dewar must never run out of liquid nitrogen, even briefly, and the storage box must only be removed from the dewar for brief periods of time (preferably less than 3 minutes) so that the contents of the cryovials do not rise above a temperature of approximately -130 °C.

Thawing Agar Strain

CAUTION: The next steps should be performed under subdued lighting until the cryoprotective agent has been removed and the vial is placed under normal growth conditions.

1. For recovery of living algae from the LN_2 storage dewar a 400 mL volume of water is pre-warmed to approximately 37 °C.
2. The storage rack is retrieved from the LN_2 storage dewar and the cryovial is removed from the rack and quickly inserted into a cryovial float in the 37 °C water bath.
3. The cryovial is gently agitated during thawing and left in the water bath until all ice has just melted (generally under 2 minutes).
4. If a significant amount of algae has remained adhering to the agar, then it may be possible to remove the methanolic solution from above the agar with a disposable pipette without disturbing the algae on the surface of the slant. When the liquid has been removed, very slowly add fresh culture medium to fill the vial.
5. Leave the vial undisturbed for several minutes, then remove the liquid gently with a disposable pipette and once again add fresh culture medium.
6. After the solution sits undisturbed for several minutes, gently remove the liquid. Place the cryovial under normal growth conditions. A successfully cryopreserved culture will produce a fresh lawn on the culture surface within a few weeks and may be transferred to a fresh slant when desired.

NOTE: If the algae do not remain adhering to the agar surface when the solution is first thawed, then it may be necessary to subject the cryovial to centrifugation before decanting the liquid in each wash. The room-temperature centrifugation should be as gentle as possible to avoid damaging the fragile algal cells.

PROCEDURE FOR STRAINS THAT GROW PREFERENTIALLY IN LIQUID MEDIUM

Cryopreserving Liquid Strain

1. A liquid culture of the alga of interest is grown in medium that supports active growth. The culture should be cryopreserved while it remains in exponential growth.
2. Prepare the following materials in advance:
   - Culture medium diluted in methanol (MeOH) to 5 % (*v/v*)
   - Cold Nalgene 1 °C freezing container (canister) that contains isopropanol as specified by the manufacturer, placed into a 4 °C refrigerator at least a day before it is used for cryopreservation
   - An 81-position square cryo storage box designed to hold 2-mL cryovials is placed into a rack and stored in a liquid nitrogen dewar for at least several hours before it is used to store cryovials

3. Add 0.9 mL of algae in liquid culture medium into a 2-mL cryovial. Then 0.9 mL of the 10 % MeOH solution is added to the vial and the contents quickly, but gently, mixed. (**CAUTION:** Algal cultures should be kept in subdued light any time they are directly exposed to a methanolic solution).
4. The pre-chilled freezing canister is removed from the refrigerator, the cryovial is placed into one of the vial holder locations in the canister, and the lid is placed back onto the canister. The canister is then placed into a -80 °C freezer.
5. After at least 1.5 hours, but not as long as overnight, in the -80 °C freezer, the freezing canister is removed. The cryo storage box is immediately removed from the rack in the liquid nitrogen dewar and the cryovial is transferred from the canister to the box. The cryo storage box is then placed back into the rack, which is placed into the LN_2 storage dewar for short-term or long-term storage.

IMPORTANT: The storage dewar must never run out of liquid nitrogen, even briefly, and the storage box must only be removed from the dewar for brief periods of time (preferably less than 3 minutes) so that the contents of the cryovials do not rise above a temperature of approximately -130 °C.

Thawing Liquid Strain

CAUTION: The next steps should be performed under subdued lighting until the cryoprotective agent has been removed and the vial is placed under normal growth conditions.

1. For recovery of living algae from the LN_2 storage dewar a 400 mL volume of water is pre-warmed to approximately 37 °C.
2. The storage rack is retrieved from the LN_2 storage dewar and the cryovial is removed from the rack and quickly inserted into a cryovial float in the 37 °C water bath.
3. The cryovial is gently agitated during thawing and left in the water bath until all ice has just melted (generally under 2 minutes).
4. The cryovial is immediately subjected to centrifugation (as gentle as possible) to pellet the algae, and the methanolic liquid is gently decanted.
5. The vial is then filled with fresh culture medium and left undisturbed for several minutes. It is then again subjected to gentle centrifugation, and the liquid is removed as before.
6. Fresh culture medium is placed into the cryovial to suspend the algae and the culture is transferred to a larger volume of fresh culture medium and grown under normal culturing conditions.