

## CRYOPRESERVATION OF CHLORELLA

### Cryopreservation procedure for strains that grow preferentially on agar slants:

1. A Proteose agar slant (1.5% agar) 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. Culture medium diluted with methanol to a final concentration of 5.0% (v/v) MeOH is prepared.

A Nalgene 1deg C Freezing Container (canister) that contains isopropanol as specified by the manufacturer is placed into a 4 deg C refrigerator at least a day before it is used for cryopreservation.

An 81-position square storage box designed to hold 2-ml cryovials (also known as a “Mr. Frosty”) 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 5.0% 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 between 1.5 to 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 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 deg C freezer.

5. After at least 1.5 hours in the –80 deg C freezer, the freezing canister is removed. DO NOT allow the culture to go as long as overnight at –80 deg C. The 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 box is then placed back into the rack, which is placed into the liquid nitrogen storage dewar for short-term or long-term storage.

6. 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 cryovials do not rise above approximately –130 deg C.

7. For recovery of living algae from the dewar a 400-ml volume of water is pre-warmed to approximately 37 deg C. The storage rack is removed from the liquid nitrogen dewar and the cryovial is removed from the rack and quickly inserted into the 37 deg C water bath.

8. The cryovial is gently agitated during thawing and left in the water bath until all ice has just melted (generally under 2 minutes). If a significant amount of algae has remained adhering to the agar, then it may be possible to remove the solution from above the agar with a disposable pipette without disturbing the algae on the slant. When the liquid has been removed, very slowly add fresh culture medium to fill the vial. Leave the vial undisturbed for several minutes, then remove it gently with a disposable pipette and add fresh culture medium. After the solution sits undisturbed for several minutes, gently remove the solution. 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.
9. 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.
10. No cryopreserved samples are stored permanently in a  $-80$  deg C freezer.

Alternatively Chlorella could be grown on a media that was not as nutrient rich so that the culture's rate of growth would be slower and the cells would be more capable of sustaining the shock of cryopreservation.

### Proteose Medium

Equivalent to Bristol Medium (Bold 1949) with 1.0 g per Liter of Bacto-Peptone. pH approximately 6.8

#### Directions

For 1 L Total

1. To approximately 900 mL of dH<sub>2</sub>O add each of the components in the order specified while stirring continuously. Measure the pH. It should be approximately 6.8.
2. Add 1.0 g Bacto-Peptone.
3. Bring total volume to 1.0 L with dH<sub>2</sub>O.
- \*For 1.5% agar medium, add 15.0 g of agar into the flask; do not mix.
3. Cover and autoclave medium.
4. Store at refrigerator temperature.

1	NaNO <sub>3</sub>	10 mL/L	2.50 g/100 mL dH <sub>2</sub> O	2.94 mM
2	CaCl <sub>2</sub> ·2 H <sub>2</sub> O	10 mL/L	0.25 g/100 mL dH <sub>2</sub> O	0.17 mM
3	MgSO <sub>4</sub> ·7 H <sub>2</sub> O	10 mL/L	0.75 g/100 mL dH <sub>2</sub> O	0.30 mM
4	K <sub>2</sub> HPO <sub>4</sub>	10 mL/L	0.75 g/100 mL dH <sub>2</sub> O	0.43 mM
5	KH <sub>2</sub> PO <sub>4</sub>	10 mL/L	1.75 g/100 mL dH <sub>2</sub> O	1.29 mM
6	NaCl	10 mL/L	0.25 g/100 mL dH <sub>2</sub> O	0.43 mM

### **Cryopreservation procedure for strains that grows preferentially in liquid medium:**

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. Culture medium is diluted in methanol to a final concentration of 20% (v/v) MeOH.

A Nalgene 1deg C Freezing Container (canister) that contains isopropanol as specified by the manufacturer is placed into a 4 deg C refrigerator at least a day before it is used for cryopreservation.

An 81-position square storage box designed to hold 2-ml cryovials (also known as a “Mr. Frosty”) 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. 1.5 ml of algae in liquid culture medium is placed into a 2-ml cryovial. Then 0.5 ml of the 20% 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 deg C freezer.
5. After at least 1.5 hours, but not as long as overnight, in the -80 deg C freezer, the freezing canister is removed. The 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 box is then placed back into the rack, which is placed into the liquid nitrogen storage dewar for short-term or long-term storage.
6. 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 cryovials do not rise above approximately -130 deg C.
7. For recovery of living algae from the dewar a 400-ml volume of water is pre-warmed to approximately 37 deg C. The storage rack is removed from the liquid nitrogen dewar and the cryovial is removed from the rack and quickly inserted into the 37 deg C water bath. The cryovial is gently agitated during thawing and left in the water bath until all ice has just melted (generally under 2 minutes).
8. The cryovial is immediately subjected to centrifugation (as gentle as possible) to pellet the algae, and the liquid is gently decanted. The vial is then filled with fresh culture medium and left undisturbed for several minutes. It is then again subjected to gently centrifugation, and the liquid is removed as before. Fresh culture medium is placed into the cryovial to suspend the algae and the culture is transferred to a larger volume of medium under normal culturing conditions.

9. No cryopreserved samples are stored permanently in a  $-80$  deg C freezer.

Alternatively *Chlorella* could be grown on a media that was not as nutrient rich so that the culture's rate of growth would be slower and the cells would be more capable of sustaining the shock of cryopreservation.

Here methanol is being used both as a cryopreservant to increase membrane fluidity and as a dehydrating agent

### Proteose Medium

Equivalent to Bristol Medium (Bold 1949) with 1.0 g per Liter of Bacto-Peptone. pH approximately 6.8

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2. Add 1.0 g Bacto-Peptone.
3. Bring total volume to 1.0 L with dH<sub>2</sub>O.

\*For 1.5% agar medium, add 15.0 g of agar into the flask; do not mix.

3. Cover and autoclave medium.
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