Post-thaw viability assessment of cryopreserved microalgae & cyanobacteria

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The traditional approach to cryopreservation involves exposure of cells to sub-zero temperatures using gradual (slow), and controlled rates of temperature reduction in the presence of a cell penetrating CryoProtective Agent (CPA). Through this process, the effects of thawing and reinitiating normal metabolic activity can all result in damage to the algal cells, which may be lethal, or repairable. Whilst the regeneration of a healthy culture within an appropriate time-frame is adequate for comparative purposes, it is important to obtain absolute levels of post-cryopreservation viability for the optimisation of preservation protocols. The most common methods employ either use of vital staining as a proxy for viability or, more robustly, methods that depend on the division and regrowth of individual cells that have been subjected to the cryopreservation protocol.

Vital staining

A variety of stains may be employed and for algae most frequently used for algae are Fluorescein diacetate (FDA) and Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE).

Fluorescein diacetate is a fluorochrome capable of producing fluorescent products after enzymatic modification. Viable cells, with an intact membrane and enzymatic activity, will fluoresce green, while non-viable algal cells are colourless or in the case of algae fluoresce red due to auto-fluorescence of chlorophyll.

Carboxyfluorescein diacetate succinimidyl ester is a non-fluorescent, membrane permeable molecule. When inside a viable cell, due to enzymatic modification, CFDA-SE becomes highly fluorescent. In addition, it loses permeability to the cell membrane and it can remain detectable inside the cell for several days.

1. Control viability levels should be assessed on material prior to initiating the cryopreservation process following steps 6 - 9 below.

2. Stored vials should be transferred from the cryostore/refrigerator to a small dewar containing liquid nitrogen using long forceps and transported to the laboratory for thawing.

   If vials are stored in liquid-phase nitrogen (LN_2) there is a possibility that nitrogen may leak into the vial. Full safety equipment and most importantly eye protection must be worn as there is a small risk of explosion of the vial during warming (although it has been noted at the CCAP collection at SAMS that even when >10% of some batches of samples contain liquid nitrogen, an explosion has never occurred). However, if leakage has occurred then inevitably axenic strains will have been contaminated.

Additional Notes

This method has been successfully employed for a wide variety of algae including: cyanobacteria, unicellular greens, Prasinophytes and diatoms.

Apparatus: Incubators with controlled light/dark cycle; a class I biological safety cabinet; a small (1-l) dewar; a heated water-bath; storage cryostat/ refrigerator; long forceps, microscope with fluorescence and phase-contrast optics; safety equipment.

Culture medium: medium appropriate for the taxon and its osmotic origin.

Plasticware/glassware: test-tubes; glass universal vials (20-ml); Schott bottles for agar; disposable pipettes; 2-ml cryogenic tubes; Petri dishes (50-mm).

All chemicals should be Analytical grade.

Additional information:

***Day JG** (2014) Cryopreservation of cyanobacteria. In: Cyanobacteria: An economic perspective. Naveen K Sharma, Ashwani K Rai & Lucas J Stal (eds) WILEY Publications. pp 319-328.*

***Day JG** (2007) Cryopreservation of Microalgae and Cyanobacteria. In: Day JG & Stacey GN (eds) Cryopreservation and Freeze-drying Protocols. Humana Press. pp 139-149.*

***Day JG and Brand JJ** (2005) Cryopreservation Methods for Maintaining Cultures. In: Algal Culturing Techniques. Andersen RA (ed) Academic Press, New York. pp 165-187.*

3. To recover cultures, the cryovials are thawed by placing in a pre-heated water-bath (40^{o}C) and agitating until the last ice crystal has just melted.

   For most marine taxa it is important not to prolong their incubation at 40^{o}C. Alternative, slower warming e.g. in a 25^{o}C water-bath may be beneficial for some strains, but in general rapid warming is optimal as it avoids/ minimises ice crystal regrowth.

4. On thawing rapidly transfer to a clean laminar flow/biological safety cabinet and wipe the outside of the vial with 70% (v/v) ethanol.

   Note: there may be high levels of viable bacterial and fungal spores in liquid nitrogen that may contaminate recovered cultures.

5. Using a disposable plastic pipette transfer the 1-ml of thawed culture from each of the three thawed samples to test-tubes containing 9-ml of the appropriate fresh sterile medium. Cover in aluminium foil and re-label with strain designation and date. Incubate at the appropriate temperature for the taxon for 24 h.

   This dilution minimises any potentially deleterious effects that sensitive taxa may experience due to longer-term exposure to the cryoprotectant solution. To prevent further biochemical-based injuries it is optimal to incubate for a period in the dark; furthermore, this incubation phase allows lethally damaged cells to die, thus reducing the incidence of false positives.

6. Prepare the vital stain. Fluorescein diacetate (0.001% w/v) is prepared by first dissolving 25-mg of FDA crystals in a few drops of acetone and making up to the final volume (25-ml) with methanol. Alternatively, CFDA-SE is freshly prepared in the form of a 5-mM stock-solution in DMSO.

7. Decant 3 x 1-ml aliquots from the test tubes in step 5 into test-tubes and add 50-µl of FDA stock-solution to each tube in turn, or alternatively add 0.5-µl of CFSE stock-solution to the samples to give a final concentration of 2.5-µM. Incubate at room temperature for 1 - 5 min.

   It is may be necessary to empirically optimise the staining procedure as some cells require higher concentrations of stain and/or longer incubation prior to observation.

8. For both the vital stains (FDA or CFDA-SE) observe under excitation-fluorescence. Cells with functioning esterase activity cleave the stain which then fluoresces intensely yellow/green under UV illumination; non-viable algal cells appear colourless, or red due to the auto-fluorescence of chlorophyll (Fig. 1).

   One needs to count at least 50 - 100 cells per sample to obtain a realistic estimate of viability levels. This method and assay is readily scalable to using flow cytometry, which has the advantage of generating much more data in terms of cell numbers. In addition, the detectors can detect yellow/green fluorescence, which can be visually masked in algae with large chloroplasts by the auto-fluorescence of chlorophyll.

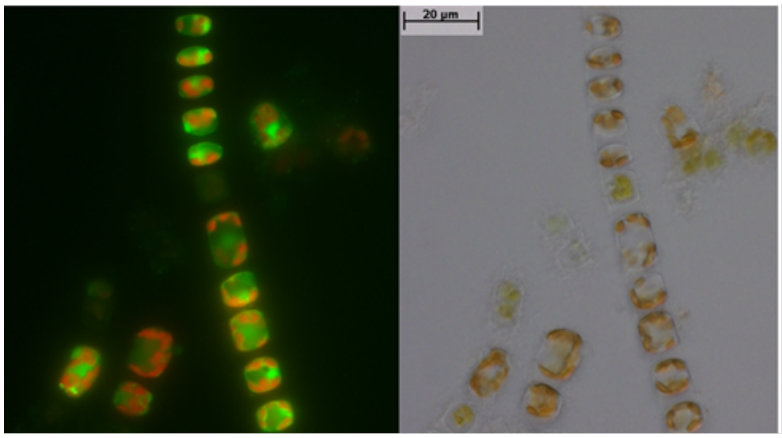

Fig. 1 **Epifluorescence and phase-contrast micrographs of *Skeletonema marinoi*. Live cells stained green correlate with morphologically normal cells under phase contrast. Dead and damaged cells remain unstained, or where cells were intact appeared red due to auto- fluorescence of chlorophyll.** (Day et al. in prep)

9. Viability is expressed by number of FDA positives (under fluorescence) against the total number of cells observed within the field(s) viewed.

   % viability = (No. post-treatment FDA +ve) / (Total No. of cells observed in field(s) of view assayed) X 100.

   Ensure that any dilutions are factored into the calculations.

   Note: if the control samples had less than 100% viability then this should be factored into the absolute post-thaw viability levels, e.g. If control samples had viability levels of 80% and post-thaw viability levels were 80%, then in reality 100% of the cells capable of surviving have survived.

Plating and Colony Forming Units (CFU) counts

Assessment of control (100%) viability level

1. Perform a haemocytometer count, or use a Coulter counter, to obtain the cell density of the culture to be cryopreserved.

2. Using 1-mL aliquots and logarithmic dilutions in sterile medium dilute to a defined culture cell density that would result in 50 - 500 cells in 1-ml, (e.g. for a culture with a cell density of 5×10^7 dilute the culture by 10^5 i.e. through 5 logarithmic dilutions).

3. Transfer 1-ml aliquots of the dilution obtained in step 2, and place in replicate (3) identical Petri dishes (50-mm diameter).

   The number of algal units dispensed should be such that between 50 and 500 colonies will be produced.

4. Pour approximately 2.5-ml of molten medium containing agar (1% w/v) into the Petri dish and agitate gently to insure uniform mixing.

   Note: you need to hold the agar at 40^oC, i.e. just above the gelation temperature, prior to dispensing. Many algae are heat sensitive and higher temperatures will kill the algal cells, alternative low temperature gelling alternatives may be employed if necessary. The agar rapidly gels, so all manipulations need to be performed quickly. It is optimal to restrict to small flasks/bottles containing ~ 50-ml agar).

   For some taxa it is possible to use spread–plates, which remove any potential issues associated with heat shock on contact with molten agar.

5. After gelation, seal the plates with Parafilm or Clingfilm to prevent excessive dehydration.

6 Incubate the plates, inverted, under standard algal culture to prevent condensation forming on the agar surface, as this can result in an algal lawn forming across the agar.

7. Depending on CFU density count the number of colonies present on each plate after 7 - 14 days (Counting on one occasion).

   Note: periodically check plates for growth, if there is a risk that colonies will merge, and then count as soon colonies are distinguishable. For some taxa growth is slow and you should check for growth using a dissecting microscope with 10 - 50x magnification.

8. Calculate the mean 100% viability level.

Assessment of frozen/thawed material

1. Stored vials should be transferred from the cryostore/refrigerator to a small dewar containing liquid nitrogen using long forceps and transferred to the laboratory for thawing.

   If storage was in liquid phase liquid-nitrogen (LN_2) then it is possible that nitrogen may have leaked into the vial. Full safety equipment and most importantly eye protection must be worn as there is a small risk of explosion of the vial (although it has been noted at the CCAP collection at SAMS that >10% of some batches of samples contain liquid nitrogen, an explosion has never occurred). However, if leakage has occurred then inevitably axenic strains will have been contaminated.

2. To recover cultures, the cryovials are thawed by placing in a pre-heated water-bath (40^{o}C) and agitated until the last ice crystal has just melted.

   For most marine taxa it is important not to prolong their incubation at 40^{o}C. Alternative, slower warming e.g. in a 25^{o}C water-bath may be beneficial for some strains, but in general rapid warming is optimal as it avoids/minimises ice crystal regrowth.

3. On thawing rapidly transfer to a clean laminar flow/biological safety cabinet and wipe the outside of the vial with 70% (v/v) ethanol.

   Note: there may be high levels of viable bacterial and fungal spores in liquid nitrogen that may contaminate recovered cultures.

4. Using a disposable plastic pipette transfer the 1-ml of thawed culture into the first test-tube of a logarithmic dilution series. Each test-tube should contain 9-ml of the appropriate fresh sterile medium. Mix each test-tube before subsequently transferring 1-ml between each test-tube until diluted to a defined culture cell density that would result in 50 - 500 cells in 1-ml.

5. Then transfer 3 x 1-ml aliquots of the dilution obtained in step 4 and place in 3 x replicate Petri dishes (50-mm diameter).

   The number of algal units dispensed should be such that between 50 and 500 colonies will be produced if all cells survived the cryopreservation process.

6. Pour approximately 2.5-ml of molten medium containing agar (1% w/v) into the Petri dish and agitate gently to insure uniform mixing.

   Note: you need to hold the agar at 40^{o}C, i.e. just above the gelation temperature, prior to dispensing. Many algae are heat sensitive and higher temperatures will kill the algal cells, alternative low temperature gelling alternatives may be employed if necessary. The agar rapidly gels, so all manipulations need to be performed quickly. It is optimal to restrict to small flasks/bottles containing ~ 50-ml agar).

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9. Depending on CFU density count the number of colonies present on each plate after 7 - 14 days (Counting on one occasion).

   Note: periodically check plates for growth, if there is a risk that colonies will merge, and then count as soon colonies are distinguishable. For some taxa growth is slow and you should check for growth using a dissecting microscope with 10 - 50x magnification.

10. The percentage viability of the cryopreserved culture is then determined as:

    % viability = (post-treatment colony count) / (control colony count) X 100.

    Note: take into account that frozen/thawed material has been diluted 1:1 with cryoprotectant solution prior to cryopreservation, thawing and logarithmic dilution.

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