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Cryopreservation Protocol for *Chlamydomonas reinhardtii*

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[Abstract] Cryopreservation is commonly used for storing viable cells, tissues, organs or organisms at ultralow temperatures, and usually involves immersion in liquid nitrogen at -196 °C. Here we provide a detailed cryopreservation protocol for *C. reinhardtii* based on Crutchfield's work (Crutchfield *et al.*, 1999), with minor changes (Yang and Li, 2016). In this study, we compared the cryoprotection effect of two common cryopreservation agents (CPAs), methanol and DMSO. Furthermore, the two-step cryopreservation process was divided into five stages to study the factors affecting the survival rate at each stage. We found that the use of methanol as the CPA, combined with the cooling process outlined here (cooling from 25 °C to -55 °C at a rate of 1 °C/min), were indispensable for cell survival after cryopreservation. The thawing process described here (thawing at 35 °C for 5 min) was also important for increasing the survival rate.

[Background] Nowadays, cryopreservation is used frequently for the storage of transgenic lines or mutation lines of *C. reinhardtii,* and for experimental needs involving this organism. Morris *et al.* (1979) discussed the effects of different CPAs in cooling, the relationships between temperature and survival rate with or without the CPAs, and the cooling rate. They found that with the addition of methanol the half-lethal temperature was the lowest of all the CPAs tested (-14.4 °C), while that of DMSO was -4.9 °C (Morris *et al.*, 1979).

Through storage in 7% (v/v) DMSO overnight at room temperature, followed by storage at -70 °C, Johnson and Dutcher (1993) gained the highest viabilities, nearly 10% in *C. reinhardtii* cultures. However, the survival rate may be restricted in *C. reinhardtii* cell lines, especially in the cell line CC-125 we used, with viabilities of only 0.34%, although the authors claim was caused by liquid culturing. Nevertheless, this method was time consuming and resulted in low cell viabilities. Crutchfield *et al.* (1999) reported a two-step cooling procedure for the cryopreservation of *C. reinhardtii* using 5% methanol as the CPA, which retained relatively high viability (> 40%).

Three different methods for improving survival rate were compared in this study. We followed the protocols mentioned above, and made detailed analyses at each stage of the cryopreservation process. The different effects of methanol and DMSO are also discussed, the results agreeing with the work of previous authors.

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Materials and Reagents

1. 1.5 ml and 4 ml Eppendorf tubes
2. 50 ml flask
3. Cryovials (VWR, Nalgene®, catalog number: 5000)
4. Filter membrane (Filter pore size: 0.2-0.3 μm) for tissue culture
5. 0.22 μm membrane filters (EMD Millipore, model: SLGV033RB)
6. *Chlamydomonas reinhardtii* CC125 was purchased from the Chlamydomonas Resource Center at the University of Minnesota (http://www.chlamycollection.org/cart/)
7. TAP medium (Gorman and Levine 1965, http://www.chlamycollection.org/methods/media-recipes/tap-and-tris-minimal/)
8. Isopropanol, purity ≥ 99.5% (Sangon Biotech, catalog number: A507048)
9. Liquid nitrogen
10. Trypan blue (Sigma-Aldrich, catalog number: T6146)
11. Methanol (MeOH) (AR), purity ≥ 99% (Chongqing Chuandong Chemical, catalog number: methanol)
12. Dimethyl sulfoxide (DMSO) (Sangon Biotech, catalog number: A503039)
13. Iodine (AR) (Sangon Biotech, catalog number: A500538)
14. Potassium iodide (AR) (Sangon Biotech, catalog number: A100512)
15. Lugol's iodine solution (see Recipes)
16. CPA stock solution (see Recipes)

Equipment

1. Thermostatic rocking incubator (Shanghai Shipping, model: SPH-211B)
2. Hemocytometer (Qiujing)
3. Freezing container (Cryo 1 °C freezing container) (VWR, Nalgene®, model: 5100)
4. Autoclave
5. Laminar flow hood
6. Microscope (OLYMPUS, model: CX31)
7. Water bath (Amersham Bioscience)
8. Portable liquid nitrogen tank
9. Centrifuge (Hettich, model: D-78532 Tuttlingen)
   Note: This equipment has been discontinued, other types with the same centrifugal force can be used as substitutions.
10. -80 °C freezer (Thermo Fisher Scientific, model: 8607)

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Software

1. Microsoft Office Excel

Procedure

A. Cell culture conditions
   1. Cell culture is grown using 20 ml of TAP medium in a 50 ml flask. 1 ml of stationary-phase cell culture is transferred into 20 ml fresh medium every week. The culture conditions are adjusted to a speed of 120 rpm shaking at 25 °C using a Thermostatic rocking incubator, and the culture is illuminated continuously with white fluorescent light (PAR = 30 μmol photons m^{-2} s^{-1}). Cultures at a cell density of approximately 0.9-2.0 x 10^7 ml^{-1} (3-5 days after transfer, late linear phase) are used for the experiment.

B. Calculating the cell density of *C. reinhardtii* and volume of cell culture needed in a 2 ml cryovial
   1. 3-5 d after transfer, take 1 ml cell culture out of the 50 ml flask for cell density estimating.
   2. Measure cell density using a hemocytometer: mix 10% volume (10 μl) of Lugol's iodine solution with 90 μl cell culture (diluted if necessary) and add 8 μl of the mixture to each chamber.
   3. Count five small squares in each chamber as a means of calculating the mean number of cells per chamber. The cell number per microliter = mean numbers of cells in five small squares x 5 x 10^4 x dilution factor (10/9, if not further diluted). Optimally, the number of cells should be approximately 0.9-2.0 x 10^7 cells per ml.
      Note: Steps B2 and B3 can be substituted by other cell counting methods.
   4. Calculate the volume of cell culture needed in a 2 ml cryovial and confirm that the final density in the cryovial is no more than 3.3 x 10^6 cells per ml (it was exactly 3.3 x 10^6 in our experiment). It has been reported that when the final cell density in a cryovial is more than 3.3 x 10^6 cells per ml, the survival rate after cryopreservation decreases significantly (Piasecki *et al.*, 2009). Cell culture volume in 2 ml cryovial = 3.3 x 10^6 cells per ml x 1.8 ml/the number of cells per ml. Supplement with TAP medium at: volume of TAP medium = 0.9 ml-cell culture volume.

C. Preparation and conditions for the cryopreservation process
   1. Precool the CPA stock solution to 4 °C.
   2. Put 250 ml isopropanol in the lower department of the two-compartment freezing container, precool it to 4 °C (chill for more than 30 min).
   3. Sterilize all the equipment and tubes used by ultraviolet sterilization and autoclave.
   4. Carry out all further operations for the further cultivation of *C. reinhardtii* cells in a clean and sterile environment (we suggest using a laminar flow hood) to avoid contamination.
   5. Avoid exposing the CPA (or culture containing CPA) to bright light, because methanol becomes toxic to cells after illumination (Crutchfield *et al.*, 1999).

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Note: C. reinhardtii stains CC124, CC3395, CC1690 (http://www.chlamycollection.org) were cryopreserved by this protocol, which works well too.

D. The cryopreservation process

1. The cryopreservation process was based on Crutchfield's work (Crutchfield *et al.*, 1999), with minor changes. We divided the process into five stages, shown in Figure 1. The treatment 4 CC'LTR in Figure 1 is a typical cryopreservation process in this field.

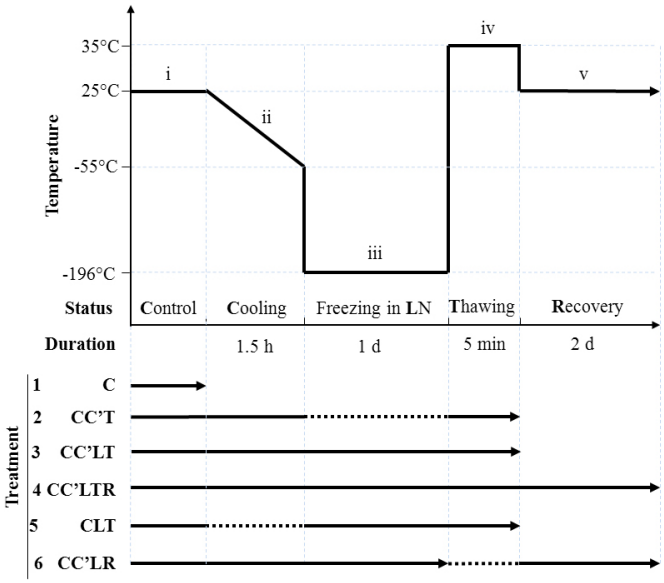

Figure 1. Cryogenic treatments and cryopreservation processes of *C. reinhardtii*. The process was divided into five stages: (i) Control (normal growth) (C); (ii) Cooling from 25 °C to -55 °C at a rate of 1 °C/min (C'); (iii) Freezing in liquid nitrogen (L); (iv) Thawing at 35 °C for 5 min (T); and (v) Recovery growth for 2 days under normal growth conditions (R). The processes that the organism underwent in each treatment is indicated by a solid line; for an omitted step, a dotted line is used. There were six different treatment combinations: (1) C: Control (normal growth) only; (2) CC'T: Control, Cooling and Thawing; (3) CC'LT: Control, Cooling, Freezing in liquid nitrogen (LN) and Thawing; (4) CC'LTR: Control, Cooling, Freezing in LN, Thawing and Recovery growth; (5) CLT: Control, Freezing in LN and Thawing; (6) CC'LR: Control, Cooling, Freezing in LN, Incubation at room temperature for 30 min and Recovery growth. (Yang and Li, 2016)

2. Put 0.9 ml CPA stock solution (we used two other CPA controls to compare with methanol in our experiment, Table 1) and a corresponding volume (see step B4) of cell culture and TAP into a 2 ml cryovial, close the cap, mix gently. The total liquid volume in a 2 ml cryovial should be 1.8 ml and the final cell density 6.6 x 10^6 cells per ml.
3. Put the cryovials into the upper compartment of the freezing container. Close the container and place it in a freezer at -80 °C, leaving it undisturbed for 1.5 h (the 'Cooling' phase in Figure 1).

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4. Remove the cryovials from the freezing container, put them into liquid nitrogen immediately, store for 1 day ('Freezing in LN' in Figure 1).
   Note: In our cryopreservation practice, C. reinhardtii survived in storage for at least 6 months.
5. Remove the cryovials from the liquid nitrogen, then quickly transfer them to a water bath at 35 °C for 5 min ('Thawing' in Figure 1).
6. Centrifuge the cryovials at 1,000 *x g* for 2 min. Then discard the liquid supernatant, add 1 ml of fresh TAP to the cryovials for culturing (preventing contamination). Loosen the cap of the cryovials, mix their contents twice a day. The culture conditions should be the same as in the Control phase, but without shaking ('Recovery' in Figure 1).

E. Calculation of survival rate

To assess the integrity of the cell membranes, the Evans blue dye test was used. The viability of cultures after cryopreservation is reflected by the percentage of cells that excluded the Evans blue dye. The method follows Crutchfield *et al.* (1999) with minor changes.

1. Mix equal volumes of Evans blue dye (0.1% w/v in water) and cell culture (diluted if needed), leave undisturbed in an Eppendorf tube for 5 min.
2. Add about 8 µl mixture to each chamber of a hemocytometer to enable counting under a microscope. At least 200 cells were examined at 400x magnification in a chamber, both chambers were counted independently to determine whether the cells had taken up the Evans blue dye.
3. Calculate the survival rate as follows: the ratio of the number of cells that retained their original green color to the total number of cells. Each of the treatments outlined above was performed five times. The survival rate for each treatment (Figure 1) in our experiment is shown in Table 1.

Note: The Evans blue dye method works well when counting cells immediately after thawing. After further culturing or a long time after thawing, the alive cells migrate fast, which makes them difficult to count.

Table 1. Survival rate of *C. reinhardtii* in culture medium, culture medium plus 5% DMSO, and culture medium plus 5% MeOH after cryogenic treatments (Yang and Li, 2016)

Treatment No.	Cryogenic Treatment	Survival rate (%)		
		CM	CM + DMSO	CM + MeOH
1	C	> 90	> 90	> 90
2	CC'T	0	0	75.9 ± 2.5
3	CC'LT	0	0	41.3 ± 2.7
4	CC'LTR	0	0	> 41.3 ± 2.7*
5	CLT	0	0	0
6	CC'LR	0	0	> 24.2 ± 3.0*

CM, culture medium; CM + DMSO, culture medium plus 5% DMSO; CM + MeOH, culture medium plus 5% MeOH. Values are means ± standard deviation (n = 5). '*' represents those samples in which the survival rate was calculated immediately after thawing without further culturing.

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Data analysis

The survival of *C. reinhardtii* after cryopreservation was assessed by Evan's blue method (see Procedure E) (Crutchfield *et al.*, 1999). For each treatment (2-6) in Table 1, five samples were counted. The mean and standard deviation was calculated by functions 'AVERAGE' and 'STDEV' respectively in Microsoft Office Excel.

Notes

1. These experiments were repeated three times on different dates (2014.7, 2015.5 and 2015.6), the survival rate in CC'LT treatment being 55.38%, 34.6%, 41.3%, respectively. The second time, the CPAs used had been stored at 4 °C for more than three months, so if fresh CPAs are used, the viability seems to rise. Estimation by eye under a light microscope of whether or not a cell was stained with Evan's blue will introduce some random errors.
2. Additional fluorescent lighting will be needed in the Thermostatic rocking incubator to reach the required illumination intensity.
3. Make sure the *C. reinhardtii* cells are well cultured: the liquid should be yellow-green or green and uniform, running under the microscope, and mainly comprising single cells, with no aggregates. We used the cells 3-5 days after transfer from liquid culture. If cultured from cryopreservation, 2 or more liquid transfers may be necessary.
4. Avoid light in the steps with CPAs. The CPAs stocks should be stored at 4 °C, for no longer than 1 month.
5. Please handle liquid nitrogen and the -80 °C freezer with care to avoid injury.

Recipes

1. Lugol's iodine solution
   1 g I_2, 2 g KI, with distilled water added to total volume of 20 ml
   Dilute 10-fold when used
2. CPA stock solution
   CPA stock solution: 10% MeOH in TAP medium
   Control: TAP only or 10% DMSO in TAP medium

Acknowledgments

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