Cryopreservation of clonal and polyclonal populations of *Chlamydomonas reinhardtii*

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**Abstract**

Long-term preservation of laboratory strains of *Chlamydomonas reinhardtii* has historically involved either liquid nitrogen cryopreservation, which is expensive and labor intensive, or storage on agar plates, which requires frequent transfer to new plates, and which may leave samples susceptible to contamination as well as genetic drift and/or selection. The emergence of *C. reinhardtii* as a model organism for genetic analysis and experimental evolution has produced an increasing demand for an efficient method to cryopreserve *C. reinhardtii* populations. The GeneArt™ Cryopreservation Kit for Algae provides the first method for algal storage at \(-80^\circ\)C; however, little is known about how this method affects recovery of different clones, much less polyclonal populations. Here, we compare postfreeze viability of clonal and genetically mixed samples frozen at \(-80^\circ\)C using GeneArt™ or cryopreserved using liquid nitrogen. We find that the GeneArt™ protocol yields similar percent recoveries for some but not all clonal cultures, when compared to archiving via liquid N2. We also find that relative frequency of different strains recovered from genetically mixed populations can be significantly altered by cryopreservation. Thus, while cryopreservation using GeneArt™ is an effective means for archiving certain clonal populations, it is not universally so. Strain-specific differences in freeze–thaw tolerance complicate the storage of different clones, and may also bias the recovery of different genotypes from polyclonal populations.

**Keywords:** *Chlamydomonas reinhardtii*; cryopreservation; GeneArt™; \(-80^\circ\)C; clonal; polyclonal

**Introduction**

*Chlamydomonas reinhardtii* is a single-celled, chlorophyte alga with a well-annotated genome, reliable methods for genetic manipulation, an extensive library of mutants, and a rich history of experimental investigation [1]. As a model organism, *C. reinhardtii* has been used to shed light on biological processes ranging from photosynthesis and cell motility to taxis and sexual reproduction [2–6]. *Chlamydomonas reinhardtii* has also been the subject of research into biofuel production [7] and into the genetics of adaptation during laboratory experimental evolution [7–9]. Each of these different lines of inquiry requires the use of archived samples that consist of either single strains or mixtures of strains produced by induced or spontaneous mutation. A reliable method for archiving either isogenic or heterogenic cell cultures in a state where they are genetically stable and can be easily distributed would accelerate research along multiple fronts.

Before the advent of the first reliable method of cryopreservation, *C. reinhardtii* clones were stored either in liquid batch culture or as colonies on agar plates at low temperature [1, 10]. These procedures allow cell lines to be stored for up to 6 months...
but require cells to be transferred to fresh liquid or solid medium, leaving populations susceptible to genetic drift and selection [11] and references therein, as well as to bacterial contamination [10]. The development of a liquid nitrogen (LN) cryopreservation protocol [12], and subsequent modifications of that protocol utilizing dimethyl sulfoxide and methanol reagents [13–16] represent an improvement over these older methods, but at a significant cost in labor and materials. A simple, reliable method of cryopreservation at −80°C, such as those used routinely with other microbial models, has long been desired. The GeneArt™ Cryopreservation Kit for Algae (Catalog # A24228, Thermo Fisher Scientific) provides the first method for cryopreserving C. reinhardtii at −80°C that allows archived algae to be stored in Ultra-Low Temperature freezers. However, little is known about how the GeneArt™ protocol affects relative recovery of different laboratory strains, much less how it may affect recovery of genetically heterogeneous populations of C. reinhardtii produced by mutagenesis or by laboratory evolution. Recent work on cryopreservation of mixed populations of laboratory-evolved Bakers’ yeast [17] and Escherichia coli [18] suggests that differential survival can alter genotypic frequencies, with consequences for the evolutionary trajectory of populations revived from storage at −80°C. To fill these knowledge gaps, we assessed a previously unreported aspect of the kit’s efficacy: the survival rate of an isogenic (i.e. clonal) sample during freeze–thaw in comparison with the survival rates of different strains present in a heterogenic (polyclonal) sample. We then compared over a 15-day period the relative recovery of C. reinhardtii cells with clonal and polyclonal cultures cryopreserved using either the GeneArt™ protocol or the LN₂ protocol of Sayre [19].

Materials and methods

Strains and culture conditions

Strains were obtained from the Chlamydomonas Culture Collection, University of Minnesota (Chlamydomonas Resource Center, RRID: SCR_014960), see Table 1. Reference wild-type strain CC-1690 (Sager 21 gr), mt+, [20, 21] was used for the isogenic (i.e. monoclonal) cultures, and five strains with differing antibiotic resistances were used for pooled polyclonal cultures. A small volume of each control sample was removed and serially diluted to 2000 cells ml⁻¹, then 50 μl of this dilution was spread plated onto TAP agar in triplicate, such that approximately 100 colonies might be observed upon growth. After incubating experimental samples at room temperature for 40 min, 240 μl aliquots were transferred into three cryovials per sample, and placed directly into −80°C storage, inside an empty Mr Frosty freezing container (Catalog #15-350-50, Thermo Fisher). After 3 days, the cryovials were removed from cold storage and thawed for 1 min in a 35°C water bath. Each sample was diluted to a concentration of 2000 cells ml⁻¹, then 50 μl of these dilutions were plated onto TAP agar in triplicate for colony counting.

Table 1: Phenotypes of Chlamydomonas strains used in this study

| Strain   | Antibiotic resistance | Genetic cause of antibiotic resistance | Mating type | Citation(s)                  |
|----------|-----------------------|----------------------------------------|-------------|-----------------------------|
| CC-87    | Kan                   | A to G change at base 1340 of chloroplast 16S rRNA gene | Mt-         | [22]                        |
| CC-119   | Str                   | A to G change at base 474 of 165 rRNA gene | Mt-         | [22]                        |
| CC-504   | Ery                   | Altered protein in large subunit of chloroplast ribosome | Mt+         | (23, 24)                    |
| CC-1690  | None determined       |                                        | Mt-         | (20, 21)                    |
| CC-2355  | Chl                   | Change in RIB1 region of 23S chloroplast rRNA | Mt+         | (25)                        |
| CC-3673  | Ani                   |                                        | Mt-         | Chlamydomonas Genetics Center, Duke University, 1998 |

Clonal survival assay using GeneArt™

An isogenic culture of C. reinhardtii strain CC-1690 was inoculated into 150 ml of TAP medium and grown for 3 days. As outlined in the GeneArt™ Cryopreservation Kit for Algae manufacturer’s protocol, samples of this culture were diluted into triplicate vials of precondition medium (45 ml TAP:1 ml Reagent A) to a final cell density of 1.3 × 10⁵ cells ml⁻¹. The pre-conditioning medium was incubated for an additional 3 days under the same environmental conditions as above, after which cell density was measured in triplicate by hemocytometer to be within 2.7–2.8 × 10⁵ cells ml⁻¹ for each of the replicates. Each sample was separated into two vials: one to be cryopreserved and one to serve as a nonfrozen control. All control and experimental samples were centrifuged at 1452g for 5 min, then the supernatant was removed and the resulting cell pellets were suspended in either Reagent B (experimental samples) or TAP media (control samples) to a final concentration of 2.5 × 10⁷ cells ml⁻¹. A small volume of each control sample was removed and serially diluted to 2000 cells ml⁻¹, then 50 μl of this dilution was spread plated onto TAP agar in triplicate, such that approximately 100 colonies might be observed upon growth. After incubating experimental samples at room temperature for 40 min, 240 μl aliquots were transferred into three cryovials per sample, and placed directly into −80°C storage, inside an empty Mr Frosty freezing container (Catalog #15-350-50, Thermo Fisher). After 3 days, the cryovials were removed from cold storage and thawed for 1 min in a 35°C water bath. Each sample was diluted to a concentration of 2000 cells ml⁻¹, then 50 μl of these dilutions were plated onto TAP agar in triplicate for colony counting.
Polyclonal survival assay using GeneArt™

Five uniquely antibiotic-resistant strains of *C. reinhardtii* (CC-87, CC-119, CC-504, CC-2355, CC-3673; Table 1) were inoculated into 150 ml TAP in separate 250 ml flasks and incubated to log-phase growth under the culture conditions described above. As outlined by the GeneArt™ Cryopreservation Kit for Algae, each was inoculated into preconditioning medium (45 ml TAP: 1 ml Reagent A) to a final concentration of 1.3 \( \times 10^5 \) cells ml\(^{-1}\). After 4 days of growth, the cell density of each strain was measured by hemocytometer, and equal cell numbers from each strain were mixed into a final volume of 45 ml. A control sample was removed from this culture and plated directly onto TAP agar at several dilutions for strain identification. The remaining volume was centrifuged at 1452 \( \times g \) for 5 min, the supernatant was discarded, and the cell pellets were resuspended in Reagent B to a final concentration of 2.5 \( \times 10^5 \) cells ml\(^{-1}\). After incubation at room temperature for 40 min, 240 \( \mu l \) aliquots of the sample were transferred to cryovials and placed into –80°C storage inside an empty Mr Frosty™ freezing container. After at least 4 days at –80°C, each cryovial was removed from storage and thawed for 1 min in a 35°C water bath. The thawed sample was then plated onto agar for various dilutions for strain identification.

After 5 days of growth on TAP agar at 23°C on a 14:10 light cycle, 205 colonies were randomly selected from each set of both control and experimental plates, and every colony was inoculated into a 5 ml test tube of fresh TAP. As each strain exhibits a unique antibiotic-resistance profile, strain identification could easily be performed by testing viability against a panel of antibiotics (Fig. 1). To do this, each row of a 96-well plate was filled with a 200 \( \mu l \) volume of either TAP medium, TAP + 100 \( \mu g/ml \) Str, TAP + 100 \( \mu g/ml \) Kan, TAP + 150 \( \mu g/ml \) Ery, TAP + 200 \( \mu g/ml \) Chlor, or TAP + 10 \( \mu g/ml \) Ani. To every second column, we inoculated with 50 \( \mu l \) of dense (~3 \( \times 10^6 \) cells ml\(^{-1}\)) culture, the first column serving as a sterile medium control. A strain’s pattern of growth in relation to its known antibiotic susceptibilities revealed its identity. In rare instances where the pattern of growth was ambiguous, the sample was discarded from the analysis.

Method for reduced volume reactions

In an effort to conserve costly reagents, reduced volume reactions were used throughout the course of our experiments. According to the manufacturer’s protocol, a single kit is designed for the preservation of five strains, each with 20 separate aliquots. By reducing the volume of the preconditioning medium from 46 ml (45 ml TAP: 1 ml Reagent A) to 15.333 ml (15 ml TAP: 0.333 ml Reagent A), the utility of a single kit could be expanded from 5 to 15 strains. All other instructions in the manufacturer’s protocol remain unchanged. Reduced volume reactions have been successfully used to archive model strains CC-87, CC-119, CC-504, CC-1690, CC-2290, CC-2355, and CC-3673, as well as strains experimentally evolved in our laboratory: B2-01, B2-03, B2-04, B2-10, B2-11, BS-05, BS-06, K1-01, and K1-06 [8, 26] without failure.

Long-term survivorship following either GeneArt™ or liquid nitrogen cryopreservation

Monoclonal survival assays

GeneArt™ Monoclonal Survival Assay: Isogenic cultures of *C. reinhardtii* strains CC-1690, CC-87, and CC-119 were inoculated into 150 ml of TAP medium and grown for 3 days. According to the GeneArt™ Cryopreservation Kit for Algae manufacturer’s protocol, samples from each culture were diluted into flasks of preconditioning medium (15 ml TAP: 0.333 ml Reagent A) to a final cell density of 1.3 \( \times 10^5 \) cells ml\(^{-1}\). Cells in preconditioning medium were incubated for additional 4 days, then cell density of each culture was measured by hemocytometer. Cell densities for CC-1690, CC-87, and CC-119 were measured to be: 5 \( \times 10^6 \) cells ml\(^{-1}\), 1.06 \( \times 10^7 \) cells ml\(^{-1}\), and 6.8 \( \times 10^6 \) cells ml\(^{-1}\), respectively. An appropriate amount of each culture was pipetted into a 15 ml Falcon tube and pelleted via centrifuge at 1452 \( g \) for 5 min in order to achieve a final cell density in Reagent B of 5.5 \( \times 10^6 \) cells ml\(^{-1}\). After incubating each sample at room temperature for 40 min, 240 \( \mu l \) aliquots were transferred into four cryovials per sample and placed directly into –80°C storage, inside an empty Mr Frosty™ freezing container (Catalog #15-350-50, Thermo Fisher). Cryovials for each sample were thawed at four

![Figure 1: Different C. reinhardtii strains exhibit characteristic patterns of antibiotic resistance and sensitivity. An individual strain’s characteristic pattern enables it to be identified in mixed culture and its frequency determined relative to other strains present. The Chl resistance of strain CC-2355 is best ascertained during heterotrophic (dark) growth, but in order to screen polyclonal cultures in parallel a light–dark cycle was utilized. Although this slightly diminished the stringency of the selection, the growth differences were clearly distinguishable by eye.](https://academic.oup.com/biomethods/article/6/1/bpab011/6278925)
The results of a paired t-test showed a significant decrease in the number of CFU observed on agar after GeneArt™ cryopreservation (P < 0.001). A 95% confidence interval for the difference between the means of the two treatment groups was calculated at 72.9 ± 12.0 CFU. On an average, 30% of CC-1690 cells survived cryopreservation at −80°C (Fig. 2).

Effect of cryopreservation on polyclonal samples

We also tested the hypothesis that the relative frequencies of different strains in a polyclonal sample were altered during cryopreservation by using repeated G-tests of goodness-of-fit.
These tests compared the observed distributions of strains in the post freeze sample groups with the observed distributions in the prefreeze sample groups, and generated statistics for hypothesis testing. First, each paired group of pre- and postfreeze samples was tested individually for significant differences, then samples were pooled together and tested for significant differences collectively.

The results of repeated G-tests of goodness-of-fit indicated that for each set of paired samples, and for the pooled samples as a whole, the strain frequencies in a polyclonal sample were significantly altered by cryopreservation. Furthermore, the direction and magnitude of the change in strain frequencies were largely repeatable (Fig. 3 and Table 2). For each set of observations, the null hypothesis that the post freeze strain frequencies were equal to the prefreeze strain frequencies was rejected. In each case, a significantly higher proportion of Kan-resistant (CC-119) isolates was recovered postfreeze. When strains are pooled together, the significance of this result increases dramatically (Table 2).

**Table 2: Pre- and postfreeze recovery of different C. reinhardtii strains from polyclonal cultures**

| Replicate | Treatment | Str | Kan | Ery | Chl | Ani | df | G     | P-value |
|-----------|-----------|-----|-----|-----|-----|-----|-----|-------|---------|
| 1         | Postfreeze| 15  | 113 | 27  | 21  | 27  | 4   | 67.2  | <0.00001|
| 2         | Postfreeze| 33  | 59  | 47  | 33  | 32  |     |       |         |
| 3         | Postfreeze| 50  | 50  | 38  | 41  | 48  |     |       |         |
| Pooled    | Postfreeze| 13  | 113 | 29  | 8   | 17  | 4   | 132.5 | <0.00001|

Five different strains can be discriminated on the basis of their antibiotic resistance and sensitivities (Table 1 and Fig. 1). We performed statistical analysis of approximately 200 randomly selected colonies from both pre- and postfreeze plating (as described in ‘Polyclonal survival assay’ section). Repeated G-tests of goodness-of-fit indicate that cryopreservation using the GeneArt protocol biases recovery of different genotypes.

**Long-term survivorship of C. reinhardtii following either GeneArt or LN2 cryopreservation**

Strain collections typically consist of individual clones, whereas mutagenesis and evolution experiments typically produce mixtures of clones. Cryopreservation of either sample type is aimed at creating a long-term resource. The experiments described in sections ‘Effect of cryopreservation on the viability of a clonal sample’ and ‘Effect of cryopreservation on polyclonal samples’ were conducted over 72 h using GeneArt protocol only. To assess the relative efficacy of each method for long-term storage, we cryopreserved single clones and mixtures of these clones using either the GeneArt protocol or the LN2 protocol of Sayre [19], then assayed cells’ survivorship over a 15-day interval (Fig. 4).

Overall, we found that the LN2 protocol (Fig. 4, solid columns) allowed for higher percent recovery of all monoclonal cultures relative to the same strains prepared using GeneArt protocol (Fig. 4, hatched columns). CC-1690 and CC-119 responded better to cryopreservation under LN2 than did strain CC-87 (Fig. 4). After 15 days, CC-119, CC-1690, and CC-87 exhibited survivorships of 36, 18, and 4%, respectively, whereas survivorship of polyclonal cultures was 21%. With the exception of CC-1690, long-term survivorship of C. reinhardtii strains cryopreserved using GeneArt was poor (Fig. 4, hatched columns). In contrast, survivorship of CC-1690 using GeneArt was within range values reported in both Figs 2 and 4.

We further tested whether long-term cold storage biased recovery of genetically different C. reinhardtii strains. For this experiment, we chose the Kan-resistant strain, CC-87, and the Str-resistant strain, CC-119. These strains exhibited pronounced differences in survivorship as clones using the LN2 protocol, though they were indistinguishable using GeneArt protocol. Interestingly, over a 15-day interval the relative proportion of genotypes recovered did not change dramatically from the
Figure 4: Percent recovery of monoclonal and polyclonal cultures for LN2 and GeneArt™ cryopreservation methods. Percent recovery for monoclonal and polyclonal cultures cryopreserved using the LN2 method (solid columns) and GeneArt™ kit (hatched columns). The time point for 5 days postfreezing is not shown due to human error during the thawing process of the LN2 samples. The polyclonal sample consists of strains CC-87 and CC-119.

Figure 5: Recovery of *C. reinhardtii* strains from polyclonal cultures cryopreserved using the LN2 and GeneArt™ protocols. (A) Strain recoveries from polyclonal samples cryopreserved under the LN2 protocol. (B) Strain recoveries from polyclonal samples cryopreserved using the GeneArt™ kit. Absolute counts of CFU are shown within the bars. Kan-resistant strain, which is CC-87. Str-resistant strain, which is CC-119.
proportions scored prior to freezing using either LN$_3$ or GeneArt$^\text{TM}$ (Fig. 5A and B). This result stands in contrast with results presented in Fig. 3 where polyclonal cultures cryopreserved using GeneArt$^\text{TM}$ were stored for ~72 h at ~80°C. We speculate that this discrepancy may be attributable to the use of only two rather five input strains.

**Discussion**

Our experiments indicate that the GeneArt$^\text{TM}$ Cryopreservation Kit for Algae can provide a short-term storage alternative to agar plates or to LN$_3$ cryopreservation for clonal samples; for some strains (e.g. CC-1690), the procedure may also be suitable for long-term storage. CC-1690, a member of the Sager lineage [27], is a widely adopted laboratory strain that was used to create EST libraries for cDNA sequencing in the Chlamydomonas Genome project [21]. Our GeneArt$^\text{TM}$ data for this strain contrast with a recent study that found postfreeze viability of C. reinhardtii in GeneArt$^\text{TM}$ reagents was dramatically lower than that obtained by LN$_3$ preservation, as measured by a postthaw growth curve in liquid TAP [28]. It is noteworthy that the authors of [28] measured the growth rate of a population after recovery from cryopreservation, whereas we measured absolute number of surviving cells after recovery. Our finding that a relatively high number of CC-1690 cells survive despite initially slower growth suggests a transient period of reduced reproductive capacity in surviving cells after they are revived. The discordance between these results may also be attributed to the use of different strains or different culture conditions. Overall, we find that the survivorship of CC-1690 at ~80°C (ca. 25%) is in the range of values previously reported for LN$_3$ (5–40%) [13, 15, 29]. In contrast, we found that two other laboratory strains, CC-119 and CC-87, responded poorly to GeneArt$^\text{TM}$ cryopreservation; for these strains, our data agree with the assessment given by Scarpbrough and Wirschell [28]. Thus, prior to considering use of this protocol for any other strain than CC-1690, researchers are urged to empirically estimate survivorship. A mixture of genotypes (a polyclonal sample) can be expected to arise in a variety of research contexts including samples obtained from environmental sources, from laboratory experimental evolution, or from samples that have been mutantized. Our data show that different genotypes can exhibit different levels of survivorship following cryopreservation, regardless of which protocol is employed. Thus, the relative abundance of different genotypes in genetically heterogeneous samples may not be reliably conserved in frozen stocks. Differences in freeze–thaw tolerance may be explained by among-strain genetic differences governing this trait; indeed, such differences have been shown among strains of other microbes [30–32]. In the bacterium E. coli, freeze–thaw tolerant strains have been generated by IS150 (transposon) insertions into the cls gene, which codes for an essential phospholipid utilized in the cell membrane, as well as by IS150 insertions into the uspA-uspB intergenic region, which is flanked by two universal stress–response proteins. Further experimentation revealed that the mutations in cls conferred increased membrane fluidity in the culture conditions of the experiment, permitting tolerance to temperature fluctuations [33]. Chlamydomonas reinhardtii strains used in the experiments reported here were intentionally chosen to be distantly related so that we could test for the effect of genetic heterogeneity. The differences we report in strains’ tolerance to freezing may be related to differences in their capacities to form plasmalemma structures associated with freeze tolerance [34], or to differences in stress–response proteins, such asAsp-Glu-Ala-Asp (DEAD)-box RNA helicase proteins [35] and Hsp70, variants of which appear to underlie the psychrophilic habit of Antarctic sea-ice Chlamydomonas sp. ICE-L [35, 36].

For researchers using monoclonal strains, low postfreeze viability may not be an important issue as long as some cells survive. However, our results serve as a cautionary tale to investigators working with mixed C. reinhardtii populations who may need to recover genotypes from cryopreserved samples at their original frequencies. In addition, our data further illustrate how genotypic variation can drive major differences in algal responses to stress in the form of freeze–thaw tolerance.

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**Author contributions**

J.B. and C.L. performed the experiments, carried out statistical analyses, and wrote the paper. E.C., F.R., and M.H. helped write the manuscript.

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**Data Availability Statement**

Raw data used to produce figures and tables in this report are available upon request.

**Conflict of interest statement.** The authors declare that they have no conflict of interest.

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