Management and monitoring of cryopreserved microalgal & cyanobacterial master stock-cultures

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Additional Notes

This SOP is based on the 40 years' experience of managing cryopreserved materials in the CCAP, which has been based at SAMS since 1986. The authors acknowledge the invaluable contributions to current and former CCAP staff, affiliated researchers and students.

Apparatus: Refrigerators capable of long-term stable maintenance of cryogenic storage temperatures; monitoring system; a database that can document and record movement of samples from initial cryopreservation to eventual use/ dispatch to users.

Additional information:

World Federation for Culture Collections *(2010) Guidelines for the establishment and operation of collections of cultures of microorganisms.* *http://www.wfcc.info/guidelines/*

Day JG, Pröschold T, Friedl T, Lorenz M and Silva PC *(2010) Conservation of microalgal type material: approaches needed for 21st century science. Taxon 59, 3-6.*

Benson EE *(2008) Cryopreservation of Phytodiversity: A Critical Appraisal of Theory & Practice, Critical Reviews in Plant Sciences, 27, 141- 219 .*

Day JG and Stacey GN *(2008) Biobanking. Molecular Biotechnology 40, 202-213.*

OECD *(2007) OECD Best Practice guide - lines for Biological Resource Centres.* *www.oecd.org/sti/biotech/38777417.pdf*

The application of cryopreservation of microalgae can obviate complications associated with traditional serial transfer culture methods, such as: adaptation to the *in vitro* environment, genetic mutations, chromosomal abnormalities, mixing of strains by mis-transfer/mislabeling, contamination and accidental loss of cultures. Where cells are stored at ultra-low temperatures, below the point at which ice crystals can form, they are effectively in "suspended animation" and are, in theory, functionally and genetically stable in storage for many hundreds of years. However, statements on sustained viability of cryopreserved cells are based on the assumption that their storage temperature will be reliably maintained at an ultra-low temperature, most commonly in liquid-phase nitrogen (-196^{o}C). Material stored in the vapor-phase nitrogen, or in electrically operated refrigerators do not achieve this temperature and the actual storage temperature may fluctuate over time, as a result of the frequency of opening the vessel for removal/addition of samples. Critically the storage temperature should not rise above the point where damage could occur and viability may be reduced (commonly assumed to be in the range -90 to -120^{o}C). This SOP lists the key criteria that need to be managed, monitored and documented to ensure the long-term value of any cryopreserved materials.

1. Compliance with legislation. Operations must be carried out safely and compliant with the various legislation and regulations that control the management and exploitation of genetic resources (see Annex 1 for further detail). Additionally, Health and Safety responsibilities associated with the materials stored and cryogenic storage facilities must be competently assessed, managed and documented to conform to local legislation.

2. Strain designation & documentation.

   As with any biological resource, base-line information needs to be held on the strain. All isolates/strain require a unique identifier, or strain number (whilst taxonomic classification may change as more data and taxonomic understanding accumulate the strain number remains constant). Other data that needs to be held include: hazard status, taxonomic identity (genus & species), geographical origin, date of isolation, name & affiliation of isolator, culture medium preference and cultivation regime. In addition, linkage to other relevant molecular, metabolomic, or functional data add value to the conserved material.

3. Methodology used to cryopreserve the material.

   Full details of the pre-culture regime, culture age/density and methodology employed to cryopreserve the material.

4. Number of replicate samples cryopreserved.

The number of replicate samples cryopreserved depends on a number of factors, not least the cooling unit employed. Controlled rate freezers come in a wide variety of sizes and specifications and in some cases can cool several hundred cryovials simultaneously; passive freezers vary in size but generally hold 10-20 cryovials. At the CCAP collection at SAMS batches of 15 cryovials per culture are the norm, but for type specimens it is advisable to generate a larger batch of material (see Day et al. 2010).

5. Quality control (data on efficacy of the approach).

Viability assessment is a key component of the procedure. The most practicable approach is to set an acceptable standard e.g. recovered samples can regenerate a healthy "normal" culture within 3 weeks. A better approach is to have data on the capacity of individual cells that were cryopreserved to divide and regenerate a healthy culture. Absolute viability levels provide data that can be used to assess both efficacy of the original method employed and quality of the storage regime (see SOP on viability assessment). Data on whole genome stability, metabolic functionality, or capacity to generate product (metabolite of interest) can all be employed to provide Q/C data. At the CCAP collection at SAMS, each batch of material is initially located in the Working/Distribution bank. Viability is assessed by removing three tubes after a minimum of overnight storage in the cryobank. Where viability is deemed acceptable, two cryovials are relocated to the Master bank.

6. Storage facility (cryostore/cryobank).

The choice of storage facility will be dictated by the number of samples to be held, budgets, compliance/regulatory requirements etc. There are a variety of "refrigerators" available that hold samples in liquid nitrogen (vapour and/or liquid phase), nitrogen-free storage, or electrically cooled ultra-low temperature storage units. It is important to hold materials in more than one refrigerator, ideally in two different buildings or geographic locations. Where possible in the main facility one should have a Master Bank and a Working /Distribution Bank (Fig. 1). At the CCAP collection at SAMS the Master Bank holds two replicate samples from each batch that has been successfully cryopreserved and the Working Bank 10 cryovials. (see 10. Audit trail).

Working/Distribution banks and the Master Bank should be located in separate "refrigerators". Furthermore, there should be clear demarcation and segregation between samples that are held as constituents of the Banks and any materials that are experimental in nature. The experimental and banked material should ideally be located in separate inventory stacks (Fig. 2), (see 9. Sample management and stock control).

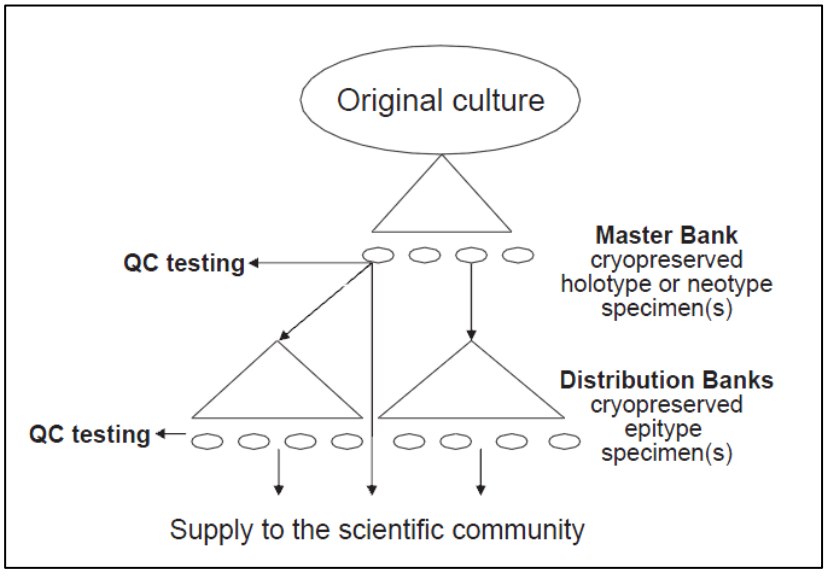

Figure 1 Cryo-storage of type specimens (Day et al. 2010)

7. Storage temperature monitoring.

Monitoring and management of the temperature regime in the storage vessel is critical to the long-term retention of viability/ sample value. Appropriate automated alarm systems should be supplemented by routine checks by competent personnel. At the CCAP collection at SAMS the cryostore is checked at least twice daily by security staff who will report anything unusual, e.g. a build-up of ice, or excessive level of condensation on the vessels. In addition, levels of liquid nitrogen are checked weekly and the vessels filled every two weeks (The storage vessels used will when refilled be thermally stable for in excess of 6 weeks). An emergency plan to deal with major problems should be formulated (see below).

8. Labelling minimum standards.

It is essential that the cryovials are appropriately labelled; they should as a minimum have the strain number and the date of cryopreservation. Where batches of more than one strain are being produced and banked it is important to use cryovials with different coloured tops or inserts for each taxon/strain to speed the processing of the samples. Clarity of writing is critical, as is the use of indelible marker pens. Modern methods such as the use of Barcoding allow automated and human-error-free management of the samples. Irrespective of the labelling approach employed, the connection of the vial to a database that allows retention of all relevant data associated with the sample is critical for Q/C and management of the conserved material.

9. Sample management and stock control.

The management and monitoring of samples within the storage inventory is critical to ensuring best practice. As samples are added they should be simultaneously logged into the database managing the cryopreserved materials (both purpose designed and proprietary stock-managing databases are available). From the experience of the CCAP collection at SAMS those that allow visualisation of a storage box/drawer in the inventory are most practicable (Fig. 2b). It is also extremely important to ensure that Master stock- cultures experience the minimum possible level of disruption i.e. do not co-locate Working Banks and Master Banks in the same inventory system/cryostat. If at all possible separate any experimental materials from either Working Banks or Master Banks to minimise possible human-error mis-transfers, or potential thermal shocks due to the increased amount of manipulations within the inventory drawer or stack.

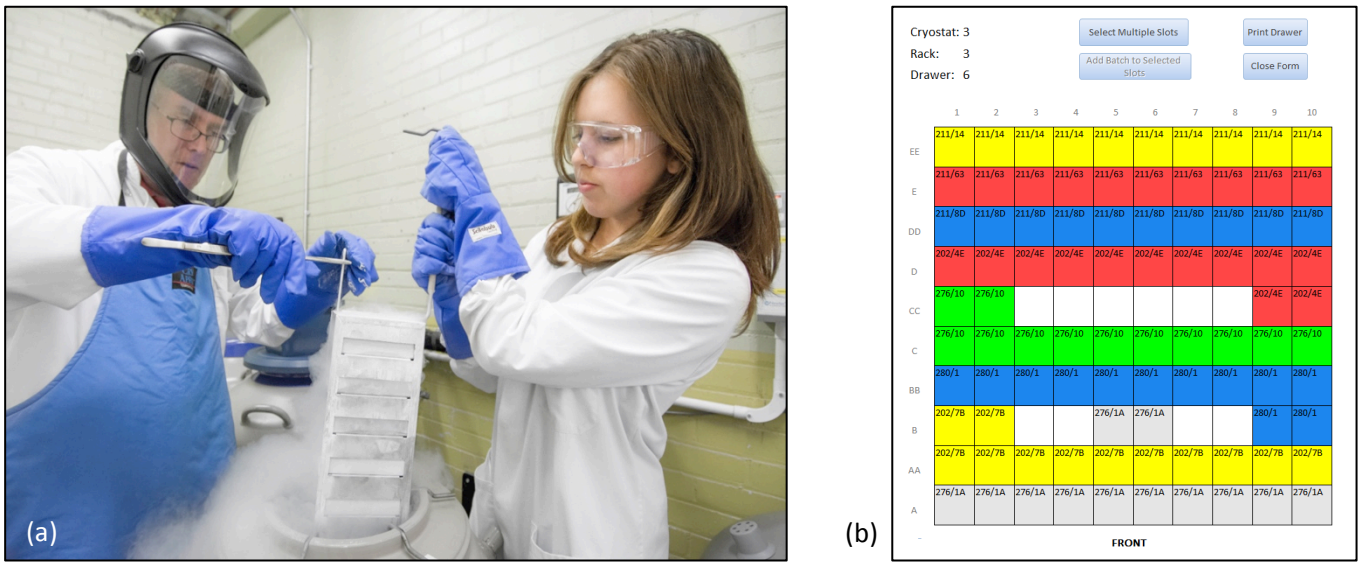

Figure 2 Cryo-storage inventory manipulation (a); management and visualisation of samples in a storage drawer (b)

10. Audit trail.

It is important to ensure that all movements and manipulations of the stored samples are monitored and logged. This includes who undertook the manipulation, what was done and where any individual cryovial has been located over the duration of its storage. Note the recommended working-life of different storage systems vary, but something in the range 8-10 years is not unusual; therefore cryopreserved samples have and will continue to be relocated/transferred between

storage vessels. Experience from the EnAlgae project has demonstrated that history of samples may have a profound effect on absolute viability levels, but not necessarily on their capacity to regenerate an acceptable, normal culture.

11. Management, responsibilities and training.

    Clear management responsibilities are a key pre-requisite to the maintenance of high standards. Only those who have been trained should have access rights to both the physical cryostore and the management database. All location changes should be verified by a second, competent individual. Experience from the EnAlgae project, interrogating archive sample data, has demonstrated that human errors can result in a significant level of mistakes in the location mapping of samples and stock-levels.

12. Emergency plan.

    It is important to ensure that there is a clear emergency plan in place to deal with any catastrophic failure in refrigeration. Routine visual inspection in addition to any remote monitoring and alarm systems is a pre-requisite. Clear lines of responsibility and communication are needed as is coverage over weekends and public holidays. The use of multiple refrigerators to hold samples of any given strain can reduce, but not completely remove, the risk of loss. In addition, as is the case in the CCAP collection at SAMS, spare refrigerators into which samples can be transferred provide insurance against sample loss.

EnAlgae Output WP1A4.05

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Annex 1: Additional information on regulations relevant to the commercial exploitation of microalgae & cyanobacteria

Action	Requirement	Law, Regulation, Convention	Further information
Collecting in the field	Prior Informed consent from a recognised authority	Convention on Biological Diversity (CBD)	http://www.cbd.int
	Mutually agreed terms	Convention on Biological Diversity (CBD)	http://www.cbd.int http://www.cbd.int/abs/instruments/
	Consent from the land owner	Property law	
Import	Non-indigenous plant pathogens require licenses from country authority	Quarantine regulations	
	Human, animal and plant pathogens can often only be imported to specified laboratories	Health and Safety	
Handling: Manipulation; Growth	Containment dependent on hazard	Control of Biological Agents - Health and Safety EC Directive 2000/54/EEC on Biological Agents	http://eur-op.eu.int/opnews/395/en/r3633.html
Genetic manipulation	Containment of manipulated organisms	EEC Directives 90/219/EEC. Contained use of genetically modified microorganisms (GMO's), *L117 Volume 33, 8 May 1990. EEC Directives 90/220/EEC. Release of GMO's, *L117 Volume 33, 8 May 1990. Cartagena Protocol on Biosafety	http://www.biodiv.org/biosafety/protocol.asp http://biosafety.ihe.be/Menu/BiosEur1.html http://biosafety.ihe.be/Menu/BiosEur1.html
Deposit as part of a patent process	Long-term storage and compliance with the Budapest Treaty	Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure	http://www.wipo.int/treaties/en/registration/budapest/
Storage	Appropriate containment	Health and Safety Licence to hold pathogens Security	
Export to another country	Some plant and animal pathogens require export licences	Quarantine regulations	
	Dangerous organisms with potential for dual use	Export Licences for dangerous organisms, Biological and Toxin Weapons Convention (BTWC)	http://binas.unido.org/binas/reqs.php3 http://www.opcw.nl/fact/rel_conv.htm http://www.dfat.gov.au/isecurity/pd/pd_4_96/pd9.html
Distribution	Packaging and transport considerations	IATA Dangerous Goods Regulations (DGR), Universal Postal Union Convention (UPU) United Nations Sub-Committee of Experts on the Transport of Dangerous Goods (UNSCETDG)	http://www.iata.org/cargo/dg/dgr.htm http://www.upu.int/ http://www.unece.org/trans/danger/danger.htm
	Sovereign rights over the strains	Convention on Biological Diversity	http://www.cbd.int
	Access and benefit sharing	Bonn Guidelines	http://www.cbd.int
	Intellectual Property Right ownership Customer licensed to receive organism?	Copyright	http://www.wipo.org
	Dangerous organisms export	EU Council Regulation 3381/94/EEC on the Control of Exports of Dual-Use Goods from the Community	http://eur-op.eu.int/opnews/395/en/r3633.html See national Export Offices