

# TRANSLATING CRYOBIOLOGY PRINCIPLES INTO TRANS-DISCIPLINARY STORAGE GUIDELINES FOR BIOREPOSITORIES AND BIOBANKS: A CONCEPT PAPER

Erica E Benson<sup>1\*</sup>, Fotini Betsou<sup>2</sup>, Barry J Fuller<sup>3</sup>, Keith Harding<sup>1</sup> and Olga Kofanova<sup>2</sup>

<sup>1</sup>Damar Research Scientists, Drum Road, Cuparmuir, Fife, Scotland, UK

<sup>2</sup>Integrated Biobank of Luxembourg, 6, rue Ernest Barblé, L-1210 Luxembourg

<sup>3</sup>University Department of Surgery, Royal Free and University College Medical School, UCL, London UK

\*Corresponding author: [e.benson-damar@tiscali.co.uk](mailto:e.benson-damar@tiscali.co.uk)

## Abstract

Low temperatures are used routinely to preserve diverse biospecimens, genetic resources and non-viable or viable biosamples for medical and clinical research in hospital-based biobanks and non-medical biorepositories, such as genebanks and culture, scientific, museum, and environmental collections. However, the basic knowledge underpinning preservation can sometimes be overlooked by practitioners who are unfamiliar with fundamental cryobiological principles which are more usually described in research literature rather than in quality and risk management documents. Whilst procedures vary, low temperature storage is a common requirement and reaching consensus as to how best it is applied could facilitate the entire biopreservation sector. This may be achieved by encouraging an understanding of cryoprotection theory and emphasizing the criticality of thermal events (glass transitions, ice nucleation, thawing) for sample integrity, functionality and stability. The objective of this paper is to inspire diverse biopreservation sectors to communicate more clearly about low temperature storage and, raise awareness of the importance of cryobiology principles to field newcomers and biopreservation practitioners, by considering how the principles may be translated into evidence-based guidelines for biobank and biorepository operations.

**Keywords:** biobank, biorepository, biopreservation, cryobiology, storage guidelines

## INTRODUCTION

The preservation of biological samples, germplasm and culturable organisms at low temperatures, ranging from refrigerated chilling and freezing, to cryostorage in liquid nitrogen (LN) has historical timelines that span decades (29,30,114). Consequently, the application of cryobiology has charted different paths (44) defined by the type and utility of scientific collections, biological resources and biospecimens, culminating in biobanks, that for example, service personalized medicine (58). Correspondingly, modern genebanks and culture collections underpin biodiversity studies and the sustainable use of genetic, biological and biotechnological resources (10,29,114). The Biological Resource Centre (BRC) concept was created by the Organization for Economic Co-operation and Development (OECD) for the biotechnology sector but it now has wider relevance (92). Although at the technical level, low temperature storage is routine in most biopreservation communities, its' underpinning science is sometimes overlooked as practitioners do not necessarily have a specialist knowledge of cryobiology, leading in some circumstances to incorrect assumptions. Advances in biomolecular techniques and sensitive, high-throughput analyses demand stringent, routine biobank operations (3,13,31,33,38,100) of which low temperature storage is a core component

(11,13,15,16,51,72,76,85,86,112,113). BRC criteria require biological resources recovered from storage to be authentic, free from contamination, stable and fit-for-purpose (92) and upholding these principles concerns most biopreservation communities. This involves standardizing and validating procedures (10,11,13,16,108) and using performance indicators (PIs) which are the results of quality control (QC) assays or tests performed on samples (17). Biopreservation needs to satisfy well defined PIs for sample type, storage regime and utility (13,31,38,100) and repositories have regulatory, quality and risk management obligations towards stakeholders and end users (20,25,26,27,43,93,116,117,118,119,124). Thus, storage conditions and cold chains need to be safe, reliable *and* operate within Quality Management Systems (QMS).

### *Translational perspectives*

The overarching aims of this paper are to explore how low temperature preservation cross-cuts thematic boundaries and consider how the biopreservation goals of maintaining quality and stability can be *translated* into basic methodological guidelines. There is a general realization that greater connectivity is needed to bridge the gap between cryobiology science and its applications. This is personified, by two interconnected perspectives: (a) the cryobiology research scientist who explores the fundamental issues attributed to ‘life in the frozen state’ and (b) the biobanker, or collection curator, tasked to ensure that preserved samples retrieved from storage are of the highest possible quality and fit-for-purpose. Contemporary biobanks often conserve a multiplicity of samples under different storage conditions, ranging from extracted DNA stored at -80°C, to viable cell lines cryopreserved in LN, to tissues preserved in formalin and histology substrates. As a step towards achieving consensus about low temperature storage guidelines, the authors have compiled a series of concepts within the framework of biospecimen science (9,15,16,17,36,74,78,88). The incentive is to connect the experiences of different sectors and use their combined knowledge to facilitate technical practices (21,29,30,44,60,63). To emphasize the importance of the translational paradigm (10, 90), the scientific and quality management principles representing different disciplines and sectors have been purposefully intermixed, an approach that accentuates how cryobiological principles can influence critical steps in storage protocols – noting however, that each sector is affected by policies and regulations. These will dictate levels of stringency regarding technical operations and quality management and for certain biopreservation communities, infrastructure, resources and cost restrictions will limit capacity to action some guidelines.

### *Transcending traditional thematic boundaries*

There are benefits in fostering the reciprocation of expertise regarding cryobiological research and translating its outcomes into best practices and evidence-based, risk management and QMS. The ‘melding’ of knowledge helps to break down traditional disciplinary boundaries, reveals their connectivity and stimulates wider debate about low temperature preservation. In this paper, the cross cutting of thematic boundaries has been encouraged by: (a) involving co-authors with diverse perspectives; (b) interacting with the Society for Low Temperature Biology (SLTB, <http://www.sltb.info/>); the Society for Cryobiology (SfC, [www.societyforcryobiology.org](http://www.societyforcryobiology.org)) and the International Society for Biological and Environmental Repositories (ISBER, <http://www.isber.org/>) and (c) engaging a team of 17 reviewers representing different biopreservation communities. The importance of transcending thematic boundaries is evidently explained by Scientific Collections International (SciCOLL, [www.scicoll.org](http://www.scicoll.org)) which identifies the pressing needs of the museum sector as: (a) improving interoperability among scientific collections in different disciplines; (b) creating/enabling opportunities for researchers in different disciplines to undertake next generation research needing access to collections across scientific disciplines; (c) promoting the development and use of standards and best practices; (d) catalyzing the development/implementation of data management policies and practices that improves access to and interoperability among collections in different disciplines. System-wide guidelines (13) are relevant for biobanks and biorepositories preserving multiple taxa and specimen types, as is the case for environmental biobanks (97), zoos and botanical gardens preserving samples in centralized facilities. Some culture collections and biobanks conserve different forms of specimens of diverse origin, for which system-wide guidelines can enhance interoperability, as exemplified by the System Wide Genetic Resources Program (13,104).

This paper is in two parts, the first introduces ‘core’ concepts; the second is a compilation of generic low temperature guidelines, for which the definition of the Clinical and Laboratory Standards Institute (2005) is used (23). A guideline is “*A document developed through the consensus process describing criteria for a general operating practice, procedure, or material for voluntary use. A guideline may be used as written or modified by the user to fit specific needs.*” As sectoral differences in terminology can create barriers, typological explanations and definitions of terms have been included as concepts. Target readers are researchers, practitioners, field newcomers, students and stakeholders across all biopreservation fields.

## CORE CONCEPTS

This section examines what we consider to be five ‘core’ concepts which may be used to guide more robust, comprehensible and translatable applications of cryobiology that the biopreservation community can take, merge and modify for their specific requirements.

1. *Biopreservation typology* - sharing trans-disciplinary perspectives as to what defines different types of biorepositories and biobanks and understanding how they have evolved across diverse thematic sectors.
2. *Biospecimen and cryobiological process chains* - identifying and understanding the impacts on outcomes of different biopreservation steps and their associated practices.
3. *Use of terms and definitions* - highlighting the importance of talking the same, clear language regarding cryobiological and quality/risk management nomenclature and using it in a non-ambiguous and appropriate context.
4. *Biospecimen science and observing the principles of cryobiology* - exploring how best to apply cryobiological knowledge to successfully preserve biospecimens and biological resources, and retrieve them from storage as fit-for-purpose.
5. *Translating cryobiological principles into generic storage guidelines* - using generic guidelines to inform the development of evidence-based, biopreservation protocols and their associated practices in the context of quality and risk management systems.

We do not claim that these are the only core concepts which can be proposed; rather, we have used them to fix reference points on which to construct nascent, scientifically informed guidelines. These provide a basic platform which conveys how a knowledge of cryobiology principles may be incorporated across sector specific biopreservation guidelines.

### *Concept 1. Biopreservation typology*

Historically, scientific, natural history and medical pathology collections may be considered progenitors of modern environmental and clinical biobanks (95,97,114). Therapeutic, diagnostic and interventional medicine adopted the generic ‘banking’ model prefixed with a description of their holdings. Correspondingly, the first seedbanks promoted the concept of service collections which collected and distributed plant genetic resources and thus formed the basis of modern seed biopreservation guidelines (39,98). The International Seed Testing Association (ISTA, [www.ista.org](http://www.ista.org)) founded in 1924, with members in over 70 countries, publishes standard seed testing procedures. Modern genebanks representing biodiversity across various taxa are repositories of replicable, genetic resources (germplasm) and hereditary materials (DNA, RNAs). For instance, the *In Vitro* Genebanks (IVGBs) of the Consultative Group on International Agricultural Research (CGIAR, [www.cgiar.org](http://www.cgiar.org)) use a range of biopreservation methods (13,31,75,98,99).

The first microbial collections established by Kral in 1869 for industrial purposes recognized the need to maintain strains under controlled laboratory conditions (114). The concept of the BRC, as a service provider of high quality biological materials *and* their associated information emerged from the culture collection community to meet the needs of the biotechnology era (92). In the microbial sector the majority of microbial resource centres are aligned to the World Federation for Culture Collection (WFCC, <http://www.wfcc.info/>), this is also the case for animal cell collections and some stem cell banks. Regional level organizations such as the European Culture Collections' Organization (ECCO, [www.eccosite.org](http://www.eccosite.org)) comprise collections that hold yeasts, filamentous fungi, bacteria and

archaea, phages, plasmids recombinant DNA constructs, animal cells, viruses, plant cells, algae and protozoa. The biopreservation landscape also includes large scale international research infrastructures, such as the EU funded road map project, Microbial Resource Research Infrastructure project (MIRRI, [www.mirri.org](http://www.mirri.org)).

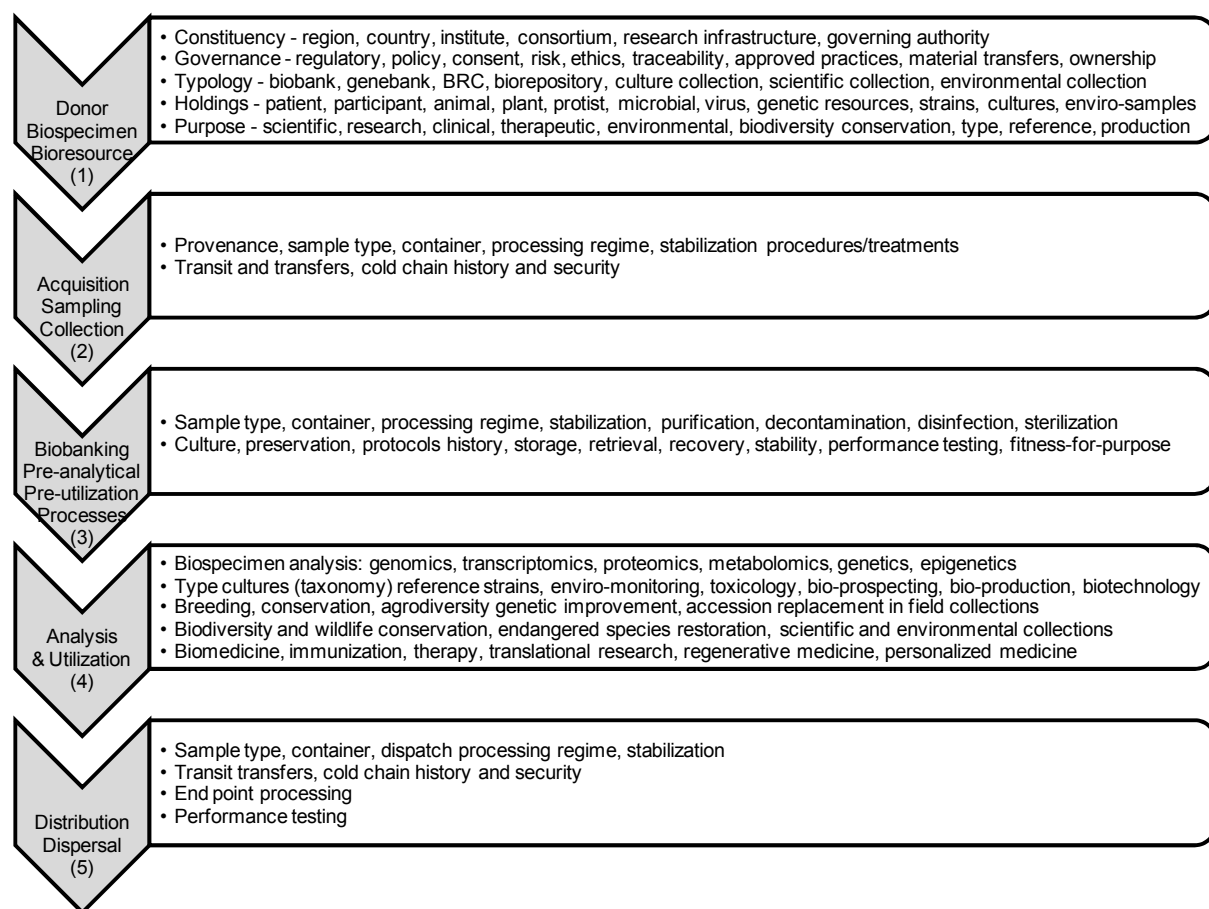
**Diverse typologies.** Terminology varies across biopreservation sectors therefore, two generic terms, biorepository and biobank, are used in this paper, albeit terminology overlaps where they have been adopted by different communities. Based on the BRC model, a *biorepository* refers to a specialized facility in which biological resources (biospecimens, cultures, genetic resources, germplasm, DNA) are collected, processed and stored, usually for the long term, and distributed to end users. A BRC supports research and offers services to clients and stakeholders representing different sectors (agriculture, aquaculture, biodiversity, biotechnology, forestry, horticulture, conservation, environmental protection/management, veterinary or human medicine). According to the OECD (92) definition, BRCs (*inter alia* biorepositories) must meet the high standards of quality and expertise demanded by the international community of scientists and industry. The third edition of ISBER's Best Practices for Repositories (63) gives the definition of a biobank or biorepository as: "*an entity that receives, stores, processes and/or distributes specimens as needed. It encompasses the physical location as well as the full range of activities associated with its operation.*" Based on this more general definition, the term biobank can encompass various types of repository (cell banks, genebanks, tissue banks, culture collections).

Biorepositories and biobanks should sustain appropriately stringent procedures (with respect to their *modus operandi*) for the safe receipt, quality management, storage and distribution of their holdings, for which definitions also vary. The Convention on Biological Diversity, Article 2 'Use of Terms' (46) states: "*Biological resources include genetic resources, organisms or parts thereof, populations, or any other biotic component with actual or potential use or value for humanity.*" Whereas, the OECD (92) defines biological resources as "*living organisms, cells, genes and their related information that are the essential raw materials for the advancement of biotechnology, human health care and research and development in the life sciences.*" ISBER (63) defines "*biospecimen-resource as a collection of biological specimens that is acquired for a defined purpose for which management responsibility is led by the custodian of the collection and the biospecimen resources may be stored in a repository or laboratory depending upon the numbers of specimens contained therein.*" Defining typologies that satisfy diverse biopreservation sectors requires careful consideration moreover, not all biorepositories have a well defined purpose, for instance where collections are merged, or their utility changes when new technologies become available. This may be the case for some scientific and museum collections thus, the Global Genome Biodiversity Network (GGBN), Tree of Life, Policies and Practices Task Force Team is working to update the 4<sup>th</sup> edition of ISBER's Best Practices to include non-human and non-medical specific biorepository issues, including the clarification of terms regarding biospecimen, bio-sample and biological resource. To make more straightforward this paper, the generic terms 'biospecimen' and 'biological resource' are used.

## *Concept 2. Biospecimen and cryobiological process chains*

The 'Biospecimen Process Chain' (BPC) is the summation of all the processes and connected procedures that a biospecimen or biological resource undergoes, as it progresses from the point of collection to pre-analytical processing, maintenance, storage, retrieval, recovery and dispatch to end users. Sample characterization may be included at different stages and extend to downstream applications as defined by the biospecimen or biological resource. The journey of a biospecimen is 'signposted' by management and operational perspectives (regulatory, ethical, quality and risk management) that are sector specific. A generic, five-step process chain is represented (Fig. 1) in the context of hierarchical (i.e. regulatory to technical) frameworks and their associated procedures, each of which may concern cryobiological manipulations. At the governance level (Fig. 1) regulations can limit the duration of time that samples are held in cryobanks, as might be the case for biospecimens from clinical trial participants or, samples collected from regulated regions such as Antarctica that fall within the jurisdiction of the Antarctic Treaty. Similarly, approved practices e.g. the Convention on Illegal Trade in Endangered Species (CITES) have to be in place when storing germplasm from endangered taxa, and biodiversity conservation is undertaken in accord with the Convention on Biological Diversity (CBD). Technical detail is also important, for instance sampling container can be

considered as a standard pre-analytical code (SPREC) element because its intrinsic properties can interfere with post-storage analysis (Fig. 1). Similarly, some plastic containers are incompatible with cryogenic storage because they are not resilient to LN. Pugh *et al.* (97) considered the general requirements of environmental biobanks, those of relevance to cryobiological principles are: (a) long term storage (decades) under conditions that ensure stability and security; (b) well organized sample inventory and tracking systems that allow rapid retrieval of specimens and their associated data for future researchers; (c) storage vessels, containers, freezers and facilities that minimise the potential risks of contamination and changing the properties or composition of the specimen; (d) conditions, procedures and equipment that allow the production of aliquots for analysis that minimize the risks of incurring changes to the sample and the preserved specimens.



**Figure 1.** A five-step generic process chain presented in various biopreservation frameworks. Block arrows (1-5) represent five generic steps of a simplified biospecimen/biological resource process chain, from the point of entry Stage 1 (donation) to Stage 5 (distribution and dispersal). The attached text bars give examples of the various frameworks (governance, operational, technical) and procedures (sampling, analysis, performance testing) that can impact on representative holdings (microbial, human, plant) of biospecimens and biological resources as they journey through the biopreservation process chain as indicated for different types of repository (biobank, genebank, culture collection). BRC = Biological Resource Centre.

### Concept 3. Use of terms and definitions

An unambiguous understanding of terminology will help to establish low temperature storage best practices and guidelines across different communities. This is exemplified by the common use of ‘freezing’ and ‘thawing’, terms that are liberally used to describe the taking of biomaterials to, and recovering them from, low temperatures. A more exacting chemical definition of freezing is ‘*the process by which a liquid becomes a crystalline solid at a temperature which is sufficiently cold*’. In biophysical terms, it is an event, in relation to ice formation that occurs over a narrow temperature range, rather than over a complete thermal history of progression to extreme low temperatures.

However, for the purposes herein, we will use the term ‘freezing’ to convey the concept (which is generally understood by the public) of cooling, achieving ice crystallization, and then continuation of cooling of the icy matrix until the much lower preservation temperature is reached. Target preservation temperatures stabilize biospecimens and biological resources during storage. The rationale is equally true for the reverse process, thawing, this is where samples are progressed back up the temperature scale, eventually reaching the equilibrium melting point where the disorganization of crystalline ice occurs. These definitions may seem overly pedantic, but they become important when referring to preservation in the absence of ice (vitrification) and their inappropriate usage can cause misunderstandings in protocol descriptions.

Tables 1 and 2 convey the importance of defining and using terms appropriately and consistently, not only across the diverse sectors that use low temperature preservation, but also between researchers and practitioners; for a comprehensive glossary of cryopreservation terms, see Shaw and Jones (106). Points of nomenclature may seem trivial, but they can be a source of confusion in scientific discussions between different disciplines. A case in point is when the terms freezing and thawing are used inappropriately to describe steps in vitrification protocols, the aim of which is to avoid ice formation. The unsuitable use of terms can also lead to misunderstandings during validation, proficiency testing and technology transfers, especially when collaborators are dispersed across large consortia on different sites.

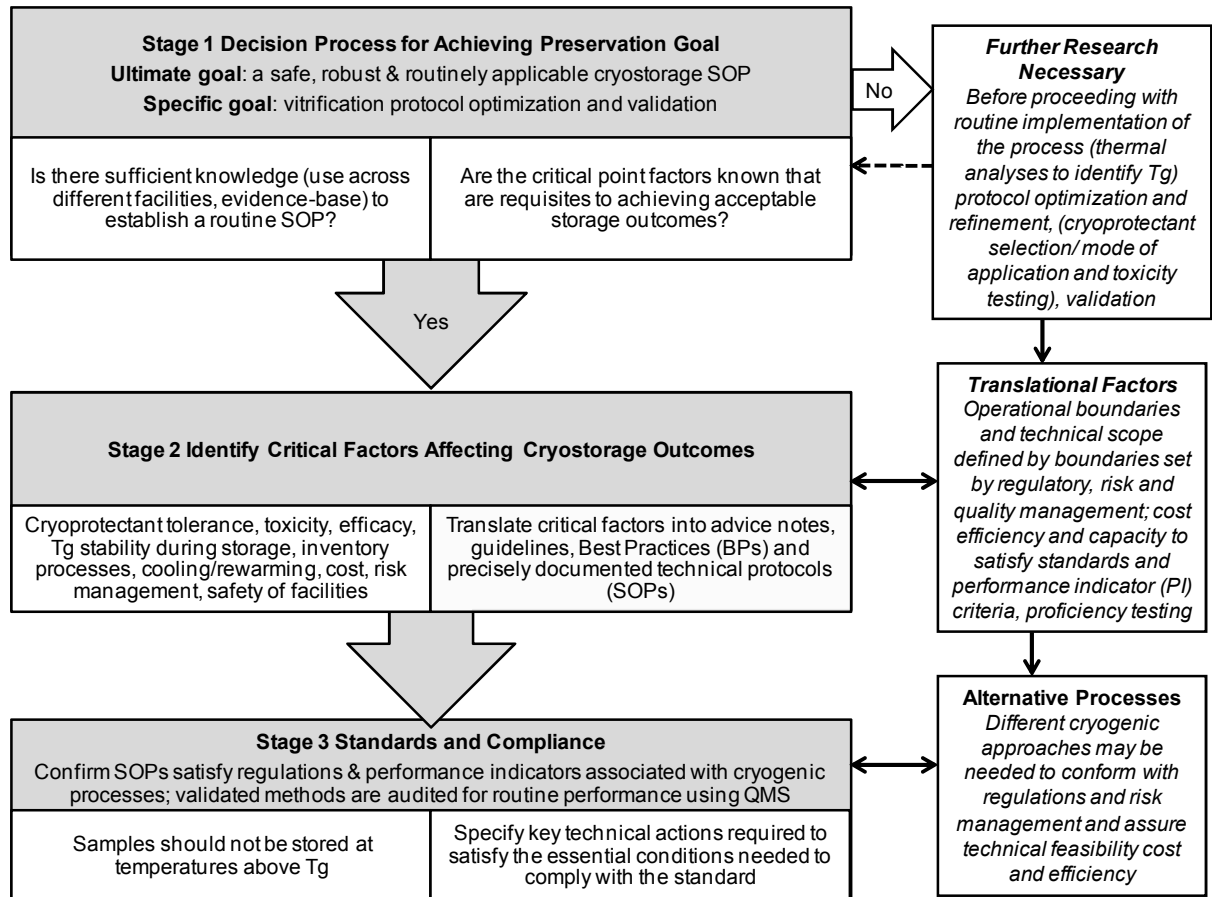
Researchers similarly benefit from an explicit understanding of formal quality and risk management nomenclature, facilitating cooperation with managers working within logistical, operational, regulatory and accreditation frameworks that use a particular vocabulary to define precise procedures (Table 2). An exact comprehension of terms is necessary to describe work flows used for accreditation, validation, proficiency testing, risk management and record keeping and is essential for writing accurate, unambiguous, standard operating procedure (SOPs) and handbooks. Unequivocal ontology is needed for information management systems, interfacing sample inventories for electronic barcodes, Laboratory Information Management Systems (LIMS) and Standard Pre-analytical Variable codes (SPRECs). Proper use of terms supports the interoperability of virtual infrastructures and consortium projects (76) and enhances the cycle of audits and corrective action plans that drive up standards. Unambiguous definitions and nomenclature are prerequisites for biopreservation education, work place training and technology transfer and they properly inform regulators, stakeholders and the wider public about cold preservation technologies. Typical examples that demonstrate the appropriate use of terms (63) are: “specimen” (a specific tissue, or blood collected from an individual or donor); “sample” (a single unit, containing material derived from one specimen, that can be stored) and “derivative” (a product or analyte derived from an original sample).

#### *Concept 4. Biospecimen Science and observing the principles of cryobiology*

High-throughput processing and large scale -omics analyses for biomarker discovery (35,123) prompted the creation of the new discipline of Biospecimen Science to meet the rigorous needs of translational and personalized medicine (100). Central to which is the processing, tracking and preservation of huge numbers of samples by the most effective and safest means possible. Whilst Biospecimen Science has evolved in the medical sector, it provides a common framework that may help build consensus regarding the development of low temperature storage guidelines across other biopreservation communities. Table 3 summarizes the technical and regulatory practices that are employed by different biopreservation communities. Despite the taxonomic diversity (microorganisms, humans), evolutionary range (prokaryotes, eukaryotes), and differences between specimens (viable and non-viable, pathological and non-pathological primary, derivative and analyte) and biological resources (somatic, regenerative, reproductive cells, DNA, whole organisms) there are marked similarities across storage technologies (Table 3). Sectoral differences in procurement, logistics, policy, regulatory practices and stakeholders/end user needs will, however profoundly dictate biopreservation operations. Nevertheless, some problems affect all biopreservation sectors, such as the tolerance or sensitivity (recalcitrance) of certain specimens and biological resources to low temperature storage (10). Thus, Concept 4 emphasizes the relationship between observing cryogenic principles and achieving successful storage outcomes. It considers how cryoprotection, low temperature stabilization and preservation are achieved across different biopreservation communities

and shows how cryobiological principles may be identified as critical factors that can be embedded as technical points in procedures, SOPs, QMS and risk management guidelines (Figs. 1, 2).

Cryoprotective treatments enable viable cells, tissues and organisms to survive exposure to LN temperatures (42) and their optimization and translation into SOPs is critical to developing successful biopreservation methods (Fig. 2). Full discussions on cryobiology theory are beyond the scope of this paper, readers wishing to gain greater insights regarding specific taxa, disciplines and cryobiological research are directed to other literature (5,8,29,30,44,99). Concept 4 draws attention to the cryobiological principles that are generally applicable to biopreservation and uses sector specific examples to illustrate how they may be incorporated as critical points in technical, operations and associated practices; these are presented as follows:



**Figure 2.** A decision pathway of the stages involved to achieve a low temperature storage biopreservation goal using a vitrification-based protocol as an exemplar. Different arrows indicate: the main decision pathways (large open arrows); junctures where further research may be necessary regarding how a protocol is developed (dashed line arrow); examples of translational factors and alternative processes that support the decision pathway (black line arrows) and *vice versa* (black line, two way arrows) examples demonstrating where critical factors, QMS, risk and regulatory issues may influence the design and planning of translational biopreservation research projects.

Medium term storage (MTS) at suboptimal growth temperatures. This involves reducing the growth of *in vitro* plants, by lowering the temperature (~4°C for temperate species; ~10-15°C for tropical species) at which the cultures are maintained; the practice can also be applied to some protists and microorganisms. The objective is to enhance maintenance efficiency and reduce the risks and costs of repeated subculturing at standard growth temperatures. Success is dependent upon monitoring the health of MTS accessions and their timely regeneration and rejuvenation (13).

Cold chains, transfer conditions and logistics. Interim ‘holding’ at intermediate low temperatures is sometimes necessary to stabilize samples during their acquisition, pre-analytical processing, transfer and delivery to and from a central storage facility. Thus, low temperature guidelines should extend across the entire cold chain and include the selection of PIs that evaluate the impacts of collecting

procedures and cold chain transportation on sample stability (10,57,69,128). In the case of medical samples, such as blood, pre-analytical variables encountered during sampling and prior to storage are critical factors. Samples should be stabilized during transit (e.g. in dry shippers, on ice, wrapped in gel packs) to and from cryostores and to the processing laboratory. Providing pertinent, detailed documented information about the timing of events and recording the interim temperatures to which samples have been exposed during collection, stabilization and processing before and after storage is crucial, but may not be easily achievable. Logistics are particularly challenging for biodiversity and wildlife biospecimens and biological resources sampled from remote and extreme environments (Table 3). For some environmental/biodiversity biobanks the continuous monitoring of temperature *and* humidity throughout transit (including airports, customs) is necessary. The risks of compromising sensitive materials from areas with difficult transport logistics and limited infrastructures may be managed using specialist couriers and tagging cryovials and dry shippers with a Radio Frequency Identification Device (RFID).

Physiological acclimation. Whilst the principles of cryoprotection are generally applicable, there are some distinct taxonomic differences. In some microbial, protist and plant species, cryoprotection can be enhanced, or substituted by inducing natural acclimation or simulating stress adaptations (99,10). These ‘pretreatments’ include osmotic additives or biochemicals (trehalose, proline, abscisic acid) that increase tolerance to stress; alternatively cold acclimation (cold hardening) regimes enhance natural resilience to low temperatures.

Cryoprotection in the frozen state. Two primary biophysical factors are involved in freezing injury, colligative damage and intracellular ice (82). The chemical *sensu stricto* use of the term ‘colligative’ refers to the modulation of solute concentration, and as such colligative-acting cryoprotectants do not have to be cell penetrating as non-permeating additives can also act colligatively. However, the term colligative is more commonly used in the cryopreservation literature to describe the mode of action of cell-penetrating cryoprotectants that are applied to offset the damaging solution effects incurred during cryopreservation. Here we introduce the more precise term of ‘colligative-acting’ for cryoprotectants (cell-penetrating or non-penetrating) that modulate solute concentration during cryopreservation. These additives, applied in conjunction with optimal cooling protect cells against damaging solution effects, harmful volume changes and lethal, intracellular ice. During controlled rate cooling ice is initially formed extracellularly, creating a differential water gradient across the membrane with the consequence that water moves outside the cell (42,82). This has a protective effect because the amount of intracellular water available to form ice crystals is reduced. Although cells are able to survive in extracellular ice, it can be harmful for larger cells or complex tissues; whereas, intracellular ice is almost always lethal with rare exceptions. As water is expelled from cells during slow cooling the concentration of solutes is increased, the cells shrink and the freezing point is depressed. Thus, the main principles of controlled rate cooling are that ‘just the right amount’ of water is removed to prevent intracellular ice formation but, at the same time, the toxic concentration of solutes is avoided by applying colligative-acting, permeating cryoprotectants (e.g. dimethyl sulphoxide [DMSO]). These additives prevent the harmful cell volume changes that are caused by solution effects and they depress the freezing point, so that when nucleation does occur it is not as injurious. Colligative cryoprotectants also enhance cell viscosity and impede the mobility of water molecules; because they are equally distributed across membranes, a more uniform cooling rate is achieved which improves recovery in heterogeneous tissues of variable water contents. Colligative-acting agents are often combined with non-penetrating, osmotic additives (polyols, sugars) that also remove potentially freezable water from the cell, and these are often the cryoprotectants of choice for plant cryopreservation (10).

Cryoprotection in the glassy state. Colligative cryoprotection in combination with slow cooling raises the  $T_g$  at which the cells within the icy matrix vitrify, although extracellular freezing still occurs, this is generally considered as preservation in a ‘partial’ glassy state. In contrast, totally ice free cryogenic storage is achieved by increasing medium and cell viscosity to the point at which ice formation is inhibited, inside and outside the cell i.e. the entire system becomes vitrified at storage temperatures (10). This type of ‘operational vitrification’ (commonly termed vitrification in the literature) is not a true physico-chemical equilibrium and is metastable, consequently rewarming must be optimized to avoid: (a) glass relaxation, which creates tension fractures that can damage fragile structures; and (b) devitrification which risks ice nucleation and the formation of damaging ice crystals during warming. The operational vitrified state is achieved by manipulating cell viscosity using two



main approaches: (a) the addition of penetrating cryoprotective additives at high concentrations; (b) the removal of water by osmotic dehydration and/or evaporative desiccation. Such vitrification involves rapid cooling rates, the direct plunging of samples into LN and optimizing rewarming to ensure glass stabilization around the characteristic  $T_g$ , which is critical. Operational vitrification is commonly used (Table 3) to cryopreserve microbial, animal and plant cells and tissues (10,30,44,99). Plant Vitrification Solution 2 (PVS2) developed by Sakai and colleagues (101) has been particularly successful for the cryostorage of diverse plant taxa. Vitrification additives are often the same as those used to preserve cells in the frozen state, but they are applied at higher levels which can be toxic, therefore, their application has to be carefully optimized. The Liquidus Tracker has been designed to vitrify sensitive, biological samples that cannot be preserved using controlled rate or ultra rapid cooling required for vitrification (127). The instrument tracks the concentration of cryoprotectants and slowly increases their delivery during cooling to outpace potential ice nucleation.

Droplet methods. Rapid heat transfers during the cooling and rewarming of droplets respectively reduce the potential for ice nucleation and devitrification. Droplet freezing and droplet vitrification are more commonly used to cryopreserve plant germplasm (99,94,102). The procedure involves applying cryoprotectants to tissues or cells suspended in  $\mu\text{L}$  droplets (DMSO for droplet freezing; PVS2 for droplet vitrification), these are deposited on aluminium foils which are directly plunged into LN. During rewarming, the foils are removed from cryovials and directly immersed in liquid medium at ambient or controlled temperatures, the tissues dislodge and are recovered in medium. Nanoliter droplet vitrification is being developed for oocytes using automated technologies (130), the aim is to achieve higher rates of cooling/warming than is possible for standard vitrification protocols.

Encapsulation methods. These involve encapsulating cells, tissues and organisms in calcium alginate beads and exposing them to chemical cryoprotection, osmotic dehydration and/or evaporative desiccation; the protective principle is vitrification. Encapsulation has been applied to microbial, protistan, plant, animal and human cells, tissues and organisms (11,28,41,80,99). Alginate beads are usually plunged directly into LN, although some protocols include a slow cooling step (28). PVS2 treatments combined with alginate encapsulation (encapsulation-vitrification) are used to cryopreserve storage recalcitrant plant germplasm (101).

Cooling regimes. These are the physical means by which a sample is cooled to a terminal (final) storage temperature; achieved using a range of procedures, including rapid, 'snap' freezing of non-viable samples, controlled, stepwise cooling (for structurally labile/sensitive non-viable specimens or viable cells), and the direct immersion of viable, vitrified cells into LN. Passive (e.g. Mr Frosty™) and computer programmable cooling devices precisely control cooling rates, hold times and ice nucleation (Table 3). Ultra rapid cooling (also referred to as ultra rapid freezing) involves plunging cryoprotected, viable biological resources into LN without intermediary cooling. This method is usually applied in droplet freezing/vitrification, encapsulation/dehydration, vitrification protocols and to highly desiccated microorganisms or plant germplasm such as orthodox seeds, dormant and acclimated vegetative tissues.

Controlled rate cooling. This precedes the introduction of a viable sample into a LN cryostore, allowing the operator to precisely control cooling rates and ice nucleation. Manipulating the point of extracellular ice crystallization above the temperature of homogeneous ice nucleation ( $-40^\circ\text{C}$ ) is a critical factor because it creates the conditions that allow intracellular water to move outside the cell (see above). The operator includes a 'hold' ('soak time') of controlled duration to commence around the temperature extracellular ice is 'seeded'. This may also be considered as heterogeneous ice nucleation, the temperature of which is dependent upon the thermal properties of the cryoprotectant biological system and/or the induction of ice by the operator. More robust cells may not require seeding. Controlled rate cooling resumes (following the ice nucleation-soak time) to an intermediate transfer temperature, at which another hold may be included to facilitate cryovial transfer. The terminal transfer temperature is usually between  $-35^\circ\text{C}$  to  $-40^\circ\text{C}$ , or below in some protocols, after which samples are transferred to LN for long term storage.

Terminal storage temperatures and critical cryogenic factors. The most common storage temperatures (Table 3) are hypothermic/chilling ( $4^\circ\text{C}$ ); subzero medium-low range temperatures ( $-20^\circ\text{C}$ ,  $-70^\circ\text{C}$ ,  $-80^\circ\text{C}$ ) and cryogenic (ultra low) temperatures achieved using electrical freezers ( $-140^\circ\text{C}$  to  $-150^\circ\text{C}$ ) or LN (vapour/liquid) usually to  $-130^\circ\text{C}$  dependent upon sample position in the vapour phase, to the minimum temperature ( $-196^\circ\text{C}$ ) of LN liquid phase (3). Non-viable biospecimens are

stored at cryogenic, freezing or chilling temperatures (Table 3), the stability and longevity of storage is sample and temperature dependent. Cryogenic storage in LN is usually required to conserve viable cells, tissues and organisms with the exception of some microorganisms; orthodox seeds and dormant buds that can be preserved in short, medium to long term storage at hypothermic/chilling or medium-low range subzero temperatures (Table 3). Temperatures are maintained using various equipment (refrigerators, static freezers, cryogenic freezers, isopentane baths, manual/auto-fill LN devices) the choice of which is based on sample type, rate of attrition and level of stability required to satisfy specific PIs (Table 3).

The significance of maintaining the glassy state. It is generally accepted that the most stable conditions are achieved using cryogenic storage in liquid or vapour phase LN (5,62). For long term stability cryopreserved biomaterials need to be stored at temperatures less than the critical T<sub>g</sub>, for which safe limits (10,63,129) are variously reported as -130°C to -132°C, i.e. below the T<sub>g</sub> of pure water. Wowk (129) reports that T<sub>g</sub>s are relevant to cryopreservation in the *totally vitrified* state and in the *frozen state*, in which cells survive in glasses between ice crystals. In the majority of samples that are thought of as *frozen* (i.e. with a large content of crystalline ice), there will still be a small component that forms a 'glassy' matrix. Current knowledge suggests that cells capable of functional recovery exist in the vitreous state at ultra low temperatures, even though they are surrounded by a 'wall' of ice, this explains why storage below the critical T<sub>g</sub> is crucial for both frozen and totally vitrified samples. It is precautionary to place precious materials in the bottom of storage vessels or in 'archives' that have low LN-loss systems and/or longer standing times (113).

Storage longevity. Qualifying what is meant by the term 'storage stability' is complicated as the condition ranges from maintaining the integrity of the glassy state to the preservation of genetic stability and functionality (see below). With respect to storage longevity, generally, the lower the storage temperature, the greater the stability of the sample and the longer it can be preserved before its deterioration (4,5,80). Terminal storage temperature largely dictates the length of time (longevity) a sample can be preserved and longevity dictates the future utility of samples. For example, it can define the type of storage regime, applied to active (MTS) and base collections (LTS) in *in vitro* plant genebanks (13) and the management of master and working stocks in animal, microbial and protistan culture collections. In these scenarios prolonged maintenance in serial culture can lead to genetic and phenotypic variation, therefore cryopreserving the original, master, reference and depository strains provides the only means of stabilizing the culture in its original state (113).

The biochemical and physiological (pH, growth regulators, hormones, co-factors, chelating agents, nutrients) properties of viable or culturable cells, tissues and microorganisms and, how they are stabilized before and during storage can influence longevity, as exemplified by the moisture content status of seeds (125,126). The stability of clinical, derivative samples, analytes and sub-cellular extracts is affected by preparative procedures, as is the case for RNA, for which storage solution composition (alcohol vs water-based buffer) can influence storage outcomes. *Importantly, conclusions drawn on one type of biospecimen cannot be extrapolated to other biospecimen types*, for example, what applies to RNA, may not apply to DNA, which is a more robust macromolecule. This is similarly the case for orthodox (storage tolerant) and recalcitrant (storage sensitive) microbial and protistan strains and plant and animal germplasm.

Longevity, technical and logistic considerations. Deviations from SOPs and suboptimal or laborious protocols compromise efficient practices, exposing biospecimens and biological resources to undesirable conditions that could affect their storage longevity. Non-conformity in the technical operations that maintain critical storage temperatures predispose samples to instability. This is the case for protein extracts in saturated urea buffers that are stored in -20°C freezers for which the upper temperature limit should not be > -18°C. This is because the freezing point of protein/urea buffers can be as low as -16°C, a temperature frequently monitored in the routine operation of -20°C freezers and potentially resulting in a failure of protein extracts to reach their critical freezing point and predisposing them to degradation. Appropriate choice of freezer technology is also crucial, for instance, 'frost-free' -20°C freezers continually operate cyclic temperatures, which can shear purified DNA samples and cause deterioration in stored nucleic acids (23). Variations in the use and maintenance of different storage facilities may be critical factors, as demonstrated for -20°C freezers which are not always managed as rigorously as -80°C freezers. Solutions and analytes are often temporarily stored at -20°C, in which case the upper alarm limit has to be chosen carefully based on a

prior knowledge of thermal lability. Contamination risks need to be evaluated regarding the logistics of holding and transit vessels and during sample transfers to and from the final storage vessel. For cryogenic storage, the prevention of contamination for samples held in the liquid phase of LN (10,115,116,117) should be addressed as long term storage vessels can accumulate ice sludge debris that act as reservoirs for microbial flora (10, 113).

Rewarming, thawing and significant thermal properties. Rewarming and thawing of respectively vitrified or frozen samples are significant factors that should take into consideration post-storage processing, handling logistics and analysis (Table 3). Whilst it is to be expected that the precise control of rewarming, thawing and thermal cycling is critically important for viable cells and culturable organisms, this can *also* be the case for non-viable specimens. Specific cases include, solid pathology tissues being placed and stored in an Optimal Cutting Temperature (OCT) compound at -80°C; and being aware that OCT compounds contain alcohols and poly ethylene glycol that may have an impact on the chemical structure of certain sample macromolecules (103,121).

Thermal resilience of containers. The physical-thermal properties of containers used during sample procurement should be considered; in general vacutainers and cryo-storage tubes should be thoroughly tested to ensure that they are able to withstand storage temperatures and thermal freeze/thaw cycles. The type of plastic from which the storage vessels/vials are made (homopolymer v random copolymer) and the method of sterilization (irradiation v clean production) may affect the rigidity, fatigue strength, depolymerisation and degradation of the plastics over repeated freeze/thaw cycles. Container properties could affect the quality of the samples (unpublished data), thermal resilience of vessels is especially pertinent for samples/biological resources held in very long term storage, as would be the case for base genebanks, archive collections and master cell lines of culturable organisms.

Thermal cycling and temperature fluctuations. The number of successive freeze/thaw cycles (105) that a biospecimen undergoes during its process history can affect sample stability, this is a particular issue for aliquots that are repeatedly retrieved and returned from 'bulk' stored samples for analysis (24,79). This is exemplified by the technique used to remove bacterial strains that are stored in glycerol in -80°C freezers, which can involve retrieving the cryovial, scraping a sample from the surface (without complete thawing) and putting the cryovial back into storage. Viability and growth of microbial strains can be compromised, dependent upon species tolerance, number of samplings and the time the vial remains at ambient temperatures, leading to variable and suboptimal storage outcomes. Temperature deviations occur each time cryovials are added to, or retrieved from a cryobox stored in LN and these routines can have consequences, particularly for labile samples. Short, repeated temperature excursions may cause microfractures across cell membranes and should be avoided by the use of cryocarts (34) although cost restrictions may limit their use in some institutions. Thawing and rewarming needs careful optimization, particularly in the case of vitrified biological resources (10,18,56,70,83), labile biospecimens and environmental samples exposed to complex cold chain logistics (Table 3).

Recovery of functionality and post-storage genetic stability. Post-storage recovery procedures are biospecimen and biological resource specific, their purpose is to restore functional capacity following storage (Table 3). Quality assurance (QA) measures require that strains, cell lines and cultures retrieved from cryobanks recover metabolic functions, retain their genetic integrity and, as appropriate, are totipotent and capable of replication or reproduction (10,13,22,29,113,114). Typical assessments of biomolecular stability include normal morphology, secondary metabolite production capacity (microbial strains); antibody secretion (animal cell lines) and totipotent/regenerative capacity (replicable cultures, germplasm). Assessments of genetic integrity and functionality span several levels (phenotypic, cytological, biochemical, molecular, genetic/epigenetic) and include: evaluation of totipotency and morphological characters, biometric studies of phenotypes, histological, cytological and karyological testing, confirmation of functional biochemistry (metabolites/proteins, proteins/enzymes), -omics analyses, biomarker studies, investigations of genome structure, DNA damage and repair, chromatin analysis, analysis of DNA adducts (methylation, oxidation) and confirmation of genetic modifications (22,51,55).

The optimization of long term storage parameters to ensure the preservation of genetic and epigenetic stability for large numbers of taxon-specific samples and genetic resources is a foremost challenge and has significant technical, logistic and cost implications. This is especially true for environmental, biodiversity and museum collections that hold a vast array of taxa and, for which

rigorous pre-testing of samples before committing them to long term storage in biobanks is essential. Variations in sample and biospecimen quality and suboptimal techniques can also contribute to storage instability (10), in the longer term their single or cumulative effects can compromise the fitness-for-purpose of stored samples (see below).

Physiological-molecular basis of recovery and cryoinjury. Baust and Baust, and colleagues (5,6,8) encourage a paradigm shift in cryopreservation research to take advantage of advances in molecular technologies. They identify diverse molecular/cellular responses, including delayed onset, cryopreservation-induced cell death as being contributory factors in storage failure (7). A delayed decline in post-storage recovery associated with free radical production has been observed in plant meristems recovering from cryopreservation (54) suggesting parallels with animal tissue (7,9). The potential for temporal shifts in gene expression being used as biomarkers for apoptosis and oxidative stress in the study of plant cryopreservation has been considered (52). Hubálek reviews the physiological mechanisms of CPA action in microorganisms (61) and Chen *et al.* (22) have undertaken physiological studies on cryopreserved fish spermatozoa. The cryobionomics concept proposed by Harding (51,54) examines links between cryoinjury, recovery and stability, providing a framework to assess cryostorage risks and their impacts on reintroductions into the environment.

Variability. It is important, to identify, reduce and where possible eliminate uncontrolled sources of variation introduced by low temperature manipulations (93). Standard pre-analytical variable codes identify pre-analytical variation (11,16,73,113) which is attributed to those processes that take place between the point of biospecimen collection and their use in experimental analyses and research. By definition, pre-analytical variation, including that associated with storage, must be within the control of operations undertaken by the biorepository (9,13,73). Pre-analytical variation does not however, encompass variation attributed to the intrinsic properties of samples (biological, genetic), personnel or uncontrolled environmental parameters. Where feasible, actions should be put in place to identify, reduce or eliminate these other sources of variability. Careful annotations, labelling and rigorous tracking systems can help identify, manage and reduce process-chain variation. High-throughput, automated operations simplify quality control and reduce operational and person-to-person variability, as does training, well described SOPs, proficiency testing and validation. If corrective actions are not possible efforts should be made to identify the source/extent of the variation.

Performance indicators (PIs) and sample auditing. PIs confirm that biospecimens and biological resources recovered from storage are functional and fit-for-purpose (Table 3). In biodiversity sectors they include biomarkers for biochemical functionality, developmental capacity (totipotency) and genetic/epigenetic stability as well as taxa-specific descriptors that confirm trueness-to-type (27,51-55,65,66,107). For protistan and microbial cultures the ‘three pillars’ of BRCs - stability, purity and authenticity are observed (92,113); for human cells, assessments of DNA damage, cellular replication and the stability of specific markers are used to refine preservation practices (1,59).

Salient examples of auditing preserved samples in therapeutic clinical treatments is where patients need to receive the rewarmed product as soon as it has been recovered; samples may experience slightly different thermal histories because of their small size, although this generally does not rule out the value of an audit sample (defined as a sample processed with identical concurrent preservation steps, and recovered solely for PI assessment at the same time as the therapeutic samples). Major deviations in thermal history or end-storage temperature need to be identified and recorded as a *Serious Adverse Event* (SAE). Whether these in turn will impact on the downstream behaviour of the stored products will depend upon their specific applications, and they can be important for both cellular and non-cellular biospecimens. In therapeutic (hospital-based) cryobanking, it is potentially possible that significant storage damage may have ensued, leading to a *Serious Adverse Reaction* (SAR) in the patients treated with the product that has been exposed to suboptimal preservation regimes, which must also be identified and recorded.

Performance indicators (PIs) and risk management. There is a fundamental connection between assessing post-storage performance and choosing appropriate PIs and risk management (see guidelines). PIs should be fit-for-purpose, which means that any QC assay applied should have been previously validated and provide the information required for the intended use (17). For example, RNA Integrity Number (RIN) may be an appropriate PI for next generation sequencing of an RNA sample, but not for a targeted qRT-PCR test. In all situations, a sample of products stored at low temperatures experiencing deviations in SOPs must be audited for the relevant performance outcomes

(Table 3) and a risk analysis made to determine if it will be acceptable to use the stored biospecimens, biological resources or products, or whether they should be discarded. Risk analysis can also define if a biospecimen is acceptable without conditions or under specific conditions (using the results of a specific PI). With respect to the cryostorage of cardiac valve allografts, Bessone and colleagues (14) reported on work that modelled a transient increase in storage temperature (from below  $-100^{\circ}\text{C}$  to  $-40^{\circ}\text{C}$ ) over 3 days before return to the stable end-storage temperature ( $-147^{\circ}\text{C}$ ). This deviation had been noted for a larger batch of heart valves undergoing a clinical storage protocol, because of a technical failure. The authors showed that by a number of indices, the valves experiencing the deviation in SOP demonstrated significant structural and functional impairments making them unsuitable for application (14). Whilst Bessone *et al.* (14) consider the criticality of applying robust performance criteria in a therapeutic context their study illustrates an important principle that has relevance across other biopreservation communities.

Regulatory considerations and quality management systems (QMS). There exist several examples of the regulatory, convention and policy frameworks in which different biopreservation sectors operate (Table 3). Very few are regulated to date and essentially those that are concern therapeutic end uses (e.g. umbilical cord blood and stem cells with therapeutic potential applications, or bone banks). The conservation of agrobiodiversity as global public goods (13) is governed by international treaties (104); the extent and stringency of regulatory and QMS requirements varies across sectors and applications (Table 3). General QA parameters are distinguished from those that are explicit to practitioner and end user needs, as exemplified by specific post-storage stability and functionality criteria (Table 3) dictated by the type of biospecimen or biological resource. Certain biorepositories, such as production biobanks, require particular quality measures because cells and cell substrates are used in the manufacture of medicines or for therapeutic purposes. Addressing their specific needs falls outside this paper's remit because they operate in distinct regulatory frameworks. These necessitate meticulous operational practices to meet end user requirements, in brief they include rigorous control of procurement, traceability, purity (axenicity) and specialized containment measures are needed to control inadvertent pathogen transmission through cells and biological media components (113). Procedures are implemented that offset the risks of genetic/phenotypic changes being incurred during passage and that mitigate the risks of cell tumorigenesis and the oncogenicity of cell DNA (113). Nonetheless, low temperature storage practices impinge on many of these factors, in which case the cryogenic concepts and generic guidelines highlighted herein may have value as baseline knowledge. In the remaining research-orientated biobanks, culture collections, genebanks and other types of biorepository, QMS is usually implemented on a volunteer basis, although resource-poor repositories may not be able to support full QMS or ISO accreditation. In these circumstances, aspirational planning to achieve the best possible quality outcomes, as dictated by infrastructure and budget boundaries is important.

In those sectors that are regulated, cryobiological principles are translated into official binding recommendations against which biobanks are audited for compliance. In non-regulated areas, the way in which cryobiological principles can be translated into Best Practices (BPs), SOPs and thereafter into QMS, is through the formal processing of method validation, QC and proficiency testing (10,12,13,17,52). For example, biospecimens and biological resources should be processed and stored according to the temperatures stipulated in their individual SOPs and evidence-based best practices. Storage facilities should be limited by controlled access, and have systems (log books, data loggers connected to automated temperature monitoring equipment) in place to record temperature fluctuations during sample retrieval/withdrawal and storage. Temperature stability profiles are related to longevity at different storage temperatures and during cold/cool chain delivery (41,80); they may be considered as QC elements (17,33). Method validation should employ evidence-based selection of critical factors (e.g. duration of time a sample is exposed to DMSO or other agents, medium composition, thawing delays) that will influence how robust a procedure is when it is applied on a routine basis by different operators. Thereafter, inter-biobank/biorepository audit and validation exercises on processing protocols and proficiency testing on QC methods can be implemented to assess competency (13,17,96).

Quality standards, fitness-for-purpose and acceptance criteria. Fitness-for-purpose spans several hierarchical levels, ranging from defining the quality of an individual sample to accrediting an entire institution as a service provider. In terms of quality standards, the fitness-for-purpose of a particular

product or process is demonstrated by an institution as a documented quality system. In culture collections, cell banks, genebanks and biobanks this may include the accreditation of formal ISO quality standards (Table 2) that are operated to ensure consistent service provision (10,13,113,114). Regarding microbial collections, current good manufacturing practices (cGMP) are also used for product QA and some BRCs adopt ISO 9001 certification or national standards that are specific for BRCs. At the ‘sample’ level evaluating fitness-for-purpose following low temperature storage is a core requisite across all biopreservation sectors and has to be initially defined, relative to the requirements of stakeholders, beneficiaries and clients/end users. Critically, fitness-for-purpose is defined by acceptance criteria that have to be decided up-front, after which the next stage is to perform bibliographical searches (17) and research (fundamental, applied, collaborative) that will translate performance outcomes into robust biopreservation methods (Fig. 2). This may be achieved by scrutinizing storage, stability and PI assessment methods with respect to reproducibility and robustness, this approach identifies critical factors associated with suboptimal storage procedures. Acceptance criteria used to assess the fitness-for-purpose of samples after storage can influence QC outcomes (17).

Documentation and traceability. Accurate documentation and rigorous tracking and record keeping of samples stored at low temperatures enhance sample handling efficiency, and minimize disturbance to critically controlled low temperature facilities (113,114). Choosing storage containers and labels that withstand the expected duration of storage at a specified terminal temperature offsets handling problems at a later time, particularly for samples that are held for years/decades in storage. Different levels of traceability (numbers/codes attributed to operator, accession, cell bank, strain, QC-reference, media batch, sterilization numbers) may be attached to stored samples; for production biobanks, referencing steps in process chains ensures traceability across procedures and QC audits (113).

Future proofing. Future proofing biopreservation practices with respect to storage longevity and quality issues is becoming an issue of considerable interest. In the case of long established scientific collections it is particularly relevant as they may possess unique and as yet unknown cellular and genetic information that can only be revealed by modern technologies. Natural history museums and herbarium collections may have ‘untapped’ potential regarding their molecular-genetic significance, particularly in the current biodiversity crisis. In these situations the utility and fitness-for-purpose criteria of historic archive collections and contemporary biospecimens held in low temperature storage may change as new molecular technologies add value to historical accessions, an outlook that has relevance across biopreservation sectors.

### *Concept 5. Translating cryobiological principles into generic storage guidelines*

Concept 5 bridges the ‘gap’ that defines the translational ‘bench-to-bedside’ paradigm, which, although developed by the medical sector, is now a broadly applied framework that focuses research targets on practical outcomes. The boundaries of translational research are typically (but not always) delineated by policies and regulations that address stakeholder and end users needs. Whilst storage protocols are not (so far) usually under regulatory control (with the exception of certain therapeutic applications, health and safety provision) they are influenced by policies, conventions and regulations, and storage practices may need to comply with overarching ethical, consensual and risk management guidelines (Table 3). Pertinent examples are secure sample containment (e.g. double bagging to avoid risks of contamination in cryostores), cold chain security and the maintenance of critical temperatures during transit (41). A knowledge of sample T<sub>gs</sub>, and the prospective selection of limits of storage duration (with recorded procedures to direct disposal or other end uses) at terminal temperatures has practical applications (80). However, thermal analysis requires specialist expertise and equipment and the characterization of T<sub>gs</sub> may not be a feasible option for some institutions. Ensuring the stability of samples under specific low temperature regimes requires evidence-based QC measures (17) regarding sample longevity at different storage temperatures (80). Another example of how knowledge of cryobiological principles can facilitate the development of biobank SOPs is in the processing and cryostorage of cell types that have a differential tolerance to cryostorage (Table 3) Although many cells may typically maintain a high level of viability when preserved using a common protocol, others (e.g. erythrocytes, thrombocytes) have a different behaviour and specific storage methods have to be optimized support recovery. This is the case for seeds classified by storage behaviour (orthodox,

intermediate and recalcitrant) and for which studies are required that assess their sensitivity to storage parameters (10,91).

## GENERIC LOW TEMPERATURE AND CRYOGENIC STORAGE GUIDELINES

Concept 5 considers how cryobiological principles may be translated into generic biopreservation guidelines (13,44,45,48,63,92,97), these are not intended to be binding or prescriptive, rather the rationale for their production is to encourage knowledge sharing across different biopreservation practices (Table 3). These guidelines are not intended to substitute for technical handbooks as this level of specialist detail is outside the scope of a trans-disciplinary document. Their proposed use is to: (a) facilitate quality and risk management practices; (b) encourage the setting of standards by identifying critical cryogenic factors in preservation protocols; (c) illustrate the importance of defining limits of tolerance for acceptable values; (d) highlight the use of PIs in testing the reliability and effectiveness of low temperature storage parameters and (e) catalyze the planning and work-up of 'bespoke' handbooks for repositories or collaborating research infrastructures. The following guidelines are distilled from information derived from different sectors; they are presented mainly as the sequence of operations, procedures and techniques that use low temperature storage. Quality and risk management measures are placed within guidelines to demonstrate the relationships between cryobiological principles and biopreservation practices.

Biosecurity. As defined by the OECD (92) for microbial collections, and as appropriate for other biorepository holdings, biosecurity concerns measures that need to be in place to prevent the loss, misuse or intentional release of dangerous pathogens or toxin producing organisms that are held in storage or transferred by BRCs.

*Guidelines – Identify biosecurity issues, establish risk management for personnel, samples and facilities and put emergency/incident response plans in case of a containment breach.*

Risk management. This concerns all aspects of personnel and sample safety. Staff working at ultra low temperatures must be informed about the safe use of LN and dry ice and their particular hazards (asphyxiation, cryogenic burns, frostbite, explosion of pressurized cryogenic liquids, filling of cryovials with LN), safety equipment (O<sub>2</sub> sensors/alarms) and personal protective equipment (PPE). Sample safety requires low level LN and temperature monitors, alarms and sensors to monitor storage at a stable temperature under conditions that do not compromise integrity, stability and containment. This paper only provides general guidance, risk management should be considered in full detail by institutions and personnel.

*Guidelines - Put in place stringent risk management procedures for personnel, samples and facilities. Establish emergency and incident response planning for back up, evacuation and prioritization and relocation of samples, specimens and resources in the event that storage facilities/vessels become compromised or catastrophically fail due to technical and/or power failures, emergencies, or a force majeure. As appropriate, create alternative backup storage capacity, extra storage space, and duplicate collections in another location, with procedures in place for transferring samples from a failed to an alternative facility.*

Accession Management. An accession is a unique identifiable, authenticated, sample maintained in storage for conservation and use, it is identified by a unique identifier (accession) number by the collection curator. When managing new accessions, especially in long term storage, up-front decisions are required to ascertain the number of samples that should be conserved. Dussert *et al.* (37) have developed a probabilistic tool for plant genetic resources that calculates the minimum percentage recovery that ensures the probability to recover a minimum number (target level) of plants from the cryopreserved sample size.

*Guidelines - Confirm with donors the reliability of biospecimen and biological resource 'passport' information, classify incoming accessions using tested markers and descriptors and code as 'tentative' until characterized. Storage inventories should be sufficiently robust to allow trouble-free sample location and traceability of accessions held in cold/cryostorage, together with accompanying passport, regulatory and sample (e.g. taxonomic) information; data should remain active so long as the sample remains viable/fit-for-purpose. Loss or purposeful removal of an accession from storage*

should be recorded and documented. Measures, checks, and cross-checks should be documented to counteract risks of non-compliance along the regulatory chain, including responsive updating of regulatory obligations, in line with new policies, agendas and laws. A database should 'tag' accessions with restrictions presented as pop-ups which have to be approved when ordering and filling out orders. Regenerate accessions as appropriate (e.g. for threatened species) and back up (duplicate) accessions (13,40,98).

Quality management. This involves identifying critical factors that affect low temperature storage and using pilot, proof of performance and future proofing studies to reveal problems before a preservation protocol becomes routine. The service/maintenance of equipment and storage units related to low temperatures, including full monitoring log sheets is required. Quality management concerns setting QC standards, proficiency testing, identifying non-pre-analytical and pre-analytical variables and highlighting the impact of cryobiological principles on storage outcomes in quality documents (manuals, BPs, SOPs and SPRECs).

*Guidelines - Apply validation, QC and proficiency testing to storage protocols and procedures as appropriate for: (a) type and use of biospecimen/biological resource; (b) mode of biopreservation (e.g. snap, controlled rate cooling, freezing, vitrification); (c) storage temperature (2-10°C, -20°C, -80°C, -196°C) as suitable for the low temperature preservation of non-viable samples and the cryopreservation of viable cells, tissues and organisms; (d) longevity (short, medium, long). Define acceptable levels of tolerance for specific storage treatments and corroborate using pertinent PIs.*

Pre-analytical variables. These variables (e.g. time before preservation, preparative centrifugation, cell fractionation, evaporative dehydration, osmotic pretreatments) can affect the ability of biospecimens and biological resources to withstand storage.

*Guidelines - Use SPRECs to: (a) identify specific variables that have deleterious or positive effects on storage outcomes and (b) optimize pre-analytical variables to improve post-storage outcomes and PIs. Pre-analytical variation is attributed to the processes that take place between the point of biospecimen collection and their use and, by definition, must be within the control of biobank or culture collection operations.*

Sample procurement, preparation, size and volume. These parameters can influence tolerance to preservation and storage outcomes and include: donor status, collecting regimes, timing and conditions of sampling (e.g. from the field) or procurement (e.g. from other repositories or research laboratories), intermediate holding temperatures and length of time before stabilization. Initial sampling vessels may not be suitable for storage, long term use or standardization and may be incompatible with automated cold storage processing platforms. Carry-over tests may be required for frozen samples that are handled using automation. Sample size/volume can affect cryoprotectant efficacy and thermal behaviour (conductivity, ice nucleation, ice crystal size/growth; glass formation, stability, relaxation).

*Guidelines - Store samples at the appropriate, optimal terminal storage temperature as soon as is practicable. Be aware that procurement and preparative processes can affect tolerance to storage treatments and that precautions may be necessary to counteract deleterious or variable effects including: (a) being aware that some manipulations, non-pre-analytical and pre-analytical variables may influence thermal behaviour/responses to storage treatments; (b) using consistent procedures; (c) recording departures from routine practices; (d) working within standardized/acceptable process times; (e) using consistent procurement and preparative practices (sample size/volume, centrifugation regimes); (f) using task-appropriate containers (g) optimizing procedures that stabilize samples during interim handling stages (humidity, temperature, ice/4°C, ambient, controlled environment).*

Containment. The prevention of microbial/pathological contamination occurring as a result of biopreservation practices is crucial. Containment can be compromised by exposing samples to contaminated liquid or vapour phase LN, cross infection of samples stored in the same cryotank, LN permeating into cryovials, contaminations from water baths used in rewarming or arising from other adventitious and environmental sources (10). Containment measures require careful consideration regarding the cleanliness of the storage container/facility including: type of vessel, security of caps when submersed in LN (internal threads, double bagging), low temperature resilience of vessels, closure integrity and the product shelf life of storage containers.

*Guidelines - Ensure containment under conditions that: (a) satisfy manufacturers' recommendations for safe and appropriate use (resilience of cryovials to LN, avoidance of cryovials filling with LN, noting that some protocols involve direct exposure to LN [94,102]); (b) state and*



guarantee the lifetime of a technical product; (c) measure/document physical parameters related to thermal resilience at the storage temperatures used; (d) assess capacity to withstand repeated freeze/thaw cycles; (e) risk-manage containment breach (safety/security of cryovials/closures, long term deterioration of vessel plastics, effects of pH on vessel stability); (f) instigate corrective measures (e.g. use of internally threaded cryovials); (g) as appropriate, set up quarantined and/or delineated storage to control the spread of pathogens and infectious agents; (h) locate storage vessels in clean, secure and safe environments in order to reduce the risk of introducing adventitious microbial flora and dust into vessels from the surrounding area; (i) use a clean source of LN.

**Cryoprotection.** Cryoprotectants are diverse, small molecules such as alcohols, polyols, and DMSO that are able to modify water behaviour at low temperatures and have a high biocompatibility. They are used to preserve viable cells, tissues and organisms and are applied as single or multiple additives, or combined with special treatments (e.g. alginate encapsulation). Cryoprotectant efficacy depends upon biospecimen-type, tolerance to their deleterious effects and mode of action, usually defined as colligative, osmotic or physiological. It is necessary to optimize additive application and removal in order to stabilize stored samples and offset devitrification risks. Cryoprotectants also provide 'biological' protection e.g. antioxidants, membrane stabilizers.

*Guidelines - As appropriate, perform cryoprotectant toxicity trials (undertaken when establishing new protocols for cells, tissue, and organisms that have not been cryopreserved before, and for which standard methods have been found unsuccessful). Identify critical factors e.g. additive concentration, loading temperature and application regime (gradual or all at once), duration of exposure, cryoprotectant unloading, dilution and washing away the cryoprotectant, and optimize each step before proceeding to routine cryopreservation. Ambiguities in how a protocol is written or translated can lead to miss-interpretation therefore, clearly describe cryoprotectant protocols and include all required levels of detail e.g. reagent quality, preparation (w/w, v/v) in culture media, buffers, pH adjustments, sterilization method, (filtered, autoclaved) in BPs and SOPs. Use knowledge of cryoprotectant mode of action and thermal behaviour to draw attention to critical steps, guide treatment optimization, confirm the stability of frozen and vitrified states and identify critical temperatures (T<sub>g</sub>, ice nucleation, melting points) that need to be monitored to maintain thermal stability during handling, storage and transit.*

**Biopreservation Protocols.** These are used to preserve biological resources and biospecimens at low temperatures, for which the main methodologies (a-e) are described.

(a) **Snap freezing.** The rate of cooling affects ice crystal size and growth, the more rapid the rate the smaller the ice crystals that are formed. Snap freezing usually involves reducing the temperature of non-cryoprotected, non-viable biospecimens to well below the freezing point of water. This is undertaken as quickly as possible so as to 'fix', at a specific point in its process chain history the structural and biochemical integrity of the sample.

*Guidelines - Maximize rapid heat transfer using vessels with high thermal conductivity and by plunging samples into cold and ultra cold cryogens (liquid phase LN, dry ice, isopentane cooled in dry ice, in LN or LN 'slurry' or 'slush'). Some labile samples may not be resilient to extremely fast rates of cooling which can cause undesirable cryo-induced artifacts (e.g. altered structure) in which case, freezing at higher temperatures (-80°C) may be required. Optimize thawing regimes and handling logistics to offset temperature fluctuations that might cause ice crystal growth during transfers to and from storage and during thawing.*

(b) **Ultra rapid freezing/cooling.** These protocols are critically dependent upon achieving ultra rapid rates of cooling/rewarming, they can involve the direct exposure of cryoprotected, viable samples to LN as is the case with droplet freezing and droplet vitrification (94,102).

*Guidelines - Use supporting structures (e.g. aluminium foils in the case of droplet vitrification or freezing) or cryovials that can withstand extreme low/high temperature cycles, and that have a high thermal conductivity. If direct exposure to LN is necessary use stringent risk management procedures to counteract cryogenic hazards to operators/personnel; take precautions against contamination should samples come into direct contact with LN.*

(c) **Controlled rate cooling/freezing.** This involves the controlled rate cooling (between -0.25 to -20°C/min depending on species/cell biology) of cryoprotected, viable samples to an intermediate terminal temperature, followed by transfer to long term storage in liquid or vapour phase LN. Each stage is a critical point that can affect storage outcomes.

*Guidelines - Use evidence-based knowledge of cryoprotectant mode of action (colligative/osmotic/biological) and thermal behaviour to identify critical parameters (heterogeneous/homogeneous ice nucleation [freeze-induced dehydration], Tgs, melts, freezing point depression [supercooling]) to design cooling programmes. Optimize each step of the thermal programme (cooling rate, number of cooling steps [ramps], ice nucleation [‘seeding’] temperature, [dependent upon sample/cryoprotectant]; ‘soak’ time [hold] duration at seeding temperature [to facilitate removal of intracellular water by freeze-dehydration], hold time at the terminal transfer temperature before placing in LN). Rationalize handling logistics to prevent inadvertent rewarming during transfers to and from the cooling chamber and cryostores. To ensure consistent ice nucleation, choose cryovials with high thermal conductivity; regularly service programmable freezers, position thermocouples/temperature probes in appropriate locations (sample, chamber and/or dummy specimen). Service/maintain cryogenic equipment and storage units and use monitoring logs.*

(d) Liquidus tracking. This process is ‘slow’ vitrification, it prevents ice crystals from forming within samples and permits vitrification without using ultra rapid cooling rates. This is achieved by increasing cryoprotectant concentration at the same time samples are cooled. The advantages being that higher concentrations of cryoprotectant can be tolerated at lower temperatures and cryoprotectant cytotoxicity decreases with temperature (127). Liquidus tracking is particularly advantageous for the vitrification of larger or sensitive cells/tissues.

*Guidelines - Use knowledge of the biophysical properties of cryoprotectant solutions to calculate the liquidus curve (concentration of cryoprotectant required at a given low temperature to prevent ice nucleation). Apply a gradual and progressive increase of cryoprotectant concentration as cooling proceeds to the point that an amorphous glassy state is achieved. Correlate this with knowledge of thermal behaviour to confirm Tgs and ensure that cryoprotectant additions meet the requirements of the liquidus curve. Optimize handling logistics to prevent inadvertent rewarming and devitrification at critical Tg temperatures (e.g. during transfers to and from cryostores). Carry out progressive cryoprotectant dilution during rewarming to avoid high toxic levels of cryoprotectant whilst inhibiting ice nucleation at higher subzero temperatures i.e. reverse the liquidus curve.*

(e) Vitrification. This is the process by which the viscosity of a protective additive and viable sample are increased to the point at which an amorphous vitreous state is formed at a glass transition temperature (Tg), usually by the direct introduction of the cryovial into LN.

*Guidelines - Use a knowledge of thermal behaviour to identify Tgs, and critical cryoprotectant concentrations and cooling and warming rates to respectively achieve vitrification and to avoid the deleterious consequences of devitrification. Optimize handling to prevent inadvertent glass relaxation as this can cause structural cracks that damage fragile samples or fracture complex structures. Optimize transfer regimes to avert devitrification occurring at critical Tg temperatures, during transfers to and from the cooling chamber and cryostores. Choose appropriate cryovials that can withstand direct exposure to LN over the required storage period.*

Storage temperature and longevity. As temperature is reduced metabolism becomes sufficiently slow to reduce degradation, the lower the temperature the longer samples remain in a stable/viable state. The Tg of pure water (-132°C/-130°C) is considered as the threshold temperature at which all molecular motion and metabolism ceases and biophysical injuries may be avoided. For human and mammalian systems the presence of CPAs tends to raise the Tg to around -120°C (5) depending on the mixture. However, operational Tgs may be higher for dehydrated, desiccated or vitrified cryopreserved plant germplasm (10,91). Storage above Tg may be acceptable for the long term preservation of some samples used for molecular analyses (protein extraction for western blots), but evidence suggests that if recovery of viable cells is required, functionality is lost within a few weeks of storage above Tg at -80°C (81). Some vitrification solutions and treatments (encapsulation/dehydration) have Tgs above -130°C, in theory permitting preservation at higher temperatures if there is equipment which can maintain this stable temperature. Practically, for most cases vapour phase LN provides such a stable environment.

*Guidelines - Choose a terminal storage temperature that is appropriate for sample type, duration and purpose of storage (short term, medium term, long term); operate within acceptable tolerance limits for glass stabilization and sample attrition as verified by PIs and storage stability parameters. Put in place operational and risk management strategies, (temperature monitoring, surveillance measures) to detect and prevent inadvertent sample rewarming above critical chilling, freezing and*

*vitrification temperatures, based on a knowledge of cryoprotectant/sample thermal behaviours (T<sub>g</sub>, melt, nucleation temperatures).*

Storage facilities. These comprise the equipment manufactured to maintain a critical storage temperature for which overarching performance factors are thermal reliability, consistency and uniformity. Choice of facility is largely dictated by current available technologies and cost, which must be balanced against knowledge of the thermal characteristics and biological stability at the technology-restricted storage temperature. Emergency disaster plans and business continuity plans should be in place in the event that a low temperature storage facility fails, including measures for the emergency retrieval of labelled priority collections which may be delineated in colour-coded racks/inventories to alert staff about priority evacuations. *Guidelines (a-c) are collated at the end of this section.*

(a) Incubators, growth chambers and environmental rooms. These maintain animal, microbial, protistan or plant cultures in: (a) proliferative, slow or minimal growth; (b) intermediary storage before samples are transferred to lower temperatures; (c) cold acclimation facilities (d) pregrowth (culture) treatments. They are also used to stabilize chemicals, reagents and environmental biospecimens. Maintaining the uniformity of environmental parameters (photoperiod, light intensity, temperature and humidity) over time and in different locations is critical, especially for smaller chambers. Special considerations are the use of dual compressors and backup systems, door safety features to prevent personnel from becoming trapped and control measures to prevent microbial spoilage at high relative humidity (RH).

(b) Mechanical refrigerators, freezers. These maintain a range of temperatures (4°C, -20°C to -150°C [commonly -70°C/-80°C]) but, because they are dependent upon electrical power, backup, emergency response plans are required in the event of failure. Critical issues are temperature variations in different storage locations (refrigerator shelving); thermal cycling during sample loading and unloading; frost accumulation/defrosting regime (avoidance of thermal cycles in frost-free systems), equipment calibration, maintenance, depreciation and guaranteed product lifetime.

(c) Liquid nitrogen cryostores. Because of the presumed cessation of molecular mobility at ultra low temperatures below the T<sub>g</sub>, preservation in LN is considered the most effective, long term storage method. Cryostore management is dependent upon *vapour* or *liquid* phase storage for which critical issues are the prevention of contamination in samples stored in liquid phase LN on the one hand, and, on the other, the avoidance of temperature fluctuations and gradients at temperatures above the T<sub>g</sub> of water (-130°C to -132°C) in the vapour phase (10,63,129). Safety alarms for LN level monitoring and automatic/manual fill routines need to be calibrated and regularly tested and back up protocols in place in the event of system failure or an interruption in LN supply.

*Guidelines - These are applicable for all types of storage facilities. Take measures to maintain constant terminal storage temperature stability; longevity is reliant upon achieving consistent, thermal uniformity across samples preserved in different locations of a storage vessel. These can vary according to LN phase, equipment specification, design and maintenance. Use PIs to check for resilience to fluctuating storage temperatures on biological degradation, which will depend upon sample lability. Verify the acceptability of thermal tolerance limits for specific facilities, sample types, biopreservation regimes and post-storage fitness-for-purpose criteria. The deposition of dust and water condensation should be minimized during the opening of storage containers. The prevention of contamination occurring in some samples (non-axenic plant, protist, microbial cultures) may not be easily achievable and in such cases storage regimes have to balance efforts to avoid contamination before, in, and following removal from LN storage, and the suppression of microbial flora after rewarming. In these situations, it may be prudent to maintain non-axenic cultures in a separate cryostore. Routine maintenance of primary and backup systems should be included in budgets as a cost effective, risk management measure because they extend the lifetime of compressors, display panels, control units and alarm systems.*

Low temperature surveillance. The calibration of cryogenic and low temperature storage monitoring equipment is undertaken according to manufacturers' recommendations and facility requirements, noting that thermal sensors can fail or erroneously display a constant temperature reading thus logs need to be regularly maintained to verify equipment performance. The careful positioning of thermal probes is required and multiple sensors reveal thermal gradients within freezers and cryotanks and temperature differentials between samples, open shelves and inventory spaces.

*Guidelines - Link monitors to alarms and personnel notification systems (mobile phones, pagers, where appropriate); use a second, independent thermal monitor to verify the accuracy of temperature measurements; ensure the low temperature storage facility is maintained in an infrastructure that supports the critical thermal tolerance range of the stored samples. Maintain, calibrate, test and routinely replace temperature surveillance systems, alarms and data loggers.*

Cold storage inventories, tracking, logistics and retrieval. These are the physical infrastructures in which samples are stored and organized in low temperature storage, together with their manual labelling and electronic tracking (barcoding) systems. Traceability and records management at low temperatures presents particular challenges, including label and bar code resilience to LN. The logistics of introducing and removing samples (opening/closing of freezer/refrigerator doors) from cryotanks requires efficient locating and handling protocols to avoid thermal gradients and temperature fluctuations. Other parts of the inventory are affected when samples are added to and removed from vessels, therefore deviations in thermal history should be logged and reported.

*Guidelines - Use permanent labels (indelible inks, laser etched barcodes, metal or plastic tough tags for LN racks, towers, vessels, cryovials) that are able to withstand storage conditions, and extreme fluctuations in temperature over the storage period. Organize storage inventories and label and track samples in a way that enhances the efficiency of sample input and retrieval. Apply a high degree of redundancy, i.e. include extra components which are not required, but which function to offset risks should other components fail. To decrease the risks of mislabelling include, at least three levels of identification: 1<sup>st</sup> storage container/cryovial label; 2<sup>nd</sup> identification by position in the storage vessel and 3<sup>rd</sup> colour code of the vial. Codes may be incorporated that prioritize saving the most precious samples in the event of a catastrophic failure of a storage vessel. Use thermocouples/data loggers to record thermal fluctuations that are specifically caused by door opening (frequency and duration) and utilize the data to optimize routine sample handling and improve inventory logistics. Record inadvertent temperature fluctuations and sample thaws. Preserve small audit or sentinel samples to test storage inventory sampling before protocols are adopted for routine, large scale; test samples held in long term storage.*

Cold chain security. Shipping frozen or vitrified samples at ultra low temperatures e.g. -79°C in dry ice or  $\leq -150^{\circ}\text{C}$  in dry-shippers; is potentially hazardous and careful risk management is required for solid CO<sub>2</sub> and LN-charged dry shippers. The preparation and packaging of samples for distribution and shipping is required to comply with regulations (International Air Transport Association, IATA). Samples are susceptible to temperature fluctuations during transit, therefore shipping logs and manifests should include details of critical point low temperatures (T<sub>gs</sub>, melting, devitrification). The stability of LN in dry shippers should be pre-tested with respect to the amount of LN needed to fully charge the shipper and maintain the required temperature during transit. This should take into account contingencies for delays or administrative hold-ups during transit or, on receipt.

*Guidelines - Apply cold chain risk management and QC practices to ensure that cryogenics (LN, dry ice, gel packs) are in sufficient supply to securely maintain chilled, refrigerated, frozen and vitrified samples during transit. Include safety margins for anticipated delays and contingencies for labile consignments. Use data loggers to monitor thermal history during shipment; instigate a quality assurance procedure to track temperature fluctuations during transit. If this is not possible, alternatively measure and document the sample temperature on dispatch and arrival. Use a test (QC-sentinel sample to trial for instability/stability during a test shipment) audit run to assess stability/instability and PIs before and after consignment.*

Thawing and rewarming. Controlled thawing (frozen samples) and rewarming (vitrified samples) after storage is critical as suboptimal regimes predispose cells to osmotic stress and mechanical damage propagated by ice crystal growth. Low temperature glasses are metastable, so even small changes in energy and molecular mobility may be sufficient to allow vitrified water molecules to form ice during rewarming, with loss of sample stability. Material properties of low temperature glasses are relaxation, fracturing and cracking, these can occur during rapid rewarming and they can cause structural damage, especially in large and complex samples, cells and tissues.

*Guidelines - Use measures to offset deleterious thermal transitions, changes in water state and osmotic phenomena which can occur when samples are removed from storage. Examples include the rapid thawing (at 35-45°C in warm air incubator or water bath) of frozen samples to the point at which all visible ice has melted and transfer to optimal post-thaw handling temperatures for a*

controlled time to offset ice crystal growth. Rewarming of vitrified samples is dependent upon cryoprotection regime, and sample size; options include: (a) rapid rewarming (40-45°C) to circumvent ice crystallization as the sample rewarms through the T<sub>g</sub>; (b) slow rewarming at ambient room temperatures (20-25°C) for a ~1 min to reduce the risks of the 'glassy matrix' fracturing; (c) two-step slow/rapid rewarming at ambient temperatures for a ~1 min, followed by rapid rewarming at 40-45°C (water bath) for 1-2 minutes to prevent glass fracturing and devitrification proceeding via ice crystallization. Monitor warming profiles by thermocouple in a 'dummy' sample where appropriate, to facilitate evidence-based SOPs.

Inadvertent thermal cycles. Repeated exposure of samples to alternating episodes of melting/freezing and vitrification/devitrification can be caused by: (a) undesirable (uncontrolled) and inadvertent thawing/rewarming episodes occurring during storage manipulations; (b) locational/positional fluctuations in temperature gradients in mechanical freezers and cryotanks; (c) equipment or operational failure; (d) thermal cycling in preserved, bulk samples from which aliquots are periodically removed for analysis; (e) cold chain temperature fluctuations; (f) inappropriate steps in a warming protocol. The deleterious consequences of repeated thermal cycling vary according to sample type and temperature tolerance limits (upper/lower), stability criteria, PIs, sample attrition, the number of thermal cycling episodes and storage longevity.

*Guidelines – Adopt measures that reduce freeze/thaw and cooling/rewarming cycles that could have deleterious consequences for thermal stability. If feasible, apply thermal analysis (DSC) to identify critical T<sub>g</sub> and devitrification temperatures that could predispose samples to cryogenic instability and loss of viability. Minimize or eliminate the need for repeated freeze/thaw cycles of bulk samples by using smaller aliquots or dispensing one-sample aliquots. For master stocks (mammalian and microbial cell lines) or active and base collections (genebanks) deploy different storage vessels and cryotanks. Instigate QC related to temperature surveillance to monitor the effects of multiple thermal cycles, on critical PIs (sample integrity, analyte measurement, viability, genetic/epigenetic stability).*

Recovery procedures. These concern the revival of viable, cells, tissues, organs and organisms after storage. Critical factors include the amelioration of osmotic, ischemic and oxidative/photooxidative stresses, necrosis and apoptosis, and optimizing the removal, unloading, washing and dilution of cryoprotectants. Recovery media supplemented with antioxidants, chelators, growth factors and hormones and sequential transfers to different media, osmotica and environmental regimes (low light reduces photooxidation) support the sustained recovery of survivors and resumption of functionality.

*Guidelines - Optimize post-storage manipulations and treatments to ameliorate storage stress, support sustained recovery and the resumption of functionality.*

Recovery assessments. Contemporary approaches to biopreservation take into account the role of biomolecular mechanisms and how they contribute to achieving successful recovery after storage (6,8,10). As pioneered by Baust and Baust and colleagues (5,9) these include delayed onset, cryopreservation-induced cell death (a phenomenon that can 'reverse' positive viability results) and the recognition that apoptosis is involved in recovery responses. Increasingly, post-storage assessments use assays and diagnostic probes to elucidate the complex molecular basis of cryopreservation recovery and failure.

*Guidelines - Viability and recovery assessments can only be fully assessed by measuring the end functions for which cells, tissues, organs and organisms will be used after low temperature storage, this is best achieved using multiple endpoint approaches as described by Van Buskirk (122). Define time periods of any cryopreservation-induced delayed onset cell death. Be aware that viability assays are prone to interference and anomalous interpretation and their standardization can be problematic. Check for interference (causing false positive or false negative outcomes) and validate tests appropriately and when feasible apply proficiency testing (17). Unambiguous confirmation (cell division, colony forming units, motility, regrowth, germination, regeneration, totipotency, acceptance of PIs) of functionality is required to demonstrate successful storage.*

Post-storage viability tests and functional assays. For microorganisms, methylumbelliferyl (4MU) substrates and analysis of colony morphology and culture characteristics provide useful criteria for viability/recovery assessment. Examples of assays used in medical biopreservation have been reviewed (122), and together with tests and post-recovery morphological/growth assessments applied in other fields they are listed as follows, noting that selection of a test will depend on sample type and taxa (microbial, protist, plant, animal). They include: (i) *cytological live/dead assays*: (a) LDH assay

(BioVision; CytoScan<sup>®</sup> LDH-cytotoxicity assay kit); (b) Trypan Blue exclusion assay; (c) bioluminescence, ToxiLight<sup>®</sup> BioAssay kit; (d) fluorescence microscopy (fluorescein diacetate, FDA UV/microscopy); fluorescent (fluorescein-based) probes, Calcein-AM, CFDA-AM, BCECF-AM, SYTO<sup>®</sup>, SYBR-14<sup>®</sup>, ethidium bromide/propidium iodide; **(ii) viability-mitochondrial/metabolic activity probes**: (a) Resazurin derivative - AlamarBlue; (b) MTT assay; (c) JC-1 dye; (d) tetrazolium salt reduction assays; **(iii) apoptosis probes**: (a) Caspase activation, fluorogenic substrates Vybrant<sup>®</sup> FAM caspase assay kit - flow cytometry, Image-iT LIVE<sup>®</sup> caspase detection kit – microscopy, CaspGLOW fluorescein caspase staining kit; (b) RT-PCR of caspase activity, (c) Annexin V/PI/Hoechst stain or Annexin V/Sytox green/Hoechst stain; **(iv) oxidative stress linked to apoptosis, stress signalling**: (a) ROS detection kits, MitoSOXRed reagent; Image-iT LIVE<sup>®</sup> Green ROS; (b) DCF fluorescence (measurement of intracellular ROS generation); (c) volatile ROS markers (54) (d) antioxidants (22,65), (e) DNA adducts (66); **(v) viability/functional assays specific to cell type**: (a) Sperm - JC-1, motility; (b) cardiomyocytes - Calcein AM, cell beating; (c) hepatocytes drug-metabolising enzyme assay (CYP3A4; ECOD; FMO; UGT; ST); (d) PBMCs - LPA (lymphocyte proliferation assay); (e) seed germination (TTC); (f) plant morphogenetic totipotency (TTC) from cells/callus, via organogenesis or somatic embryogenesis; (g) regrowth from original plant meristems/dormant buds; (h) microbial and protist cell division; **(vi) molecular genomics, proteomics, transcriptomics, metabolomics assays for stress/protein profiles.**

*Guidelines - Validate/verify viability, recovery and fitness-for-purpose assays and participate in proficiency testing to ensure consistency and reliability across different operators; corroborate initial findings with sequential, longer term evaluations to confirm sustained survival. Use multiple levels of assessment (viability assays, apoptosis markers, stress markers) and definitive indicators of fitness-for-purpose (cell growth, division, confluence, colony former units, totipotent and morphogenetic competence e.g. in plants, seed germination, somatic embryogenesis, shoot regrowth or regeneration).*

**Stability.** Preservation methods and associated practices (tissue culture, cryoprotection, use of anticoagulants, chelators, antioxidants, cold chain processing) should not affect the fitness-for-purpose of stored samples. Stability can be considered at different levels (cryogenic, biophysical, structural, cytological, biochemical, molecular, immunological, genetic, epigenetic, phenotypic, reproductive capacity). Specific measures include confirming genetic stability (89) in offspring/cell lines derived from stored germplasm and cryobionomics (51).

*Guidelines - Apply risk management and QC procedures to identify and mitigate any storage and recovery manipulations that could predispose samples to storage instability. Track changes in sample PIs before and after storage, identify SAEs (for clinical samples) and alert operators if preservation outcomes (e.g. viability) fall below an expected level. Monitor documentary evidence of post-storage outcomes and feed back to end users as dictated by type of bioresource or biospecimens. In a medical/therapeutic setting these may be identified as SARs; for microbial/protistan production strains they may manifest as a loss in ability to produce secondary metabolites after storage. For plant genetic resources they can include molecular evidence of genetic/epigenetic instability. As a precautionary measure for large scale and batch processed cells or highly labile biospecimens, it is recommended to preserve small audit (sentinel) samples during the same storage run. These can be recovered from storage and tested for instability/stability over set timelines, dependent upon sample and storage method.*

**Performance indicators (PIs).** These are reliant upon sample type and utility and they qualify that a sample is fit-for-purpose; they are used in QC to pinpoint critical factors in storage regimes and identify suboptimal or non-compliant storage practices. Performance indicators may encompass not only relative viabilities post-preservation (which tend to be thought of as ratios of the untreated sample), but also the total functional biomass which has been successfully preserved. PIs are used to ensure that preservation methods do not interfere with or have deleterious effects on the future utility or analysis of stored samples, they must be fit-for-purpose, i.e. relevant, informative and validated.

*Guidelines - Apply a quality assurance protocol (as appropriate, linked to risk management procedures) that tracks for changes in sample PIs before and after storage, and alerts operators if storage outcomes fall below an expected level. As a precaution, preserve during the same run, a small audit (sentinel) sample that can be recovered and tested for PIs prior to recovering the main sample. Participate in proficiency testing.*

Verification and authentication. Sample identity should be confirmed following retrieval from storage and to substantiate duplicate accessions; authentication is particularly important for samples that have been held in very long term storage.

*Guidelines - Instigate an overarching quality assurance protocol to verify the authenticity of samples retrieved from low temperature storage. Measures used to confirm authenticity will depend upon the sample, biospecimen or bioresource type and the level of identification criteria (e.g. taxonomic characters [for microbial/protistan strains] phenotype descriptors [for crop plant genetic resources]; cell morphology, genetic fingerprints [for individual DNA/RNA specimens, cells, tissues]). Authenticity testing should concentrate on the most relevant test criteria as pre- and post-storage analyses are costly and time consuming.*

A vitrification protocol is used as an exemplar (Fig. 2) to demonstrate how the translational paradigm and generic guidelines can be utilized to choose: (a) the appropriate research strategy, (b) identify critical point factors and (c) pinpoint key actions and processes that are required to satisfy standards and regulatory conditions. This approach can be refined to help develop guidelines for different types of storage regimes (Table 3) and indicate where decisions are necessary to implement research findings appropriately (Fig. 2).

## DISCUSSION

Translational low temperature research, underpinned by cryobiological principles supports quality and risk management practices which can be both technically and economically feasible, and compliant with overarching biobanking and biorepository regulations. Generic guidelines (Concept 5), process chains (Fig. 1) and translational roadmaps/decision paths (Fig. 2) can help to identify critical factors that affect successful storage outcomes. The unambiguous use of scientific, quality and risk management terminology (Tables 1 and 2) is important in achieving robust guidelines that are relevant to multiple users. No one section of the scientific community is able to provide comprehensive answers that meet all the challenges involved in defining guidelines across sectors. However, the three societies (SfC, SLTB, ISBER) and other stakeholders in the applications of cryobiology have many shared and overlapping interests and objectives; seeking to develop consensus, generic guidelines for low temperature biopreservation is one step forward. This is particularly timely as biobanking materials for biochemical, ultra structural or genomic interrogation increasingly requires stringent levels of quality and traceability and cross correlated ancillary information. This is becoming ever more important as regulatory compliance is mandated, whether in biobanking for therapy, genetic conservation or biotechnological application. Such information includes for example, a history of the individual donor, choosing and applying what is regarded as a satisfactory end storage temperature (e.g. -80°C or -196°C) and accepting cooling/warming profiles that are dictated by both sample format and rigorous, post-storage, fitness-for-purpose criteria.

The molecular-omics era places greater emphasis on maintaining genetic/epigenetic stability and assuring the authenticity of biospecimens and biological resources stored at low temperatures. Historically, cryopreservation research has focused on scrutinizing the biophysical changes that are required to achieve the recovery of viable cells; this has been generally achieved by optimizing cryoprotection and cooling regimes with less emphasis being placed on the provision of corollary data. In these scenarios, end storage temperatures of -80°C may not be compatible with long term, stable biopreservation, although they may be used for short periods so long as there is an adequate knowledge of the rate of attrition. This information is vital, as it is used to choose the appropriate storage temperature and refine protocols to ensure that sustained, acceptable levels of functionality and viable cell recovery are achievable for specific storage regimes. However, the increasing sophistication surrounding the molecular questions that are being asked of ‘banked’ samples presents particular challenges. Where viable *and* functional cells are required to be recovered, low temperature preservation practices need to support and permit a greater understanding of ‘true’ biological capabilities, for example, in response to agonists, therapeutic agents or environmental impacts. In a contemporary context, all the interested parties involved in using low temperature preservation technologies are likely to converge with respect to the development and implementation of storage quality and risk management guidelines. Tangible ways are now needed to support successful

communication concerning the application of cryobiology in biopreservation across diverse communities. Examples are joint educational activities, mutual break-out sessions and workshops in scientific meetings (109,110,111), organization of transdisciplinary working groups (e.g. ISBER Biospecimen Science Work Group) and published, cryobiological SOPs (48).

## CONCLUSIONS AND FUTURE SCOPE

We conclude, that it will be greatly enabling if all the stakeholders involved in using biobanks, biorepositories and genebanks can collaborate and share knowledge, not only with respect to fundamental and applied research, but also regarding Biospecimen Science and the development of low temperature storage guidelines. Such an undertaking will enhance the utility and impact of cryobiology and biobanking, now and for the future. Biorepositories and biobanks have a role in raising awareness as to the importance of best practices, both at the institutional level and across different communities of practice. This involves engaging with a broad spectrum of stakeholders, cryobiology researchers, curators and laboratory managers, as well as an awareness of cost implications and working within institutional boundaries. It is hoped that this paper may have future utility as a foundation document which can help build sector specific, 'bespoke', SOPs, guidelines and BPs. Follow up, customized publications may include 'how-to-do-it' protocols which include 'translational' notes explaining the relevance of cryobiological principles across biopreservation practices.

## Abbreviations and acronyms

**AT** = Antarctic Treaty; **BCECF-AM** = 2',7'-bis-(2-carboxy ethyl)-5-(and-6)-carboxy fluorescein acetoxy methyl ester; **BP(s)** = Best practice(s); **BPC** = Biospecimen process chain; **BRC** = Biological Resource Centre; **BT** = Budapest Treaty; **Calcein-AM** = Calcein acetoxy methyl ester; **CBD** = Convention on Biological Diversity; **CFDA-AM** = 5-carboxy fluorescein diacetate acetoxy methyl ester; **GCP** = Good clinical practices; **CITES** = Convention on Illegal Trade in Endangered Species; **CoCP** = Colligative cryoprotectant; **CP** = Cryoprotectant; **CPA** = Cryoprotective additive (also see CP - cryoprotectant); **CRC** = Controlled rate cooling; **CRF** = Controlled rate freezing; **CRP** = Critical point factor; **CYP3A4** = Cytochrome P450 3A4; **DCF** = 2',7'-dichlorofluorescein; **DES** = Desiccation; **Di** = Diagnosis; **DMSO** = Dimethyl sulphoxide; **DNA** = Deoxyribonucleic acid; **DR** = Droplet freezing (DRV droplet vitrification); **DSC** = Differential scanning calorimetry; **ECOD** = 7-ethoxycoumarin O-deethylase; **EN** = Encapsulation; **FDA** = Fluorescein diacetate; **FMO** = Flavin monooxygenase; **GLP** = Good laboratory practices; **GMP** = Good manufacturing practices (GMP) cGMP (current GMP); **GPA-PGR** = Global Plan of Action for the Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture; **GSPC** = Global Strategy for Plant Conservation; **HEFA** = Human Embryo and Fertilization Authority (UK); **HTA** = Human Tissue Act (UK); **IATA** = International Air Transport Association; **ISBER** = International Society for Biological and Environmental Repositories; **ISO** = International Standard Organization; **ITPGRFA** = The International Treaty on Plant Genetic Resources for Food and Agriculture; **JC-1** = 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; **LDH** = Lactate dehydrogenase; **LIMS** = Laboratory information management systems; **LN** = Liquid nitrogen; **LPA** = Lymphocyte proliferation assay; **LTS** = Long term storage; **MC** = Moisture content; **MHRA** = Medicines and Healthcare Products Regulatory Agency; **MSDS** = Material safety data sheet; **MTA** = Material transfer agreement; **MTS** = Medium term storage; **MTT** = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NG** = Nagoya Protocol (United Nations); **NIST** = National Institute of Standards and Technology (US); **OCT** = Optimal cutting temperature; **OECD** = Organization for Economic Co-operation and Development; **OsCP** = Osmotic CryoProtectant; **OSD** = OSmotic Dehydration; **PBMCs** = Peripheral blood mononuclear cells; **PI** = Propidium iodide; **PI(s)** = Performance indicator(s); **PIS** = Post-storage performance indicators; **PPE** = Personal protective equipment; **PT** = Proficiency testing; **PVS2** = Plant Vitrification Solution number 2; **QC** = Quality control; **QM** = Quality management; **QMS** = Quality management systems; **RAPD** = Randomly amplified polymorphic DNA; **Re** = Research; **RFID** = Radio frequency identification device; **RIN** = RNA integrity number; **ROS** = Reactive oxygen species; **RT-PCR** = Reverse transcription polymerase chain reaction; **SAE** = Serious adverse event; **SAR** = Serious adverse reaction; **SfC** = Society for Cryobiology; **SLTB** = Society for Low Temperature Biology; **SOP(s)** = Standard operating procedure(s); **SPREC(s)** = Standard pre-analytical variable code(s); **ST** = Sulfotransferase; **Tg** = Glass transition temperature; **TTC** = Triphenyl tetrazolium chloride; **Th** = Therapy; **UGT** = Uridine 5'-diphosphoglucuronosyltransferase; **UN** = The United Nations; **URF** = Ultra rapid freezing; **UV** = Ultraviolet; **V** = Vitrification.



**Table 1.** Definitions and explanations of common cryobiological terms and technical practices supp

Terms	Definitions and explanations
Cold Chain	A temperature controlled supply chain defined by the critical temperature(s) required to preserve resource procurement and their transfer or delivery to and from the biopreservation facility.
Colligative	A term describing the relationship between water and solutes, colligative properties are characterized irrespective of size, structure and chemical properties. Relates to cryoprotection and cryoinjury during
Controlled rate cooling	A method that controls the rate at which a biological sample is cooled using defined parameters: temperature(s) before final transfer to LN. Also known as 2-step, stepwise, slow or controlled rate or programmable freezers or other self-made or commercial device (Mr Frosty™) which can achieve a usually involves the controlled induction of ice nucleation in the sample ('seeding').
Controlled rate warming	Control of the rate at which a biological sample is warmed from cryogenic temperatures using environment (air, water bath, electric incubator) and temperature.
Cryocarts	A portable, controlled environment workbench, designed for the loading of biological samples into handling and transporting of large numbers of samples between cold storage facilities.
Cryoinjury	Damage caused by freezing, attributed to 2 biophysical factors: (i) ice causing mechanical damage, and (ii) dehydration causing harmful cell volume changes, excessive solute concentration. Biomolecules considered significant components in cryoinjury (e.g. delayed onset, cryopreservation-induced cell death).
Cryopreservation	The conservation of living cells, tissues, organs and organisms at ultra low temperatures (also known as vapour phase LN to the minimum temperature of -196°C (liquid phase)).
Cryoprotectant	Cryoprotectant(s) (CPAs); a single compound or mixture of additives that allows living cells, tissues to survive at ultra low temperatures. Categorized into two main types: (i) <i>penetrating colligative</i> cryoprotectants that prevent excessive solute concentration; these CPAs depress freezing point, so that any ice formed will not form at ultra low temperatures; (ii) <i>non-penetrating osmotic</i> cryoprotectants, these dehydrate cells, minimizing water damage. Numerous biochemical protective modalities (antioxidants, membrane protection, glass stabilizers) are used in conjunction with CPAs, i.e. their cryoprotective benefits outweigh their harmful effects.
Cryoprotectant addition/removal	CPA addition steps, incremental and final CPA concentrations; temperature and time of exposure to CPA concentration), osmotic buffers (e.g. sugars, polyols), temperature and duration of washes, centrifugation
Crostability	Stability (physical, biophysical, biochemical, molecular, genetic) of biological samples stored at low temperatures
Cryostorage	Storage of biological samples in cryovials, cryotubes, containers, straws or bags at ultra low temperatures (shelves, canisters) of a cryostorage tank or Dewar (specialized vacuum insulated vessels that hold LN)
Devitrification	The process by which a vitrified sample converts from a glassy state to ice or liquid water. The vitrified sample easily revert back to a liquid and/or devitrify to form ice.
Dry shipper	A container used for the safe transport of biological samples, usually at the temperature of LN vapour.
Eutectic point	The point at which a whole system solidifies and there is no further change as all available water has frozen at a given temperature and concentration and it applies to two or more substances that are able to form solid solutions at a minimum temperature.

Enthalpy	A measure of the total energy of a thermodynamic system equivalent to the sum of its internal energy and the energy of its surroundings. The unit of measurement is the Joule. Total enthalpy, cannot be measured directly but can be determined from the change in enthalpy ( $\Delta H$ ) in endothermic (melting) and negative in exothermic reactions (freezing).
Equilibrium melting point	In aqueous systems, the highest temperature at which ice crystals can co-exist with a cold solution. The equilibrium melting point is determined by differential scanning calorimetry (DSC), where the enthalpy of melting can be identified. The numerical value of this temperature is close to, but not identical with, the highest temperature of ice formation on cooling dissolved solutes. For a given solution, this temperature is close to, but not identical with, the highest temperature of ice formation on cooling.
Freezing	At standard atmospheric pressure, the process by which an aqueous liquid becomes a <i>crystalline</i> solid, in which ice is formed. In biological systems it occurs intracellularly and extracellularly. In preservation, it is achieved by ultra rapid freezing, the direct immersion of cryoprotected (usually) viable/replicable biological samples into liquid nitrogen at temperatures less than $-70^{\circ}\text{C}$ , usually of non-cryoprotected, non-replicable/non-viable samples; (iii) slow cooling to a terminal temperature usually of LN. Equilibrium freezing in controlled rate (slow cooling) is achieved by remaining in osmotic balance with the extracellular solution in the presence of ice crystals. Non-equilibrium freezing is achieved by dehydration and colligative cryoprotectant penetration before cooling with limited change in osmotic pressure. Cryoprotectants are applied.
Glassy state	An amorphous state lacking organized structure that has the mechanical and physical properties of a solid.
Glass transition $T_g$	The temperature ( $T_g$ ) at which a glass is formed and a fluid becomes so viscous that it appears to be a solid.
Ice nucleation	Also termed 'seeding'; the point at which ice crystals are initiated in a sample. Crystallization occurs on the presence of support stable 'ice' nuclei. Below the $T_g$ (around $-39^{\circ}\text{C}$ to $-40^{\circ}\text{C}$ ) of homogenous ice nucleation water molecules act as 'templates' (surfaces, impurities, preformed crystals) support heterogeneous ice nucleation above $-40^{\circ}\text{C}$ .
Liquid nitrogen	The element N, in its liquid state at atmospheric pressure, that acts as a cryogenic coolant used to cool samples to $-196^{\circ}\text{C}$ or, vapour phase at variable temperatures above $-196^{\circ}\text{C}$ , depending on proximity from the liquid phase.
Liquidus tracking	A process of cooling cells or tissues whilst continuously increasing the concentration of dissolved solutes to suppress ice nucleation at any given point. This can proceed until the mixture has increased both in solute concentration and temperature, nucleate on further cooling, and the mixture achieves a stable glassy state. The required increase in solute concentration is calculated for a specific cryoprotectant from physical principles.
Osmosis	Osmosis is the flow of a solvent across a semi-permeable membrane which permits the passage of the solvent but not the solute. The pressure that must be applied to a solution to prevent the flow of a solvent across the semi-permeable membrane is called osmotic pressure.
Melting	At standard atmospheric pressure, the process by which a solid becomes a liquid at a specific temperature.
Preservation	Controlled storage under conditions that prevent or reduce sample deterioration.
Warming	Increasing the temperature of a sample; particularly used to describe the rewarming of vitrified samples.
Storage	Preservation of biological samples under specific, low temperature conditions for future use.
Super cooling	Freezing point depression below the temperature of ice formation, water supercools to freezing points below $-20^{\circ}\text{C}$ .
Thawing	The warming of frozen samples to a temperature at which ice melts and water converts to a liquid.
Thermal analysis	Study of thermal phenomena using a Differential Scanning Calorimeter (DSC) in order to optimize storage conditions. Enthalpy (enthalpy) and water state transitions are monitored as a function of time and temperature; phase transitions are identified.

vitrification and revealed as exotherms (latent heat of ice nucleation - energy is released) or endotherms. Energy released on freezing is the enthalpy of fusion and is equivalent to the energy required to melt. Thermal hysteresis occurs, in which melting and freezing temperatures are different. Tgs are revealed by DSC. Devitrification is manifest as melt endotherms and ice nucleation exotherms. Vitrified samples are also highly viscous samples.

Vitrification

Solidification of solutions (liquids) without crystallization; the transformation of a liquid into a glassy state accompanies ice crystal formation; an amorphous metastable state at a critical glass transition temperature is observed in highly viscous samples.

Definitions are based on scientific literature and biobank guidelines/best practices from different sectors as represented in references 10,13,15,16,29,63,92,93,106,114.

**Table 2.** Definitions and use of quality and risk management terms used in biobanks

Terms	Definitions and explanations
Accident	An event/deviation that has the potential or actuality to cause harm or deleteriously affect operations and investigated to prevent, correct or limit adverse outcomes.
Accreditation	The process by which an accredited, external auditor awards a certificate of competence.
Adverse outcome	A detrimental effect or complication that could affect operations and performance indicators.
Audit	A formal and documented review of personnel, procedures, records and protocols to evaluate operating procedures (SOPS) regulations and statutes.
Authentication:	Proof and verification of correct biological sample identity.
Best Practice (BP)	A protocol, method or process that is the most effective in realizing a particular outcome, as compared to other methods tested for compliance with explicit, required outcome(s). A BP should be achieved without compromise. It is the most effective means of producing the best possible result. A BP is a method, technique or procedure that is superior to those achieved by other means, and that are used as a benchmark or point of reference. When improvements are discovered, they are reviewed regularly.
Good Clinical Practices (CGCP)	A set of internationally recognized ethical and scientific quality requirements which must be followed in the reporting clinical trials that involve participating human subjects. They provide assurance that the subjects are protected, and that the results of clinical trials are credible and accurate. The principles of CGCP are defined in ICH Q10 2005/28/EC. GxP is an abbreviated term used in guidelines for lab samples from clinical studies.
Good Laboratory Practices (GLP)	A quality management system for research laboratories and organizations used to ensure uniformity, reliability and integrity of their testing procedures. Applied in physiochemical, chemical, pharmaceutical, non-pharmaceutical and other industries.
Good Manufacturing Practices (GMP)	A production and testing practice that helps to ensure a quality product. Many countries have laws that require companies must follow GMP procedures; complying with GMP is mandatory aspect in pharmaceutical industry.
Certification	A process through which an organization grants recognition to an individual, organization, process, product or service based on criteria (see Accreditation).

Critical point factor	A factor or issue affecting a successful outcome.
Deviation	Unintended/intended departure from a BP, SOP or regulation that requires formal reporting, explanation and corrective action.
Error	A departure from a BP or SOP attributed to technical or document mistakes; requiring formal, documented corrective action and reporting of consequences.
Guideline(s)	Established principles of practices for particular processes, usually established through consensus or agreement. A guideline is a course of action and it aims to streamline particular processes according to a set routine or procedure. <i>mandatory, guidelines are non-binding and they cannot be enforced.</i>
Hazard	Any formally identified substance or procedure that can cause harm, the effects of which are mitigated through a hazard analysis; (ii) identification of critical control points; (iii) establishing critical limits for each control point; (iv) stabilizing corrective actions; (vi) record keeping; (vii) applying proper practices; (viii) reviews.
Incident (see Accident)	An unplanned deviation from a BP, SOP, safety protocol or regulation that affects or puts at risk people, property, facility and that requires the instigation of an incident response plan and a formal report.
Informed consent	A decision to participate in research, taken by a competent individual who has received and understood the risks and benefits of the decision without inappropriate influence. Also see Material Transfer Agreement (MTA).
ISO	International Organization for Standardization. The designation and logo of the International Organization for Standardization to biobanking are: ISO9001 Quality management systems – Requirements ISO17025 General requirements for the competence of testing and calibration laboratories ISO15189 Medical laboratories – Particular requirements for quality and competence ISO Guide 34 General requirements for the competence of reference material producers.
Material Safety Data Sheets (MSDs)	Information sheets provided by manufacturers and suppliers providing details of the properties and hazards of a substance. Handling risks, they inform operators about personal safety equipment (PPE) and clothing (PPC).
Material Transfer Agreement (MTA)	An agreement that controls the transfer of materials and data between two organizations which specifies the terms and conditions. It defines the rights and obligations of the donor, provider and recipient regarding use, they can be used by research communities of practice (e.g. for plant genetic resources). MTAs may be presided over by conventions such as the Convention on Biological Diversity and the Nagoya Protocol and treaties, e.g. The International Treaty on Plant Genetic Resources for Food and Agriculture Organization of the United Nations.
Procedure	A series of steps intended to achieve a specific outcome when followed as instructed.
Performance indicator	A measurement used to evaluate the success of a particular activity or outcome and for which there is a target value.
Process chain	All the procedures in a repository involved in the security, safe management storage, dispatch and receipt of samples.
Proficiency test(ing)	An important way of meeting the requirements of ISO/IEC 17025 in the area of quality assurance of laboratories. It involves bodies that laboratories participate in proficiency testing programs for all types of analyses under their scope. Proficiency testing involves a group of laboratories or analysts performing the same analyses under the same conditions.
Quality Management System (QMS)	A QMS is designed to make sure processes are defined within SOPs, recorded in accessible form and monitored to ensure that a facility meets its QA obligations.

Quality Assurance (QA)	Evidence of action confirming that work is done effectively using systematic accounting of action product, service meets its quality requirements.
Quality Control (QC)	Testing to monitor samples, equipment, processes, procedures to prove that they are operating to n
Regulation	Regulations concern administrative legislation of different kinds. They constitute or constrain differentiated by primary legislation put in place by parliaments or elected legislative bodies or ju disseminated by government authorities, they can include, industrial self-regulation.
Risk	Risk is the chance, high or low of harm being caused by a hazard.
Risk assessment	Risk assessment, informs of preventative safety measures and in the event that an incident does and deal with their consequences as effectively as possible.
Risk management	Risk management is a formal (usually statutory) process that identifies and categorizes risks.
Serious adverse event	An undesirable experience that is associated with the use of a medical product in a patient.
Serious adverse reaction	An unintended response associated with the procurement or human application of tissue or incapacitating and which can result in/or prolongs, hospitalization or morbidity.
SPREC	A code that identifies the pre-analytical variables of a biospecimen attributed to processes that take and their use, by definition they must be within the control of biobank or culture collection operation
Standard	A technical standard is an established requirement, rule or norm that is defined in technical, c process. It is usually described in a formal document that establishes the consistent and uniform c procedures) that are required to attain and satisfy the standard.
Standard Operating Procedures (SOPs)	A written document or instruction that details all the steps and activities involved in a process detailed, documented standard procedures that are consistently and commonly used by bioreposito
Tolerance limits	Limits that define the max-min range of acceptable values used to test the efficacy of a procedure or emergency actions. For example, acceptable times and temperatures of cryoprotectant exposure limits for performance indicators (see performance indicator above).
Validation	A process by which a procedure is checked against a specific criterion, confirming that it meets the that gives a high level of assurance that a process, technique protocol, method, service, or syste often involves an acceptance of fitness for purpose by end users and stakeholders. It is usually a working together cooperatively to validate a common process, procedure or protocol.
Verification	Verification is usually an internal quality process that ensures a procedure complies with required compliance of a product, protocol, method, service, or system with regulations, specifications (spec during development or scale-up phases and before going 'live' with a procedure.

---

Definitions are based on scientific literature and biobank guidelines/best practices from different sectors as represented 10,13,15,16,29,46,63,92,93,106,113,114,117,118,119.

**Table 3.** Comparisons of preservation regimes and performance indicators applied to biospecimens

Biopreservation sector community of practice sample type & use (references)	Typology <sup>1</sup> sample category, taxa biospecimen, bioresource	<sup>2</sup> Preservation regime □ freezing- thawing cooling-rewarming cryoprotection	<sup>3</sup> Terminal storage (T°C)	<sup>4</sup> S
Environmental & Scientific Collections (11,46, 63,67,95,97)	Polar algae viable samples	Sample stabilization during transfer from remote locations (cold chain security)	4°C, -20°C	ST,
Non-viable and/or non-replicable biospecimens for environmental monitoring	Fauna tissue samples and extracts (liver, kidney, blubber, horn, hair, birds eggs)	Snap freezing in LN, transfer to terminal storage (T°C), rewarm at ambient or controlled rewarming @ 35-40°C	-20°C, -80°C, -150°C, LN <sub>vapour/liquid</sub>	ST,
Viable germplasm	Marine toxicology sea urchin embryo-larvae for eco-assays	CRC (control of seeding), CoCP + OsCP to test optimal regime thawing @ 16°C	-35°C	N/A
Wildlife & Biodiversity Conservation (19,29,44,46)	Fauna	CRC (control of seeding), CoCP + OsCP transfer to terminal storage (T°C), rewarm at ambient (T°C) or controlled rewarming @ 35-40°C	LN <sub>vapour/liquid</sub>	LT
Viable samples, gametes, embryos, oocytes, replicable cells (clonal, somatic)	e.g. viable, replicable germplasm, cells from animal biopsies	CRF (Mr Frosty™ to -80°C), CoCP DMSO thaw @ 38°C	LN <sub>liquid/vapour</sub>	ST,
Non-replicable, non-viable biospecimens (genetic diversity, wild-life forensics, veterinary health care)	e.g. tissue samples (liver, kidney, egg, horn, hair)	Snap freezing in LN, transfer to terminal storage (T°C), rewarm at ambient or controlled rewarming @ 35-40°C (T°C)	-20°C, -80°C, -150°C, LN <sub>vapour/liquid</sub>	ST,
Biological Resources & Genetic Resources (13,29,30,31,44,46,51,53, 66,84,99,120)	Culture Collections			
Prokaryotes, eukaryotes, replicable and non-replicable, somatic cells, tissues, organs	Viral suspensions	Direct transfer to storage T°C, CP (dehydration may be required), thaw @ 37°C	4°C, -20°C, -70°C, LN <sub>liquid/vapour</sub>	ST, LT

	Microbial- bacteria	CRF, CoCP glycerol, DMSO, thaw @ ambient T°C	-80°C, LN <sub>liquid/vapour</sub>	MT
	Protists-algae	CRF, CoCP methanol, DMSO, thaw @ 40°C; EN+ OSD + DES transfer to LN, rewarm @ 45°C	LN <sub>liquid/vapour</sub>	LT
	Protists-fungi	CRF, CoCP DMSO, glycerol, thaw @ 40°C	LN <sub>liquid</sub>	LT
	Higher plant cells	CRF, CoCP DMSO, glycerol, OsCP sucrose, thaw @ 40 °C	LN <sub>liquid</sub>	LT
	Genebanks			
Viable replicable plant germplasm	<i>In Vitro</i> Plant Genebank (cryobank) germplasm, somatic embryos, shoot meristems	CRF+CoCP+OsCP, V, CoCP+OsCP, PVS (DMSO, sucrose, glycerol, ethylene glycol), direct transfer to LN, rewarm @ 45°C EN + osmotic dehydration + desiccation to a critical MC, direct transfer to LN, rewarm @ ambient T°C	LN <sub>liquid/vapour</sub>	LT
	Recalcitrant seed germplasm excised embryos, embryonic axes			
	Seed Bank Orthodox seeds	Evaporative dehydration to a critical MC, direct transfer to storage facility or LN, rewarm/thaw @ ambient T°C	-20°C, LN <sub>liquid/vapour</sub>	LT
	Clonal Genebank Dormant buds	Collected optimal season, cold-stored (~4°C) optimal MC, CRF	LN <sub>liquid/vapour</sub>	LT
Viable replicable animal germplasm	Live Stock Genebanks animal germplasm spermatozoa	CRF CoCP (glycerol, DMSO, ethylene glycol) thaw @ ~35°C, URF + DF, CoCP (DMSO), thaw rapidly @ ~60°C	LN <sub>liquid/vapour</sub>	MT
	animal germplasm embryos	CRF CoCP+OsCP (DMSO, sucrose, glycerol), 2-step rewarm @ ambient and 35°C	LN <sub>liquid/vapour</sub>	MT
DNA - protocol appropriately selected for sector, taxa and utility	Isolated DNA, (generally applicable)	DNA in buffer, transferred to freezer or LN, rewarm @ ambient T°C	-20°C, -80°C, LN <sub>liquid/vapour</sub>	ST, MT
Human Clinical Therapy and Research	Tissues (Re; Di) (49)	Snap frozen in LN, instantly homogenized in lysis buffer at the moment of extraction	-80°C, LN <sub>liquid/vapour</sub>	ST LT
Nucleic acids, cells, tissues and organs, representing different, medical applications:	Non viable cells (Re; Di) (32)	Cell pellet in PBS or lysis buffer or RNALater or other stabilization buffer/matrix	-20°C, -80°C	ST,

Application context  
 Re = Research  
 Di = Diagnostic  
 Th = Therapy

Viable cells (Re; Th) (77,80)	Progressive rate freezing in cryopreservation media; rapid thawing at 37°C	LN <sub>liquid/vapour</sub>	MT
Viable cells (50)	Ultra rapid cooling vitrification in small volumes; rapid rewarming at 37°C	LN <sub>liquid/vapour</sub>	MT
Tissues (Th) (87,127)	Progressive rate freezing; slow cooling ice-free solidification by Liquidus Tracking; controlled stepwise rewarming	LN <sub>liquid/vapour</sub>	MT
Tissues ( Re; Th) (2)	Chilling, packing in melting ice;	4°C	ST
Tissues ( Re; Th) (127)	Cold continuous perfusion	Ambient, 4°C	ST,
Organs ( Re; Th) (64,68)	Chilling, packing in melting ice; Cold continuous perfusion	Ambient, 4°C	ST,
DNA (Re; Di) (71,90)	DNA in stabilizing, buffer, transferred to freezer, rewarm at ambient T°C	-20°C -80°C	LT
RNA (Re; Di) (47)	RNA in buffer, transferred to freezer, rewarm on ice	-20°C -80°C	LT

Examples are either generic or based on specific protocols representing different sectors as governed by their indicative regulatory framework.

<sup>1</sup>Sample type: generally defined as a biospecimen (e.g. non-viable or non-replicable sample) or a bioresource (e.g. viable culture, germplasm, DNA). Medical applications: Re = Research; Di = Diagnosis; Th = therapy.

<sup>2</sup>Preservation Regime: CoCP = Colligative CryoProtectant, CP = CryoProtectant (± cryoprotectant), CRC = Controlled Rate Droplet Freezing, EN = Encapsulation (+DES = DESSiccation + OSD= OSmotic Dehydration), OsCP = Osmotic Cryoprotection, V = Vitrication. <sup>□</sup> Clarifies appropriate use of terminology for freezing (freezing/thawing), MC= moisture content.

<sup>3</sup>Terminal storage temperature (T°C): is the temperature at which the sample are finally preserved in storage, in the case of LN (132°C the T<sub>g</sub> for pure water) to a minimum of -196°C (liquid phase storage).

<sup>4</sup>Storage duration: categorized in the appropriate disciplinary context as ST = short term (hours to days) MT = medium term (months to decades) LT = long term (decades to multiple decades).

<sup>5</sup>Post-storage Performance Indicators (PIS): specific PIs will be required for certain types of biospecimen and bioresource.

<sup>6</sup>National/international regulatory, convention and policy frameworks: examples linked to biopreservation practices. AT = Antiquities Act; CBD = Convention on Biological Diversity; CITES = Convention on Illegal Trade in Endangered Species; GPA-PGR = Global Plant Genetic Resources for Food and Agriculture; GSPC = Global Strategy for Plant Conservation; HEFA = Human Embryonic Fetal Act (UK); HTA = Human Tissue Act (UK); ITPGRFA = The International Treaty on Plant Genetic Resources for Food and Agriculture; FDA = Food and Drug Administration; NIST = National Institute of Standards and Technology (US); NG = Nagoya Protocol; UN = United Nations



**Acknowledgements:** The authors gratefully acknowledge JG Baust; ISBER (Biospecimen Science Working Group) and colleagues in SLTB and SfC. We are grateful to the 17 reviewers (John G Baust, David Gilham, Kirstin Goldring, Brian Grout, Ryan Guest, Lynn Healy, Ann Linda Van Kappel, Joachim Keller, Vitaliy Kholodny, Maurizio Lambardi, Thomas Leya, Jacky Mackenzie-Dodds, Yuri Petrenko, Matt Ryan, H Martin Schumacher, Glyn Stacey and Erik Woods) for their guidance and insightful critiques.

## REFERENCES

1. Anchordoquy TJ & Molina MC (2007) *Cell Preservation Technology* **5**, 180-188.
2. Baicu S, Taylor MJ, Chen Z & Rabin Y (2008) *Cryobiology* **57**, 1-8.
3. Baird PM & Frome RJ (2005) *Cell Preservation Technology* **3**, 256-266.
4. Ballesteros D, Estrelles E, Walters C & Ibars AM (2011) *CryoLetters* **32**, 89-98.
5. Baust JG (2007) in *Advances in Biopreservation*, (eds) JG Baust & JM Baust, CRC Press/Taylor & Francis, Boca Raton, London pp 1-14.
6. Baust JG & Baust JM (2007) *Advances in Biopreservation*, CRC Press/Taylor & Francis, Boca Raton, London.
7. Baust JM (2002) *Cell Preservation Technology* **1**, 17-31.
8. Baust JM, Snyder KK, Van Buskirk RG & Baust JG (2009) *Biopreservation and Biobanking* **7**, 3-12.
9. Baust JM, Vogel MJ, Van Buskirk R & Baust JG (2001) *Cell Transplantation* **10**, 561-571.
10. Benson EE (2008) *Critical Reviews in Plant Sciences* **27**, 141-219.
11. Benson EE, Betsou F, Amaral R, Santos LMA & Harding K (2011) *Biopreservation & Biobanking* **9**, 399-410.
12. Benson EE & Harding K (2012) in *Plant Cell Culture Protocols*, (eds) VM Loyola-Vargas, N Ocho-Alejo, 3<sup>rd</sup> edition, Humana Press, New York pp. 191-226.
13. Benson EE, Harding K, Debouck D, Dumet D, Escobar R, Mafla G, Panis B, Panta A, Tay D, van den Houwe I & Roux N (2011) *Global Public Goods Phase 2: Parts I, II, III*, SGRP, Rome.
14. Bessone V, Pizarro MD, Izaguirre MF, Biancardi ME, Furno G, Baumgartner N, Rodriguez JV & Quintana AB (2011) *CryoLetters* **32**, 69-80.
15. Betsou F & ISBER Working Group on Biospecimen Science (2009) *Cancer Epidemiology, Biomarkers & Prevention* **18**, 1017-1025.
16. Betsou F et al & ISBER Working Group on Biospecimen Science (2010) *Cancer Epidemiology, Biomarkers & Prevention* **19**, 1004-1011.
17. Betsou F, Gunter E, Clements J, DeSouza Y, Goddard KAB, Guadagni F, Yan W, Skubitz A, Yeadon & Chuaqui R (2013) *Journal of Molecular Diagnostics* **15**, 1-16.
18. Bull M, Lee D, Stucky J, Chiu YL, Rubin R, Horton H & McElrath MJ (2007) *Journal of Immunological Methods* **322**, 57-69.
19. Caamaño JN, Rodriguez A, Muñoz M, de Frutos C, Diez C & Gómez E (2008) *Cell Preservation Technology* **6**, 83-86.
20. Cambon-Thomsen A, Rial-Sebbag E & Knoppers BM (2007) *European Respiratory Journal* **30**, 373-382.
21. Carter A & Betsou F (2011) *Biopreservation & Biobanking* **9**, 157-163.
22. Chen YK, Liu QH, Li J, Xiao ZZ, Xu SH, Shi, XH & Ma DY (2010) *Cryobiology* **61**, 189-193.
23. Clinical and Laboratory Standards Institute (2005) *Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods; Approved Guideline*. CLSI document MM13-A, (ISBN 1-56238-591-7) **25**, 1-51.
24. Comstock GW, Burke AE, Norkus EP, Gordon GB, Hoffman SC & Helzlsouer KJ (2001) *Clinical Chemistry* **47**, 139-142.
25. Council of Europe (1979) Recommendation No. R (79) 5 of the committee of ministers to the member states concerning international exchange and transportation of human substances. Adopted by the Committee of Ministers on 14 March 1979 at the 301st meeting of the

- Ministers' Deputies. [http://www.coe.int/t/dg3/health/recommendations\\_en.asp#policy](http://www.coe.int/t/dg3/health/recommendations_en.asp#policy) (accessed 28/03/2013).
26. Council of Europe (1994) Recommendation No. R (94) 1 of the committee of ministers to member states on human tissue banks. Adopted by the Committee of Ministers on 14 March 1994 at the 509th meeting of the Ministers' Deputies. [http://www.coe.int/t/dg3/health/recommendations\\_en.asp#policy](http://www.coe.int/t/dg3/health/recommendations_en.asp#policy) (accessed 28/03/2013).
  27. Council of Europe (2006) Recommendation Rec(2006)4 of the Committee of Ministers to member states on research on biological materials of human origin. Adopted by the Committee of Ministers on 15 March 2006 at the 958th meeting of the Ministers' Deputies. Appendix to Recommendation Rec(2006)4, Guidelines, Chapter I, Article 2, Recommendation 4. <https://wcd.coe.int/ViewDoc.jsp?id=977859> (accessed 28/03/2013).
  28. Day JG & Harding K (2008) in *Plant Cryopreservation: A Practical Guide*, (ed) B Reed, Springer, New York pp 95-116.
  29. Day JG, Harding K, Nadarajan J & Benson EE (2008) in *Molecular Biomethods Handbook*, (eds) JM Walker & R Rapley, 2<sup>nd</sup> edition, Humana Press, Totowa pp 915-945.
  30. Day JG & Stacey G (2007) *Cryopreservation and Freeze Drying Protocols*, Methods in Molecular Biology, Volume 368, 2<sup>nd</sup> edition, Humana Press, Totowa.
  31. de Vicente MC & Andersson MS (2006) *DNA Banks - Providing Novel Options for Genebanks? Topical Reviews in Agricultural Biodiversity*, IPGRI, Rome.
  32. Debey-Pascher S, Hofmann A, Kreuzsch F, Schuler G, Schuler-Thurner B, Schultze JL & Staratschek-Jox A (2011) *The Journal of Molecular Diagnostics* **13**, 452-460.
  33. Diekmann U, Spindler R, Wolkers WF, Glasmacher B & Müller T (2011) *Cryobiology* **63**, 104-110.
  34. Doerr D, Stark M, Ehrhart F, Zimmermann H & Stracke F (2009) *Biotechnology Journal* **4**, 1215-1220.
  35. Downey P & Peakman TC (2008) *International Journal of Epidemiology* **37**, 146-150.
  36. Draber P, Draberova E & Novakova M (1995) *Journal of Immunological Methods* **181**, 37-43.
  37. Dussert S, Engelmann F & Noirot M (2003) *CryoLetters* **24**, 149-160.
  38. Elliott P & Peakman TC (2008) *International Journal of Epidemiology* **37**, 234-244.
  39. Ellis RH, Hong TD & Roberts EH (1985) *Handbook of Seed Technology for Genebanks: Principles and Methodology*, Handbooks for Genebanks: No. 2, IBPGR, Rome.
  40. Engels JMM & Visser (2003) *A Guide to Effective Management of Germplasm Collections*, Handbooks for Genebanks 6, IPGRI, Rome.
  41. Erro E, Bundy J, Massie I, Chalmers S-A, Gautier A, Gerontas S, Hoare M, Sharratt P, Choudhury S, Lubowiecki M, Llewellyn I, Legallais C, Fuller B, Hodgson H & Selden C (2013) *Bioresearch* **2**, 1-11.
  42. Fuller BJ (2004) *CryoLetters* **25**, 375-388.
  43. Fuller BJ & Dijk S (2008) *CryoLetters* **29**, 175-179.
  44. Fuller BJ, Lane N & Benson EE (2004) *Life in the Frozen State*, CRC Press, London.
  45. Geiseberg JV & Basque T (2011) *Biopreservation & Biobanking* **9**, 373-377.
  46. Glowka L, Burhenne-Guilmin F, Synge H, McNeely JA & Gundling L (1994) *A Guide to the Convention in Biological Diversity*, IUCN Gland and Cambridge.
  47. Grotzer MA, Patti R, Geoerger B, Eggert A, Chou TT & Phillips PC (2000) *Medical & Pediatric Oncology* **34**, 438-442.
  48. Guerin JS, Murray DW, McGrath MM, Yuille MaA, McPartlin JM & Doran PP (2010) *Biopreservation & Biobanking* **8**, 3-63.
  49. Gurusamy KS, Farquharson MJ, Craig C & Davidson BR (2008) *Biometals* **21**, 373-378.
  50. Gvakharia M & Adamson GD (2011) *Fertility and Sterility* **95**, 2552-2553.
  51. Harding K (2004) *CryoLetters* **25**, 3-22.
  52. Harding K & Benson EE (2012) in: *Conservation of Tropical Plant Species*, (eds) NM Noor, HF Chin, BM Reed, Springer, New York pp 121-136.
  53. Harding K, Friedl T, Timmermann H, Lorenz M, Müller J, Day JG & Benson EE (2008) *CryoLetters* **29**, 15-20.
  54. Harding K, Johnston JW & Benson EE (2009) *Agricultural & Food Science* **18**, 3-16.
  55. Harding K, Müller J, Day JG, Lorenz M & Friedl T (2010) *CryoLetters* **31**, 640-472.

56. Harish G, Venkateshappa C, Mahadevan A, Pruthi N, Srinivas Bharath MM & Shankar SK (2011) *Biopreservation & Biobanking* **9**, 379-388.
57. Heins M, Heil W & Withold W (1995) *European Journal of Clinical Chemistry & Clinical Biochemistry* **33**, 231-238.
58. Hewitt RE (2011) *Current Opinion in Oncology* **23**, 112-119.
59. Ho CK, Choi S-W, Siu PM & Benzie FF (2011) *Biopreservation & Biobanking* **9**, 343-348.
60. Holland NT, Smith MT, Eskenazi B & Bastaki M (2003) *Mutation Research* **543**, 217-234.
61. Hubálek Z (2003) *Cryobiology* **46**, 205-229.
62. Hubel A, Aksan A, Skubitz, APN, Wendt C & Zhong X (2011) *Biopreservation & Biobanking* **9**, 237-244.
63. ISBER (2012) *Biopreservation & Biobanking* **10**, 76-161.
64. Jochmans I, Moers C, Smits JM, Leuvenink HG, Treckmann J, Paul A, Rahmel A, Squifflet JP, van Heurn E, Monbaliu D, Ploeg RJ & Pirenne J (2010) *Annals of Surgery* **252**, 756-764.
65. Johnston JW, Benson EE & Harding K (2009) *Plant Physiology & Biochemistry* **47**, 123-131.
66. Johnston JW, Pimbley I, Harding K & Benson EE (2010) *CryoLetters* **31**, 1-13.
67. Johnstone C, Block, W, Benson EE, Day JG, Staines H & Illian, JB (2002) *Polar Biology* **25**, 553-556.
68. Karam G, Compagnon P, Hourmant M, Despins P, Duveau D, Noury D & Boudjema K (2005) *Transplant International* **18**, 657-663.
69. Kisand K, Kerna I, Kumm J, Jonsson H & Tamm A (2011) *Clinical Chemistry & Laboratory Medicine* **49**, 229-235.
70. Kleeberger CA, Lyles RH, Margolick JB, Rinaldo CR, Phair JP & Giorgi JV (1999) *Clinical & Diagnostic Laboratory Immunology* **6**, 14-19.
71. Knebelberger T & Stöger I (2012) *Methods in Molecular Biology* **858**, 311-338.
72. Kokkat TJ, McGarvey D, Lovecchio LC & LiVolsi VA (2011) *Biopreservation & Biobanking* **9**, 349-354.
73. Lehmann S & ISBER Working Group on Biospecimen Science (2012) *Biopreservation and Biobanking* **10**, 366-375.
74. Loken SD & Demetrick DJ (2005) *Human Pathology* **36**, 977-980.
75. Loyola-Vargas VM & Ochoa-Alejo N (2012) *Plant Cell Culture Protocols*, 3<sup>rd</sup> edition, Humana Press, London.
76. Macleod AK, Liewald DCM, McGilchrist MM, Morris AD, Kerr SM & Porteous DJ (2009) *European Respiratory Journal* **33**, 419-425.
77. Majado MJ, Salgado-Cecilia G, Blanquer M, Funes C, González-García C, Insausti CL, Parrado A, Morales A, Minguela A & Moraleda JM (2011) *Transfusion* **51**, 799-807.
78. Malo C, Gil L, Gonzalez N, Cano R, de Blas I & Espinosa E (2010) *Cryobiology* **62**, 17-21.
79. Männistö T, Surcel HM, Bloigu A, Ruokonen A, Hartikainen AL, Järvelin MR, Pouta A, Väärasmäki M & Suvanto-Luukkonen E (2007) *Clinical Chemistry* **53**, 1986-1987.
80. Massie I, Selden C, Hodgson H & Fuller B (2013) *Tissue Engineering Part C: Methods* **19**, 189-195.
81. Massie I, Selden C, Morris J, Hodgson H & Fuller B (2011) *CryoLetters* **32**, 158-165.
82. Mazur P (2004) *Life in the Frozen State*, (eds) B Fuller, N Lane, EE Benson, CRC Press, Boca Raton, London pp 3–65.
83. Mazur P & Seki S (2011) *Cryobiology* **62**, 1-7.
84. McLellan M & Day JG (1995) in *Cryopreservation and Freeze Drying Protocols*, Methods in Molecular Biology, Volume 38, Humana Press, Totowa.
85. Mills FJ (2009) *Biopreservation & Biobanking* **7**, 115-117.
86. Mills FJ & Swanberg G (2012) *Biopreservation & Biobanking* **10**, 1-3.
87. Mirabet V, Carda C, Solves P, Novella-Maestre E, Carbonell-Uberos F, Caffarena JM, Hornero F, Montero JA & Roig RJ (2008) *Cryobiology* **57**, 113-121.
88. Moore HM, Compton CC, Lim MD, Vaught J, Christiansen KN & Alper J (2009) *Cancer Research* **69**, 6770-6772.
89. Müller J, Day JG, Harding K, Hepperle D, Lorenz M & Friedl T (2007) *Am J Bot* **94**, 799-808.
90. Mychaleckyj JC, Farber EA, Chmielewski J, Artale J, Light LS, Bowden DW, Hou X & Marcovina SM (2011) *Journal of Translational Medicine* **10**, 91.

91. Nadarajan J, Mansor M, Krishnapillay B, Staines HJ, Benson EE & Harding K (2008) *CryoLetters* **29**, 95-110.
92. OECD (2007) *Best Practice Guidelines for Biological Resource Centres*, OECD, Paris.
93. OECD (2009) *Guidelines on Human Biobanks and Genetic Research Databases*, OECD, Paris.
94. Panis B, Piette B & Swennen R (2005) *Plant Science* **168**, 45–55.
95. Paredes E & Bellas J (2009) *Cryobiology* **59**, 344-350.
96. Poloni F et al & ISBER Working Group on Biospecimen Science (2011) *Biopreservation & Biobanking* **9**, 415-417.
97. Pugh RS, Becker PR, Porter, BJ, Ellisor MB, Moors AJ & Wise SA (2008) *Cell Preservation Technology* **6**, 59-72.
98. Rao NK, Hanson J, Dulloo ME, Ghosh K, Nowell D & Larinde M (2006) *Manual of Seed Handling in Genebanks*, Handbook for Genebanks No. 8, Bioversity International, Rome.
99. Reed BM (2008) *Plant Cryopreservation: A Practical Guide*. Springer, New York.
100. Riegman PHJ, Morente MM, Betsou F, de Blasio P & Geary P (2008) *Molecular Oncology* **2**, 213-222.
101. Sakai A, Hirai D & Niino T (2008) in *Plant Cryopreservation: A Practical Guide*, (ed) BM Reed, Springer, New York pp 33–57.
102. Schäfer-Menuhr A, Müller E & Mix-Wagner G (1996) *Potato Research* **39**, 507–513.
103. Serrato D, Nieto-Aguilar R, Garzón I, Roda O, Campos A & Alaminos M (2009) *Histology & Histopathology* **24**, 1531-1540.
104. SGRP, System Wide Genetic Resources Programme (2003) *Booklet of CGIAR Centre Policy Instruments, Guidelines and Statements on Genetic Resources*, SGRP-IPGRI, Rome.
105. Shao W, Khin, S & Kopp WC (2012) *Biopreservation & Biobanking* **10**, 4-11.
106. Shaw JM & Jones GM (2003) *Human Reproduction Update* **9**, 583-605.
107. Skyba M, Urbanová M, Kapchina-Toteva V, Košuth J, Harding K & Čellárová E (2010) *CryoLetters* **31**, 249-260.
108. Smith D & Ryan MJ (2008) *CryoLetters* **29**, 63-72.
109. Society for Low Temperature Biology-SLTB (1999) *CryoLetters* **20**, 133-162.
110. Society for Low Temperature Biology-SLTB (2008) *CryoLetters* **29**, 43-72.
111. Society for Low Temperature Biology-SLTB (2008) *CryoLetters* **29**, 157-179.
112. Stacey GN (1999) *CryoLetters* **20**, 141-146.
113. Stacey GN (2004) in *Life in the Frozen State*, (eds) B Fuller, N Lane, EE Benson, CRC Press, London pp 437-452.
114. Stacey GN & Day JG (2007) in *Cryopreservation and Freeze Drying Protocols*, Methods in Molecular Biology, Vol 368, (eds) JG Day & G Stacey, 2<sup>nd</sup> ed, Humana Press, Totowa pp 1-14.
115. Tedder RS, Zuckerman MA, Brink NS, Goldstone AH, Fielding A, Blair S, Patterson KG, Hawkins AE, Gormon AM, Heptonstall J & Irwin D (1995) *The Lancet* **346**, 137-140.
116. Tomlinson MJ (2005) *Human Reproduction* **20**, 1751-1756.
117. Tomlinson MJ (2008) *CryoLetters* **29**, 165-174.
118. Tomlinson MJ & Pacey AA (2003) *Human Fertility* **6**, 100-105.
119. Tomlinson MJ & Sakkas D (2000) *Human Reproduction* **15**, 2460-2463.
120. Towill, LE & Ellis DD (2008) in *Plant Cryopreservation: A Practical Guide*, (ed) BM Reed, Springer, New York pp 421-442.
121. Turbett GR & Sellner LN (1997) *Diagnostic Molecular Pathology* **6**, 298-303.
122. Van Buskirk RG (2007) in *Advances in Biopreservation*, (eds) JG Baust & JM Baust, CRC Press/Taylor & Francis, Boca Raton, London pp 123-142.
123. Végvári Á, Welinder C, Lindberg H, Fehniger TE & Marko-Varga G (2011) *J Clin Bio* **1**, 24.
124. von Versen R, Mönig H-J, Salai M & Bettin D (2000) *Cell Tissue Banking* **1**, 181-192.
125. Walters C (2004) *Biophysical Journal* **86**, 1253-1258.
126. Walters C, Wheeler L & Stanwood PC (2004) *Cryobiology* **48**, 229-244.
127. Wang L, Pegg DE, Lorrison J, Vaughan D & Rooney P (2007) *Cryobiology* **55**, 138-147.
128. Woodrum D, French C & Shamel LB (1996) *Urology* **48**, 33-39.
129. Wowk B (2010) *Cryobiology* **60**, 11–22.
130. Zhang X et al. (2012) *Nanomedicine* **7**, 553–564.