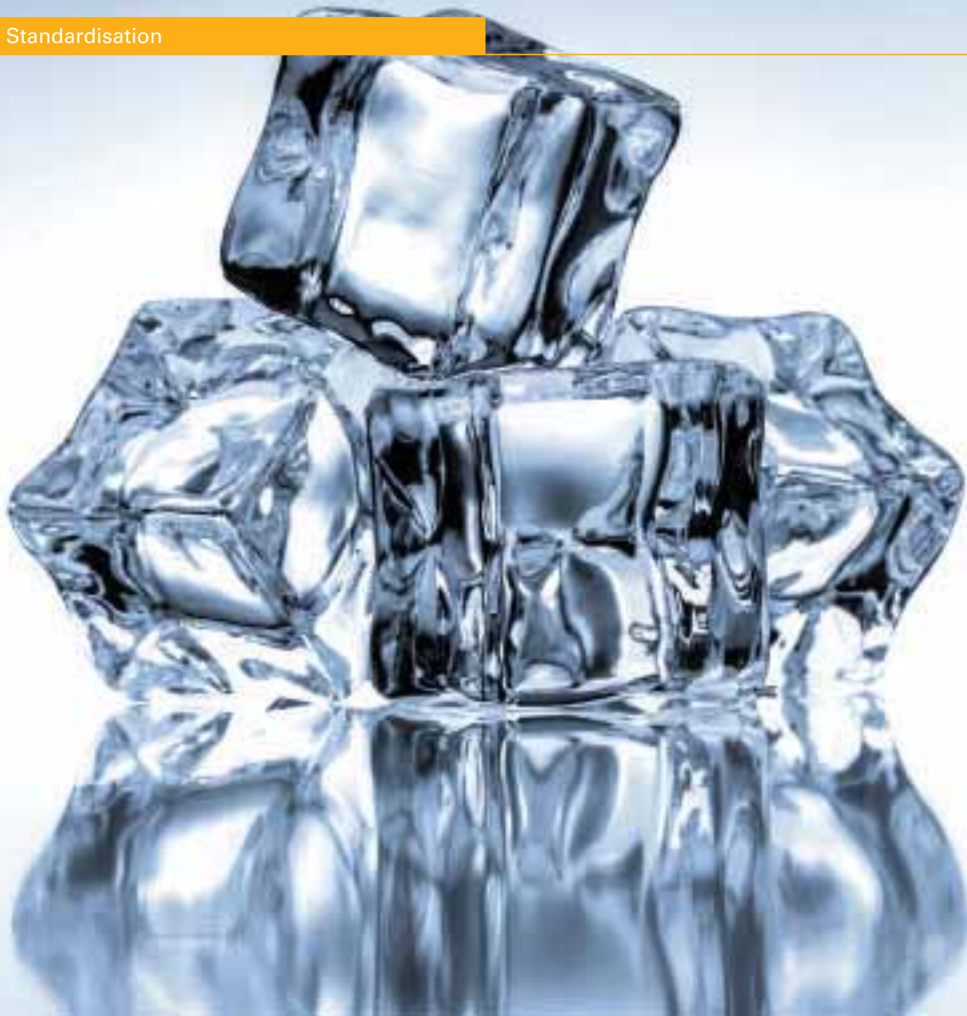

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Playing it Cool

With pharma investing heavily in cell-based research and drug development, it is becoming imperative to take extra precautions in maintaining temperature stability. Although recognition is widespread, efforts to standardise processes are still in their early days

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Modern cell biology research dates back to the early 1950s when it first became possible to maintain, grow and manipulate cells outside of a living organism. During the same time period, Ernest Polge discovered that living cells and tissues could be preserved through the use of very low temperatures, and the field of cryopreservation was born. Today, the art of preserving and manipulating cells for therapeutic purposes is one of the fastest growing areas of modern science.

Many large pharmaceutical companies are on target to invest billions of dollars in the stem cell therapy industry in the next decade. Cells are fragile and can be sensitive to even minute changes in temperature – this is particularly true of stem and primary cells, which form the basis of many of these promising new therapies (1,2). For this reason, great pains are taken to protect cells from undesired changes in temperature during handling, packaging, transport and storage. Despite the widespread recognition that stringent temperature requirements are a necessary part of cell research, efforts to standardise temperature control still have a very long way to go.

Temperature Challenge

Biobanking

Biobanks have become an increasingly important resource for scientists worldwide, particularly those in fields that must have highly detailed, well-documented information about the donor or source material. Although biobanks are heavily regulated and have stringent quality control guidelines, there is little maintenance over how biomaterials get there, at what temperature they have been shipped, or how the sample has been handled prior to shipment or arrival.

Biobanks of human samples fall into various categories, depending on the downstream use of the stored material. Disease-based biobanks are often found at or near a hospital setting; in this case, stored specimens may include human blood cells, tumour cells, or other tissue specimens from both diseased and healthy individuals. Population-based biobanks, on the other hand, are generally situated in an independent setting and usually store samples from healthy volunteers.

While some of the larger biobanks are capable of on-site sample collection and processing, many others have to rely on off-site facilities, followed by transport to the biobank for long-term storage. Alternately, both harvesting and preliminary processing of cells may be done on-site, while more time-intensive or specialised processing must be done elsewhere. These diverse scenarios present many opportunities for variability in the way temperature-sensitive biological samples are handled, and the need for specific guidelines is a great concern (3-5).

Preventing Degradation

Temperature control during collection, isolation and preparation of cells intended for biobanking is crucial, but even when those dynamics are well-controlled, cells are exposed to other temperature-related hazards once they are ready to be sent to a biobank. Shipping is frequently a weak point in any biobanking workflow, since packages are often under the care of a contract company with little in-depth knowledge of sensitive biological materials. Cells should be stored in an appropriate container, with the temperature carefully monitored along the route.

Samples are often protected from degradation during shipping by being kept at or below 4ºC, so high-quality thermal packaging should be used to ensure that temperature is maintained. Frozen samples need to be packed in sufficient dry ice to cover any unforeseen shipping delays, with records indicating how long the samples spent outside of a controlled-temperature environment. Any changes in temperature during shipping should be documented, including how often it occurred and for how long. Ambient temperature at the destination site must be factored into the shipping process, as well as ensuring that cells arrive in a suitable manner, and at a time when technicians are readily available to process the samples in question.

Cells stored in a biobank may be transferred from large freezers to automated processing systems, with many samples requiring more than one transfer. Again, samples need to be monitored and protected from temperature fluctuations during such transfer. When cells are removed from storage for distribution, whether from a biobank or a manufacturing facility, the same care must be taken regarding temperature requirements.

Sample Handling

Small variations in sample handling can affect survival and function of the banked cells, and thus affect the quality of data they generate. Sometimes, diseases are associated with very small changes in DNA (for example, single-nucleotide polymorphisms) or in protein bioactivity, and using reliable samples to study such biomarkers becomes crucial (6,7). Inadequate temperature control can also affect the reproducibility of research or clinical study results. Recently, Professor Kevin Barnham called for increased standardisation of pre-analytical sample handling, which he blamed for unacceptable variability in a blood-based diagnostic assay for Alzheimer's disease (8). His group identified the problem as stemming from protocol-based differences in the temperature at which the blood samples were processed.

In the field of cell-based therapy, lack of reproducibility is arguably an even more serious problem, since it translates directly into changes in efficacy and dosage of the drug product (9,10). The Islet Cell Resource Center, which provides human pancreatic islets for FDA-approved transplantation protocols, recently published a comprehensive study analysing the results of over 2,000 islet shipments. It identified the optimisation of temperature control as the most important factor monitoring the quality of human islet samples (3).

Peripheral blood mononuclear cells (PBMCs) form the basis of many T cell-based therapies, including cancer, HIV, diabetes and rheumatoid arthritis. In many cases, these cells are cryopreserved prior to their use in the clinic. For many years, overnight shipping of whole blood samples from which PBMCs are derived was standard practice. In 2007, the HIV Vaccine Trials Network overhauled the standard operating procedures at all 28 of its clinical trial sites when rigorous studies by Dr Marta Bull *et al* proved that PBMC needed to be cryopreserved within eight hours of venipuncture to retain their optimal function and viability (11).

Cryopreservation Protocols

Fluctuations in temperature during shipping and storage of biological materials may be remedied by innovative temperature control technology, improved oversight, and standard operating procedures with stricter requirements. However, much of the loss of sample integrity that plagues cell-based research occurs during the cryopreservation process – therefore, the protocols, procedures and equipment deserve particular consideration due to the magnitude of the importance to biobanking, cellular therapy and cell research in general.

Cryopreservation protocols contain many modifications based on the cell type being preserved, but, on the whole, the process has been based on the same precept since the early 1970s – namely that the ultimate survival of a frozen and thawed cell is dependent on the cooling and thawing rates, and on how these parameters affect solute distribution and ice formation (12).

Cell Freezing

Cooling of cells from room temperature to 0ºC slows cellular metabolism with little effect on cellular integrity.

However, as cooling continues from 0ºC to -20ºC, extracellular ice crystal formation increases the solute concentration of the culture medium. Water moves out of the cells, beginning the process of dehydration and cell shrinkage. If the cooling rate is too rapid, intracellular ice crystals can form, damaging the cell before it can dehydrate. Ideally, the cooling rate should be slow enough to prevent intracellular ice formation, but fast enough to prevent serious dehydration affects. Numerous studies have identified the optimal cooling rate for most cells to be -1ºC/min (12-14).

Even with this slow cooling, the survival rate for most cells in their normal growth media is very low. Cryopreservatives are therefore added to lower the freezing point of the media, depressing extracellular ice formation while the cell dehydrates. Vitrification is an alternative method to slow-cooling; it relies on flash freezing cells so that there is literally no time for ice crystal formation. This technique also relies on the presence of cryopreservatives. Many different cryopreservatives have been evaluated for their effectiveness in preserving cell viability. Dimethylsulfoxide continues to be the most common choice, but toxicity issues, especially in the cell therapy field, fuel the pursuit of a more optimal alternative (15).

Frozen cells are usually stored at -70ºC to -90ºC for a short time (from days to months) before being transferred to long-term storage in either vapour or liquid-phase liquid nitrogen (-150ºC to -196ºC). Temperature should not be allowed to increase during this transfer, since transient warming events will affect cell viability and possibly function.

Cell Thawing

A great deal of time, money and energy has been spent on optimising cryopreservation techniques. These efforts often concentrate on improving freezing rates, freezing and storage temperatures, storage vessels and freezing media. In terms of effectiveness in preserving cell viability and function, the reasoning behind these approaches may be somewhat misguided if this is the sole focus and cell thawing is overlooked. The causes behind post-thaw cell death have as much to do with the mechanics of thawing as with freezing. Although it has been acknowledged since the 1970s that thawing rate affects cell viability (12), to date, little research has been carried out in this area, and there are few, if any, automated temperature control solutions for the thawing portion of any given cryopreservation protocol.

In contrast to the literally thousands of scientific papers devoted to optimising freezing techniques, there are surprisingly few on the biophysical aspects of thawing. We now know that post-thaw cell death or apoptosis is often a delayed effect of mechanical and chemical stresses that build up during both the freezing and thawing processes. Apoptotic inhibitors added post-thaw can mitigate the effects of this stress, resulting in higher cell survival rates (13). It has also been shown that actively thawing cells at higher temperatures – and therefore faster thawing rates – results in higher post-thaw cell viability (14). Slower thawing rates and repeated freeze-thaw cycles lower cell survival rates by allowing ice to re-crystallise (16).

The current accepted methodology for thawing cells is not standardised; most often, cells are thawed manually in a 37ºC water bath or rolling vials between hands. The 'end-point' of cell thawing is subjective and relies on an individual researcher's observation, with little regard to the optimal temperature and thaw rate for a given cell type. This can introduce significant variability into the results that are obtained. Driven in part by the FDA's oversight into cell therapy, there is now an emerging need to develop a standardised cell thawing device.

Standard Solutions

If there are many temperature-related hazards and pitfalls in the biopharma industry, there are also many solutions. Good Manufacturing Practice guidelines, developed by the FDA for the burgeoning cell therapy industry, can carry over to eliminate many of the inconsistencies in temperature management that exist in basic research (17). Quality assurance oversight programmes developed for large-scale clinical studies provide guidelines for temperature standardisation and management that can be adapted to smaller-scale studies.

Having the right temperature control technology is just as important as having clear, well thought out quality assurance procedures. When freezing tissues, proteins, or cell lysates and cells, many laboratories still use outdated options, including dry-ice filled Styrofoam boxes and ice buckets, or snap-freezing in a dry ice/ethanol slurry. Modern technology offers more sophisticated solutions that are still relatively inexpensive, but provide better temperature consistency. Thermo-conductive racks and trays, for example, which hold Eppendorf tubes or cryogenic vials, provide uniform, highly reproducible freezing rates by transmitting heat from the sample to the dry ice beneath it (18). The tray itself provides a barrier that prevents potential contamination from the ice or dry ice slurry being used to maintain temperature.

Positive Updates

The technology involved with controlled-rate cell freezing has also seen significant updates in recent years. This cryopreservation method has long relied on automated programmable freezers, which are highly accurate and have extremely reproducible freezing rates. Smaller, liquid nitrogen-free versions are available to help cut the extortionate costs and alleviate some of the maintenance issues. In addition, passive freezing devices have now come of age, offering low-cost, portable, maintenance-free temperature control, that has been shown to be just as effective as programmable freezers in maintaining freeze rate. Alcohol-free, controlled rate freezing containers such as CoolCell® containers have now been adopted in clinical trials (19), and are even recommended by American Type Culture Collection for handling induced pluripotent stem cells (20).

Progress has also been made in the capacity to safely transfer and transport cells and other temperature-sensitive biological products. Where gel packs and dry-ice filled Styrofoam boxes were once the only option for shipping and handling these valuable materials, several different companies now offer innovative advances in temperature control technologies.

While there is still a long way to go, efforts to improve temperature standardisation and reproducibility are gaining momentum. The clinical requirements of rapidly advancing fields like cell therapy stand to benefit academic research as well. It is hoped that increased awareness of the unique importance of temperature management to the field of cell biology will bring a brighter future to both research and patient care.

References

1. Golab K *et al*, Challenges in cryopreservation of regulatory T cells (Tregs) for clinical therapeutic applications, *International Immunopharmacology* 16(3): pp371-375, 2013
2. Olson WC, Smolkin ME and Slingluff CL, Shipping blood to a central laboratory in multicenter clinical trials: Effect of ambient temperature on specimen temperature, and effects of temperature on mononuclear cell yield, viability and immunologic function, *Journal of Translational Medicine* 9(26): pp1-11, 2011
3. Kaddis JS, Hanson MS and Niland JC, Standardized transportation of human islets: An Islet Cell Resource Center study of more than 2,000 shipments, *Cell Transplantation* 22(7): pp1,101-1,111, 2013
4. Vaithilingam V, Barbaro B, Oberholzer J and Tuch BE, Functional capacity of human islets after long-distance shipment and encapsulation, *Pancreas* 40(2): pp247-252, 2011
5. Vaught JB, Caboux E and Hainaut P, International efforts to develop biospecimen best practices, *Cancer Epidemiology Biomarkers and Prevention* 19: pp912-915, 2010
6. Malm JL *et al*, Developments in biobanking workflow standardization providing sample integrity and stability, *Proteomics* 95: pp38-45, 2013
7. Cao E, Chen Y, Cui Z and Foster PR, Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions, *Biotechnology and Bioengineering* 82(6): pp684-690, 2003
8. Watt AD *et al*, Variability in blood-based amyloid beta assays: The need for consensus on pre-analytical processing, *Journal of Alzheimer's Disease* 30(2): pp323-336, 2012
9. Valeri CR and Pivacek LE, Effects of the temperature, the duration of frozen storage, and the freezing container on *in vitro* measurements in human peripheral blood mononuclear cells, *Transfusion* 36: pp303-308, 1996
10. Antonenas V *et al*, Fresh PBSC harvests, but not BM, show temperature-related loss of CD34 viability during storage and transport, *Cytotherapy* 8(2): pp158-165, 2006
11. Bull M *et al*, Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials, *Journal Immunological Methods* 322: pp57-69, 2007
12. Mazur P *et al*, Interactions of cooling rate, warming rate, and protective additive on the survival of frozen mammalian cells, *Ciba Foundation Symposium on the Frozen Cell*, 1970
13. Bissoyi A, Nayak B, Pramanik K and Sarangi SK, Targeting cryopreservation-induced cell death: A review, *Biopreserv Biobank* 12(1): pp23-34, 2014
14. Chua KJ and Chou SK, On the study of the freeze-thaw thermal process of a biological system, *Applied Thermal Engineering* 29: pp3,696-3,709, 2009
15. Morris C *et al*, Chronic Malignancies and Lymphoma Working Parties of the EBMT. Should the standard dimethyl sulfoxide concentration be reduced? Results of a European Group for Blood and Marrow Transplantation prospective noninterventional study on usage and side effects of dimethyl sulfoxide, *Transfusion*, June 2014
16. Deller RC, Vatish M, Mitchell DA and Gibson MI, Synthetic polymers enable non-vitreous cellular cryopreservation by reducing ice crystal growth during thawing, *Nature Communications* 5: p3,244, 2014
17. Buckler RE, Ehrhardt R and Thompson M, Back to the Future, *European Biopharmaceutical Review* 67: pp10-14, 2014
18. Reducing the variability of pre-analytical handling of biological samples, BioCision Biotech International, 2010
19. Foussat A *et al*, Effective cryopreservation and recovery of human regulatory T cells, *BioProcess International* 12(S33): pp34-38, 2014
20. iPSCguide ATCC, ATCC stem cell culture guide: Tips and techniques for culturing stem cells, 2014

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