# Right cell, right result with controlled-rate freezing

Advances in embryology that have characterised medical progress over the past quarter of a century have relied on the application of methods such as controlled-rate freezing, as Paul Lakra and Geoffrey Planer explain.

Twenty-five years ago, in the spring of 1984, Britain triumphed on the ice when Jayne Torvill and Christopher Dean won gold as Olympic ice-skating champions in Yugoslavia. At nearly exactly the same time, a less well-known triumph, but also a frozen one, occurred when an Australian team using equipment made by a British company helped the first baby ever to be born from a frozen embryo.

On 28 March 1984, Zoe Leyland was born in Melbourne after Alan Trounson and Carl Wood, the doctors who managed her birth, had decided to try 'test tube' fertilisation of a frozen embryo. Her mother had hormonal stimulation and produced 11 eggs, which were frozen using a new type of controlled-rate freezer made by a London company.

Professor Trounson set something of a trend and since then, of the three million or so babies born via *in vitro* fertilisation (IVF) techniques, some 20% (about 600,000 people) are estimated to have been created from controlled-rate frozen embryos.

However, it is not just embryos that need freezing down carefully to survive liquid nitrogen temperatures (–196°C). It was found that other valuable cells had to be precision frozen, in cryoprotectants, using different temperature ramps before they could be stored and then thawed successfully. In the years that followed, as the biological sciences in general expanded, the need for low-temperature cell and tissue storage also increased. Simultaneously, the need for control, monitoring and alarming of the equipment used with these valuable specimens increased. In turn, the technology developed allowing internet-based logging, control and feedback to scientists to improve dramatically the whole environment for important cell lines.

Controlled-rate biological freezer being used for cell preservation.

## Pioneers in preservation

Pioneering husbandry in the area was carried out when fowl sperm was cryopreserved for the first time in 1949 by a team of scientists in the UK led by Dr Christopher Polge. The process moved into the human world in the 1950s with pregnancies obtained after insemination with frozen sperm. The first calf derived from the transfer of frozen bovine embryos was reported by Ian Wilmut in Cambridge in 1973.

Early haematopoietic stem cell transplants were undertaken in the 1970s, with some bone marrow work being performed using the original controlled-rate freezing machine (Planer) between 1968 and 1971. The ability of cryoprotectants (glycerol in early cases) to protect cells from freezing injury was discovered accidentally. Freezing injury has two aspects: direct damage from the ice crystals, and secondary damage caused by the increase in solute concentration as progressively more ice forms.

In 1963, at Oak Ridge National Laboratory in the USA, Peter Mazur showed that lethal intracellular freezing could be avoided if cooling was slow enough to permit sufficient water to leave the cell during progressive freezing of the extracellular fluid. The controlled-rate freezing procedure,

Stem cell laboratory with LN2 vessels and controlled-rate freezers prior to installation.

originally suggested nearly 40 years ago by British scientist Professor David Pegg, was a breakthrough, and most IVF laboratories worldwide now use controlled-rate freezers. The same 'freezing down' techniques were used for other types of cell prior to frozen storage in instances where post-thaw viability of the sample was important (eg bone marrow, semen, oocytes, botanical seeds, skin, ovarian tissue, heart valves, blood vessels, stem cells and other cell lines).

## Better cell viability

As the medical, pharmaceutical and biological sciences expanded, so too did the range of freezers for safe storage of the associated cells, and colder mechanical freezers and more sophisticated liquid nitrogen-based equipment evolved. The preservation of biological matter was clinically important because increasing amounts of human and animal tissue needed to be stored. Such tissue was required for research and regenerative medicine purposes in cancer treatment, vaccine production, stem cell work, human fertility, regenerative medicine as well as in animal husbandry, botanical studies, aquaculture and seed and fungi banking in conservation.

Cryogenic storage at very low liquid nitrogen temperatures is presumed to provide an indefinite longevity to cells, although the actual shelf-life is rather difficult to prove. Estimates based on the accumulation of radiation-induced DNA damage during cryogenic storage tentatively suggest a maximum storage period of 1000 years. In experiments with dried seeds, researchers found that there was noticeable variability in deterioration when samples were kept at different 'frozen' temperatures – even very cold ones. Temperatures below the glass transition point (Tg) of water (around –136°C) appear to be accepted as the range where biological activity very substantially slows down, and –196°C (liquid phase of nitrogen) is the preferred temperature for the storage of important specimens.

Hence, while refrigerators, deep freezers and extra-cold deep freezers – all similar to domestic ones – can often be used, the ultracold environment of liquid nitrogen at –196°C is increasingly demanded for successful preservation of the more complex or more important biological samples. Freezing in liquid nitrogen is also safer in that equipment failure very rarely results in fast warming of samples. As there is little point in freezing samples if they prove suboptimal on thawing, controlled-rate freezing was used more often as the importance of a cell's specific environment became more apparent.

In the IVF world, after Zoe Leyland's birth, freezing embryos was considered mainly as a back up for some time and as less desirable than using 'fresh'. However, as follow-up studies were undertaken it appeared that controlled-rate frozen embryos developed into equally healthy children compared with the 'fresh' ones. Recent studies from Denmark, Australia, the USA and Finland have indicated they could be even healthier.

So, what might the future bring? New techniques have shown that it is also sometimes possible to preserve important and difficult-to-freeze living cells by cooling so rapidly that ice cannot form – the main problem to overcome for such difficult samples. However, this process is still limited to very small samples and requires high concentrations of potentially toxic cryoprotectant chemicals. Such methods, termed vitrification, have been developed for a restricted range of cell types including spermatozoa and embryos. However, these methods require skilled clinical scientists to implement them and are incompatible with the higher standards of laboratory practice because it is difficult to record the actual freezing process – and additionally such methods are not easily repeatable.

Another new approach, which is applicable to larger samples such as cartilage, hopes to achieve a type of 'vitrification' through the use of a controlled variable concentration of cryoprotectants during the freezing process. This idea was suggested a few years ago but thus far the equipment, if any, has been experimental. The tracking of a 'liquidus' involves a programmed increase in cryoprotectant concentration, coincident with reductions in temperature at a rate that prevents supercooling and allows the tissue cryoprotectant concentration to follow the liquidus curve. This avoids both ice crystal formation and the cytotoxic effects of exposing tissues to highly concentrated cryoprotectants at high temperatures. Experiments with the technique have proved successful in cryopreserving animal cartilage, and the process is now being investigated further with a view to human replacement.

Looking at even larger samples, attempts to cryopreserve vital organs have been uniformly unsuccessful to date, and currently the storage of most

organs is limited to temperatures just above freezing for a maximum of a few days. Ovarian cryopreservation, however, has been studied quite extensively, primarily due to its clinical application in fertility preservation. Perhaps this new 'liquidus tracking' technique will make progress towards that goal and even the preservation of larger organs. This could be the route by which whole organs such as heart, liver or kidney might one day be cryopreserved for later transplantation.

## Challenges remain

Apart from the increasing importance each year of cryopreservation, the actual management of cold-stored biological material grows with the increasing numbers of samples under storage. The UK has long been a world leader in legislative control of human fertilisation. The Human Fertilisation and Embryology Authority (HFEA) has followed a fine line in allowing clinical techniques to develop while excluding public fears of science-fiction scenarios (eg human cloning and animal-human hybrids).

Comparatively recently, the European Union (EU) has introduced the Human Tissue Directive, which had to be implemented in all member states by April 2006. This wide-ranging legislation impacts on all forms of work with human tissue, particularly how it is handled and stored. The EU Directive includes a requirement to 'monitor' samples. With many cells stored at low temperatures, logging systems, such as those available from Planer that can also issue multiple warnings about changing conditions, are becoming mandatory. Such systems need to be extremely robust and reliable in their environments.

Installation of systems following the introduction of the EU Directive often reveals to laboratory managers obvious potential improvements in working practices. Instances such as leaving refrigerator or incubator doors open for periods of time, which results in temperature recovery times of half an hour or more, are not unknown! The impact of unnoticed errors can be even worse for fully controlled environments such as in $CO_2$ incubators.

For nearly four decades the electronics and other manufacturing industries have been paying attention to every detail in their processes, trying bit by bit to make everything better. They have done this by measuring everything they do, ensuring every process has quality data that can be analysed, and reducing the variation in every activity (called Six Sigma). In this way, the significance of every variation is revealed and can be categorised depending on whether or not it impacts on the final product. This is how improvements have been made that have led to the reliability of modern electronic equipment. If we look at a biomedical laboratory as an example, how many processes generate quality information that can be analysed? Monitored storage conditions and controlled-rate freezers can, but what about all the manual operations performed by skilled scientists?

In its widest sense, IVF has become a recognised 'industry' in its own right, and other cell-based disciplines (eg work with stem cells and mouse embryos) are heading in the same direction. The regulations enshrined in the EU Directive and similar initiatives from the US Food and Drug Administration (FDA) are being implemented and gradually those at the grass-roots level will be taking up techniques from industry to improve their processes – driven both by these regulations and the desire for better yields.

Six Sigma methods are common in the motor industry but are rarely if ever implemented in a laboratory or clinic. As individuals, scientists probably do not even feel that they are part of an 'industrial' type of process, albeit one where the output is a cured patient, a successful trial, a new vaccine or a healthy mother and baby!

Storage Dewars set up for web-based monitoring.

### SPECIALIST IN CELL SAFETY

Planer specialises in the measurement and control of physical parameters related to cell safety. Based near London's Heathrow Airport, the company has sold thousands of state-of-the-art electronic and software products worldwide since 1973. It pioneered the development of controlled-rate freezing and now supplies incubators, integrated monitoring equipment and software for the viable processing, freezing and preservation of medical and biological specimens to customers who depend on the onward viability of their cell samples.

### Studies on the health of cryopreserved embryos using controlled-rate freezers


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