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WHITE PAPER No. 60

Cell Thawing Protocol Standardization – Guide for More Reproducible Cryopreservation Results

Executive Summary

Thawing frozen cells is an essential, but sometimes undervalued element in all areas of cell-based research and production. Standardization of the cryopreservation process – including a standardized cell thawing protocol – helps to use cell stocks to their fullest extent for reproducible, reliable results.

Content overview

Introduction

The freezing and thawing of cells has long been one of the standard techniques in cell biology. The process, also known as cryopreservation, has made it possible to effectively stop the cellular clock, which adds significant reproducibility to experiments carried out at different times, because it allows very similar batches of cells to be used months, years, or even decades apart (figure 1). It also helps in saving time, resources, and costs [1].

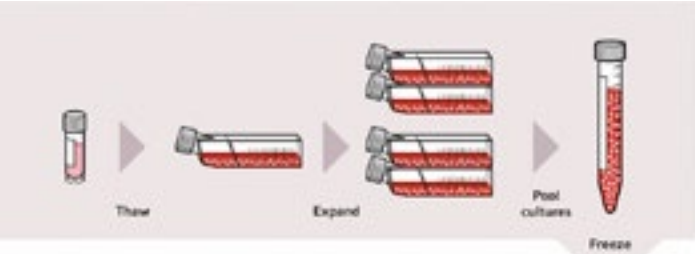

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Figure 1: Schematic overview of a two-tiered cell banking system: The establishment of a master cell bank and a working cell bank including cryopreservation at the lowest passage is good cell culture practice.

The key challenge throughout the entire freezing-storage-thawing process is to ensure high cell survival and, perhaps more importantly, predictable and reproducible cell behavior after cryopreservation. To achieve this, many different guidelines and protocols have been developed over time.

During cryopreservation, the main cause of cell death is the formation of intracellular ice. When frozen incorrectly, ice crystals can be formed inside cells and can damage cell membranes and organelles, thereby significantly reducing the chance of cell recovery.

To help prevent intracellular ice formation, there are two essential requirements of every cell freezing protocol. The first is the addition of a cryoprotective agent to the freezing medium. These compounds, such as dimethyl sulfoxide (DMSO) or glycerol, penetrate cell membranes and usually lower the freezing point and the glass transition temperature of the medium, thereby preventing ice crystal formation inside the cells [2,3].

The second requirement is slow cooling. Regardless of cell type, cooling cell suspensions at a rate of 1 °C per minute over the critical temperature range between 4 and –70 °C is commonly seen as optimal for cell survival [3]. This is because cooling cells slowly ensures that low-solute ice crystals form outside the cell first, thereby increasing solute concentration in the remaining medium and draining the cell through osmosis. This in turn leads to less ice formation inside the cells [3].
After controlled freezing to a temperature of at least –70 °C, vials can be transferred to liquid nitrogen storage (–150 to –196 °C, figure 2) or specialized freezers operating at –150 °C for long-term preservation [4].

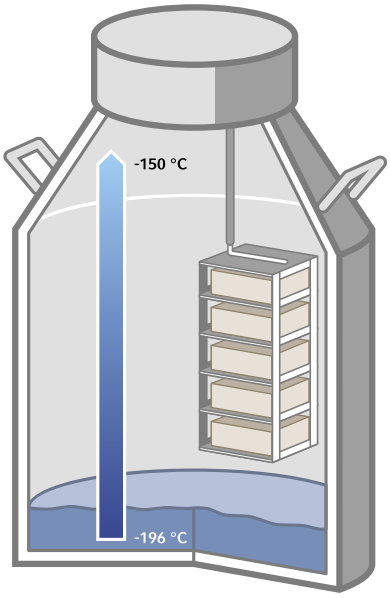


Figure 2: Liquid nitrogen storage is recommended for long-term cell storage.

▶ Click here for more information

Many textbooks and online resources give detailed information on the principles and procedures that help scientists achieve standardized, reliable cell freezing, but often pay little attention to considerations associated with the cell thawing process. The following sections aim to give an overview of the principles of cell thawing, describe different methods, and discuss considerations for improved standardization of a cell thawing protocol.

How to thaw cells

Whereas cell freezing needs to occur at a slow, controlled rate, thawing frozen cells works best when it is done quickly. The disappearance of ice around the cell does not have the same damaging effects as ice formation, so it is preferable to bring the cells back to normal culture conditions as soon as possible, where (in the case of adherent cells) they can anchor to a surface.

A typical cell thawing protocol usually begins with retrieving vials from the liquid nitrogen storage. Here, it is important to be familiar with all the normal precautions for working with liquid nitrogen (figure 3) [5]. In addition, if a vial did not seal properly and was stored in the liquid phase, liquid nitrogen may have seeped into the vial over time, leading to a rapid build-up of pressure inside the vial shortly after removal from the liquid nitrogen – so wearing adequate face protection is essential.

Figure 3.: Care should be taken to prevent injury when collecting cells from liquid nitrogen storage.
Image source: Minerva Studio/shutterstock.com

Next, the vials must be thawed. A frequently used rule of thumb is that upon beginning the cell thawing process in a standard cryovial containing a 1 mL cell suspension, all ice needs to have disappeared within a few minutes. Rapid heating prevents localized recrystallization during thawing, which can cause cellular damage [6].

Sometimes thawing within a few minutes is not possible, for example due to time spent on finding different vials or due to the location of the liquid nitrogen storage. In this case, leaving vials at the lowest possible temperature followed by rapid thawing is preferable to both slow thawing and to leaving the cells in thawed freezing medium for longer than necessary [8].

When all ice has disappeared, any further adverse effects of the cryoprotective agent on the cells need to be minimized. There are two ways of doing this. Firstly, cells can be cultured directly, for example in a T-flask, while ensuring the freezing medium is diluted by at least a factor of ten with normal culture medium. Alternatively, it is possible to dilute the freezing medium with normal medium, centrifuge the tube, discard the medium, and resuspend the cells in fresh culture medium [1,4,8].

In order to check that the cell thawing protocol has been successful, it is recommended to determine the percentage of viable cells (e.g. with a trypan blue staining and a cell counter) [9]. To improve standardization, it is good practice to continue observing the culture over several days for any abnormalities in shape or growth rate.
Poor or inconsistent growth might be a sign of problems with the cell stock or the cryopreservation process, so early detection can help to minimize experimental errors.

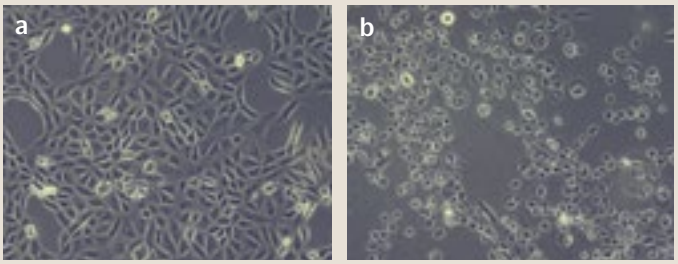
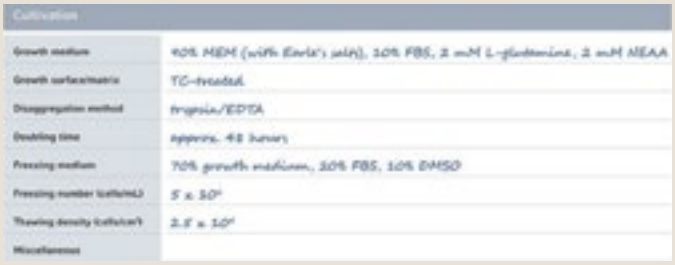


Figure 4: Observe the culture after thawing for abnormalities in shape. Example of normal (a) and abnormal (b) morphology of cells (Vero cells, 10 x)



▶ **Use our template „Cell profile“ in order to record clearly and consistently the important details of cultivation**

Cell thawing methods

Different methods and equipment exist to thaw cells in cryovials.

Table 1. Overview of the strengths and weaknesses of different cell thawing methods.

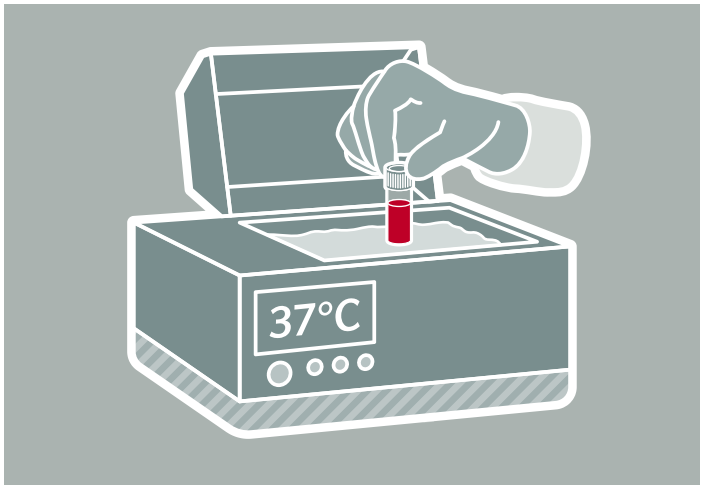
	Warming speed	Contamination prevention	Documentation	Cost
Water bath	+	–	–	+
Hand warming	–	+	–	+
Bead bath	–	(+)	–	+
Dedicated devices	+	+	+	–

Waterbath

Probably the most commonly used method for thawing vials of frozen cells in the lab is by using the communal water bath (figure 4). Water ensures rapid heating as it has good conductivity, while also preventing local overheating inside the vial. A water bath is also part of the standard equipment used in the cell culture lab on a day-to-day basis, so there is no need for additional preparation or investment.

When warming cells using a water bath, or indeed any other method of thawing, it is important not to expose the cells to temperatures higher than 37 °C. Even though the total temperature change during warming can be more than 200 °C, exceeding 37 °C locally inside the vial can quickly lead to undesired effects, and even to cell death [10].

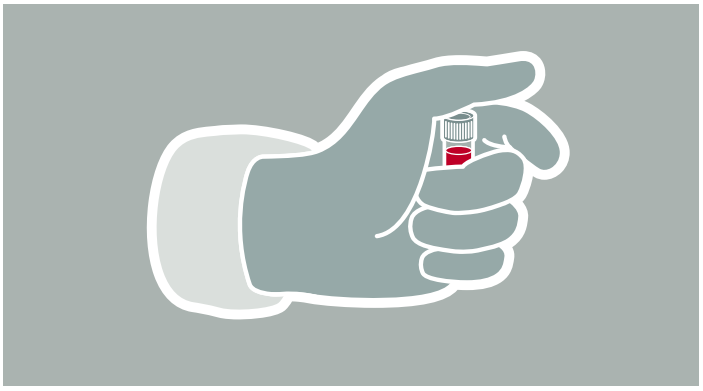
A key drawback of water baths however, is the contamination potential due to contact between the water and the sample. Keeping the top of the vial dry during thawing can be difficult due to the water level, the need to check the vial continually, and the need to agitate the sample to prevent thermal gradients [6].



Hand-warming

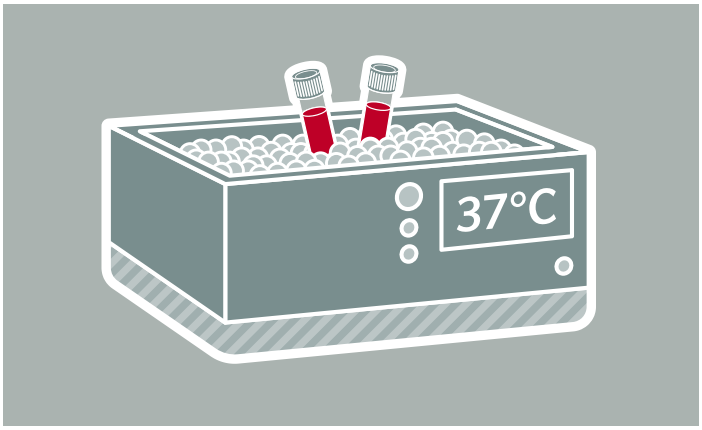
Warming cryovials by hand can be tempting as a cell thawing method because it does not require any equipment, body temperature is similar to a water bath, and because it enables the user to monitor and agitate the vial continuously. However, it is important to note that heat transfer is substantially less efficient and more variable compared to a vial submerged in water, which means that warming a vial by hand is likely to be slower and less reproducible.

Hand-warming is also impractical when thawing more than two vials at the same time, and can lead to even slower cooling rates. In addition, exposing the skin, even through nitrile gloves, to temperatures close to –196 °C can cause serious injury. For these reasons, hand warming is not recommended.



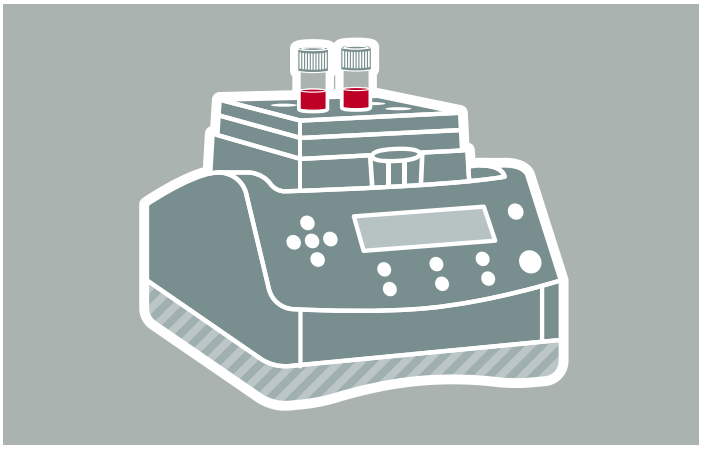
Bead bath

Bead baths are an alternative to a water bath, for example when warming up cell culture media or when keeping cultures warm outside the incubator. However, similar to hand warming or air warming (e.g. in an incubator), beads do not provide the efficient heat transfer of a water bath as there is not as much surface area in contact with the vessel, making it unlikely that cells can be thawed reliably or quickly enough. Bead baths are often promoted because they do not require refill, however accumulation of dust and spillages may still require cleaning and refills over time to prevent contamination.



Dedicated devices

Over the last decade, several manufacturers have developed specialized devices that can achieve the required rapid warming rate without using a water bath. Most are designed for cryovials alone, but systems that can handle larger volumes are also available. Dedicated devices perform the thawing process in a reproducible procedure, which offers important benefits in situations where there is a higher demand for standardization and documentation of the thawing process. They are also well suited for situations where water baths are impractical [6,7]. The most important drawback to dedicated thawing devices is the upfront investment required. Whereas water-bath thawing usually requires no extra investment, specialized thawing equipment adds extra costs to the cell culture workflow. In addition, some devices are only capable of thawing one vial at a time, making them potentially incompatible with some cell culture protocols.



Considerations prior to selecting a thawing method

Lab-specific considerations

When comparing the different methods of cell thawing, there are inevitably many lab-specific considerations that play a role. One example is when carrying out cell culture in a cleanroom. In this environment, water baths could be a source of air contamination and alternative methods should be considered [11].

Another specific situation affecting the choice of thawing method is when working in cell culture labs where documentation and tracing of every working step is required, or where a constant warming rate is needed [6,12]. Of course, the available budget for lab equipment will also play a key role in whether cell thawing is carried out with existing equipment or using dedicated devices.

Standardization in cell thawing

Regardless of the method chosen, standardization brings great benefits to results – whether it concerns work in a regulated environment or fundamental research. For example, having a consistent cell survival rate reduces the need to thaw excess cells to ensure sufficient cell stock. This is particularly important when working with low passage numbers as the amount of available cells is likely to be limited.

Protocol standardization also affects the behavior of cells after plating. For example, some cell types are more likely to undergo differentiation when exposed to DMSO for longer [13]. Variations in the freezing or thawing protocol can therefore lead to differences in the cells' phenotype after cryopreservation and to variation in results.

Cell type-specific considerations

Many aspects of freezing and thawing apply to all cell types, however it is important to be aware that some aspects may vary for certain cell types, and so cell type-specific thawing protocols should be used where possible. Many commercial cell lines have been thoroughly studied and have optimized cryopreservation protocols, but for less common cell types a literature search or small-scale optimization study is recommended.

When it comes to cell thawing, one aspect that varies per cell type and should be monitored closely is the time it takes before the cells resume normal growth and a normal response to stimuli. For example, when the rate of proliferation is a key parameter in a study, it is essential that experiments are not started with cells that have not yet fully recovered from the thawing process – something that can vary greatly between cell types. Similarly, when expression of a specific protein is measured, the cells' ability to produce this protein needs to have fully returned [8].

Summary

For consistent results in the cell culture lab, it is important to aim for a high level of standardization in every aspect of cell culture. Cell thawing can be performed using different methods – and choosing the right cell thawing protocol for every specific cell type, lab, or application is key to setting the right standards for reliable data and reliable products.

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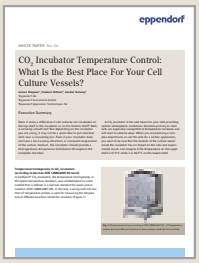

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