Chapter 2

Cryopreservation of Hepatocytes

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Abstract

The use of cryopreserved hepatocytes has increased in the last decade due to the improvement of the freezing and thawing methods, and has even achieved acceptance by the US Food and Drug Administration for use in drug metabolizing enzyme induction studies. This chapter provides an overview of the theories behind the process of cryopreservation as well as practical advice on methods to cryopreserve hepatocytes, which retain functions similar to fresh cells after thawing. Parameters, such as cell density, cryoprotectants, freezing media, storage conditions, and thawing techniques, should be critically considered. Special emphasis is put on human hepatocytes, but information for the cryopreservation of animal hepatocytes is also described.

Key words Cryopreservation, Human, Animal, Hepatocytes, Purification, Plateability, Thawing, Recovery

1 Introduction

As a result of the improvement of the cryopreservation of human hepatocytes and their increased plateability after thawing, their application to different assays has been extended dramatically. Early cryopreservation methods only allowed for short-term incubations (i.e., less than 6 h) with thawed cell suspensions. Improved methods have resulted in plateable cryopreserved hepatocytes that can be used in long-term assays, such as drug metabolizing enzyme induction studies, time-dependent inhibition studies, long-term drug metabolism, hepatotoxicity, and bile transporter function assays [1–3]. The quality of thawed plateable hepatocytes from different species is apparent as highly confluent cultures with morphologies similar to that of fresh cells (Fig. 1). The confidence in cryopreservation methods has reached a level such that regulatory agencies accept fresh and cryopreserved cells data interchangeably [4].

The method of cryopreservation is based on preventing cellular damage due to ice crystal formation and chemical changes in cells as they cool and eventually freeze. If cells are frozen using a

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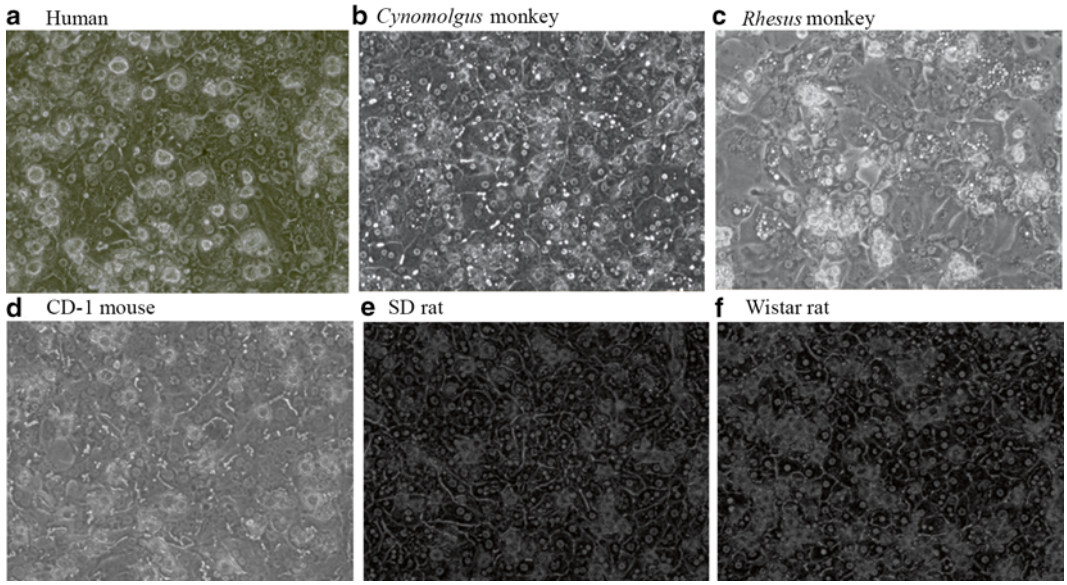

Fig. 1 Morphology of cultures of primary hepatocytes from different species. The morphology and confluency of thawed cryopreserved (**a**) human, (**b**) *Cynomolgus* monkey, (**c**) *Rhesus* monkey, (**d**) CD-1 mouse, (**e**) SD rat and (**f**) Wister rat hepatocyte cultures indicates optimal health of the hepatocytes. Hepatocytes that are well cryopreserved would retain the typical polygonal cell shape with defined plasma membranes, clear (multi-) nuclei and high confluency (10× magnification)

constant slow decline in temperature, regardless of the rate, the cells lose viability and functions [5]. This is because water in the medium starts to freeze before intracellular water, resulting in a higher osmolarity in the medium. Water leaves the cells by osmosis, causing them to shrink. Although a certain amount of shrinkage is not detrimental, if the temperature continues to decrease at a slow rate, too much water leaves the cells and they start to collapse on themselves (i.e., plasmolysis). Excessive loss of intracellular water causes precipitation of solutes, changes in pH, and denaturation of proteins, which causes them to die [6]. In contrast to slow freezing, rapid freezing simply freezes all water and does not allow intracellular water to leave the cells by osmosis. The intracellular water forms ice crystals, which disrupt membranes, leading to the demise of the cells. As a result of the understanding of the effects of freezing, methods for preventing cryo-injury due to ice crystals or biochemical imbalances due to dehydration have been developed [7]. These have been shown to produce cryopreserved hepatocytes with the highest and reproducible viability (Table 1). In this chapter, the entire process of cryopreservation, from selection of the best fresh hepatocytes to their thawing, is described. The procedures described represent the most current practice based on available published information rather than procedures from a specific laboratory. The procedures may be further optimized for best results by investigators interested in cryopreservation of their hepatocytes of interest.

Table 1
Yield and viability of primary human hepatocytes from different donors

Human donor number	Experiment number	Age (years)	Gender	Race	Yield (million)	Viability (%)
1	1	35	M	H	7.9	95
2	1	26	F	C	6.0	98
3	1	47	F	C	5.3	95
4	1	25	M	C	5.8	79
5	1	64	F	H	7.0	93
6	1	15	M	C	6.0	75
7	1	17	M	C	4.4	97
8	1	21	F	C	6.4	89
9	1	59	F	C	8.3	97
10	1	56	M	H	7.5	97
11	1	51	M	C	5.8	89
12	1	40	F	C	5.8	92
13	1	17	F	C	4.4	93
14	1	45	M	C	5.1	98
15	1 2 3 4	55	M	C	8.0 7.8 8.8 7.0	98 92 94 92
16	1 2 3 4	42	M	H	6.9 6.0 7.7 7.8	97 93 90 91

The cryopreserved human hepatocytes were recovered using Universal Cryopreservation Recovery Medium. Viability was determined based on trypan blue exclusion. The reproducibility of the yield and viability for each donor can be seen in the results for human donor numbers 15 and 16 where results of four independent evaluations, each with a different vial, are shown. Yield is the number of viable cells recovered after thawing (C, Caucasian; F, female; H, Hispanic; M, male)

2 Materials

2.1 Preincubation Medium

1. Complete William's Medium E. Add 5.5 mL 2 mg/mL insulin solution dissolved in water, 2.5 mL 10 mg/mL gentamycin solution and/or 5 mL 100× concentrated penicillin/streptomycin solution to 500 mL William's E Medium GlutaMAX (Life Technologies, Germany).
2. Fructose or alpha lipoic acid. If the performance of the freshly isolated cells is suboptimal (e.g., the viability is below 70 %, the

morphology is poor or there is low attachment when seeded), the preincubation medium can be supplemented with fructose or alpha lipoic acid. To this end, add fructose or alpha lipoic acid at final concentrations of 200 mM and 5 mM, respectively, to complete William's Medium E (*see* **Note 1**).

3. Glucose and insulin. If the performance of the cells after isolation is suboptimal, the preincubation medium can be modified to include glucose and insulin. For this purpose, add glucose and insulin at final concentrations of 5 mM and 1 nM, respectively, to complete William's Medium E (*see* **Note 2**).
4. *N*-Acetyl-L-cysteine. If the performance of the cells after isolation is suboptimal, the preculture medium and the culture medium after thawing can be supplemented with a final concentration of 1 mM *N*-acetyl-L-cysteine. Alternatively, a medium which already has L-cysteine as a basal supplement (e.g., William's Medium E) can be used (*see* **Note 3**).

2.2 Freezing Medium

1. Basal freezing medium. Standard basal media can be used, such as William's Medium E, Leibovitz, Dulbecco's Modified Essential Medium, Minimal Essential Medium, or Modified Earle's Medium (*see* **Note 4**). Animal component-free medium could also be considered (*see* **Note 5**).
2. Bovine or human serum. The concentration of serum can be between 10 and 90 % [8, 9] (*see* **Note 6**).
3. Dimethylsulfoxide (DMSO). The most common and effective cryoprotectant is DMSO [10]. It should be slowly added to the diluted cell suspension in freezing medium to a final concentration of 10 %. The concentration of DMSO used for cryopreserving hepatocytes from any species should be noncytotoxic and give rise to maximal postthaw recovery. This can be tested by checking the viability 30 min after adding and *prior* to freezing (*see* **Note 7**).
4. Trehalose. Use at a final concentration of 0.2 M in the freezing medium (*see* **Note 8**).

2.3 Thawing Medium

There are a number of options for thawing media, including basic and commercial types. The best option is to test each and compare the quality of the cells after thawing.

1. Standard thawing medium. Add 5 mL fetal bovine serum to 45 mL complete William's Medium E or other appropriate culture medium. This is sufficient for 1–5 cryovials.
2. 90 % Percoll solution. Add one part 10× concentrated Hank's Balanced Salt Solution (Life Technologies, Germany) to nine parts Percoll with density 1.124 g/mL. Mix thoroughly and make sure the pH is 7.4. If it is not, add 0.5 M HCl until the pH is 7.4. It is very important that there are no crystals in the

Percoll solution (*see* **Note 9**). If there are, remake the solution, but adjust the pH of the 10× concentrated HBSS before adding to the Percoll. The limits of pH are 7.2–7.6. Anything outside this range will be toxic to the cells.

3. Percoll thawing medium. Add 25 mL thawing medium to a 50 mL centrifuge tube. Then add 90 % Percoll to the 50 mL centrifuge tube according to the hepatocyte species, namely, 18 mL for rat, dog, monkey, mouse and 16 mL for human.
4. Recovery medium. Two commercial media are recommended for recovery of thawed cryopreserved human or animal hepatocytes, namely Cryopreserved Hepatocytes Recovery Medium (Life Technologies, Germany) and Universal Cryopreservation Recovery Medium (In Vitro ADMET Laboratories, United States of America).

2.4 Freezing and Storage

1. Programmable freezer. A programmable freezer allows for a controlled and precise rate of freezing. Most freezing regimens follow the freezing profile example shown in Table 2. The freezing program takes into account the increase in temperature of the cell suspension when the latent heat of fusion is released (i.e., at about –9.5 °C when the concentration of DMSO is 10 %) by counteracting it with a small shock freeze (Table 2). The cells are maintained at 10 °C for 10 min and then gradually frozen down to –30 °C, which includes the shock freeze to maintain the continued gradual decrease in temperature in the cell suspension itself. This slow freezing

Table 2
Programmable freezer temperature profile

Time (min)	Temperature (°C)
0	20
10	0
20	0
27	–9.5
27.01	–28
27.5	–25
30	–25
31	–15
41	–30
51	–100

step allows for the loss of some intracellular water, but is not enough to cause changes in solute concentrations. After this, the temperature is dropped by 70 to −100 °C in 10 min, which effectively freezes all water remaining in the cells and that prevents further loss of water.

2. Mr. Frosty freezing container (Thermo Scientific, Germany). This freezing container has 12–18 places for cryovials. It requires isopropyl alcohol and a −70 °C freezer.

3 Methods

3.1 Hepatocyte Quality Check

Cryopreserved hepatocytes are more likely to attach after thawing if the fresh cells frozen are of the highest quality (*see* **Note 10**). If the viability is lower than 80 % and the morphology of the cells is compromised (e.g., with blebs and vacuoles), the isolation procedure itself should be optimized (*see* **Note 11**) or the viability should be increased by Percoll purification.

3.2 Percoll Purification

1. Resuspend human hepatocytes in 36 mL basal culture medium and add 16 mL 90 % Percoll solution, pH 7.4 (*see* **Note 12**).
2. Mix the cells and Percoll solution thoroughly but gently by inverting the tubes 3–4 times.
3. Centrifuge the cells at 168 × *g* and room temperature for 20 min.
4. Remove the upper layer of dead cells and all of the supernatant, leaving only the cell pellet at the bottom of the tube.
5. Gently dislodge the cells in the pellet by agitating the tubes.
6. Add 5 mL basal freezing medium to the cells and gently mix the cells to result in an even single cell suspension.
7. Count the viability and density of the cells using either trypan blue exclusion or a cell counter.

3.3 Preincubation

If the cell viability after isolation is below 70 % or the morphology is poor, the performance of the cells and their recovery from cryopreservation may be improved by preincubating them with medium supplemented with ingredients known to have a beneficial effect on the cells (*see* **Notes 1–3**). This should be carried out *prior* to cryopreservation to allow the hepatocytes to recover from the effects of isolation and washing [11, 12]. The addition of fructose, alpha lipoic acid, glucose, insulin, and *N*-acetyl-L-cysteine may improve the recovery of the cells after cryopreservation, but this is dependent on the quality of the cells. If they are already highly viable (i.e., over 95 % viable) with a good morphology (i.e., clear, spherical cells with no blebs), then the improvement in viability *prior* to cryopreservation is likely to be minimal, but recovery after

cryopreservation may be improved. Low-quality cell suspensions (i.e., 50 % viability with poor morphology and cell debris) are unlikely to survive cryopreservation. In these cases, either the cells can be purified using the Percoll method to increase the initial cell viability for cryopreservation or can be used only as fresh cells.

1. The preincubation medium can be supplemented with fructose, alpha lipoic acid, glucose, insulin, and *N*-acetyl-L-cysteine to increase the success of the cryopreservation.
2. Dilute the hepatocytes to 5 to 10 million cells/mL in the preculture medium and add 30 mL to a T175 culture flask (*see* **Note 13**).
3. Place the flask in an incubator at 37 °C and 5 % CO_2 for 30 min.
4. After the preincubation, transfer the cells to a 50 mL centrifuge tube and centrifuge at 60 × *g* and room temperature for 5 min.
5. Resuspend the cells in an appropriate volume of freezing medium.

3.4 Preparation of Cells for Cryopreservation

1. Dilute human hepatocytes to 11 million cells/mL in freezing medium (*see* **Note 14**).
2. Place the hepatocyte suspension on ice-water and keep them on ice until they are cryopreserved (*see* **Note 15**).
3. Slowly add DMSO over at least 10 min to allow for its equilibration between intracellular matrix and the medium [13]. If DMSO is added too quickly, then it creates an osmotic imbalance and the cells are compromised [3].
4. Aliquot 1 mL of the cell suspension into each cryovial.

3.5 Cryopreservation with a Programmable Freezer

1. Prepare the programmable freezer for use by ensuring it has sufficient liquid nitrogen for the entire run.
2. Precool the programmable freezer to 10 °C.
3. Place the vials in a programmable freezer and start the program.
4. Once finished, transfer the cryovials to a suitable storage tank.

3.6 Cryopreservation Without a Programmable Freezer

This method has shown to be successful in the cryopreservation of hepatocytes from a number of species [13–16].

3.6.1 Method 1

1. Place the cryovials into a noninsulated rack (i.e., not a polystyrene holder with a tight fit).
2. Place the cryovials into a −20 °C freezer for 12 min.
3. Transferred to a −80 °C freezer for 1 h.
4. Plunge the cryovials into liquid nitrogen.

3.6.2 Method 2

1. Place the cryovials into cooling boxes containing isopropanol.
2. Place the Mr. Frosty box into a −80 °C freezer for 8–24 h [8, 11]. The cells will be frozen at a constant rate of 1 °C/min (*see* **Note 16**).

3.7 Storage of Cells

1. The vials of cells should be transferred to a storage container as quickly as possible to avoid cell damage by slow warming.
2. The storage container should be maintained at less than −130 °C, since some chemical reactions (i.e., proteases) are still possible at temperatures warmer than this, and they may compromise cell viability and recovery (i.e., the number of viable cells surviving cryopreservation).
3. Ideally, cells can be stored at −150 °C in a freezer (*see* **Note 17**), −160 °C in the vapor phase of nitrogen, or −196 °C in liquid nitrogen (*see* **Note 18**). Storage at −80 °C for longer than 2 h leads to lower initial postthaw viabilities. Thus, if stored correctly, the recovery (e.g., postthaw viability and attachment) can be maintained for years [13].

3.8 Thawing and Handling of Cryopreserved Hepatocytes

The success of cryopreservation depends on both the freezing process and the method of thawing and handling of the cryopreserved cells (*see* **Note 19**). Thawing can include a Percoll purification step if required. The correct Percoll concentration and centrifuge speeds will result in maximal recovery of viable cells whilst effectively removing dead cells (*see* **Notes 9** and **20**).

1. Prepare the standard or Percoll thawing medium and warm it to 37 °C and keep it in the water bath until the cells are thawed.
2. Transfer the cryovial from the storage tank to a water bath maintained at 37 °C. Transfer of the vial from the liquid nitrogen tank to the water bath should be completed with in 10 s (*see* **Note 21**).
3. Thaw the cells until all the ice is melted. It is better to slightly overthaw them, than to leave ice crystals in because these will damage the cells. Thawing should take about 90–120 s, but this will vary.
4. Transfer to the vial and the warmed thawing medium to a sterile flow hood.
5. Transfer the cells into the tube containing the thawing medium by pouring or gently pipetting.
6. Wash all the cells from the cryovial by gently adding 1 mL thawing medium and then pouring it back into the centrifuge tube.
7. Centrifuge cells in standard thawing medium at room temperature for 5 min at an appropriate speed (i.e., $60 \times g$ for rat, dog, monkey, and human, and $30 \times g$ for mouse hepatocytes).

8. For cells thawed in Percoll containing medium, make the volume up to 50 mL with extra thawing medium, but not with Percoll.
9. Close the lid, invert the tube slowly about 1–2 times to resuspend the cells, and mix the Percoll to result in an even concentration.
10. Centrifuge the cells in Percoll thawing medium at room temperature for 20 min at an appropriate speed (i.e., 168 × *g* for rat, dog, monkey, and human, and 60 × *g* for mouse hepatocytes).
11. Pour off the supernatant, loosen the cell pellet before adding medium, and resuspend the cells in appropriate culture medium (*see* **Note 22**).

4 Notes

1. Attachment of rat and human cryopreserved hepatocytes may be improved by preincubating the fresh hepatocytes with fructose or alpha lipoic acid *prior* to freezing [17–19].
2. These supplements increase the glycogen content of hepatocytes, which is broken down to glucose-6-phosphate after thawing, and thus provides an energy source which is reported to be lacking in these cells [20, 21]. Preincubating fresh cells in glucose-containing Krebs–Henseleit buffer for 30 min in a 95 % air and 5 % CO_2 atmosphere improves the attachment efficiency of both rat and human cryopreserved hepatocytes [22].
3. *N*-Acetyl-L-cysteine is an amino acid and is a precursor of reduced glutathione, a cytoprotective tripeptide found in high concentrations (i.e., 5 mM) in hepatocytes [23]. The isolation procedure and cryopreservation cause hepatocytes to lose glutathione [24, 25]. Glutathione protects against the effects of reactive oxygen species, lipid peroxidation, and prevents apoptosis [26]. Therefore, the survival from cryo-injury is increased by increasing glutathione levels. The addition of glutathione per se to the culture medium will not increase intracellular glutathione because it will not cross the plasma membrane. Therefore, hepatocytes could be incubated with *N*-acetyl L-cysteine, a precursor of glutathione, which is actively taken up into the cells by transpeptidase and then converted to L-cysteine in hepatocytes.
4. The basal medium used for freezing cells varies between laboratories, but there is no *consensus* as to which is optimal for cryopreservation. Basal media with strong buffering capacities, such as phosphate-based media, may offer an advantage in maintaining pH.

5. Animal component-free media has gained in use, either due to transport requirements or clinical reasons, such as a lack of tolerance to animal derived products [27].
6. Positive attributes of serum include quenching of proteolytic enzymes released from dead cells, cytoprotection from free oxygen radicals [28], and slowing down cell sedimentation during freezing due to its viscosity.
7. DMSO has been shown to cause toxicity if incubated with hepatocytes at high concentrations (i.e., 10–20 %) for longer than 30 min *prior* to cryopreservation, especially if maintained at 37 °C [7, 13]. This could explain why cryopreservation of large quantities of cells (i.e., more than 300 vials) may result in poorer postthaw viabilities. They are compromised because the time between addition of DMSO and the start of freezing is too long. The key is to allow the DMSO to equilibrate in the cell suspension, but not to allow the DMSO to cause toxicity. Other cryoprotectants have been included in freezing medium, such as polyvinylpyrrolidone and polyethyleneglycol, but these are used in combination with DMSO, rather than as single supplements. A drawback of these compounds is that they may interfere with the liquid chromatography-mass spectrometry analysis of test compounds after the cells have been thawed and used in a metabolite profiling assay. Washing thawed cells a number of times may reduce this effect, but because polyethyleneglycol interacts strongly with the plasma membrane, it is unlikely to be completely removed.
8. This disaccharide protects proteins and cellular membranes from inactivation or denaturation due to different stress conditions. The basis for the idea came from the fact that lower organisms, such as yeasts, bacteria, fungi, and insects, all survive freezing or drying and all have concentrated levels of disaccharides, especially trehalose [29]. Membrane stabilization may be a result of trehalose interacting with the plasma membrane to counteract the changes in membrane fluidity. An additional mechanism of cryoprotection may be due to scavenging of free radicals, which may be released during oxidative stress [30].
9. A drawback of Percoll is that it may form large crystals if it is repeatedly heated and cooled and these will prevent hepatocytes from attaching. If this occurs, wash the cells until no Percoll crystals are visible. Another key factor in using Percoll is that it is nonphysiological (i.e., osmotically equivalent to water) and requires dilution in tenfold concentrated buffer, such as Hank's Balanced Salt Solution, before use. If Percoll is diluted in water, this will result in the demise of the hepatocytes due to water entering the cells through osmosis, causing them to swell and burst.

10. Attempting to improve the quality of poor cell preparations by adding supplements or preincubating cells will not increase the postthaw recovery [17].
11. The quality of hepatocytes can be improved by considering factors, such as the transport medium used to transfer the liver to the laboratory, the time between organ procurement and the start of collagenase digestion, the condition of the liver, and the isolation time itself.
12. It has been reported that purifying rat and dog hepatocytes using Percoll *prior* to cryopreservation results in higher cell recoveries after thawing [31]. However, Percoll is known to cause a substantial loss of viable cells [31] unless the method is optimized [32]. The use of Percoll does not have to result in a significant loss of viable cells. In general, the larger the number of cells which are purified, the higher the concentration of Percoll that is needed. Although in theory, all centrifuges should spin at the same speed, this is not always true in reality. Therefore, the speed at which the cells are centrifuged may need to be adjusted according to the specific centrifuge. Hence, it is recommended that the concentrations of Percoll or centrifuge speed is adjusted for hepatocytes of different species due to differences in their sizes [33].
13. If the hepatocyte isolation or subsequent wash steps do not include oxygenation of the buffers, hepatocytes will lose adenosine triphosphate. Incubation of freshly isolated hepatocytes under an atmosphere of 95 % air and 5 % CO_2 for 30 min at 37 °C increases the adenosine triphosphate content of hepatocytes and may increase their likelihood of survival [34]. The increased energy status of hepatocytes may make them more able to undergo energy-consuming processes such as urea synthesis [35] and gluconeogenesis [5].
14. This may not be optimal for hepatocytes from other species which may have different cell volumes. Mouse hepatocytes are much larger than rat or human hepatocytes (i.e., approximately threefold [33]) and this affects the optimal freezing density. The optimal freezing density of mouse hepatocytes is between 1.2 and 1.5 million cells/mL, whereas rat hepatocytes are optimally frozen at 4–10 million cells/mL.
15. Keeping cells at 4 °C reduces the toxicity of DMSO [21] and maintains metabolizing enzyme activities [35]. Repeated warming and cooling of hepatocytes leads to activation of proteolytic enzymes *via* alterations in the cellular iron homeostasis and apoptosis [36]. Thus, repetition of the warm and cool cycles should be avoided at all times.
16. This method of freezing human and pig hepatocytes was found to be equally effective as a programmable freezer with respect

to initial cell viabilities, attachment efficiencies, and some drug metabolizing enzyme activities [8, 11, 20].

17. An electrically powered freezer has the advantage of being low maintenance, but make sure it is connected to emergency electricity in case of power failure.
18. A disadvantage of liquid nitrogen is that it sometimes leaks into the vials. This will create pressure in the vial when it is removed from the storage tank and warmed. The obvious consequence of this is that the tube explodes. Many researchers store cryopreserved hepatocytes in vapor phase nitrogen, which decreases the likelihood of exploding vials and maintains them at a temperature well below the critical threshold of −130 °C.
19. If cells are warmed slowly, for instance, if the vials are removed from liquid nitrogen and placed on water ice, intracellular ice crystals will coalesce and form larger crystals, which subsequently disrupt cell membranes. Even cells placed on dry ice are warming up (i.e., from −196 to −80 °C) and this may also cause membrane damage due to ice crystals growing. Rapid thawing in a 37 °C water bath causes the intracellular ice crystals to instantly thaw, preventing any damage due to growing ice crystals.
20. If the concentration of Percoll is too high, viable cells are lost as well as dead cells [37]. Conversely, if the concentration is too low, the viability may even decrease because the viable and dead cells are rapidly sedimented into a tight pellet, which is hard to resuspend.
21. The most convenient transfer of vials to the water bath is when the storage tank is in the cell culture laboratory. However, this is not always possible and vials may well be kept in the basement of the building. Even if cells are stored in a different room, the method of transfer needs to be quick and safe. The best way to transfer cells from the storage tank to the water bath is in a container of liquid nitrogen, with enough liquid nitrogen to ensure the vials are immersed for the entire transfer time. It is not sufficient to pour a small amount of liquid nitrogen over vials in a polystyrene box and hope that it is still there by the time they arrive at the water bath. Those who do not have good access to liquid nitrogen can opt for transferring vials in dry ice pellets. Do not use ice-water and do not allow the vials to warm above −70 °C at any time during transfer.
22. After centrifugation, pour off the supernatant and loosen the cell pellet before adding any media. If you add media first and then try to resuspend the cells, they will form clumps. Loosen the pellet by gently shaking the bottom of the tube.

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