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Cryopreservation of Red Blood Cells and Platelets

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Summary

Blood cells can be regarded as a classical field of application of low-temperature biology. Cryopreservation methods have been developed for different categories of blood cells namely red blood cells (RBCs) (erythrocytes), platelets (thrombocytes), mononuclear cells (i.e., lymphocytes, monocytes), and hematopoietic progenitor cells. This chapter outlines the four most commonly applied techniques for RBCs and two for platelets.

Key Words: Cryopreservation; red blood cells; platelets; dimethyl sulfoxide; hydroxyethyl starch.

1. Introduction

Frozen red erythrocytes, thrombocytes, mononuclear cells (i.e., lymphocytes, monocytes), and hematopoietic progenitor cells (from peripheral blood as well as from bone marrow) are being used for various diagnostic and therapeutical purposes (to *see* reviews, e.g., **refs. *1*** and ***2***). A variety of cell-specific cryopreservation protocols have evolved so far. The methods differ with regard to (1) cell concentrations, (2) protective solutions used (cryoprotectants and their concentrations), (3) temperature–time histories during cooling and rewarming, and (4) storage temperature. Additionally, some of the cryoprotectants are not well tolerated in the concentrations required (e.g., dimethyl sulfoxide [DMSO] for platelets) or lead to an osmotically induced lysis of the cryoprotectant-loaded cells when transfused into an isotonic organism (e.g., glycerol for red blood cells [RBCs]). In these cases, a washing procedure is required after thawing prior to the application/transfusion.

The four techniques for RBCs and two for platelets (PLT) described in detail in the following are:

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No.	Cell	Method	Author
1.	RBC:	High glycerol–slow cooling technique	(Meryman ***[3,4]***)
2.	RBC:	Low glycerol–rapid cooling technique	(Rowe ***[5,6]***)
3.	RBC:	Hydroxyethyl starch–rapid cooling technique	(Sputtek ***[7,8]***)
4.	RBC:	Hydroxyethyl starch–rapid cooling technique	(Thomas ***[9,10]***)
5.	PLT:	Dimethyl sulphoxide–intermediate cooling technique	(Schiffer ***[11,12]***)
6.	PLT:	Hydroxyethyl starch–intermediate cooling technique	(Choudhury ***[13]***)

Please note that (1) methods 1, 2, 3, 4, and 6 as described next are "adjusted" to whole blood donations (approx 450–500 mL), whereas 5 deals with platelets obtained by apheresis. This is usually equivalent with what can be obtained from four to five whole blood donations. (2) Deviations regarding the volume and sample geometry (e.g., by changing from bags to vials for laboratory purposes) may lead to different temperature–time histories ("rates") during cooling and rewarming; this can be very critical in the case of the rapid cooling techniques and may result in less favorable results, and (3) some of the methods have been developed at a time when sterile tube-docking devices were not available. In this case, the use of laminar air-flow cabinets are highly recommended, and as nowadays some of the "old" cryogenic bags are not available, they have been replaced by what the author thinks to be suitable as well. It is assumed for all methods that standard blood bank equipment is available, e.g., (refrigerated) bag centrifuge, scales, freezer, plasma extractor, tube stripper, clamps, and so on. A list of companies selling such devices (as cleared by the FDA's Center for Biologics Evaluation and Research) can be found at http://www.fda.gov/cber/dap/510kman.htm. Isotonic saline, ACD-A, ACD-B, CPD*, CPD-A1*, SAG-M*, PAGGS-M*, AS-1*, AS-3* are "standard solutions" that contain the "ingredients" at prescribed concentrations and can be obtained from many companies supplying blood banks. The solutions marked with an asterisk are usually contained in the blood donation/processing/storage bag system.

1.1. RBCs

Cryopreserved RBCs for transfusion are of advantage in the case of patients with rare blood groups, adverse antibody problems, and civil as well as military disasters. Additionally, they can be used for blood typing, antibody screening, and compatibility testing. In principle, three different methods have been established for clinical use: (1) the Huggins ***(14)*** technique, using glycerol in a nonionic suspension and removal of the cryoprotectant by

reversible agglomeration of the RBC, (2) the "high glycerol–slow cooling technique" according to Meryman and Hornblower ***(3,4)***, which is the dominant method in the United States. (3) The "low glycerol–rapid cooling technique" according to Rowe ***(5,6)*** and Krijnen ***(15)*** is the dominant method for the cryopreservation of RBCs in Europe. The utilization of macromolecular cryoprotectants goes back to Rinfret ***(16)*** and coworkers, and water-soluble, cryoprotective macromolecules such as albumin, dextrans, modified gelatin, polyvinylpyrrolidone (PVP), polyethylene oxide, polyethylene glycol, and hydroxyethyl starches (HES) exhibit the principal advantage of not entering into the cells. This property significantly facilitates their removal after thawing. In the case of emergencies, this step could be omitted if the additive, e.g., albumin, dextrans, modified gelatin, and HES are biodegradable and tolerated by the human organism. In 1967, Knorpp et al. ***(17)*** described for the first time the successful cryopreservation of human RBCs using HES and liquid nitrogen (LN_2), comparing the efficacy of HES to that of PVP. They preferred the colloid HES to PVP as the latter is retained to a considerable extent in the recipient (as are polyethylene oxide and polyethylene glycol). Moreover, in the case of hypovolemia, albumin, dextrans, modified gelatins, and HES serve as blood volume substitutes. We have carried out several in vitro investigations and optimizations of the HES procedure, and after in vivo experiments in dogs a successful in vivo study including seven healthy volunteers has been carried out ***(8)***. Finally, we have performed a systematic clinical trial in patients ***(18)***. Based on the work published by Robson ***(19)***, Thomas et al. ***(9,10)*** have developed another procedure for the freezing of RBCs using HES. The major differences compared to our procedure are (1) no prefreeze washing, (2) different HES modification, (3) lower HES concentration, (4) higher electrolyte concentration, (5) higher hematocrit (HCT), (6) larger freezing bag, (7) smaller sample thickness, (8) smaller volume, (9) higher viscosity, (10) different freezing container, and (11) uncontrolled thawing. For an overview regarding the nomenclature of different HES modifications and specific aspects of their usefulness for the cryopreservation of human cells, *see* http://www.sputtek.de/Info/HES.pdf.

1.2. Platelets (see *Note 1)*

Since the first reported attempt in 1956 to stop thrombocytopenic bleeding by the infusion of previously frozen platelets by Klein ***(20)***, a broad variety of in vitro and in vivo studies on cryopreserved platelets have been published. The most widely used method for the cryopreservation of platelets is a "10% DMSO–slow cooling" method by Schiffer ***(11,12)***, another one is a "low glycerol/glucose–intermediate cooling" method described by Dayian and Rowe in 1976 ***(21)***. HES-cryopreserved

platelets (frozen in the presence of 4% [w/v] HES at 1°C/min), turned out to be hemostatically effective when using this 4% HES method ***(13)***.

1.3. Mononuclear Cells (see *Note 2)*

The use of cryopreserved mononuclear cells (i.e., lymphocytes and monocytes) is well established and a routine procedure for clinical laboratory testing. Most recently, there is a growing clinical interest in cryopreserved lymphocytes for the supplemental treatment of patients after blood stem cell transplantation. Usually, they are frozen according to methods that are more or less modifications of a technique that was first described for bone marrow by Ashwood-Smith ***(22)*** in 1961 using 10% DMSO. During cooling, the heat is removed either by computer-controlled and LN_2-operated machines or in mechanical (–80°C) refrigerators. Stiff et al. ***(23)*** have demonstrated that the addition of 6% HES reduced the "original" concentration of DMSO (10%) by one-half. Cryopreserved autologous and homologous blood stem cells (in combination with high-dose chemotherapy and/or irradiation) have become a "standard" blood component for the treatment of several malignant diseases. Frozen cryopreserved mononuclear cells are used for various diagnostic purposes, e.g., human leukocyte antigen typing, detection of human leukocyte antigen antibodies in patients on waiting lists for organ/bone marrow transplantations, and mixed lymphocyte reactions/cultures. They are also of interest with respect to look-back procedures in transfusion medicine or diagnosis in patients. The methods for freezing mononuclear cells reported in the literature vary from one author to another ***(1,2)***.

1.4. Granulocytes

There have been publications in the past that claim the successful cryopreservation of granulocytes (*see* reviews, e.g., **refs.** ***24,25***). Despite reports appearing now and then in the newer literature (mostly as abstracts), it is our opinion that no clinically suitable method for the preservation of granulocytes has been found. The huge variation of the in vitro results shows how cumbersome the viability assays are, and how unsuitable they will be to predict anything that is going to happen in vivo. Membrane integrity tests (i.e., staining tests often referred to as "viability tests") measure only a *conditio sine qua non* (i.e., an intact cell membrane). However, what is the meaning of these results if tests measuring typical granulocytic functions (e.g., chemotaxis, bactericidal activity) fail to detect any significant activity? Takahashi et al. ***(26)*** have proposed some explanations why granulocytes are so unrewarding regarding their cryopreservation. Already at temperatures below –5°C without the formation of ice, a significant loss of function can be observed. This could be prevented by the addition of DMSO, whereas glycerol failed to show this effect. Because of their limited osmotic tolerance (already a twofold increase compared to isotonicity caused a

significant loss of function), they are highly susceptible to the electrolyte enrichment taking place during ice formation. Granulocytes also showed a limited tolerance to hypotonic stress, which may occur upon thawing.

2. Materials

2.1. High Glycerol–Slow Cooling Technique

1. Plasma transfer sets (e.g., Terumo 1TC*00505, Baxter 4C2298).
2. Polyvinyl chloride storage containers.
3. 400 mL 6.2 *M* glycerol solution containing 0.14 *M* sodium lactate, 5 m*M* potassium chloride, and 5 m*M* sodium phosphate (pH 6.8 after autoclaving).
4. Cryogenic freezing containers made of polyolefin, polytetrafluorethylene (i.e., Teflon®, polyimide [Kapton®]/Teflon), or ethylenevinylacetate (EVA).
5. Cardboard or metal canister for freezing.
6. Freezer (–65°C or below).
7. 37°C water bath or 37°C dry warmer.
8. 12% (w/v) hypertonic sodium chloride solution.
9. 1.6% (w/v) hypertonic sodium chloride solution.
10. 0.9% (w/v) isotonic sodium chloride with 200 mg/dL glucose.

2.2. Low Glycerol–Rapid Cooling Technique

1. Plasma transfer sets (e.g., Terumo 1TC*00505, Baxter 4C2298).
2. Polyvinyl chloride storage containers.
3. Cryoprotective solution (CPS): 35 g glycerol, 3 g mannitol, and 0.65 g sodium chloride per 100 mL.
4. Cryogenic freezing containers made of polyolefin, polytetrafluorethylene (Teflon), or polyimide (Kapton)/Teflon.
5. Heat sealer.
6. Freezing bag holder, retainer, gloves, and goggles.
7. Open LN_2 Dewar.
8. LN_2 storage tank.
9. 45°C water bath.
10. 3.5% hypertonic sodium chloride solution.
11. 0.9% isotonic sodium chloride with 200 mg/dL glucose.

2.3. HES–Rapid Cooling Technique (7,8)

1. Plasma transfer sets (e.g., Terumo 1TC*00505, Baxter 4C2298).
2. 600 mL transfer packs with couplers (e.g., Terumo Teraflex B600, Baxter 4R2027).
3. Sterile tube-connecting device.
4. CPS: 220 mL of 23% (w/w) (i.e., 25% [w/v]) HES 200/0.5 in 60 mmol/sodium chloride solution (*see* **Note 3**).
5. Platform rocking device.
6. Cryogenic freezing containers made of polyimide (Kapton)/polytetrafluorethylene (Teflon), e.g., Fresenius Gambro Hemofreeze Bag DF 1200.

7. Heat sealer.
8. Patented freezing container (*see* **Note 4**). The container can be obtained by contacting the patent holders.
9. Open LN_2 Dewar.
10. LN_2 storage tank.
11. Hand tongs, gloves, and goggles.
12. 48°C shaking water bath with pouch.

2.4. HES–Rapid Cooling Technique (9,10)

1. Plasma transfer sets (e.g., Terumo 1TC*00505, Baxter 4C2298).
2. Polyvinyl chloride storage containers.
3. CPS: 40 mL of 40% (w/v) HES 200/0.5 in isotonic saline (*see* **Note 3**).
4. Cryogenic freezing containers made of polyimide (Kapton)/tetrafluoretylene (Teflon).
5. Sterile tube connecting-device.
6. Heat sealer.
7. Aluminium freezing frame and metal clips.
8. Open LN_2 Dewar.
9. LN_2 storage tank.
10. Hand tongs, gloves, and goggles.
11. 43.5°C controlled circulating water bath.

2.5. DMSO–Intermediate Cooling Technique

1. Sampling site couplers (e.g., Terumo TC*MP1, Baxter 4C2405).
2. 300 and 600 mL transfer packs with couplers (e.g., Terumo Teruflex B-600, Baxter [300 mL] 4R2014A, [600 mL] 4R2027).
3. Heat sealer.
4. 3-, 10-, and 60-mL sterile disposable plastic syringes.
5. 18-gage needles.
6. Cryogenic freezing bag: polyolefin, polytetrafluorethylene (Teflon), polyimide (= Kapton)/Teflon, or EVA.
7. 10-mL vials of sterile DMSO.
8. 16-gage butterfly needle with 75-cm tubing.
9. Infusion pump for 60-mL syringe.
10. Platform rocking device.
11. Metal plates with clamps.
12. Ultralow temperature freezer with temperature range down to –135°C.
13. Protective bags.
14. 37°C water bath.
15. Infusion set.

2.6. HES–Intermediate Cooling Technique

1. CPS: 8% (w/w) HES 70/0.5 or HES 200/0.5, sodium chloride concentration 0.6% (*see* **Note 3**).
2. Sterile, disposable, 2-, 20-, and 50-mL plastic syringes with 18-gage needles.

3. Cryogenic freezing bags made of polyolefin, polytetrafluorethylene (Teflon), polyimide (Kapton)/Teflon, (e.g., Fresenius Hemofreeze Bag DF 200), or EVA.
4. Heat sealer.
5. Platform rocking device.
6. Closed aluminium container with 3-mm inner thickness.
7. Controlled-rate LN_2 operated freezer.
8. Hand tongs, gloves, and goggles.
9. LN_2 storage tank.
10. Punched aluminium thawing container.
11. 37°C shaking water bath.
12. 250-mL transfer pack.

3. Methods

3.1. High Glycerol–Slow Cooling Technique (3,4)

1. Prepare a RBC concentrate from whole blood using standard blood bank techniques (e.g., **ref.** *27*). RBC preserved in CPD (combined citrate/phosphate buffer and glucose containing whole blood/RBC anticoagulant/storage solution) or CPD-A1 (also contains adenine) may be stored at 1–6°C for up to 6 d before freezing. RBC preserved in AS-1 (Adsol®) and AS-3 (Nutricel®) (both are glucose and adenine containing RBC storage solutions, the latter also contains a combined citrate/phosphate buffer) may be stored at 1–6°C for up to 42 d before freezing. RBC that have undergone a rejuvenation procedure may be processed for freezing up to 3 d after their original expiration. RBC in any preservative solution that have been entered for processing must be frozen within 24 h after opening the system.
2. The combined mass of the cells and the collection bag should be between 260 and 400 g.
3. Underweight units can be adjusted to approx 300 g either by adding isotonic saline or by the removal of less plasma than usual.
4. Warm the RBC and the 6.2 *M* glycerol solution to at least 25°C by placing them in a dry warming chamber for 10–15 min or by allowing them to remain at room temperature for 1–2 h. The temperature must not exceed 42°C.
5. Place the container of RBC on a shaker and add approx 100 mL of the glycerol solution as the red cells are gently agitated.
6. Turn off the shaker and allow the cells to equilibrate, without agitation, for 5–30 min.
7. Allow the partially glycerolized cells to flow by gravity into the freezing bag.
8. Add the remaining 300 mL of the glycerol solution in a stepwise fashion, with gentle mixing. Add smaller volumes of the glycerol solution for smaller volumes of red cells. The final glycerol concentration is 40% (w/v).
9. Maintain the glycerolized cells at temperatures between 25 and 32°C until freezing. The recommended interval between removing the RBC unit from refrigeration and placing the glycerolized cells in the freezer should not exceed 12 h.
10. Place the glycerolized unit in a cardboard or metal canister and place in a freezer at –65°C or below. The freezing rate should not be less than 10°C/min.
11. Do not "bump" or handle the frozen cells roughly.

12. Storage of the frozen RBC at –65°C or colder is possible but not recommended for up to 10 yr (and more).
13. For thawing place the protective canister containing the frozen RBC in either a 37°C water bath, or 37°C dry warmer.
14. Agitate gently to speed up thawing. The thawing process takes at least 10 min, and the thawed cells should be at 37°C.
15. After the RBC are thawed, you may either use a commercial instrument (e.g., centrifuge) for batch or a continuous-flow washing device to deglycerolize cells. Follow the manufacturer's instructions precisely, especially when using a special device (e.g., Cobe Cell Processor 2991).
16. For batch washing, dilute the unit with a quantity of hypertonic (12%) sodium chloride solution appropriate for the size of the unit. Allow to equilibrate for approx 5 min.
17. Wash again with 1.6% sodium chloride until deglycerolization is complete. Approximately 2 L of wash solution per unit are required.
18. Suspend the deglycerolized RBC in isotonic saline (0.9%) with 0.2% glucose.
19. If you have opened the system for the processing, deglycerolized RBCs must be stored at 1–6°C for no longer than 24 h (in the case of a transfusion).

3.2. Low Glycerol–Rapid Cooling Technique (5,6)

1. After collection of a unit of whole blood in ACD (citrate buffer and glucose containing whole blood/RBC/platelet anticoagulant/storage solution; formulations ACD-A and ACD-B vary with regard to the concentration of the solutes) or CPD anticoagulant, the plasma is removed from the cells after centrifugation. The RBCs should be frozen as soon after the collection as possible, but preferably before they are 5 d old.
2. The remaining packed RBCs are weighed, and an equal volume by weight of the glycerol freezing solution is added at room temperature to achieve a final concentration of 14% (v/v). The freezing solution contains 28% (v/v)–35% (w/v) glycerol, 3% mannitol, and 0.65% sodium chloride.
3. After 14–30 min equilibration at room temperature (22°C), the RBC suspension is transferred into a suitable (e.g., polyolefin) freezing bag. Conventional PVC plastic bags cannot be used, as they become brittle and crack upon freezing in LN_2.
4. The bag is placed between two metal plates (holder). These are used to keep the bag in a flat configuration. The top and bottom of the bag are tucked under to allow the holder plates to close without pinching the bag.
5. Cooling is performed by complete immersion of the container in the open LN_2 filled without agitation. A freezer retainer should be used to prevent excess bulging of the container during freezing. Freezing is complete in 2–3 min when the LN_2 stops boiling.
6. The unit is stored in its metal plate holder in a LN_2 storage tank either in the vapor or in the liquid phase.
7. Thawing: on retrieval from the LN_2 storage, the unit is immediately thawed by immersing the complete unit (bag and holders) into a 40–45°C warm water bath under gentle agitation (approx 60 cycles/min) for about 2.5 min.

8. The bag is removed from the metal holding plates and it is checked that all ice has disappeared. If not, immediately reimmerse the bag into the thaw bath and knead the bag under the warm water until the ice has completely melted.
9. Following centrifugation and removal of the supernatant containing free hemoglobin, glycerol, and debris, the RBCs are washed three times using a bag centrifuge. The first wash is with 300–500 mL 3.5% sodium chloride at 4°C, the last two are with 1000–2000 mL isotonic saline (or preferably with 0.8 % NaCl containing 200 mg/dL glucose). All washes must be added slowly to the cells at room temperature with gently mixing.
10. For resuspension of the RBC after the deglycerolization, the glucose-supplemented sodium chloride solution may be used as well.

3.3. HES–Rapid Cooling Technique (7,8)

For an overview of this method, *see* http://www.sputtek.de/Info/V140.pdf.

1. 450–500 mL whole blood are collected in blood bank packs containing CPD-A as anticoagulant.
2. Plasma and buffy coat are removed using standard blood bank techniques ***(28)*** and the RBCs are leukodepleted by filtration and stored in an additive solution (SAG-M [adenine-, glucose-, and mannitol-containing saline solution]), PAGGS-S (phosphate buffer, adenine, glucose, guanosine-containing saline solution, AS 1, AS 3, and so on) (*see* **Note 5**).
3. The leukodepleted RBCs can be kept at 4 ± 4°C for a maximum of 3 d prior to freezing.
4. The first centrifugation takes place at 4000*g* for 10 min at 4°C.
5. The supernatant additive solution is removed by means of a plasma extractor.
6. The RBC concentrate is resuspended in 333 mL isotonic saline solution.
7. The suspension is then centrifuged again. The centrifugation step is repeated three times to make sure that all of the additive solution and plasma have been removed and that the "contamination" of the RBC concentrates by leukocytes and platelets is minimal.
8. After the removal of the last supernatant-purified RBC concentrates, a volume of approx 220 mL at a HCT of 85% ± 5.0% should be obtained.
9. An equal part of the CPS containing 23% (w/w) HES and 60 mmol/L sodium chloride is added to the purified RBC concentrate while mixing continuously.
10. Two aliquots of 220 mL of this suspension are transferred in two freezing bags.
11. The bags must be carefully deaerated and heat sealed below the inlet port. As the densities of the CPS and the RBC concentrates are quite similar (ca. 1.08 g/mL), the final HES concentration in the suspension to be frozen is 11.5% (w/w).
12. The two bags are then placed in two aluminium containers (*see* **Note 6**).
13. Cooling at the required cooling rate of about 240°C/min is achieved by complete vertical immersion of the containers into the open LN_2-filled Dewar. Use hand tongs, gloves, and goggles. Cooling is complete within 3 min. The total time is not critical as long as the containers are immersed for at least 3 min.

14. Lift the reusable container from the LN_2. Open it quickly and remove the freezing bag. Transfer the freezing bag to the vapor phase of a LN_2 storage tank within 30 s to avoid the risk of premature thawing. The bags must be stored in vapor phase over the LN_2 though not actually in the LN_2. Storage in the vapor phase over LN_2 below –130°C results in no time-dependent degradation.
15. Thawing is achieved by means of a shaking water bath. To guarantee thermally defined and reproducible conditions during rewarming, the bags have to be transferred from the LN_2 vapor phase into the pouch of the shaking water bath. This pouch maintains a well-defined flat geometry, and effectively transduces the shaking frequency (300 cycles/min) and amplitude (2 cm) of shaking in the water bath (48°C) to the frozen unit. After 75 s, the temperature in the bag is about 20°C and they are immediately removed from the bath and the thawing container.
16. Repeat this with the second bag prepared from this donation if needed.
17. If desired, free hemoglobin, HES, and debris can easily be removed by washing the RBC once with 300–500 mL of isotonic saline (or preferably with 0.8% NaCl containing 200 mg/dL glucose). Centrifuge refrigerated (4°C) at 4000*g* for 10 min, and remove the supernatant by means of a plasma extractor.
18. For resuspension of the washed RBC, the glucose-supplemented sodium chloride solution can be used. Standard RBC additive solutions (SAG-M, PAGGS-S, AS-1, and AS-3) may be used as well.

    The percentage recovery of intact cells, immediately post-thaw, is not used as a quality control procedure because it is only a crude indicator of quality (*see* **Note 7**).

3.4. Hydroxyethyl Starch–Rapid Cooling Technique (9,10)

1. This patented method (UK patent PCT/GB 90/0140, priority 08.02.1989) starts with a standard 450–500 mL whole donation collected in a suitable anticoagulant (e.g., CPD). In this case, the whole blood has to be filtered first, e.g., using standard blood bank techniques ***(27)*** (*see* **Note 8**).
2. Place the filtrated whole blood unit in a centrifuge and spin at 3000*g* for 20 min. Express the plasma from this bag into an attached satellite bag. It is important that all visible plasma is expelled from the filtrate bag to ensure that a packed cell volume (HCT) of at least 90% is achieved. Detach the plasma bag (*see* **Note 9**).
3. Connect the filtrate bag to the tubing of the freezing bag. Invert the bag and suspend it 2 m above ground level. Allow the packed red cells to flow into the freezing bag.
4. Express any air, which has collected in the freezing bag, back up the tubing into the previous bag together with a small volume of red cells.
5. Aseptically connect the freezing bag to the bag containing the CPS. Suspend the inverted bag 2 m above ground level and allow the HES solution to flow into the freezing bag, displacing all red cells from the tubing into the freezing bag. Roll up the nearly empty bag that has contained the cryoprotective solution to ensure that it is completely emptied.
6. Seal off the freezing bag, leaving as great a length of tubing as possible attached to it.
7. Mix the contents of the freezing bag thoroughly but carefully by repeated manual rotation and inversion of the bag for 4 min.

8. Freezing the HES/red cell mixture. Place the freezing bag into the base of the freezing frame. Ensure that the bag is lying smoothly and centrally and that the tubing/insert area is positioned within the frame recess.
9. Place the top of the freezing frame in position and secure this with six metal clips placed around the edges of the frame.
10. Carefully drop the frame vertically into the LN_2-filled Dewar so as to totally immerse the frame. Use hand tongs, gloves, and goggles. The nitrogen will boil vigorously. Bubbling should cease in approx 30 s. Leave the frame totally immersed for a further 30 s.
11. Lift the frame from the LN_2. Quickly place the frame on a steel table, unclip the frame, and remove the freezing bag. Transfer the freezing bag to the LN_2 storage tank. This stage is to be completed within 30 s to avoid the risk of premature thawing.
12. Storage of HES-cryopreserved red cells. Bags are to be stored flat. The bags must be stored in vapor phase over the LN_2n, not actually in the LN_2 (*see* **Note 10**).
13. Thawing HES-cryopreserved red cells. Check that the temperature of the water in the circulating water bath is within one degree of 43.5°C.
14. Remove the required frozen unit of cryopreserved red cells from the storage tank.
15. Place the unit in the water bath within 30 s of removing it from the storage tank, ensuring that the unit is totally immersed.
16. Holding the bag horizontally move it gently to and fro during the first minute of thawing. Rapid freezing is essential.
17. Leave the bag in the water bath for a further 9 min to allow the temperature to equilibrate and then remove the thawed unit from the bath. Blot it dry with paper towels. After thawing, the pack of HES/RBC can be stored at 4°C for 10 d without deterioration. It still contains the original amount of HES if no washing step is performed. If mixed with a diluent or optimal additive solution, storage times at 4°C are not improved over those of the mixture stored in the undiluted state.
18. Viability testing. The saline stability test may be performed 2 h after resuspension for the determination of the "2 h saline-stability value" as well. "Plasma stability" can be measured by using red cell-free nonlipemic plasma instead of the saline solution. In this case, the plasma should also be used for the determination of the blank (*see* **Note 11**).

3.5. DMSO–Intermediate Cooling Technique (11,12)

1. Platelet concentrates (PC) can be obtained from whole blood donations using standard blood bank techniques ***(28)*** or by apheresis.
2. Record the net weight of the PC.
3. Using a sampling site coupler, add 4.5 mL of additional ACD-A per 100 mL PC if the platelets have been obtained by apheresis. (This additional ACD-A is necessary to prevent platelet clumping following centrifugation because anticoagulant ratios used in automated procedures are lower than when platelets are manually collected from individual whole blood units.)
4. Allow the PC to stand undisturbed at room temperature for 1–2 h to permit disaggregation of any platelet clumps.

5. Transfer the PC to a 600-mL transfer pack and heat seal the tubing approx 20 cm from the bag. Discard extra tubing.
6. Strip the tubing several times, mixing the PC thoroughly (but gently) in between.
7. Heat seal the tubing 2–5 cm from the bag. Remove the segment.
8. Perform a platelet count on the segment and weigh the PC; record the information.
9. If gross RBC contamination of the PC is obvious, centrifuge the PC at 65*g* for 12 min.
10. Express the platelet-rich plasma (PRP) supernatant (minus the RBC button) into another 600-mL transfer pack. Obtain a specimen segment as in **steps 5–7**. Perform a platelet count on the segment, weigh the concentrate, and record the net weight and platelet count on a data sheet.
11. If the initial PC has few or no RBC, skip **steps 9** and **10** and proceed with **step 12**.
12. Calculate the platelet yield. If it is greater than 4.4×10^{11}, split the material prepared for cryopreservation equally such that each bag contains less than 4.4×10^{11} platelets. (Example: if the platelet yield = 10.5×10^{11}, split the freeze into three bags, each containing 3.5×10^{11} platelets.) Prepare a record for each bag of platelets frozen.
13. Centrifuge the PC at 1250*g* for 15 min.
14. Express the platelet-poor plasma (PPP) into another 600-mL transfer pack, leaving behind the undisturbed platelet button and approx 10 mL of plasma. Clamp the tubing between the two transfer pack; do not seal it.
15. Gently mix the platelet button with the 10 mL of plasma.
16. Using a wet folded paper towel, gently rub the platelet button until the suspension is homogeneous.
17. If the platelet button is still clumped, stop. Allow it to stand undisturbed at room temperature until the clumping has disappeared, usually for 1–2 h.
18. Using a sampling site coupler, a 60-mL syringe, and an 18-gage needle, withdraw the 10 mL of PC.
19. Unclamp the tubing between the two transfer packs and run 10–15 mL of the PPP into the now empty platelet bag. Reclamp the tubing.
20. Swirl the plasma around inside the platelet bag in order to mix any residual platelets with the plasma.
21. Using the same 60-mL syringe as in **step 18**, withdraw the residual platelet mixture.
22. Repeat **steps 19–21** until all of the platelet mixture is withdrawn and the syringe contains a final volume of 45 mL.
23. Insert a sampling site coupler into one of the ports of a platelet freezing bag and inject the 45 mL of PC (*see* **Note 12**).
24. Using the same syringe, withdraw 40.5 mL of plasma from the PPP-bag. Draw up to 4.5 mL DMSO (final syringe volume will now equal 45 mL); gently invert the syringe several times to mix the contents.
25. Disconnect the 18-gage needle from the syringe, and connect a butterfly needle with approx 75-cm tubing. Insert the 16-gage needle into the sampling site coupler already in place in the freezing bag, taking care not to puncture the bag.
26. Place the syringe in an infusion pump set to deliver 3.0 mL/min.

27. Place the platelets (in the freezing bag) on a rocker so they will be gently mixed while the DMSO mixture is being added. Turn on the rocker and the infusion pump.
28. When all of the DMSO/plasma mixture has been injected into the freezing bag (at a setting of 3.0 mL/min), the total running time should be approx 16 min), turn off the infusion pump and remove the syringe from the pump.
29. Turn off the rocker and remove the platelet freezing bag.
30. Withdraw any air in the freezing bag into the now empty 60-mL syringe. Remove the 16-gage needle from the site coupler in the freezing bag.
31. Clamp the freezing bag port below the sampling site coupler between the bag and the coupler.
32. Remove the sampling site coupler and heat seal the port.
33. Place the freezing bag flat and gently press it to check for leaks. If there is a leak, reseal the port.
34. Place the bag between two metal plates (to produce a final thickness of approx 0.5 cm). Clamp the plates together and place them horizontally in the –135°C freezer.
35. For each bag of frozen platelets, prepare one transfer pack containing 100 mL of autologous donor plasma and one transfer pack containing 120 mL autologous donor plasma.
36. Place the bags of plasma in a conventional freezer below –18°C.
37. After 24 h, the platelets may be removed from the metal plates and filed in the freezer.
38. For thawing remove one 100-mL bag and one 120-mL bag of autologous plasma from the freezer. (If autologous plasma is not available, use ABO type-specific plasma.)
39. Remove one 10-mL vial of ACD-A from the same freezer.
40. Seal each bag of plasma in an additional protective bag (in case of breakage) and thaw the bags in a 37°C water bath without agitation.
41. Thaw the vial of ACD-A in a rack in the water bath. Make sure the water level does not reach the cap of the vial so that contamination of the ACD-A is prevented.
42. As soon as the ACD-A, and the plasma have thawed, remove them from the water bath.
43. Remove the bag of frozen platelets from the –135°C freezer.
44. Seal each bag of plasma in an additional protective bag and thaw it in the 37°C water bath without agitation.
45. Remove the platelets from the bath as soon as thawing is complete.
46. Using a 10-mL syringe and an 18-gage needle, aseptically withdraw the 10 mL of ACD-A from the vial.
47. Insert a sampling site coupler into one port of the 100-mL bag of autologous plasma.
48. Wipe the coupler with an alcohol pad and inject the ACD-A through the coupler into the plasma and gently mix it.
49. Insert one end of a blood transfusion set into the remaining port of the 100-mL bag of plasma.
50. Insert the other end of the blood transfusion set into the port of the bag of thawed platelets.
51. Hang the plasma ACD-A mixture above the platelets.

52. Place the bag of platelets on a rocker (to ensure complete mixing) and adjust the roller clamp on the infusion set such that the plasma/ACD-A mixture is added to the platelets at a rate of 10 mL/min (total running time will be 10–15 min).
53. When all of the plasma mixture has been added to the platelets, remove the platelets from the rocker.
54. Carefully disconnect the infusion set from the bag of platelets and transfer the platelet/plasma/ACD-A mixture to a 300-mL transfer pack.
55. When all of the mixture has run into the transfer pack, heat seal the tubing approx 2 cm from the transfer pack. Discard the tubing and the solution administration set.
56. Discard the empty platelet freezing bag.
57. Centrifuge the thawed platelet mixture at 1250*g* for 15 min.
58. Carefully remove the platelets from the centrifuge cup and place the bag in the plasma extractor.
59. Express the DMSO-containing supernatant plasma into another 300-mL transfer pack, leaving behind the undisturbed platelet button.
60. Heat seal the tubing between the two transfer packs and discard the bag containing the supernatant.
61. Insert one end of a double-coupler plasma transfer set into one port of the 120-mL bag of autologous plasma; insert the other end into the transfer pack containing the platelets.
62. Open the plasma transfer set roller clamp and allow a small amount (10–15 mL) of autologous plasma to run in with the platelets.
63. Using a wet folded paper towel, gently rub the platelet button until the mixture is homogeneous.
64. While gently agitating the bag of platelets, open the roller clamp on the plasma transfer set, and allow the remainder of the plasma to run in with the platelets.
65. Remove the plasma transfer set from the port of the bag of resuspended platelets; discard the set, and the empty plasma bag.
66. Insert a sampling site coupler into the open port of the bag containing the platelets.
67. To obtain a sample of the resuspended platelets, wipe the coupler with an alcohol pad and insert a 3-mL syringe and an 18-gage needle.
68. Withdraw 1–2 mL of platelets into the syringe, but do not remove the syringe from the sampling site coupler. Invert the bag several times so the platelet suspension is thoroughly mixed, then inject the sample in the syringe back into the bag.
69. Repeat **step 67** two or three times (so the specimen will be a representative sample) and remove the syringe from the sampling site coupler.
70. Perform a platelet count on the sample and obtain the net weight of the final bag of re-suspended platelets. Calculate the platelet yield and the percent recovery.

3.6. HES–Intermediate Cooling Technique

Please *see* **ref.** ***13***, which was modified according to **ref.** ***29***.

1. 450–500 mL whole blood are collected in standard blood bank packs containing CPD-A as anticoagulant.

2. The blood is kept at room temperature for at least 3 h and a maximum of 1 d before the pack is centrifuged for the first time at 180*g* for 30 min at room temperature.
3. After removal of about 120–200 mL of the supernatant platelet-rich plasma (PRP) by means of a plasma extractor, a 10% volume ACD-A is added to the PRP.
4. The PC is obtained from the PRP after a second centrifugation at 180*g* for 60 min.
5. The supernatant after this centrifugation step is removed as complete as possible into an empty bag. The PC (optimum volume 20 ± 2 mL, can be adjusted by adding previously removed supernatant plasma) is resuspended within 10 min by careful kneading of the bag.
6. 20 mL of the cryoprotective solution and 2 mL ACD-A are added by means of a syringe.
7. After removing the air by means of an empty syringe the bag is heat-sealed.
8. The resulting suspension of approx 40 ± 2 mL contains approx 4 g HES/100 mL and 700,000–1,000,000 platelets per microliter. It is transferred into the freezing bag and incubated for 15 min at room temperature under agitation.
9. The bag is then placed into an aluminium container of 3-mm inner thickness (wall thickness 1 mm) and the cooling process is started using a controlled-rate freezer. The cooling rate in the temperature region between –5 and –70°C should be approx 15 ± 5°C/min.
10. When a chamber temperature of –70°C (or lower) has been reached, the freezing chamber is opened, and the bags are removed from the freezing containers and stored in the vapor phase over LN_2. Use hand tongs, gloves, and goggles.
11. For thawing, they are transferred from the LN_2 storage tank into a punched thawing container within 30 s.
12. Thawing is performed in a shaking water bath (37°C, shaking frequency approx 60 cycles/min, shaking amplitude 2 cm) within 120 s, the temperature of the thawed suspension should be 22 ± 2°C.
13. For the removal of the HES (if desired), transfer the thawed product into a 250-mL transfer pack and add approx 200 mL of isotonic saline (or autologous or AB0-identical plasma) supplemented with 10% ACD-A by means of a 50-mL syringe. Centrifuge at 580*g* for 20 min at room temperature. Discard the supernatant and resuspend the platelets in plasma.

4. Notes

1. Optimum results can only be achieved when increasing the cooling rate from 1°C/min as described in Choudhury's method ***(13)*** to cooling rates of 15 ± 5°C/min and reducing the sodium chloride concentration in the cryoprotective solution from isotonic to 120 mmol/L ***(29)***. When comparing the optimized HES method to Schiffer's technique ***(11)***, we found that both protocols are highly effective regarding the post-thaw numerical platelet recovery (approx 90%). However, functional in vitro parameters showed that the DMSO-protected frozen platelets were inferior to fresh controls but superior compared with the HES protected ones ***(30)***.

2. Generally, the cell concentrations of mononuclear cells range from 0.5×10^6 to 50×10^6/mL. The most frequently used medium is RPMI 1640 supplemented with human or fetal calf serum or plasma, and the cryoprotectant of choice is 5–10% DMSO. Cooling is performed in 1- or 2-mL vials at 1–2°C/min down to a temperature of –30°C or less by means of a programmable LN_2-operated freezer, whereas thawing is usually performed in a water bath of 37°C. Numerical recoveries reported vary from 60 to 90%. We believe that cooling rates at temperatures below –40°C are not as critical as in the upper temperature region (i.e., above –40°C) and can be increased up to 10°C/min to save time. Additionally, we do not think that a programmable LN_2-operated freezer is always required to generate the appropriate cooling rate: –80°C refrigerators may be suitable as long as provision is taken (e.g., by using card board insulations) that the cooling rates in the upper temperature region does not exceed 5°C/min. For long-term storage (e.g., months or years), however, we recommend temperatures below –123°C, which is the glass transition temperature of DMSO ***(31)***. For days or weeks and up to months, –80°C freezers may also be acceptable.
3. The solution is commercially not available at present. It can be prepared from dry HES powder or commercially available HES solutions after dialysis and freeze-drying ***(32)*** by dissolving the dialyzed and freeze-dried HES and the appropriate amount of sodium chloride in distilled water. Suppliers of the dry HES powder are, e.g., Ajinomoto, Fresenius Kabi. Suppliers of HES solutions for infusion are, e.g., Baxter, B. Braun , Fresenius Kabi, Serag-Wiesner, and Serum Werk Bernburg.
4. Sputtek, A., Mingers, B. Freezing container, European Patent 0 786 981 (Germany, Great Britain, France, Spain, Switzerland, Italy, Netherlands), Priority 17/10/1994; German Patent P 44 37 091 C2; US Patent 5,935,848; Canadian Patent 2,203,035; Japanese Patent 8-512944.
5. In the case of not leukodepleted RBCs, five washing steps have to be performed to guarantee the same post-thaw saline stability as can be obtained with leukodepleted RBCs after three washing steps.
6. The containers have a wall thickness of 2 mm. The exterior is pasted with a microporous textile tape to improve the heat transfer during the cooling process in boiling LN_2. Additionally, the closed containers produce a well-defined flat geometry of the bags and a homogenous sample thickness (approx 5–6 mm). LN_2 is not allowed to come into contact with the samples during the initial cooling process. Please note that you will not be able reproduce our results when not using the patented freezing container.
7. HES coats the surface of the red cells and may provide a scaffolding for damaged membranes so that some cells appear intact, though they will rupture if diluted with isotonic saline. Viability in terms of "saline stability" can be determined as follows: 250 µL of the RBC suspension is diluted 40-fold in a buffered isotonic saline solution. After 30 min the suspension is separated into a supernatant (destroyed RBC) and sediment (intact RBC) by centrifugation. Saline stability is then calculated using:

$$\text{Saline stability (\%)} = (1 - Hb_S/Hb_T) \times 100,$$

where Hb_T corresponds to the total hemoglobin and Hb_S to the hemoglobin in the supernatant. The determination of the two hemoglobin concentrations can be performed spectrophotometrically at 546 nm using Drabkin's solution. A correction for the HCT is not required, as the volume fraction of erythrocytes after a 40-fold dilution is less than 2%.

8. It is essential to remove white cells and platelets. Thomas et al. ***(10)*** have speculated that the contents of the white cells and platelets are highly thromboplastic, and as these cells are destroyed on freezing, thereby liberating their contents into the HES/RBC mixture, failure to filter could cause a disseminated intravascular coagulopathy when the RBCs are subsequently thawed and transfused without post-thaw washing. Modification of the protocol is required for cryopreservation of sheep red cells; CPD or CPD-A cause slight damage to sheep cells so heparin is the anticoagulant of choice, but it is vital that special care is taken when mixing during donation.
9. For cryopreservation of pig cells, which are a similar size to human red cells, Thomas et al. ***(10)*** have reported that the main requirement is to remove all the plasma by saline rinsing prior to the addition of HES. If all the plasma is not removed, progressive hemolysis occurs, possibly from complement activation. Sheep cells are spherical and only about one-third of the volume of human red cells, so the plasma is more difficult to separate. It is therefore necessary to centrifuge for about 25 min to pack the sheep red cells to 90% HCT.
10. Both human and sheep red cells have been stored in the LN_2 vapor phase, without deterioration, for up to 12 yr, as demonstrated by in vitro testing. Storage in mechanical freezers has been carried out at the following temperatures, without deterioration of the red cells as assessed by in vitro quality control tests, for the undermentioned periods: –140°C for 4 mo; –120°C for 4 mo; –100°C for 4 mo; –90°C for 5 wk (*see* **Note 7**).
11. Plasma stabilities may be slightly higher than saline stabilities because plasma factors allow a few very slightly damaged cells to recover. The percentage recovery of intact cells, immediately post-thaw, is not used as a quality control procedure because it is only a crude indicator of quality. HES coats the surface of the red cells and may provide a scaffolding for damaged membranes so that some cells appear intact, though they will rupture if diluted with isotonic saline.
12. If performing a split-freeze, repeat **Subheading 3.5., steps 19–23** until the volume in the platelet freezing bag equals 45 times the number of bags to be frozen. (Example: if it was determined in **Subheading 3.5., step 12** that the freeze is to be split into three bags, repeat **Subheading 3.5., steps 19–23** until the volume in the first platelet freezing bag equals $45 \times 3 = 135$ mL.) Mix well. Using the same syringe, withdraw 45 mL of platelet concentrate from the first platelet freezing bag and inject it into a second platelet freezing bag. Repeat until each freezing bag contains 45 mL of platelet concentrate. (Example: there will be three freezing bags, each containing 45 mL of platelet concentrate.)

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