Protocol for

Red Blood Cell Preservation by Droplet Freezing with Polyvinyl Pyrrolidone or Sucrose/Dextrose[1]

INTRODUCTION

Red blood cell (RBC) preservation of small volumes (i.e. < 1 mL) is an important tool in modern transfusion medicine for serological testing. Blood group laboratories need to have the ability to suspend, reconstitute, and then analyze rare RBCs that were collected months or years before. RBC preservation methods offer a reliable long-term supply of rare RBCs that are collected once.

Droplet freezing is one method of small volume RBC preservation.[2] It is a method that preserves RBC samples in small, individual droplets. Minute quantities of RBCs can then be recovered to obtain the minimal amount of sample needed to perform a serological test. This method uses non-permeable cryoprotective agents, like polyvinyl pyrrolidone (PVP) or sucrose/dextrose/NaCl (S+D), to protect the RBCs. Non-permeable cryoprotective agents may work by dehydrating the RBCs and minimizing intracellular ice formation.[3,4] They may also stabilize the RBC membranes and help macromolecules retain their native conformations.[5-7]

MATERIALS

Reagents

- Polyvinyl pyrrolidone (PVP; PVP40-50G, K value: 29-32)
- 22% or 30% bovine serum albumin (BSA)
- Deionized water
- 0.9% saline
- Liquid nitrogen
- Sucrose
- Dextrose
- Sodium chloride

Equipment

- Fine-tip transfer pipettes
- 5 mL test tubes (with caps)
- Serofuge
- Water bath
- Liquid nitrogen storage
- 2.5 mL cryo vials (with caps)
- Cryo-storage box
- Metal canisters
- Teflon stir bar
- Tweezers
- Funnel
- Fine metal sieve
- Safety gloves
- Safety goggles

REAGENT SETUP

PVP freezing solution

- 27 mL of BSA (22% or 30%) with 23 mL of 30% PVP in deionized water. Store at 4°C. Stable for 3 months at 4°C from date made. ▲ CRITICAL The PVP freezing solution must be adequately mixed before being used. It may look cloudy or clear immediately after mixing, but this is normal.

S+D freezing solution

- 15.4 g sucrose, 5.4 g dextrose, and 0.29 g NaCl in 100 mL of deionized. Store at -20°C. Stable for 1 year at -20°C.

PROCEDURE

Preparing of RBCs

⊕ **TIMING 1 h to 2 h**

1 | Select the appropriate whole blood sample and transfer to a 5 mL test tube. Centrifuge the sample at 1000 x g for 5 min to separate the plasma and buffy coat from the RBCs. Next, aspirate the plasma and buffy coat. Gently tap and shake the test tube to dislodge the RBCs stuck to the test tube.

2 | Wash the RBCs by filling the test tube with 0.9% saline, repeatedly inverting the test tube to resuspend the RBCs, centrifuging at 1000 x g for 5 min, and then aspirating the supernatants. Continue washing the RBCs until the supernatant is clear – that is, until no hemolysis is present.

▲ CRITICAL STEP It is essential to completely resuspend the RBCs in saline during each wash step. If this is not done, then the RBCs will be poorly washed and damaged RBCs may be frozen, which will lead to significantly higher amount of hemolysis during thawing. Also, the longer it has been since the blood was drawn, the more wash steps will be required to remove all signs of hemolysis.

3 | After the RBCs are completely washed, add one volume of PVP freezing solution for every one volume of RBCs. Mix by gently inverting the test tube. Let incubate at room temperature for 1 h.

▲ CRITICAL STEP In order to maximize the contact between the PVP freezing solution and RBCs, it may be helpful to let the test tubes rest on an incline during incubation.

■ ALTERNATE FREEZING SOLUTION A sucrose/dextrose/NaCl freezing solution is a possible substitute for the PVP freezing solution.

Freezing of RBCs

⊕ **TIMING 30 min to 1 h**

4 | Set out three metal canisters. Fill two canisters with liquid nitrogen, and place a metal sieve over the empty canister. Put a stir bar into one of the canisters with liquid nitrogen and place on a stirring plate. Turn the stirring plate on to produce a weak whirlpool, but make sure that liquid nitrogen is still in contact with all of the canister's bottom.

! CAUTION Liquid nitrogen is extremely dangerous. Be sure to use appropriate safety gear, like gloves and goggles, and to practice safe handling techniques.

5 | Gently mix the RBCs and draw them into a fine-tipped transfer pipette. Hold this pipette vertically and 15 cm above the liquid nitrogen. Slowly release droplets into the stirred liquid nitrogen.

▲ CRITICAL STEP Aim to make your droplets fall by the edge of the whirlpool. Do not release droplets into the center of the whirlpool. And do not release more than one droplet per second. If you release the droplets too quickly, then they will aggregate. Also take caution to prevent air from entering the pipette – this will make droplets that are bloated with air bubbles.

6 | To collect the droplets, pour the canister containing the droplets over the empty canister with the metal sieve. The droplets will be caught in the sieve. Use the funnel to help channel the droplets into cryovials. One of the liquid nitrogen canisters can be used to temporarily store cryovials until the all samples are frozen, at which point transfer all cryovials to long-term liquid nitrogen storage.

▲ CRITICAL STEP Puncture cryovial's cap 2 or 3 times. This prevents the vial from bursting due to pressure. As long as the droplets remain in the sieve over the liquid nitrogen vapors, they should remain frozen. Chill the cryovial and funnel in liquid nitrogen prior to transferring to prevent the droplets from sticking and thawing on contact.

Thawing of RBCs

⊕ TIMING 5 min to 10 min

7 | Fill desired number of test tubes with approximately 1.5 mL of warm saline (37°C).

8 | Transfer the desired number of droplets (approximately 20 µL/droplet) to the test tubes with warm saline. Gently shake the test tube to reconstitute the droplets. Wash the RBCs by filling the test tube with 0.9% saline, repeatedly inverting the test tube to resuspend the RBCs, centrifuging at 1000 x g for 1 min, and then aspirating the supernatants. Continue washing the RBCs until the supernatant is clear – that is, until no hemolysis is present.

9 | Dilute RBCs to desired volume or concentration. They are ready for use.

⊕ TIMING

Preparing RBCs: 1 h to 2 h
Freezing RBCs: 30 min to 1 h
Thawing RBCs: 5 min to 10 min

? TROUBLESHOOTING

Occasionally, droplets will aggregate into unwieldy clumps that have difficulty fitting into the cryovials. These clumps may also be bloated with air bubbles. In either event, they are considered suboptimal droplets and should be avoided. The best way to avoid making clumps is to be extremely patient when releasing droplets into the liquid nitrogen. Droplets released in quick succession will clump together; similarly droplets with air bubbles will float on top of the liquid nitrogen and aggregate. If some suboptimal droplets are produced, it is possible to remove them with tweezers while they rest on the metal sieve.

ANTICIPATED RESULTS

Droplet freezing with PVP or S+D is a reliable and effective technique to preserve small volumes of RBCs.[1] This technique should be useful for preserving most RBCs in droplets of about 20 µL. When using the PVP freezing solution, these droplets can then be recovered at rates that exceed 90%. S+D is another viable freezing solution, but it has a significantly worse recovery rate (55%) than PVP. If the RBCs are properly washed before freezing, then the recovery rate should not greatly vary between RBCs frozen the day after being drawn and RBCs frozen two weeks after being drawn. Different anticoagulants should not affect the recovery because they are removed during the initial wash steps.

References

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