



Standard Operating Procedure

Title:

Cryopreservation and Thawing of Red Blood Cells

PURPOSE:

To describe the procedure for processing blood samples for the isolation and cryopreservation of red blood cells (RBC) collected in EDTA tubes for G6PD Specimen Repository.

SCOPE:

This SOP applies to cryopreservation and thawing of red blood cells for establishing the Specimen Repository which will be used for successful development of G6PD diagnostic tests.

RESPONSIBILITIES:

1. The Project lead has the authority to establish this procedure.
2. The Scientific lead is responsible for the control of SOP documentation.
3. Laboratory staff is responsible for the implementation of this procedure and for ensuring that all appropriate personnel are trained.

PROCEDURES:

1. Specimen receiving

- 1.1. Blood samples from Bioreclamation and other collaboration sites will be received.
- 1.2. Whole blood with anti-coagulant will be received in cold chain at 2-8C.
- 1.3. Laboratory data management system *viz. Freezer Works* will be used for chain of custody.

2. Specimen Handling

- 2.1. Use Blood borne pathogen precautions for all samples

3. Specimen Rejection

- 3.1. Quality of specimens must be evaluated at the point of delivery.
- 3.2. Technicians must have appropriate laboratory training to assess quality before the entry of specimens into the processing workflow
- 3.3. **Unacceptable specimen criteria**
 - 3.3.1. Unlabeled or mislabeled specimens must be rejected.
 - 3.3.2. Clotted specimen must be rejected.

4. Personal Protective Equipment (PPE)

Personal protective equipment must be used for handling specimens and reagents.

- 4.1. Laboratory coat or gown
- 4.2. Eye protection
- 4.3. Latex or nitrile gloves, non-powdered preferred

5. Equipment

- 5.1. Centrifuge Vortexer (USA Scientific)
- 5.2. Test Tube Rocker (Thermo Scientific)
- 5.3. Timer
- 5.4. Micropipettes, range 20, 200, 1000µL (Rainin, Gilson or Eppendorf)
- 5.5. Pipette-Aid
- 5.6. 125 ul CombiTip and dispenser (Eppendorf)
- 5.7. 37C water bath or heat block

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- 5.8. 2–8°C refrigerator
- 5.9. Liquid nitrogen storage tank ($\leq -140^{\circ}\text{C}$)

6. Materials

- 6.1. 15 ml centrifuge tubes (VWR)
- 6.2. 50 ml centrifuge tubes (Corning)
- 6.3. Glycerolyte 57 solution (Fenwal)
- 6.4. 2.0ml Cryotubes with Locking Base (Neptune)
- 6.5. 1.5 ml cryo vial

7. Reagents

- 7.1. 500 ul CombiTip and Dispenser (Eppendorf)
- 7.2. 0.9% NaCl solution, storage at room temperature
- 7.3. Additive: 2.5% glucose, 0.9% sodium chloride, 0.27% adenine, 0.75% mannitol, storage at 4C.
- 7.4. 12% Saline, storage at room temperature
- 7.5. 0.2%/0.9% dextrose/saline, storage at room temperature

8. RBC Processing and Cryopreservation

- 8.1. Divide 15 ml of whole blood in two 15 ml tubes.
- 8.2. Aliquot 7.5 ml per tube.
- 8.3. Spin 5 minutes 2200 rpm, measure and remove plasma and buffy coat and replace plasma and buffy coat volume with additive.
- 8.4. Keep cells on ice or at 4C.
- 8.5. Spin and wash the red blood cells with 0.9% NaCl, until no more lysis(red color) is evident (3-5 times approximately).
- 8.6. Measure and transfer the pellet from the 15 ml centrifuge tube to the 50 ml centrifuge tube, resulting in total two 50 ml tubes per sample
- 8.7. Add a total of two volumes of Glycerolyte 57 as below
 - 8.7.1. Slowly add 10 ul drop of 20% Glycerolyte 57 Solution using 125ul Combipip, while vortexing gently.

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- 8.7.2 Place tubes on rocker for 10 mins.
- 8.7.3 Slowly add 50 ul per drop of the remaining 80% Glycerolyte 57, while vortexing
- 8.7.4 Mix gently for 10 minutes on test tube rocker
- 8.7.5 Combine the cells from each of the 50 ml centrifuge tubes from the same donor
- 8.7.6 Prepare 1.5 ml aliquots in cryotubes
- 8.7.7 Store cryotubes in StrataCooler at $\leq -80^{\circ}\text{C}$ for gradual freezing
- 8.7.8 Transfer cryotubes from StrataCooler to liquid nitrogen on the following day
- 8.7.9 Record in lab note book and in Freezerworks database.

9. Thawing cryopreserved RBC

- 9.1. Remove the desired cryovial from Liquid nitrogen and thaw quickly at room Temperature.
- 9.2. Gently transfer the red blood cells to a 15 ml centrifuge tube and centrifuge for 1000 x g for 10 minutes
- 9.3. Discard the supernatant containing freezing solution using 28Gauge needle and 3 ml syringe.
- 9.4. Measure the red blood cell pellet volume.
- 9.5. Over a period of 5 minutes, slowly add drop wise 0.16 ml of 12 % saline for every 0.5 ml of red blood cell. Use 125 ul CombiTip, dispensing 1 ul per drop.
- 9.6. Let the cells sit for 3 minutes at room temperature
- 9.7. Slowly add 0.5ml of 0.2%/0.9% dextrose/saline using the 125 ul combiTip and dispense 10ul per drop while vortexing.
- 9.8. Let the red blood cells sit for 2 minutes at room temperature.
- 9.9. Repeat step 9.7 until the volume in the test tube is 4 ml.
- 9.10. Centrifuge the cells at 1000 x g for 1 minute
- 9.11. Remove 0.5ml of supernatant. Resuspend the red blood cells by repeated inverting of the test tube. Note: This is for gradual replacement of salt solution.
- 9.12. Slowly add 0.5 ml 0.2%/0.9% dextrose/saline at 10ul per drop using the CombiTip, while vortexing.
- 9.13. Let the cells sit for 2 minutes at room temperature
- 9.14. Repeat the process in step 9.10-9.13 with the following volumes: 1ml, 1.5ml, 2ml, and 4ml of 0.2%/0.9% dextrose/saline solution for complete replacement. Note: allow the cells to sit for one minute after each resuspension.
- 9.15. Centrifuge the cells for 1 minute at 1000 x g and remove all of the supernatant.
- 9.16. Wash the red blood cells by filling the test tube with 10 ml of 0.9% saline repeatedly
Note: gently invert the test tubes to resuspend the cells, centrifuging for 1 minute at 1000 x g and removing the supernatant. Continue washing until the supernatant is clear, and no hemolysis is present.
- 9.17. Resuspend pellet in AB plasma. The pellet constitutes 400ul to 500ul of thawed red blood cells.
- 9.18. RBC is ready to proceed with desired experimental procedure.

10. Related SOPs

- 10.1. Sample receiving, handling and data management
- 10.2. Use and Maintenance of Liquid Nitrogen storage tank
- 10.3. Sample shipment from specimen repository