Leukopak Cryopreservation

PROTOCOL

Materials

- 1 mL & 60 mL luer lock syringes
- 150/300 mL Blood transfer bag
- 250/500/750 mL Freezing bag
- 250/500/750 mL aluminum cassettes and frames
- Transfer set
- Microcentrifuge tube
- Clamping scissors
- Plastic tubing clamps
- Single channel micropipette with sterile tips
- Cell counting chamber

Reagents

- 70% Ethanol
- AO/PI staining solution
- DMEM (serum-free)
- Wash buffer (2% FBS + 1X sterile DPBS or serum-free)
- Serum-free freezing medium (10% DMSO)

Equipment

- Biosafety cabinet, BSL-2
- Centrifuge with blood bag rotor
- Plasma extractor
- Blood boot stand
- Digital scale
- Tube heat sealer
- Dual fluorescence automated cell counter
- Controlled-rate freezer
- Liquid nitrogen storage system

Procedure

Considerations

Fresh Leukopaks are processed within 24 hours of collection unless otherwise stated.

All procedures must be performed with Universal Safety Precautions, and samples are considered potentially infectious (BSL-2 or higher is recommended).

Sanguine conducts this procedure in a biosafety cabinet to promote sterility and personnel protection. However, a closed system where specimens are not exposed to the environment ensures all steps may be conducted outside a biosafety cabinet.

An automated cell counting system with dual fluorescence capabilities is used to assess cell viability and total nucleated cell (TNC) count.

Preparation

- Turn on the biosafety cabinet and ensure a sterile environment. Spray all materials, equipment, and reagents entering the cabinet with 70% ethanol.
- Remove transfer/freezing bags and transfer sets from their packaging, and ensure all clamps are closed. Use the table below to determine the appropriate bag size:

	Transfer bag (mL)	Freezing bag (mL)	Freezing cassette (mL)
Full leukopak (>200 mL)	300	750	750
Full leukopak (<200 mL)	150	750	750
½ leukopak	150	500	500
¼ leukopak	150	250	250

Leukapheresis Product Partitioning

Follow the steps below for leukopaks needing partitioning post-leukapheresis. Otherwise, skip to the Centrifugation section.

1. Obtain an appropriate number of fresh transfer bags and transfer sets.
2. Insert the spike of a transfer set into the spike port of each leukapheresis sample bag and fresh transfer bag.
3. Gently mix the sample in the leukapheresis sample bag. Use a luer lock syringe to extract the desired sample volume and inject it into the transfer bag.
4. Repeat step 3 until the desired amount of leukapheresis sample is partitioned to the transfer bag.

Centrifugation

1. Punctured spike ports of either a sample bag or a transfer bag necessitate transferring the leukapheresis sample to a fresh bag before centrifugation:
   a. Insert the spike of a fresh transfer bag into the spike port of the bag containing the leukapheresis sample.
   b. Transfer all content into the new transfer bag using a plasma extractor.
   c. Using a tube heat sealer, make two seals that are ~ 1 inch apart on the tubing of the transfer bag. Cut between the seals to remove excessive tubing.
2. Place the transfer bags into a centrifuge following proper technique. The bags should fit snugly in the blood bag adapter. Tuck in any remaining tubing and ensure the rotor is balanced.
3. Centrifuge at 400 x g for 10 minutes at 4°C with the brake off.
4. Carefully transport the centrifuged transfer bags to the biosafety cabinet using a blood boot stand.
5. Three layers should be visible in the transfer bag (top to bottom): plasma, white blood cells (WBCs), and red blood cells (RBCs).

Plasma Reduction

6. Carefully set the transfer bag in a plasma extractor.
7. Without disturbing the layers, insert the spike of a new transfer set into the spike port of the transfer bag containing the specimen.
8. While keeping the transfer bag upright on the plasma extractor, extract most of the plasma using a luer lock syringe, leaving ~10% plasma in the bag.

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9. Detach and retain the syringe containing the plasma for a later step.
10. Break up any clumps and mix the remaining components by gently rocking the bag.
11. Determine the sample volume by weighing the transfer bag containing the plasma-reduced sample and subtracting the weight of a new matching bag with a transfer set, using an approximate density of 1.0 g/mL.
12. Determine the volume of plasma needed to return to the transfer bag by subtracting the sample volume measured in step 12 from the final volume given in the table below (excluding freezing medium):

	Plasma-reduced leukopak final volume (mL)
Full leukopak	70
½ leukopak	50
¼ leukopak	35

13. Inject an appropriate volume of plasma from the syringe in step 9 into the transfer bag, discarding the remaining plasma.
14. Gently mix the specimen with plasma. Extract ~0.2 mL of sample with a new syringe and set aside for cell counting.

Cell Counting

15. Dilute the sample collected for cell counting 1:10 with serum-free DMEM in a microcentrifuge tube. For better accuracy, mix the sample sufficiently and use a micropipette to retrieve a fixed volume (e.g., 100 µL) for dilution.
16. Follow the manufacturer's protocol to further dilute the sample before measuring on a dual fluorescence automated cell counter.
17. Assess sample viability and TNC count with AO/PI staining.
18. Prepare a label for each cryopreserved leukopak with appropriate information.

Cryopreservation

19 Affix the label(s) prepared in Step 19 to the appropriately sized freezing bag(s) for each leukopak to be cryopreserved.
20. Transfer the plasma-reduced sample from the transfer bag into the fresh freezing bag using a luer lock syringe and transfer set. Repeat until the freezing bag contains the whole specimen.
21. Slowly inject an equal volume of freezing medium into the freezing bag containing the specimen using a new luer lock syringe and gentle mixing. Refer to the table below for the appropriate volume of freezing medium:

	Freezing media volume (mL)
Full leukopak	70
½ leukopak	50
¼ leukopak	35

22. Remove air from the freezing bag using a plasma extractor, leaving no visible air pockets.
23. Prevent air from re-entering the freezing bag using tube-clamping scissors.
24. Remove the freezing bag from the plasma extractor and heat seal the tubing. Make two seals that are ~ 1 inch apart. Cut between the seals to remove excessive tubing for cryopreservation.
25. Place each freezing bag into an appropriately sized cryopreservation cassette.
26. Insert the cassette into a controlled-rate freezer and gradually freeze the specimen.
27. Transfer the cassette containing the cryopreserved leukopak to the vapor phase of liquid nitrogen for long-term storage.

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