liquidbio

Procedure

Cryopreservation of IsoFlux CTC samples for biobanking and sample storage

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SUMMARY

This document is intended to guide the user through a method for working with frozen peripheral blood mononuclear cell (PBMC) samples obtained from whole blood collections. The freezing process enables the PBMC specimens to be retained for longer periods of time and shipped as necessary to the processing site. The protocol incorporates use of the IsoFlux System and the IsoFlux Circulating Tumor Cell (CTC) Enrichment Kit.

This protocol has been validated by Liquidbio using tumor cell line spike-in experiments.

BACKGROUND

Most CTC enrichment procedures start with the collection of one or more tubes of whole blood. Once drawn, these blood tubes have a relatively brief window where they can be processed, typically 36 hours. The use of a fixative inside the tube can extend this window to 96 hours, but may come at the expense of the types of analyses that can be performed (i.e. it may limit it to just enumerating the CTCs). It is a clear advantage to be able to retain the samples for longer before processing:

- Preserve the integrity of the sample during transport and storage
- Ease the logistical burden on clinical collections team and lab processing center
- Batch process samples to reduce time and expense

The protocol here describes a method for collecting blood in BD Vacutainer® CPT™ Tubes for easy recovery of the PBMC fraction. The PBMCs can be frozen and retained indefinitely for processing at a later date. The protocol describes the methods for both the freezing and thawing process, both of which are critical to maintain the integrity of the sample. After thawing, the PBMCs can move directly into the protocol for the IsoFlux CTC Enrichment Kit.

REAGENT REQUIREMENTS*

PBS (phosphate buffered saline, Ca2+ Mg2+ -free)
RPM 1640 media

CTL-Wash™ Supplement (Immunospot, Product Number: CTLW-010)
Fetal bovine serum (heat inactivated)
DMSO (endotoxin-free tissue culture grade)
Benzonase® (Sigma, Product Number: E1014-25KU)

INSTRUMENT REQUIREMENTS

IsoFlux Instrument (Product Number: 950-0100)
Swing bucket centrifuge capable of 800xg
Water bath set to 37°C
Test tube racks
Calibrated micropipettes
Wide-bore 1mL micropipette tips, and standard tips
Pipette aid and serological pipette tips
Cryopreserve container ("Mr. Frosty" container or similar)
Cryovials
15mL and 50mL conical tubes
BD Vacutainer®CPT™ Tubes (BD, Product Number: 362761)

Note: Liquidbio has recommended use of certain reagents and supplies from third-party vendors. While these kits have been tested for suitability in the protocol, Liquidbio does not have any affiliation with the respective vendors of these kits. All issues with performance of these kits should be addressed directly to the supplier.

WARNINGS AND PRECAUTIONS

1. For Research Use Only
2. Please read the entire contents of this protocol before processing samples.
3. **Caution:** Care should be taken to collect and transfer blood samples before processing. Cells are fragile and can be damaged or lost if not handled properly.
4. **Caution:** All personnel should follow universal precautions for biological sample handling and use laboratory safety equipment (i.e., safety glasses, laboratory coat, gloves).
5. **Caution:** Microbial contamination of reagents can cause erroneous results and should be avoided.
6. **Warning:** All biological specimens, cartridges and other materials coming into contact with the specimen(s) are considered biohazardous. Handle as if capable of transmitting infection. Treat and dispose of waste using proper precautions and in accordance with local, state, and federal regulations. Never pipette by mouth.

7. **Warning:** Some of the reagents contain sodium azide as a preservative. If swallowed, seek medical advice immediately. Keep out of reach of children. Keep away from food and drink. Wear suitable protective clothing. Contact with acids liberates very toxic gas. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop.
8. Operator training is required to perform the test procedure.

BLOOD COLLECTION

Prepare Wash Buffer: Phosphate Buffer Saline (PBS without Ca2+ Mg2+) containing CTL Wash™ Supplement.

1. The BD Vacutainer® CPT™ Tubes should be stored at room temperature (18 to 25°C) upright.
2. Draw blood into CPT tubes according to the product instructions.
3. After blood collection, store CPT tube on racks upright at room temperature during transportation.
4. Store tube upright at room temperature until centrifugation. Blood samples should be centrifuged within two hours of blood collection for best results.

PBMC PREPARATION

Note: If you plan to spike in tumor cells (i.e. for protocol development or as a positive control sample), open blood tube cap carefully, spike in cancer cells, close cap completely.

1. Remix the blood sample immediately prior to centrifugation by gently inverting the tube 8 to 10 times.
2. Centrifuge tube/blood sample at room temperature (18 to 25°C) in a horizontal rotor (swing-out head) for a minimum of 20 minutes at 1500 to 1800 RCF (Relative Centrifugal Force).
3. After centrifugation, mononuclear cells and platelets will be in a whitish layer just under the plasma layer. Resuspend the cells into the plasma by inverting the unopened CPT Tube gently 5 to 10 times.
4. Open the CPT Tube and pipette the entire contents of the tube above the gel into a 15mL centrifuge tube; avoid pipetting the gel.
5. Rinse the CPT tube 2 times with 2mL Wash Buffer and add the rinse to the tube above. Add Wash Buffer to bring volume to 15mL. Cap tube. Mix cells by inverting tube 5 times.
6. Centrifuge for 15 minutes at 300 RCF. Aspirate off as much supernatant as possible without disturbing cell pellet.
7. Gently tap tube with index finger to loosen the cell pellet. Resuspend the pellet using a

p1000 pipette with a wide-bore 1mL pipette tip by slow and gentle pipetting until no visible cell aggregates is observed (*cell pellet should be re-suspended extremely gently with this technique for all steps in this protocol).

8. Add Wash Buffer to bring the volume to 10mL. Cap the tube. Mix cells by inverting tube 5 times.
9. Centrifuge for 10 minutes at 300 RCF. Aspirate as much supernatant as possible without disturbing cell pellet.

FREEZING PBMC

Media Preparation:

Prepare Freezing Buffer: 100% FBS (heat inactivated) and FBS (heat inactivated) containing 20% DMSO. For rinsing, make FBS (heat inactivated) containing 10% DMSO.

1. Gently resuspend the cell pellet in 100% FBS and bring the volume to 500uL. 2. VERY IMPORTANT: Add 500uL drop-wise of FBS containing 20% DMSO, gently swirl the tube after each drop.
3. Gently transfer the entire contents into a cryovial with the wide-bore tip. Rinse the bottom of the 50mL conical tube with 200µL of FBS containing 10% DMSO. Add the rinse to the sample slowly.
4. Close the vial and freeze. It is recommended to slowly freeze the sample (using alcohol insulated container or equivalent) at -80°C overnight and transfer to liquid nitrogen after 24 hours.

PBMC STORAGE

Samples can be stored in liquid nitrogen over long periods of time. We recommend processing within 1 year of freeze-down, but it is likely that samples are stable for years if kept at liquid nitrogen temperatures.

THAWING PBMC

Prepare Thawing Buffer: RPM 1640 containing CTL-Wash™ Supplement. Keep warm at 37°C. Just prior to use, add Benzonase® to a final concentration of 50 Unit/mL.

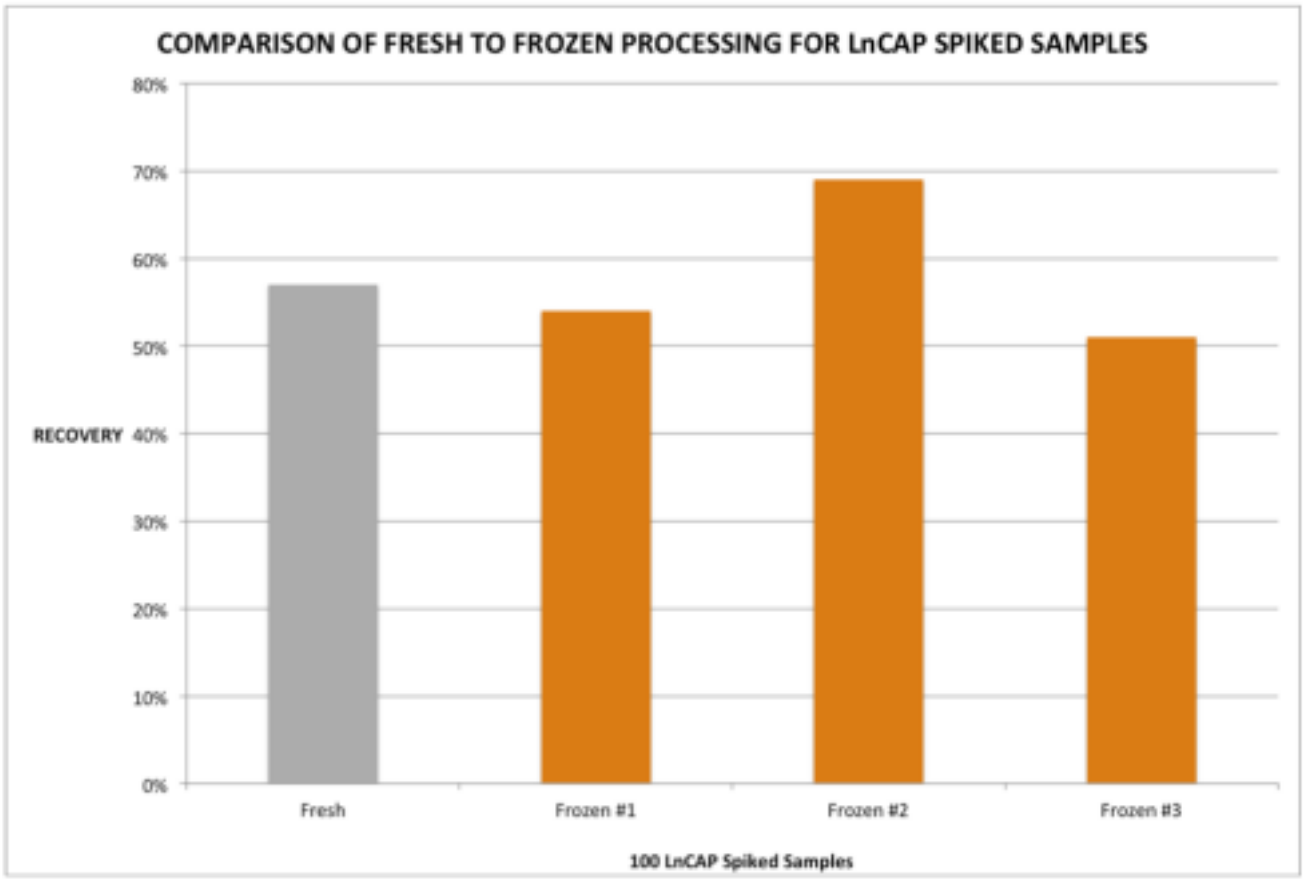
1. Remove vials from liquid nitrogen and place in 37°C water bath until cells are completely thawed. Remove from water bath.
2. Using a wide bore pipette tip, transfer the sample to a 50mL conical tube. 3. Rinse the sample vial with 1mL of Thawing Buffer and add the rinse drop-wise to the conical tube, swirling the tube after each drop.

4. Slowly add drop-wise 4mL of Thawing Buffer to the conical tube, swirling the tube after each drop.
5. Slowly add drop-wise 5mL of Thawing Buffer to the conical tube.
6. Slowly add 10mL of Thawing Buffer to the conical tube.
7. Gently swirl the tube. Incubate for 5 minutes at room temperature.
8. Centrifuge at 300xg at room temperature for 10 minutes.
9. Gently aspirate off the supernatant, being careful not to disturb the pellet. 10. Wash the sample once more with 20mL of Thawing Buffer. (If nuclease is not desired for down-stream analysis, use RPM containing CTL-Wash™ Supplement without Benzonase®)

   Note: Samples processed without Benzonase® tend to aggregate. Liquidbio has used samples that had been processed with Benzonase® for molecular analysis. No loss or inhibition was observed.
11. Centrifuge at 300xg at room temperature for 10 minutes.
12. Gently aspirate off the supernatant, being careful not to disturb the pellet. 13. Gently resuspend the pellet to final volume of ~800µL in IsoFlux Binding Buffer with 5% FBS.
13. The sample is now ready for the beads coupling step in the IsoFlux Rare Cell or CTC Enrichment protocol.

REPRESENTATIVE DATA

A prostate cancer cell line (LnCAP) was spiked into an 8mL blood sample at 100 cells/tube. One sample was processed immediately ("fresh") and three samples were frozen according to this protocol, thawed, and then processed with IsoFlux. The frozen samples gave comparable recovery rates (mean=58%, std=10%) as the fresh sample (57%).



Liquidbio LLC
www.liquidbio.co
support@liquidbio.co