

SYSTEMS BIOLOGY APPROACH TO DELINEATE MOLECULAR SIGNATURES OF PRAKRITI IN HEALTHY HUMANS

(A & B) SOP for PBMC and Plasma

1.0 Purpose

This document outlines the procedure to obtain peripheral blood mononuclear cells (PBMC) from human whole blood in Cell Preparation Tubes (CPT) with sodium citrate for transcriptome analysis.

2.0 Scope

This SOP applies to:

2.1. Collection of PBMC from subjects participating in CCRAS funded project “SYSTEMS BIOLOGY APPROACH TO DELINEATE MOLECULAR SIGNATURES OF PRAKRITI IN HEALTHY HUMANS”.

3.0 Safety

- 3.1. All blood samples should be handled and processed according to institutional Biosafety Guidelines. This procedure should be performed in accordance with all applicable safety procedures.
- 3.2. It is imperative that PBMCs are collected and processed using strict aseptic technique.
- 3.3. Laboratory personnel are required to be trained on this procedure prior to processing blood samples collected from the study. Laboratory managers are responsible for documenting the training in accordance with institutional requirements.

4.0 Reagents

- 4.1. Ca^{+2} , Mg^{+2} -free phosphate buffered saline (PBS) (Fisher Scientific, Cat. #BW17-516Q or equivalent)
 - 4.1.1. PBS can be stored either at room temperature (15 to 30°C) or refrigerator temperature (2 to 8°C).
 - 4.1.2. After opening, keep the opened bottle of PBS sterile and use the contents within 30 days or the original manufacturer’s expiration date, whichever comes earlier.
- 4.2. Fetal Bovine Serum (FBS) (Atlanta Biologicals, Cat. #S12450H, heat-inactivated [HI] or equivalent)
 - 4.2.1. The FBS used in this SOP is heat inactivated FBS. Prior to use, FBS should be stored frozen (per the manufacturer’s recommendation) in its original container and can be used until the manufacturer’s expiration date. Once thawed, FBS can be stored at 2 to 8°C and is stable for one month or the original manufacturer’s expiration date, whichever comes earlier.
 - 4.2.2. Aliquot in to sterile, labeled 50mL conical centrifuge tubes or other size aliquots appropriate for the anticipated workload. Labels should identify these tubes as “HI-FBS” and include the lot number, aliquot date, expiration date, and technician’s initials. Aliquots of FBS should be stored frozen (per the manufacturer’s recommendation) and are stable until the original manufacturer’s expiration date.
- 4.3. DMSO (cell-culture grade; Sigma, Cat. #D2650 or equivalent)
 - 4.3.1. Store unopened bottles at room temperature (15 to 30°C). Check bottle for expiration date and discard if expired.

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4.3.2. After opening, undiluted DMSO is stable at room temperature (15 to 30°C) when protected from light and moisture for three months or the actual labeled expiration date, whichever comes earlier.

5.0 Equipment and Materials *(Note: equivalent may be used)*

- 5.1. 8 mL CPT with Na citrate (BD, Cat. #362761)
- 5.2. Biological Safety Cabinet (Certified)
- 5.3. 10 mL Pipettes
- 5.4. Filtered Sterile Pipette 20ul-1000ul Universal Tips
- 5.5. Single Channel Pipette 20ul-1000ul Pipettors
- 5.6. Serological pipettes
- 5.7. Centrifuge that meets the following requirements:
 - Centrifuge must be capable of generating at least 1500-1800 x g (RCF) at the tube bottom.
 - Centrifuge with Swinging Bucket Rotor.
 - Tube buckets/adapters for 13 x 100mm and/or 16 x 125mm for selected CPT tube size.
 - Centrifuge carriers and inserts should be of the size specific to the tubes used.
 - Capable of turning off break.
- 5.8. Centrifuge tube 15 mL
- 5.9. Strata Cooler by Agilent, (Cat #400005/6) Cell freezing containers that ensure a standardized, controlled rate of -1°C/minute cell freezing in a freezer (-60oC to -90oC).
- 5.11. Cryogenic vials (Thermo Scientific Nunc Cryogenic vial, Cat. #366656; or Corning Cryogenic vial, Cat. #430488, or equivalent)
- 5.12. Refrigerator (2 to 8°C)
- 5.13. Freezer (-5°C to -20oC) Shipping/Storage
- 5.14. Freezer (-60oC to -90oC) Shipping/Storage

6.0 Procedure

6.1 Preparation

- 6.1.1. Aliquots of FBS (per 4.2.2) that may be needed the next day can be taken out from the freezer and stored at 2 to 8°C.
- 6.1.2. Mix well before use.
- 6.1.3. Once thawed, aliquots of FBS are stable for one month at 2 to 8°C or the original manufacturer's expiration date, whichever comes earlier.
- 6.1.4. Repeated freeze/thaw cycles will have an adverse effect on the quality of the FBS.
- 6.1.5. Do not refreeze aliquots that have been stored in the refrigerator (2 to 8°C).

- 6.1.6. If PBS is stored at refrigerator temperature (2 to 8°C), warm up to room temperature (15 to 30°C) prior to using to wash the cells.

- 6.1.7. Prepare freezing media (10% DMSO in FBS).
- 6.1.8. Use the following formula to calculate the amount of DMSO and FBS needed.

Freezing media = 1 part DMSO + 9 parts FBS

Examples: 10 mL = 1 mL+ 9 mL

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6.1.9. Mixing of DMSO and FBS is an exothermic reaction. It must be prepared in advance and chilled in the refrigerator (2 to 8°C) for at least 30 minutes or in an ice bath for at least 15 minutes. After the cooling period and prior to use, the freezing media should be kept at the same temperature as the Freezing Containers outlined in Step 6.1.10.

The freezing media is stable for 18 hours at 2 to 8°C after preparation.

6.1.10. The Freezing Containers should be stored per the package insert prior to use. StrataCooler should be stored at 2 to 8°C prior to use.

6.2. Cell Isolation and Plasma Collection

6.2.1. Record the following information on the write-on label of the Na citrate CPT tubes.

Volunteer ID number; Time and Date of draw

6.2.2. Whole blood is collected in Na citrate CPT tubes. Record and report the time when it is collected.

6.2.2.1. Store tubes in an upright position at room temperature (15 to 30°C) until centrifugation.

6.2.2.2. Gently invert the tubes eight to ten times to remix cells immediately before centrifuging.

6.2.2.3. Centrifuge tubes at the earliest as possible but no later than four hours after blood collection. Record and report the time when centrifugation starts. The entire process (from blood collection to freezing down cells) **should be completed within eight hours.**

6.2.3. Centrifuge tubes at room temperature (15 to 30°C) in a horizontal rotor (swing-out head). Centrifuge for 30 minutes at 1800 x g (RCF). RCF must be calculated carefully (see the equation below). Do not use brake. Care should be taken to ensure that CPT tubes are properly seated in the centrifuge insert/bucket.

RCF = Relative Centrifugal Force

RPM = Rotational Speed (revolutions per minute)

r = centrifugal radius in mm = distance from the center of the turning axis to the bottom of the centrifuge

$$\text{RCF} = \left(\frac{\text{RPM}}{1,000} \right)^2 \times r \times 1.118 \Rightarrow \text{RPM} = \sqrt{\frac{\text{RCF}}{r \times 1.118}} \times 1,000$$

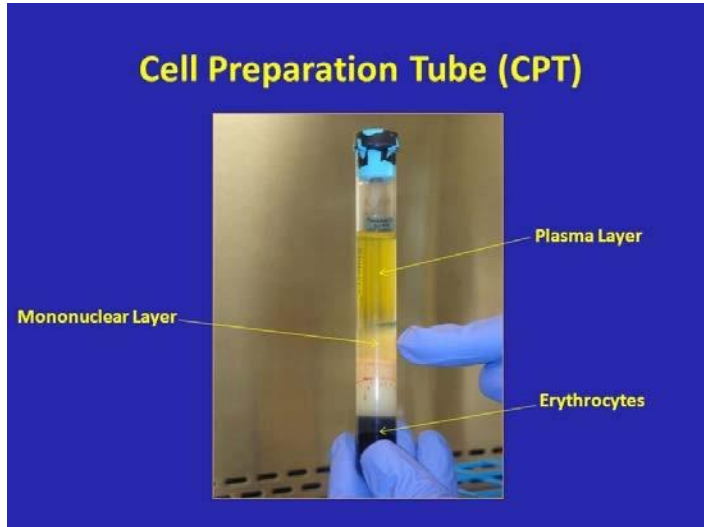
6.2.4. After the centrifuge has come to a complete stop, carefully remove the tubes and place in a rack.

6.2.5. The mononuclear cells and platelets will be in a whitish layer just under the plasma layer (see the figure below).

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6.2.6. Aspirate the plasma from each of the CPT tubes without disturbing the cell layer.

6.2.7. Collect the mononuclear cell layer from each of the tubes with a pipette and transfer them to a 15 mL plastic conical centrifuge tube with cap.



6.2.8. Add PBS equal to three volumes of the cell layers combined in each 15 mL centrifuge tube, up to a total maximum volume of 12 mL including cells and PBS. Do not overfill the centrifuge tube. Mix cells by gently inverting the tube five times.

6.2.9. Centrifuge at room temperature (15 to 30°C) for ten minutes at 300 x g (RCF). Low brake may be used. Remove as much supernatant as possible without disturbing the cell pellet by quickly decanting or “shock dumping” into the designated waste container in the Biological Safety Cabinet.

6.2.10. Resuspend the cell pellet by gently tapping the tube with index finger. Combine the cell suspensions from the same volunteer ID, time and date of draw, and add PBS to a total volume up to 12 mL, cap the tube and gently invert the tube five times.

6.2.11. Centrifuge at room temperature (15 to 30°C) for ten minutes at 300 x g (RCF). Low brake may be used. Remove as much supernatant as possible without disturbing the cell pellet.

6.3. Cell Freezing

6.3.1.1 Resuspend the cell pellet with the 2 ml freezing media by gently tapping the tube with index finger and aliquot into two cryovials.

6.3.1.2. Do not allow cells to sit in freezing solution for longer than ten minutes.

6.3.2. Aliquot 1 mL of the cell suspension into pre-labeled cryovials. Do not discard any cells. If the final aliquot has less than 1 mL, record and report the volume in the Residual Volume field for that aliquot only, not the full set of aliquots.

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6.3.3. After aliquoting is complete, place all vials in the cell freezing containers and place the freezing containers into a freezer that is approximately -20°C (range -60°C to -90°C). Record and report the time placed in freezer. Leave undisturbed for a minimum of four hours.

6.3.4. Store the cell freezing containers with the PBMCs in the freezer overnight.

6.3.5 PBMCs can be stored for two weeks in -80°C .

6.3.6. Ship the sample on dry ice by overnight shipment to School of Biotechnology, JNU.

7.0 Documentation

The project clinical site implementing this SOP will document the procedure. Any deviation from the SOP should be recorded. This documentation will be stored indefinitely or per CCRAS guidelines.

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