

GenTAC Protocol - 001

Isolate Peripheral Blood Mononuclear Cells (PBMCs) from Whole Blood Collected in BD or Greiner ACD Evacuated Tubes

Summary

Peripheral whole blood contains five general cell types; lymphocytes, monocytes, granulocytes, erythrocytes and platelets. These cells can be effectively separated from one another by taking advantage of their unique physio-chemical differences, which includes cell size, density, phagocytic potential and the ability to adhere to surfaces. The isolation of mononuclear cells by a density gradient is the most widely used separation procedure that will ensure maximum lymphocyte recovery and minimum granulocyte and erythrocyte contamination. Under optimum conditions, the mononuclear cell composition that can be harvested from normal peripheral whole blood is approximately 80% lymphocytes and 20% monocytes.

Specimen Collection

Acid Citrate Dextrose (ACD) Vacutainer Tubes (BD# 364606)

- Each 16 x 100 mm yellow stopper tube contains 1.5 mL of ACD Solution A (22.0 g/L trisodium citrate, 8.0 g/L citric acid and 24.5 g/L dextrose)
- Whole blood draw capacity is 8.5 mL
- Store out of direct sunlight at 4-25 °C (39-77 °F)
- Do not use past the printed expiration date (mm/yy = last day of month)
- Commonly used for blood bank studies, isolation of lymphocytes and DNA extraction

Acid Citrate Dextrose (ACD) Vacutainer Tubes (Greiner# - 456055)

- Each 13 x 100 mm yellow stopper tube contains 1.0 mL of ACD Solution A (22.0 g/L trisodium citrate, 8.0 g/L citric acid and 24.5 g/L dextrose)
- Whole blood draw capacity is 5.0 mL
- Store out of direct sunlight at 4-25 °C (39-77 °F)
- Do not use past the printed expiration date (mm/yy = last day of month)
- Commonly used for blood bank studies, isolation of lymphocytes and DNA extraction

Materials

Solutions

- Phosphate Buffered Saline (PBS) [Invitrogen # 14190-235 - 1000mL/btl]
 - Ca⁺⁺ and Mg⁺⁺ free
 - Store at 15 °C to 30 °C Shelf life – determined by manufacturer – ca. 3 years unopened
 - After opening, store at +2 to 8 °C. Warm to room temperature before using.
- Heat Inactivated Fetal Bovine Serum (HIFBS) [Invitrogen # 10082-147 - 500 mL/btl]
 - Store at -5 °C to -20 °C
 - Shelf life – unlimited if kept frozen
- Histopaque Ficoll-Hypaque/Lymphocyte Separation Media [Sigma # 10771-6x100 mL]
 - Specific gravity 1.077-1.080g/mL at +20 °C
 - Store at +2 to 8 °C
 - Shelf life – determined by manufacturer - 36 months unopened

- McCoys 5A Modified with, Hepes 25mM and L-Glutamine [Invitrogen # 12330-031 500 mL /btl]
 - Store at +2 to 8 °C
 - Shelf life – determined by manufacturer – ca. 12 months unopened
 - Shelf life – 5 days opened
- Pen/Strep Solution - Penicillin-10000u/mL /Streptomycin /10000mcg/mL [Invitrogen # 15140-155 - 6x100mL /btl]
 - Store at -5 to -20 °C
 - Stable at 37 °C for ~ three days
 - Shelf life – 12 months frozen
- Media A
 - McCoys 5A Modified with, Hepes 25mM and L-Glutamine
 - Formulated with 15% HIFBS
 - Formulated with Pen/Strep Penicillin-10000u/mL /Streptomycin /10000mcg/mL (1mL of Pen Strep per 100 mL of McCoys 5A media)
 - Shelf life – 5 days (See McCoys 5A)
- Bleach
- 10% Bleach Solution
 - Prepare fresh daily and discard any unused solution at the end of the day

Equipment

- Biological safety cabinet, Class II
- Centrifuge
- 1 mL sterile pipettes
- 5 mL sterile pipettes
- 10 mL sterile pipettes
- 50 mL sterile conical tubes
- 5 mL counting tubes
- Black Permanent ink lab marker
- Pre-printed cryo-labels

ACD Tubes - with Low Blood Volume

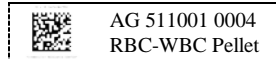
All ACD blood collection tubes (6.0 ml/8.5 ml) that have a total volume of less than 2.0 mls will be considered to have an insufficient volume for density gradient processing and must follow steps 1-4 outlined below, to yield the best possible sample. ACD blood collection tubes (6.0 ml/8.5 ml) with sufficient volume for density gradient processing and must follow steps 8-13.

1. Process blood specimens in a Class II bio-safety cabinet using approved in-house techniques and specimen handling precautions Carefully invert the ACD tube 5 to 10 times to ensure a thorough mixture of the blood and ACD solution.
2. Centrifuge each collected ACD tube of low volume at room temperature (18-25°C) for a minimum of 15 minutes at 1200g / Relative Centrifugal Force (RCF).

WARNING: Excessive centrifuge speed (over 2000g) may cause tube breakage and exposure to blood and possible injury. Use the following formula to calculate your centrifuge speed for a given g force: $x \text{ g} / \text{RCF} = 0.00001118 \times \text{radius of rotation (cm)} \times \text{rpm}^2$

3. Carefully remove the ACD tube from the centrifuge. Use a 5 ml pipette to slowly collect the upper fluid layer above the RBC/WBC cell pellet layer at the bottom of tube. Discard the fluid layer.
4. Use a 1 ml pipette to collect the RBC/WBC cell pellet layer at the bottom of tube. Dispense the concentrated cells into a cryovial that is labeled with the following cryolabel that is normally

included with the specimen shipment.



5. Record the sample ID, sequence number, material type and volume of material for each vial.
6. Store aliquoted vial in assigned rack and box of the LN2 vapor phase freezer.
7. Enter all sample ID, material type and storage location information into the NHLBI BSI RTI database.

Routine PBMC Separation Procedure

8. Process blood specimens in a Class II bio-safety cabinet using approved in-house techniques and specimen handling precautions.
 9. Mix each ACD Vacutainer tube by gently inverting the tube 5 to 10 times. Firmly hold the tube in one hand and gently rotate the stopper with the other hand and carefully remove the stopper (Caution: Glass tubes containing blood have the potential to crack or break and may present an exposure hazard).
 10. Pour the blood into a sterile pre-labeled 50 mL polypropylene conical tube. Rinse the empty ACD with 5 mL of room temperature PBS (Ca⁺⁺ and Mg⁺⁺ free) and pour the PBS blood mixture into the 50 mL conical tube. Then add a quantity sufficient volume of PBS to the whole blood to bring the total volume in the 50 mL tube to:
 - 25 mL for the BD 8.5 mL ACD tube
 - 20 mL for the Greiner 6.0 mL ACD tube
- Cap the 50 mL tube and gently mix the whole blood and PBS by inverting the tube 5 to 6 times.
11. Using a sterile 10 mL pipette slowly underlay the diluted whole blood with 12 - 13 mL of sterile room temperature Ficoll-Hypaque (FH) separation gradient medium. Take care to create a clean blood - FH medium interface (see Figure 1).
 12. Centrifuge the blood-gradient tubes for 30 minutes at 1500 rpm at room temperature. Make sure that the centrifuge brake is off and to slowly increase the speed of the centrifuge to 1500 rpm to prevent blood - density gradient admixture
 13. Carefully remove the blood-gradient tubes from the centrifuge. A well-defined white band (PBMC layer and platelets) should be seen (see Figure 2).

Figure 1

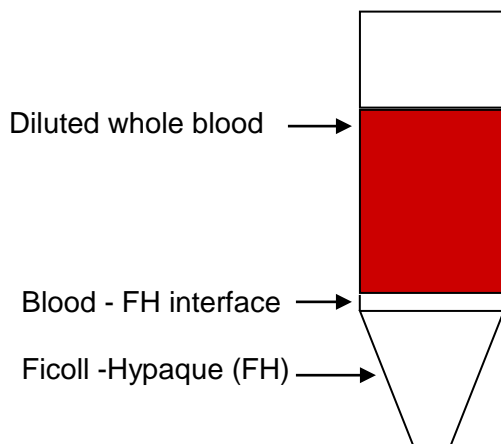
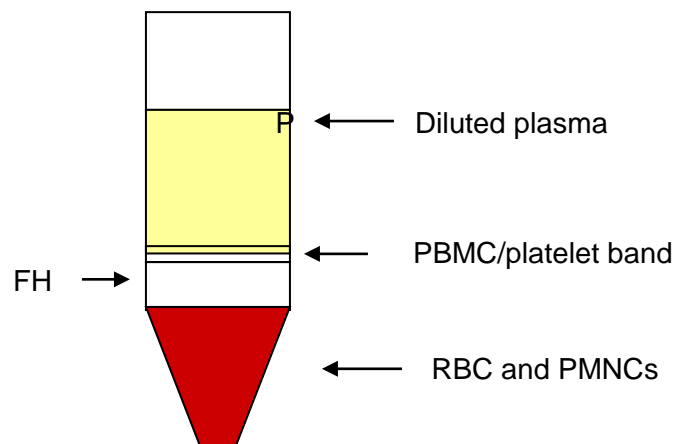


Figure 2









14. Using a 10 mL pipette aspirate and decant the upper portion of the PBS/plasma fraction to within 15 -20 mL of the PBMC/platelet band, take care not to disturb that layer of cells.
15. Then carefully position the tip of the sterile 10 mL pipette directly above the PBMC/platelet band and slowly harvest the band. To avoid PMNC and RBC contamination, aspirate as little of the FH layer and avoid touching the packed red cells below the FH layer. Transfer the harvested cell suspension to a sterile pre-labeled 50 mL tube. **Save** the RBC/WBC pellet - **Do Not** discard.
16. Dilute the harvested cell suspension with a quantity sufficient volume of PBS to bring the total volume in the 50 mL tube to 40 mL. Mix suspension thoroughly and centrifuge the suspension at 1300 rpm for 10 minutes (see step 14. If you have enough space in the centrifuge then perform the lymphocyte and RBC washes at the same time).
17. Carefully remove the tube from the centrifuge. Before decanting the supernatant, check to see if you have a cell pellet. (If you do not see a pellet on the bottom of the tube, look along the sides of the tube). If you have a cell pellet then carefully decant the supernatant into a waste container. Avoid splashing any contents from the waste container back into the 50 mL conical tube.
18. Gently re-suspend the cell pellet by finger vortexing and add 25 mL of PBS. Mix suspension thoroughly. Then centrifuge at 1100 rpm for 10 minutes.
19. Repeat step 10. Gently re-suspend the cell pellet by finger vortexing and add 10 mL of Media A. Mix suspension thoroughly and centrifuge at 900 rpm for 10 minutes
20. Repeat Step 10 and re-suspend the cell pellet in 3 mL of Media A. Perform a cell count and viability assay to determine the total number of cells and percent viable cells for each sample ID. (See GenTAC Protocol -002)

RBC/WBC Pellet

21. Add 25 mL of PBS to the RBC/WBC and gently re-suspend the cell pellet. Mix suspension thoroughly and centrifuge at 1300 rpm for 10 minutes.
22. Carefully remove the tube from the centrifuge. Using a 25 mL pipette aspirate and decant the PBS/Histopaque liquid fraction to within 2 - 3 mL of the RBC/WBC pellet. Take care not to disturb the layer of cells.
23. Gently re-suspend and measure the volume of the cell pellet with a 5 mL pipette.

Figure 3

- 24. Label three 2 mL cryovials with the sample ID labels that came with the ACD whole blood tube (see Figure 3). There are 2 labels assigned to RBC/WBC pellet samples (sequence numbers 005 and 006). If 3 labels are required, use one of the four labels that are assigned to lymphocytes. Use the label with the highest sequence number (sequence number 004).
- 25. Equally distribute the re-suspended volume into the labeled cryovials. Record the sample ID, sequence number and volume of material for each vial.
- 26. Store aliquoted vials in assigned racks and boxes of the LN2 vapor phase freezer.
- 27. Enter all sample ID, material type and storage location information into the NHLBI BSI RTI database.

	AG 511001 001 Cryo – Lymphs ACD
	AG 511001 002 Cryo – Lymphs ACD
	AG 511001 003 Cryo – Lymphs ACD
	AG 511001 004 Cryo – Lymphs ACD
	AG 511001 005 RBC-WBC Pellet
	AG 511001 006 RBC-WBC Pellet

GenTAC Protocol - 002

Count Purified Peripheral Blood Mononuclear Cells (PBMCs) with a Hemacytometer

Summary

The purpose of this procedure is to determine the cell concentration and pre-freeze viability percentage of purified peripheral blood lymphocytes. The procedure will be performed using a hemacytometer counting chamber and trypan blue stain. Counting cells by the use of a hemacytometer is a convenient and practical method of determining cell number per mL.

Materials

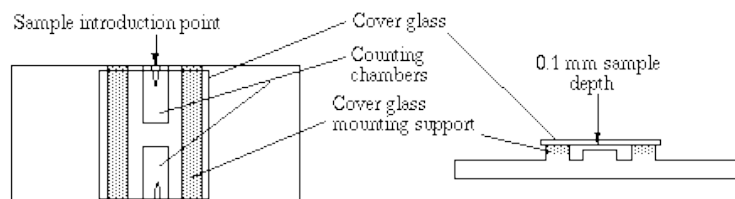
- Clean hemacytometer and cover glass, or cover slips
- Adjustable Pipettor and pipet tips
- Trypan Blue Stain 0.4% [Invitrogen # 15250-061 100mL /btl]
 - Store at 15 to 30 °C
 - Shelf life – 5 years
- Microscope
- 5 mL round bottom tubes
- Hand counter
- Cell suspension

Diagram of Hemacytometer

The hemacytometer is an etched glass chamber with raised sides that will hold a quartz coverslip exactly 0.1 mm above the chamber floor. (See Figure 1.) There are two counting chambers located directly opposite of each other. Each chamber is etched in a total surface area of 9 mm² (see Figure 2) that consists of two chambers, each of which is divided into nine 1.0 mm squares. A cover glass is supported 0.1 mm over these squares so that the total volume over each square is 1.0 mm x 0.1 mm or 0.1 mm³, or 10⁻⁴ cm³

Figure 1.

Hemacytometer

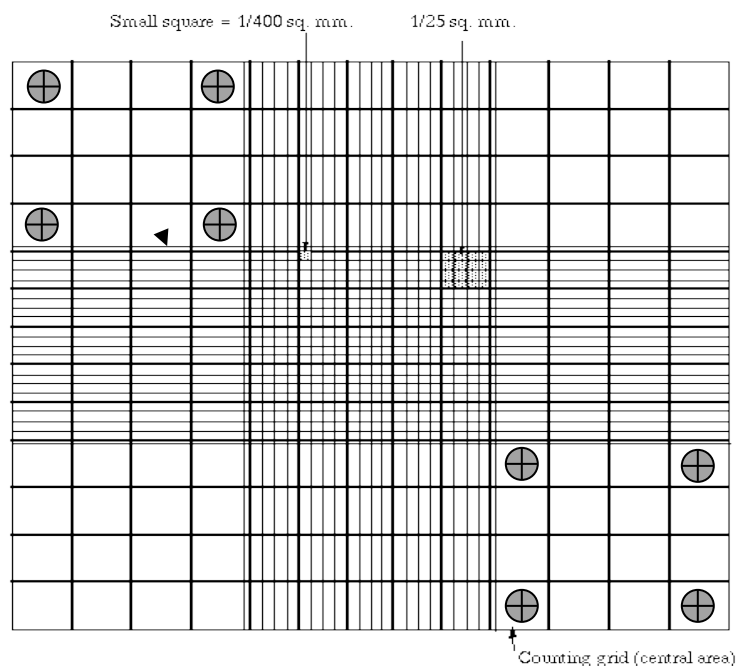


Counting Procedure

1. Do not touch the face of the coverslip or the hemacytometer with your fingers. Grease from your fingers can prevent the chamber from filling properly.
2. To prepare the counting chamber, rinse the hemacytometer's mirror-like polished surface and the glass cover slip with distilled water. Dry using Kimwipes or clean lens paper.
3. Label two small tubes with the same sample ID. (Create as many two tube sets as needed based on the total number of samples that need to be counted that day).

4. Use an adjustable pipettor to add 80 μl of trypan blue to one of the two small tubes.
5. Mix the cell suspension in the 50 mL tube and sterilely transfer approximately 50 μl of the cell suspension to the second small tube. Using an adjustable pipettor transfer 20 μl of the cell suspension to the counting tube with trypan blue. Prior to transfer check the sample ID on both tubes to make sure they are marked with the same sample ID. Mix thoroughly and allow suspension to stand for 5 minutes at room temperature.
Note: If cells are exposed to trypan blue for extended periods of time (>15 minutes), viable cells may begin to take up dye as well as non-viable cells, thus, try to do cell counts within 10 minutes after the cells are added to the trypan blue solution.
6. Place the cover slip on the hemacytometer. Use a Pasteur pipette to transfer a small amount of the trypan blue-cell suspension to one of the counting chambers on the hemacytometer. This is must be done carefully by gently touching the tip of the pipette onto the v-notched edge of the counting chamber where it meets the cover-slip. This will allow the chamber to fill by capillary action. Do not overfill or underfill the chamber. Record the sample ID on the cell counting worksheet to ensure that the cell count is applied to the correct sample ID. If needed, repeat the process for a second sample, using the remaining counting chamber.
Note: Allow the filled hemacytometer to stand for 2-3 minutes so that the cells stabilize and stop drifting within both chambers.
7. Using the 10x objective of a microscope, focus on the grid lines in chamber. (Objective power may vary based on the type of microscope being used. Always use an objective that provides the best visual field for counting). Adjust the slide by moving the field so that you view one corner, 16 square grid area surrounded by three grid lines. Count all the cells (non-viable cells stain blue, viable cells will remain opaque) in the four corner squares. (See Figure 2.) Record the count and sample ID.
8. Then adjust the hemacytometer to the 16 square grid area diagonally opposite. Count all the cells (non-viable cells stain blue, viable cells will remain opaque) in the four corner squares. (See Figure 2.) Record the count.

Figure 2.



9. The cell concentration is calculated as follows:

- Add the counts from each set of four squares.
- Divide by 2 to obtain an average
- Multiply the averaged count by the dilution (n x 5)
- Multiply the above total number by 4
- Multiply the above total by 3 (account for the resuspension volume)
- Multiply the above total by 10000 to obtain count x 10⁶

Example

- $35 + 33 = 68$
- $68 \div 2 = 34$
- $34 \times 5 = 170$
- $170 \times 4 = 680$
- $680 \times 3 = 2040$
- 2040×10000
- 20,400,000 total cells = 20.4×10^6

10. Cell viability can be calculated by counting a total of 100 cells (viable + non-viable)

Example - If one counted 90 viable +10 non-viable the viable percent is 90%

11. Record the cell count and viability for each vial by sample ID and sequence number for data entry into the NHBLI BSI RTI database.

Aliquot and Freeze PBMCs

3. After determining the cell count and viability of each cell suspension, spin down each 50 mL tube of counted cells at 1000 rpm for 10 minutes.
4. Carefully remove each tube from the centrifuge. Before decanting the supernatant, check to see if you have a cell pellet. (If you do not see a pellet on the bottom of the tube, look along the sides of the tube). If you have a cell pellet then carefully decant the supernatant into a waste container. Avoid splashing any contents from the waste container back into the 50 mL conical tube.
5. Gently re-suspend the cell pellet by finger vortexing. Look carefully at the pellet to ensure that all cells have resuspended
6. Place the cell suspensions, Recovery™ Cell Culture Freezing Medium (FM) and labeled cryovials in a tray of crushed wet ice. Make sure that all items are deeply submersed in the wet ice. Let them equilibrate for 10 - 15 minutes. **Note:** It is necessary to keep all cell suspensions, FM and cryovials cold to help minimize the toxic effects of the DMSO formulated in the FM.
7. During the equilibration period start the Planer Kryosave 750 Plus Controlled Rate Freezer to allow the chamber of the freezer to pre-cool.
8. Once the cell suspensions, FM and labeled cryovials are cooled the addition of the FM can proceed. To each cell suspension, slowly drop-wise (every 1-2 seconds) add 2 mL of the pre-cooled FM while applying a constant gentle mixing action to allow time for the DMSO to penetrate the cells.
9. When the FM addition step has been completed, return the DMSO treated cell suspension in wet ice. Before dispensing the treated cell suspension, verify that the sample ID number on the conical tube and the cryo-vials are the same. Then dispense 1.0 mL into each labeled cryo-vial(s). As you aliquot make sure that the cell suspension is thoroughly mixed. Return filled vials to wet ice after completing the aliquot step.
10. Repeat Step 8 for each cell suspension. After all aliquoting has been completed, make a control vial for the control rate freezer by adding 1 mL of FM to an unlabeled cryovial.
11. Immediately transfer the DMSO treated cells and control vial to the Planer Kryosave 750 Plus Controlled Rate Freezer (see GenTAC Protocol 004).

GenTAC Protocol – 004

Operate Planer Kryosave 750 Plus Controlled Rate Freezer

Summary

The objective of cryopreservation is to minimize damage to biological materials, such as tissues, mammalian cells, bacteria, fungi, plant cells, and viruses, during low temperature freezing and storage. Liquid nitrogen (LN₂) control rate freezing permits the machine-monitored freezing of cells at 1-2 °C per minute, which is the optimal freezing rate for lymphocytes in a DMSO containing medium. Most instruments include a feature which compensates for the release of heat by the sample at the temperature of crystallization. This usually occurs at temperatures below 0 °C due to supercooling and causes a sudden rise in sample temperature. The immediate injecting of LN₂ can help control this rise to 2-4° while maintaining the gradual cooling rate of 1 °C/min. to -30 °C and then at 8 °C/min. to a final freezing temperature of -120 °C.



Freezer Cool Down Phase

1. Prior to starting the freezing procedure, check the level of the LN₂ supply tank to ensure that there is sufficient volume of LN₂ to complete the freezing procedure.
Note: If the tank requires filling, switch the tank on the LN₂ storage freezer to the control rate freezer. Then contact Barbara Carroll (x26222, barb@rti.org) in Facilities and Maintenance to request that the empty tank be refilled as soon as possible.
2. Double check all electrical and personal computer (PC) connections. Log on to the PC that operates the Planer Kryosave 750 Plus Controlled Rate Freezer.
3. Ensure that the liquid nitrogen supply valve is open. The knob from the liquid nitrogen tank should be turned fully to the open position (as indicated on the knob itself). It is normally kept closed.
4. Open freezing chamber door and make sure the previously used control sample has been removed from the temperature probe. Check the chamber probe connection to ensure that the probe plug is firmly seated into its outlet.
5. On the PC desktop, double click on the Planer Kryosave 750 Plus controlled Rate Freezer DeltaTv6 icon to open the freezing program.
6. Click OK on the About DeltaT dialog box.
7. Enter user name (**RTI**) and password (**password**), click OK.
8. On the DeltaT KryoApplication screen, click on File and then highlight and click on Open Profile.
9. On the Select Profile to Load screen, click on RTI 1.KPF to highlight the program and then click Open.
10. Switch on the Freezer. The power switch is located on the top rear of the chamber (see mark on top of freezer). Close and lock the chamber door. The interior circulation fan will come on.
11. Click on the traffic light icon on the DeltaT KryoApplication tool bar. In the Add Samples dialog box uncheck "Samples Required". Then click on "Start Run".
12. The RTI 1.KPF freezing program (preset freezing graph) will be displayed on the PC monitor

screen.

13. Directly below the 15 minute indicator of the freezing graph there is a message space that will provide a series of 3 text messages that indicates the freezing program is downloading properly. The first message will read “waiting for response”, the second “downloading profile” and the third “going to start”. The program download sequence takes about 15 seconds before the solenoid valve starts to inject LN₂ into the freezing chamber to begin its cool down phase.
14. At this point the program will bring the freezing chamber to +4 °C and hold that temperature until the samples are placed in the chamber.
Note: LED lights adjacent to green start button will begin flash (long flashes) and a beeping alarm (short beeps) will sound when the chamber reaches its start temperature of + 4 °C. It will continue to beep until you start the freeze cycle.
15. Prepare the cell suspensions for freezing as directed in the GenTAC – 003 Protocol.

Start Freeze Cycle

16. Unlock door and open the freezing chamber.
17. Remove the rack of DMSO treated cell samples from the wet ice tray. Place the rack of samples in the freezing chamber using the location markers on the floor of the chamber as a starting guide. Ensure that the open side of the rack faces the recirculation fan.
18. Insert the temperature probe and screw the cap onto the sample control vial making sure that the probe does not touch the sides or the bottom of the vial. The tip of the probe should lie in the middle of the liquid cell suspension in the cryovials. Adjust location of the rack if necessary to position probe.
19. Close and lock the chamber door. The interior circulation fan will come on.
20. Wait five minutes for the chamber to stabilize to the start temperature. Then begin the freezing program by pushing the green start button. 
21. The freezer will now begin its freeze cycle. (See Table 1 for detailed information concerning the freeze cool down phase and freeze cycle.)
22. When the sample probe temperature reaches -100 °C the freeze cycle is complete.
Note: At the end of the freeze cycle the LED lights adjacent to green start button will begin flash (short flashes) and a beeping alarm (long beeps) will sound.
23. Press the red stop button on the front panel and the beeping should stop and the lights should go out. 
24. Open the chamber door and quickly remove the vials of frozen cells and transfer them to their designated long term storage location in a LN₂ vapor phase freezer.
25. After storing the frozen cell vials in their proper location close the freezer lid and push the “automatic fill” keypad on the temp controller of the LN₂ freezer. This will help the freezer to return to its designated storage temperature.
26. Rename and save the completed graph in the appropriate folder located in the “planar data”

folder on the GenTAC project share server using a study approved file name. (i.e. GenTAC 112207). In addition, use the Windows print screen function to capture the image of the freeze profile. Paste this image in a new Word document and save this file with the same file name as described above except with the .doc extension. Then log off the PC, shut off the Kryosave 750 Plus Controlled Rate Freezer power switch and close the valve of the LN₂ supply tank.

Table 1
Control Rate Freezer

Step #	Phase	Temperature Range	Procedure Description
1.	Start Cool Down Phase	Room temperature to +3 °C(c)	Pre-cool chamber to +3 °C
2.	Equilibrate start temperature	+3 °C (c+s)	Place chilled DMSO samples in chamber – equilibrate chamber and samples to +3 °C
3.	Start liquid phase cooling	Start temp +4 °C (c+s) / End -4 °C (s)	When chamber and samples are equilibrated to +4 °C begin liquid phase freezing at 1 °C degree per minute (15 minutes)
4.	Begin phase change (super-cooling)	Start temp -3 °C @ 25 °C per minute (s)	When samples reach -4 °C begin liquid/solid phase change at 25 °C degree per minute. This phase will fast-cool the cells to compensate for "latent heat" and help maintain a linear cooling curve
5.	End phase change	End temp -45 °C (c)	Phase change stops when chamber probe reaches -45 °C
6.	Start controlled warm up	Start temp -45 °C (c) / End temp -16 °C (c)	Chamber undergoes a controlled warm up phase until the chamber probe reaches -16 °C
7.	Start solid phase 1	Start temp -16 °C (c) / End temp -35 °C (s)	When chamber probe reaches -16 °C samples resume freezing at 1 °C degree per minute until the sample probe reaches -35 °C
8.	Start solid phase 2	Start temp -35 °C (s) / End temp -100 °C	Samples start freezing at 8 °C degree per minute until the sample probe reaches -100 °C
9.	Transfer to storage		Quickly transfer to LN ₂ vapor phase storage – storage freezer should be adjacent to control rate freezer
10	Enter into BSI-II		Complete specimen data entry tasks for BSI-II
Legend – (c) = Chamber probe (s) = Sample probe (c+s) = Chamber and Sample			

GenTAC Protocol – 005

Aliquot and Freeze Plasma and Buffy Coat Samples from EDTA Vacutainer™ Tubes

Summary

Genomic DNA (gDNA) can be isolated from a variety of biological sample sources. A simple, effective technique is the isolation of leukocytes by centrifugation from anti-coagulated whole blood. During centrifugation, the blood separates into three visual layers; an upper plasma fraction, comprising ~55% of the sample volume, a lower red blood cell fraction comprising ~45% of the remaining sample volume and a thin interface layer, called the “buffy coat”. The buffy coat containing leukocytes (mononuclear and poly-morphonuclear) and platelets makes up less than 1% of the entire whole blood composition. It offers researchers a higher concentration and yield of gDNA than whole blood. The buffy coat layer is sometimes difficult to see and usually appears as a thin milky layer just above the packed red cell layer and can be hard to pipette because of its viscosity.

Specimen Collection

Ethylendiaminetetraacetic Acid (EDTA) Vacutainer Tubes (BD# - 366643)

- Each 16 x 100 mm lavender stopper tube contains 18.0 mg of spray dried K₂ EDTA
- Whole blood draw capacity is 10.0 mL
- Store out of direct sunlight at 4-25 °C (39-77 °F)
- Do not use past the printed expiration date (mm/yy = last day of month)
- Commonly used for hematology studies and to isolate lymphocytes, plasma and buffy coat

Ethylendiaminetetraacetic Acid (EDTA) (BD# - 367863)

- Each 13 x 100 mm lavender stopper tube contains 13.8 mg of spray dried K₂ EDTA
- Whole blood draw capacity is 6.0 mL
- Store out of direct sunlight at 4-25 °C (39-77 °F)
- Do not use past the printed expiration date (mm/yy = last day of month)
- Commonly used for hematology studies and to isolate lymphocytes, plasma and buffy coat

Equipment

- Biological safety cabinet, Class II
- Centrifuge
- 10 mL sterile pipettes
- Cryovials
- Pre-printed labels
- Bleach

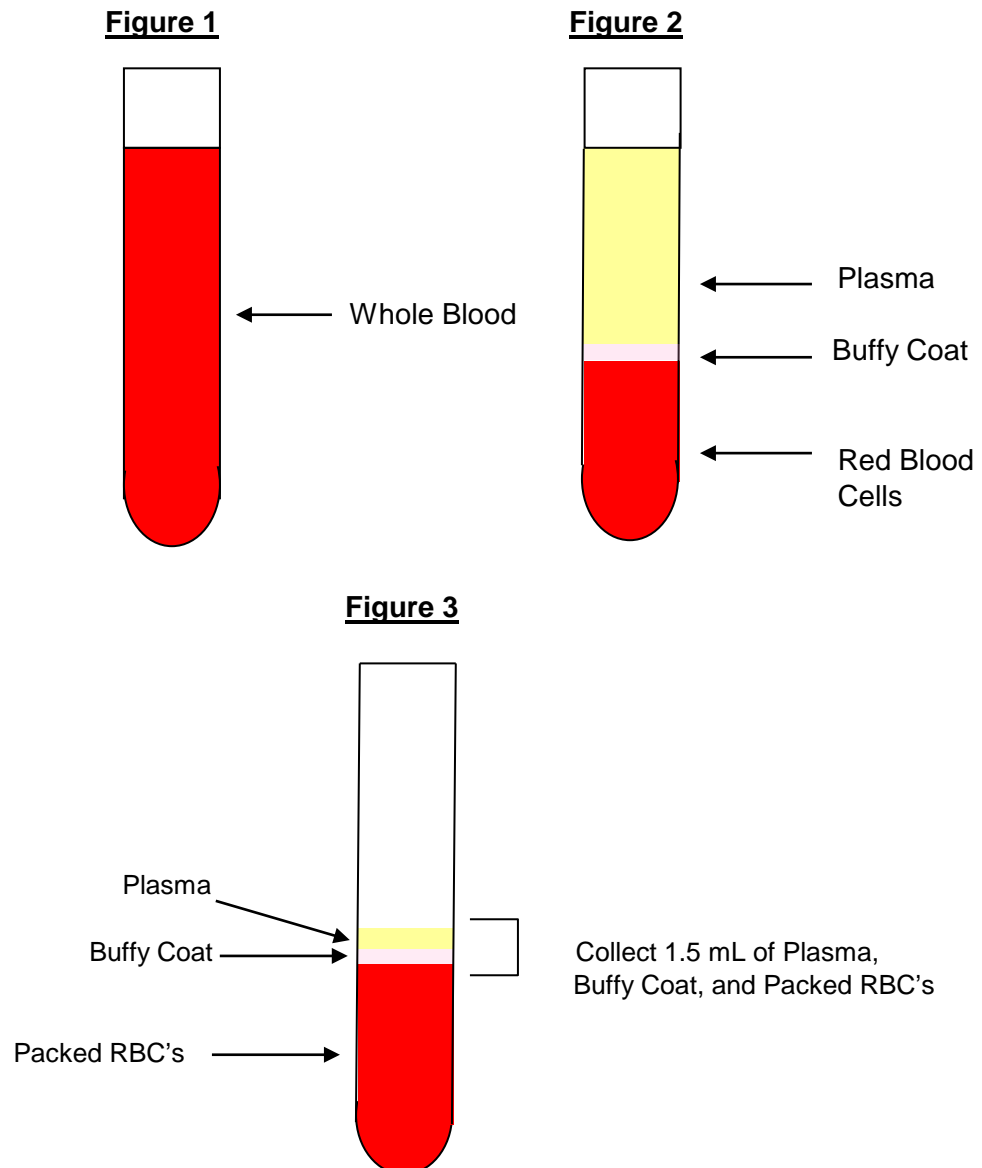
Process EDTA Tube

1. Process all blood specimens in a certified biological safety cabinet (Class I or II) using approved institutional guidelines, techniques and specimen handling precautions.
2. All EDTA tubes containing fresh whole blood should be centrifuged within 24 hours of collection, unless otherwise directed to avoid red blood cell (RBC) contamination of the separated plasma layer.

3. Centrifuge each collected EDTA tube of whole blood at room temperature (18-25 °C) for a minimum of 15 minutes at 1500g / Relative Centrifugal Force (RCF).

WARNING: Excessive centrifuge speed (over 2000g) may cause tube breakage and exposure to blood and possible injury. Use the following formula to calculate your centrifuge speed for a given *g* force: $x \text{ g} / \text{RCF} = 0.00001118 \times \text{radius of rotation (cm)} \times \text{rpm}^2$

4. The whole blood (Figure 1) will separate into three distinct layers (see Figure 2): 1) an upper layer (plasma), 2) a lower layer (RBC) and 3) a thin interface layer between the plasma and red cells that contains the buffy coat (leukocytes and platelets).

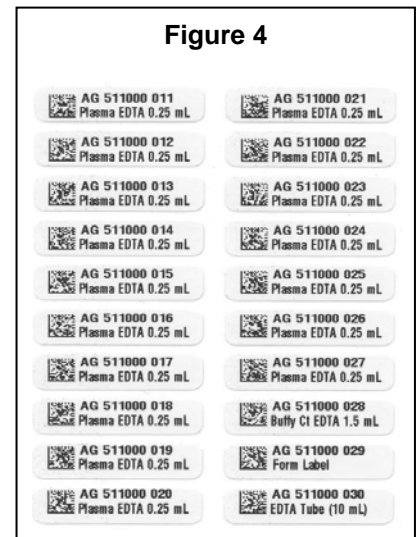


5. To obtain undiluted plasma, carefully remove the EDTA tube from the centrifuge. Remove the stopper of the EDTA vacutainer tube by grasping the tube firmly with one hand and placing the thumb under the stopper. With the other hand, twist the stopper while simultaneously pushing up with the thumb of the other hand **ONLY UNTIL THE TUBE STOPPER IS LOOSENED**. Move thumb away before lifting stopper. **DO NOT** use thumb to

push stopper off tube. Carefully remove rubber stopper from tube. DO NOT REASSEMBLE THE STOPPER AND TUBE.

Collect and Aliquot Plasma

6. Use a sterile 5 mL pipette and slowly aspirate the plasma from the EDTA tube (Figure 2) down to approximately 0.5 centimeters above the buffy coat interface (see Figure 3). Be careful **NOT** to touch, mix or disturb the buffy coat (white pinkish) interface or packed red blood cells.
7. Transfer the plasma into a sterile 5 mL tube. Before transferring the plasma, verify that the sample ID number on the vacutainer tube and the 5 mL tube are the same sample ID number.
8. Label the required number of cryovials with 2D barcode labels that are similar to the ones represented in Figure 4.
9. Using a 1.0 mL sterile pipette dispense the required volume of plasma into each labeled cryo-vial. Before dispensing the plasma, verify that the sample ID number on the plasma storage tube and the cryo-vial are the same number.
10. The plasma aliquot vials counts and volumes are as follows (i.e. based on total available volume);
 - 17 vials at 0.25 mL/vial (sequence numbers 011-027)



Collect and Aliquot Buffy Coat

11. The buffy coat is sometimes difficult to see and collect. It can be viscous and can appear as a thin milky white or pinkish colored layer just above the packed red cell layer. Some contamination of the buffy coat with the RBC layer is to be expected. However, specimens that have an extremely high WBC count will have a thicker and more defined buffy coat layer that will be easier to collect with minimal RBC contamination.
12. Label a 2 mL cryovial with the sample ID assigned for buffy coat (sequence number 018). Before collecting any buffy coat, verify that the sample ID number on the EDTA tube and the cryovial are the same.
13. To collect the buffy coat, slowly aspirate the remaining plasma and buffy coat using a slow circular motion. Collect a total volume of 1.5 mL of buffy coat (see Figure 3) and dispense the entire volume into the 2 mL cryovial.
14. Discard remaining red cells.

Transfer to Storage

15. After all aliquoting has been completed, transfer all plasma and buffy coat aliquots to -75 °C freezer storage. Note freezer/rack/box and vial locations for each aliquot and enter the information into BSI-II.

GenTAC Protocol – 006

Oragene (OG-250) Saliva Samples

Summary

The Oragene DNA Collection Kit is designed to enable researchers and clinicians to collect reliable saliva samples that contain buccal cells (stratified epithelial cells) and WBC cells. Unlike other oral collection methods, Oragene•DNA yields high-quality, high-quantity by preserving and stabilizing saliva samples for long term storage at room temperature without DNA degradation.

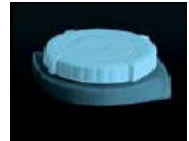
Specimen Collection

Oragene Disc Format (DNA Genotek # OG-250)

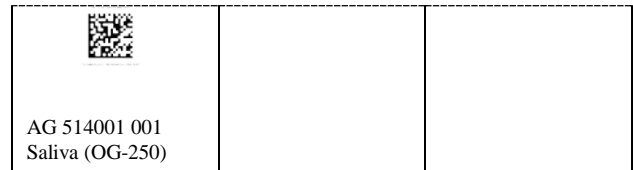
- Compact screw cap container
- Holds up 2 mL of saliva plus an additional volume of a DNA stabilizing fluid.
- Alternative collection method – 5 swab heads plus an additional volume of a DNA stabilizing fluid. (For children < 5yrs old, 5 swabs will be used to collect buccal cells and saliva. A swab will be inserted into the mouth and rubbed along the inner cheek down to the gumline. After collection the swab tips will be cut from the swab stick and placed in the bottom half of the container. The cap is then screwed on releasing the DNA stabilizing fluid.

Process Saliva

1. Remove OG-250 Disc and specimen ID label strip from the specimen bag.
2. Check that the cap is screwed tightly to the base of the disc.
3. Invert the disc 5 times to ensure that the saliva is thoroughly mixed with the stabilizing fluid. (Alternatively invert the disc 5 times to ensure that the swab heads are thoroughly mixed with the stabilizing fluid.)



4. Affix the sample ID label to the underside base of the disc (use sequence number 001). Use a permanent black lab marker and write the sample ID number on the surface of the cap.



5. Transfer the labeled saliva sample to -75 °C freezer storage. Note freezer/rack/box and container locations for each saliva aliquot and enter the information into BSI-II.

Note: If the OG-250 Disc contains swab heads, freeze the disc right side up overnight to ensure that the swabs are frozen with the stabilizing fluid. Next day transfer the frozen disc to long term -75 °C freezer storage.