CONSENSUS PROTOCOL FOR PBMC CRYOPRESERVATION AND THAWING
Version 3.0
Revision dated 1/8/02 (Changes are in Red Type)

1. EQUIPMENT/SUPPLIES/REAGENTS

1.1 Gloves (latex, vinyl, nitrile)

1.2 Lab coat or protective gown

1.3 Anticoagulated blood (EDTA, ACD or HEP)

1.4 Ficoll density gradient solution (density = 1.077), sterile and endotoxin tested. Label container with date after opening. The shelf life for Ficoll is 6 months after opening. However, discard if manufacturer's expiration date occurs before this 6-month period. It is best to purchase small volumes of this reagent and replace frequently. Examples: Ficoll-Paque, Amersham-Pharmacia, cat# 17-1440-02; Sigma Histopaque-1077 Hybri-Max, cat# H8889

1.5 Sterile Phosphate Buffered Saline (PBS), Ca^{++}-free and Mg^{++}-free or Sterile Hanks Balanced Salt Solution (HBSS)
Observe manufacturer's outdate. Label bottle with open date; use opened bottle within three months.

1.6 Fetal Bovine Serum (FBS), heat-inactivated at 56° C for 30 minutes (mix larger volumes several times while inactivating). Specific lots of heat-inactivated and filter-sterilized fetal bovine serum are reserved for ACTG. Contact Operations Center for ordering information.

1.7 Sterile Complete RPMI 1640 medium
Supplement RPMI with L-glutamine (2 mM final concentration), 100 units Pen/mL, 100ug Strep/mL, and 10% fetal bovine serum. Medium may be filter-sterilized after addition of supplements.

1.8 Dimethyl Sulfoxide (DMSO)
Store at room temperature. DMSO must be fresh and sterility maintained. The shelf life for DMSO is 6 months after opening. Label with the date upon opening. Example: Hybrimax, Sigma, cat# D2650

1.9 0.4% Trypan Blue solution. Store at 18 to 25° C. Filter as needed. Observe manufacturer's outdate. Example: Sigma # T81540 100mls

1.10 Sterile conical centrifuge tubes, 50-mL and 15-mL

1.11 Microcentrifuge tube for cell counting, 0.5 mL

1.12 Sterile pipettes, graduated and transfer

1.13 Pippetting device

1.14 Sterile pipette tips

1.15 Micropipettors of various volumes

1.16 Sterile, Cryopreservation Vials (cryovials)
1.8 to 2-mL with screw cap, external threads, and O-rings
NOTE: Some cryovials are unacceptable for use in liquid nitrogen. Please check the manufacturer's recommendations before using.
Examples: Sarstedt, cat# 72.694.005; Corning, cat# 430489

1.17 Cryo Labels specific for use in freezing and liquid nitrogen Examples: Cryotags/Cryobabies 1.50" x 0.75", Cat# LCRY-1200; Shamrock 5/8" x 1" satin cloth labels, cat# ACTG-SCPF; Pioneer 1.75" x 0.5", cat# 710; CILS 9100 labels

1.18 Laminar flow hood (minimum class 2, Type A Biosafety hood)

1.19 Centrifuge with horizontal rotor, with speeds up to 1800 X g, and equipped with aerosol safe canisters

1.20 Hemacytometer and microscope

1.21 Nalgene "Mr. Frosty" (Nalgene Cryo 1°C Freezing container, Nalgene cat# 5100-0001; Curtis Matheson Scientific, cat# 288-383; or Fisher Scientific, cat# 15-350-50); or Cryomed Freezing Chamber (Gordinier Electronics)

1.22 -20°C freezer

1.23 -70°C freezer

1.24 Liquid nitrogen storage tank with LN2-rated boxes (with holes to allow LN2 drainage). Note: storage of single vials in canes is not recommended due to safety concerns (submersion in liquid phase) and possible damage to the affixed labels.

2. FREEZING PROCEDURE

2.1 Label cryovials with LDMS-derived specimen number, PID, protocol, date of collection, VID, cell count, and LDMS specimen code. The label should be placed on the tube so that the contents are visible. Cryovials may be chilled in the freezer for at least 30 minutes prior to cell freezing.

2.2 Prepare the freezing medium: 90% fetal bovine serum + 10% DMSO; chill on ice or place in refrigerator at 4°C for at least 30 minutes. Freezing medium may be stored up to one week. (Experience from some ACTG laboratories has shown that larger volumes can be prepared, aliquotted and stored at -20°C for up to one year.)

2.3 Centrifuge the whole blood tubes at 400 X g for 10 minutes at room temperature. Remove the plasma layer and save if needed for the protocol.

2.4 Separate the PBMCs using Ficoll. Refer to the ACTG Specimen Processing document (sections 1.2.7.1, 1.2.7.2 and 1.2.7.3.) for additional information. Follow Ficoll manufacturer's instructions with respect to blood/Ficoll ratios and centrifugation time and speed. Centrifuge at room temperature with NO brake.

2.5 After centrifugation, transfer the cloudy interface layer (PBMC layer) into appropriately labeled 50-mL (15-mL if pediatric sample) sterile, centrifuge tubes by carefully aspirating the cells with a sterile, transfer pipette. Avoid aspirating the Ficoll solution by maintaining the pipette tip above the cell layer and SLOWLY drawing the cells up into the pipette.

2.6 Wash PBMCs by diluting the PBMC layer solution with at least an equal volume of PBS or HBSS. Centrifuge the cells at 200-400 X g for 10 minutes at room temperature. Discard supernatant. A second wash is recommended. If PBMCs are ready for freezing, skip to Section 2.8.

2.7 IF PBMCs are needed for LPA or VIRAL CULTURES: Resuspend washed cells in 1 to 4 mL of the appropriate complete medium. Count the cells (refer to Section 2.9) and adjust the cell concentration as needed for the assay. To freeze surplus PBMCs after setting up the LPA or viral culture, centrifuge the surplus PBMCs at 200-400 X g at room temperature for 10 minutes. Aspirate off the complete medium from the pelleted cells. Continue with Section 2.8.

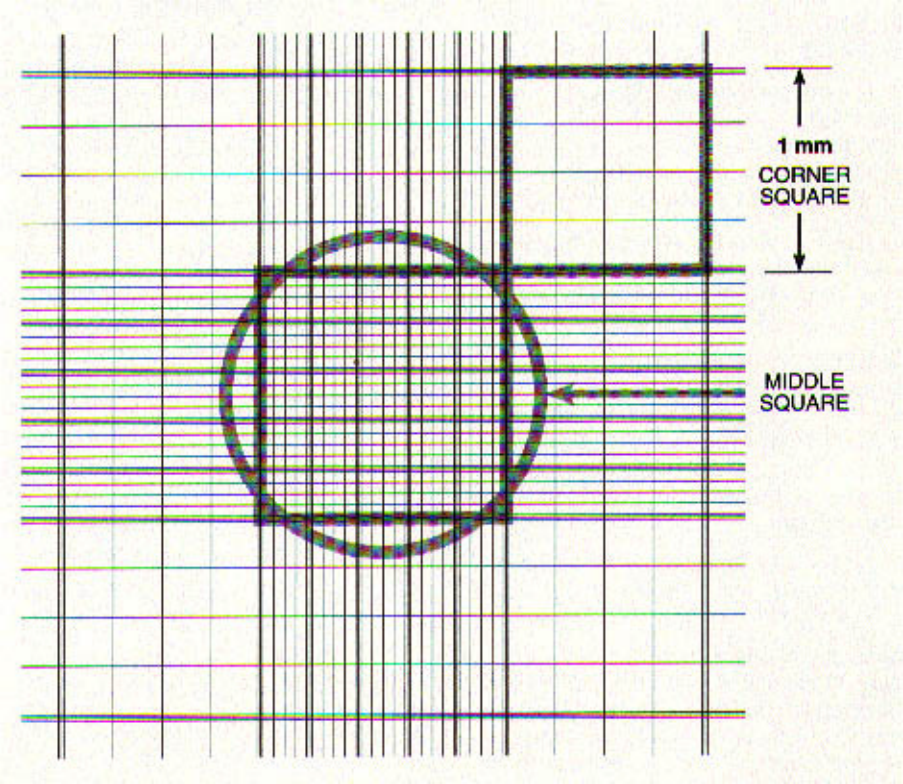
2.8 When ready to count the PBMCs, resuspend them in 1 to 4 mL of complete medium depending on the size of the cell pellet. (For example, if the PBMC pellet covers the entire bottom of the 50-mL conical tube and is greater than 1 mm thick, add 4 mL of medium. If pellet does not cover the entire bottom of the 50-mL conical tube and is less than 1 mm thick, add 2 mL of medium.) (Experience from some ACTG laboratories has shown that acceptable viability is obtained if the cell pellet is resuspended in 1 to 4 mL of cold freezing medium provided that the cells are counted and dispensed into freezing vials within 15 minutes).

2.9 Count and record the number of viable PBMCs per mL:

2.9.1 Pipette 10 μL of PBMC suspension into a 0.5 mL microcentrifuge tube. Add 90 μL of 0.4% Trypan Blue stain, making a 1:10 dilution (final concentration of Trypan Blue is 0.36%). Mix carefully to avoid aerosol formation.

Dilution Factor: $\frac{90\mu l \text{ Trypan Blue} + 10\mu l \text{ PBMC}}{10 \mu l \text{ PBMC}} = 10^1$

2.9.2 Load the hemacytometer with cell mixture (Trypan Blue + PBMC's) until the area under the cover slip is sufficiently filled. Make sure to use a cover slip that is specific for the hemacytometer. Allow the cell suspension to settle in the hemacytometer for at least 10 seconds before counting. Count the 4 large corner squares (see diagram below). Viable PBMCs will be clear; nonviable PBMCs will be blue.



Count cells in the 4 corner 1mm squares. Include cells that touch either the top line or left vertical perimeter line of any corner square. Do NOT count any cells that touch either the bottom line or right vertical perimeter line of any corner square.

2.9.3Calculate the number of PBMC/mL:

10^4 = volume conversion factor to 1 mL
10^1 = specimen dilution factor

$$\text{PBMC/mL} = \frac{\text{PBMC in all four squares}}{4} \times 10^4 \times 10^1$$

example: $\frac{88}{4} \times 10^5 = 2.2 \times 10^6$ PBMC/mL

To calculate Cell Viability:

$$\% \text{ Viability} = \frac{\text{Number of Viable Cells Counted}}{\text{Total Number of Cells Counted}} \times 100$$

2.9.4 To determine the total number of cells, multiply the number obtained above (PBMC/mL) by the cell suspension volume (mLs).

$$\text{Total Cells} = \frac{\text{PBMC}}{\text{mL}} \times \text{Volume(mLs) of PBMC suspension}$$

2.9.5 Automated counting may also be used. Follow manufacturer's instructions.

2.10 ACTG Guidelines state that the cell concentration for cryopreservation be adjusted to 5-10 x 10^6 viable cells/mL per cryovial as directed by the specific protocol. If cells were resuspended in complete medium for counting purposes, centrifuge the cells before resuspending them in cold freezing medium. Dispense 0.5 mL aliquots of the cell suspension into cryovials. Be sure that the cryovial caps are securely tightened.

2.11 Immediately place the cryovials in a slow-freeze container (e.g. "Mr. Frosty") and place the container in a -70°C freezer for 4 to 24 hours. Alternatively, place the cryovials into controlled-rate LN2 freezing chamber (Cryomed Freezing Chamber).

NOTE: To prepare the "Mr.Frosty":

- Remove the high-density polyethylene vial holder and foam insert from the polycarbonate unit.
- Add 250 mL of 100% isopropyl alcohol to the fill line. DO NOT OVERFILL. Avoid slopping the isopropyl alcohol on the labels because the alcohol can cause the ink to run.
- Replace alcohol after every fifth use and document this reagent change.
- Replace foam insert and vial holder.
- Place cryovials containing sample into holes in vial holder.
- Close "Mr. Frosty" and place in -70°C freezer.
- Pre-chilling "Mr. Frosty" in the refrigerator (4°C) prior to use does not affect cell viability

(ACTG experience).

2.12 After 4 to 24 hours in a –70°C freezer, transfer the cryovials into a LN2-rated box and place the box into vapor phase liquid nitrogen (-135°C) for long-term storage. Avoid liquid phase storage due to safety concerns and to prevent possible problems with label adhesion failure.

3. THAWING PROCEDURE

NOTE: Be sure to wear a full-face shield during the thawing procedure because cryovials containing liquid nitrogen have been known to explode.

3.1 Remove the cryovials from the liquid nitrogen container to a 37°C water bath. If liquid nitrogen has seeped into the cryovial, loosen the cap slightly to allow the nitrogen gas to escape during thawing.

3.2 Hold the cryovial in the surface of the water bath with an occasional gentle "flick" during thawing. Do not leave the cryovial unattended during the thawing process. (It is important for cell viability that the cells are thawed and processed quickly - thawing only takes a few seconds). When a small bit of ice remains in the cryovial, transfer the cryovial to the biosafety hood. Dry off the outside of the cryovial and wipe with an alcohol solution before opening the vial to prevent contamination.

3.3 Quickly transfer the thawed cell suspension from the cryovial to a 15-mL conical centrifuge tube containing 10 mL of chilled Complete RPMI medium (contains 10% fetal bovine serum).

3.4 Centrifuge at room temperature at 200-400 X g for 10 minutes. Remove the supernatant without disturbing the cell pellet. Wash once with 10 mL of room temperature PBS and centrifuge at room temperature at 200-400 X g for 10 minutes. Gently resuspend the PBMCs in the appropriate medium for the assay to be performed.

3.5 Determine the cell number and percent viable cells referring to Section 2.9.

4. PROTOCOL VERSIONS EFFECTIVE DATES

Version 1.0 Effective date:	January, 1997
Version 2.0 Effective date:	2/18/00
Version 3.0 Effective date:	8/10/01
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5. AUTHORS

Version 1.0	Virology Manual for HIV Laboratories Compiled by the Division of AIDS, National Institutes of Allergy and Infectious Diseases and Collaborating Investigators
Version 2.0	Laboratory Technologists Subcommittee of the Data Management Committee of the AIDS Clinical Trials Group
Version 3.0	Laboratory Technologists Committee of the Adult AIDS Clinical Trials Group
Version 3.0 Revision	Laboratory Technologists Committee of the Adult AIDS Clinical Trials Group