Institut für HIV Forschung
SOP #13-01 **(April 2015)**

LEUKOCYTE SEPERATION FROM LEUKAPHARESIS

1. Warm up media (R+) and ficoll (2+ bottles of media).
2. *(If plasma bag not provided, skip to step 3).* Using sterile technique transfer contents of plasma bag to two 50ml conicals. Spin for 15 minutes at 2600 RPM and **collect plasma** (1.5 ml for each cryo-preserve tube). Store in –80 ASAP.
3. Using sterile technique transfer blood product to a T75 flask.
4. **Transfer 10 ml** of the blood product to 50 ml conical. *(Skip to step 6 "add R+…") if plasma came in a separate bag).*
5. **Spin** at 2600 RPM for 15 minutes and **collect plasma** (1.5 ml for each cryo-preserve tube). Store in –80 ASAP.
6. After removing plasma (leave 2-3 ml in the tube, do not get to close to the pellet of cells) add R+ up to 45 ml and **spin for 5 minutes at 1500 RPM – SLOW START AND SLOW STOP.**
7. Remove and discard supernatant (those steps are to remove platelets) and add 45 ml of R+ again and **repeat spin**.
8. Remove and discard supernatant leaving 15 ml of volume in the tube. Resuspend by gently pipetting up and down several times, add 15 ml of R+. Mix gently again.
9. **Layer** 15ml of Ficoll-Paque under the cells/R+ mix in each tube.
10. **Spin** for 30 minutes 1500 R+ - SLOW START/STOP.
11. Gently remove tubes from centrifuge and discard supernatant leaving *at least* 10 ml above cell layer.
12. Using 10 ml pipette, gently collect the buffy coat layer, which is the middle small cloudy layer. Transfer to a new 50 ml conical tube, and discard the residual blood product (can do DNA extraction if needed).
13. Add up to 45 ml of R+ and mix well. PBMC layer will be very thick but try and dissolve it the best you can. **Spin** 1500 RPM 10 minutes **(first wash)**.
14. Prepare warm media for resuspending/PBMC request after washes.
15. After first wash – remove supernatant leaving approximately 10 ml of media and the pellet of PBMC. Resuspend by mixing cells with 10 ml pipette and pool contents of 2 tubes into 1 x 50 ml conical. Add up to 45 ml of R+ and mix gently. **Spin** 10 minutes at 1500 RPM (second wash).
16. **Repeat** step 15 (third wash).
17. Resuspend cells in 200 ml (*added with pipette so that counting calculations are accurate)* of appropriate media in a T75 flask (usually R10 to make it easy for PBMC requests) and count with nucleocounter. Make a 1:20 dilution. *Now that cells are in a large volume/container it's extremely important to mix very well before taking any aliquots!!*
18. Put cells in incubator and prepare for freezing while counting. (See Freezing passage for a detailed explanation for freezing calculations, if needed).

19. Aliquot the 9 conicals to be frozen down based on volume remaining (mL remaining / 200 vials = x). Therefore aliquot 3 conicals at 25x, 2 conicals at 22x, 1 conical at 21x, and 3 conicals at 20x. Be sure to label the conicals with these numbers.
20. **Spin** 10 minutes at 1500RPM at 4C. During spin, prepare freezing machines and freezing solution (>200ml).
21. Aspirate media. Resuspend all pellets in remaining media using P1000 micropipette. Place any conicals not being used for freezing on ice. Freeze in 3 rounds (25/25/25 for processing lab FM, 22/22/21 for LTNP FM, 20/20/20 for stratacooler).

Freezing - detailed explanation:

Requires 3 people and 3 rounds of freezing (1 for each freezing machine, and 1 for stratacoolers). Since we always freeze 200 vials no matter the yield, the concentration in each vial is simply yield / 200 vials. Note that concentration is irrelevant for freezing calculations since all we want to do is split up the volume of cells we have into 200 equal vials (instead of a set concentration/vial).

1. The 3 rounds go as follows:
    a. PL freezing machine holds 75 vials, LTNP freezing machine holds 65 vials, therefore 60 vials will be frozen by stratacooler.
    b. Each round will have 3 people freezing, so each round (75, 65, and 40) needs to be divided among those 3 people, ie. 3 separate conicals x 3 rounds = 9 conicals to spin down for freezing.
    c. Therefore, first round (PL FM) will be 25/25/25. Second round (LTNP FM) will be 22/22/21. Third round (Strata) will be 20/20/20. Make sure you label each conical with these numbers!!!
2. After counting, record yield and remove any PBMC requests. Divide the remaining volume by number of vials being frozen.
    a. This represents the volume of cells in your current T75 flask that will eventually be frozen into each vial.
3. Use this number (from step 2) to calculate how much should be aliquoted into each conical.
    a. For example: Volume in flask = 190ml. 190ml/200vials = 0.95ml/vial. Round 1 is split up into 25vials/25vials/25vials so you should aliquot 3 conicals of 0.95x25 = 23.75ml.