Isolation of Human Eosinophils

- **Yun Cao**

Ref: Praveen Akuthota,* Revital Shamri,* and Peter F. Weller Isolation of Human Eosinophils *Curr Protoc Immunol.* (2001 May); CHAPTER: Unit–7.31.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3420812/

Materials

- Human blood donor with IRB-approved protocol
- Tourniquet
- Gauze sponges
- Bandages
- Alcohol wipes
- 21G bufferfly needle (cat# BD 367281)
- Syringes (cat# BD 309653)
- Blood sample carrier container
- Biohazard bag
- 0.1 M EDTA
- Percoll Plus (cat# GE Healthcare 17-5445-01)

  (Percoll Plus is a low endotoxin reagent for density gradient centrifugation of cells, viruses, and subcellular particles. Percoll Plus is composed of colloidal silica covalently coated with silane.

  For preparation of cells, subcellular particles and larger viruses. Percoll PLUS has verified low endotoxin level, and is non-toxic and re-sterilizable, even after adjustment to physiological ionic strength. Gradients can either be preformed or spontaneously generated by centrifugation at moderate speeds in an angle-head rotor. Iso-osmotic gradients throughout cover a range of densities up to 1.3 g/ml. Low viscosity allows cell isolations on preformed gradients in only a few minutes using low centrifugal forces (200 to 1000 x g).

  Percoll PLUS is non-toxic, almost chemically inert, and does not adhere to membranes. Percoll PLUS can be stored unopened for at least five years. Preformed gradients can be stored for weeks without change in gradient shape, provided that the gradient remains unfrozen. Percoll PLUS can be buffered within the pH range 5.5 to 10 without change in properties. Both concentrated and diluted Percoll PLUS can be resterilized by autoclaving for 30 min at 120°C.)
- Water 4°C
- PBS (Ca and Mg free)
- 10 X PBS
- Sterile 60-ml Luer-lock syringes
- 50-ml conical polypropylene centrifuge tubes

- Benchtop refrigerated centrifuge for 50 ml conical tubes (cat# Eppendorf 5810R or equivalent)
- Human CD16 kit (cat# Miltenyi 130-045-701) used to label neutrophils for immunomagnetic negative selection
- Human Eosinophil separation kit (cat# 130-092-010)
- Miltenyi MACS running buffer (cat# 130-091-221)
- LS column
- Eosinophil cell culture media: 450 ml of RPMI 1640 + 50 ml of heat inactivated FBS+
- Kwikdiff (cat# 9990700/9990701)

Experimental Procedure:

A. Density gradient centrifugation

Prepare density 1.090 gm/cm^3 Percoll plus

Percoll plus	450 ml
10 X PBS	50 ml
1 X PBS	190 ml

Mix well and measure density with density meter. Aliquot 10 ml of Percoll plus adjusted to density 1.090 into 50 ml conical tube.

2. Using proper volume of container, dilute blood at least 1:2 part of 1 x PBS and mix well.
3. Layer the diluted blood to the top of density 1.090 Percoll plus. The Percoll plus used for density gradient centrifugation must be room temperature. If it is stored at 4^0C, Percoll must be brought to room temperature at least 30 min and spin down any condensate before use.
4. Set the repel button of pipet aid to slow speed. Tilt 50 ml conical tube aliquoted with Percoll plus to more than 45 degree to image the vertical line. Using 10 ml or 50 ml pipettor (depends on personal preference), put the pipet tip against the inside side wall and slowly push the repel button. When the flow reaches the surface of the Percoll plus, release the repel button for 1-2 seconds, which will decrease the risk of turbulent flow to disturb the surface of the Percoll plus. Then gently push the repel button continually, let the diluted blood slowly flow towards the top surface of the Percoll plus layer being careful not to allow any layered blood to enter or mix with the Percoll plus. When the

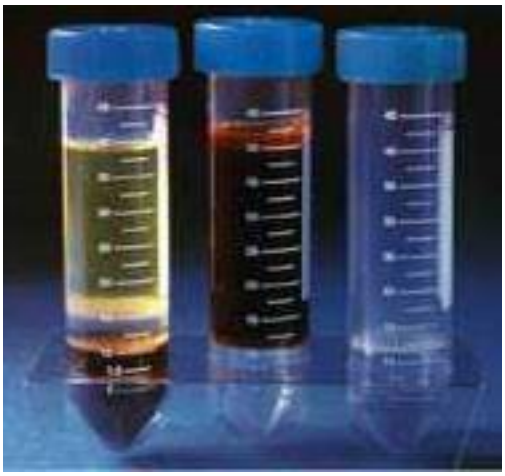
diluted blood layer reaches 1 inch thick, keep the flow slow and gradually tilt up the tube. Gradually and slowly increase the speed of the flow when the diluted blood layer reach about 2 inches thick until the diluted blood reaches the 50 ml marker.

Key point: *To reduce the disturbance of the blood flow to the surface of Percoll plus, layering of the diluted blood to Percoll plus must be done as slowly as possible. Avoid shaking the tube after blood is layered. Limit disturbance of Percoll plus surface to minimum. It will secure the best separation of the blood cells.*

5. Centrifugation of the layered tube

   Balance the samples very well

   Set centrifugation at slow start without brake: **300 x *g* for 20 min at room temperature**. Room temperature is very important. **Note:** The density of Percoll plus changes at different temperatures.



6. After centrifugation, mononuclear cells (lymphocytes and monocytes) and basophils are at the interface (white ring) between the plasma (the upper straw-colored layer) and the Percoll plus (clear layer), whereas granulocytes (eosinophils and neutrophils) and erythrocytes are in the pellet. However, with blood from eosinophilic patients —i.e., patients with some hyper-eosinophilic syndromes—lower density eosinophils may also be found between the plasma/Percoll plus interface (the mononuclear cell layer) and the pellet. In addition, some lower density eosinophils may also be present in the mononuclear layer itself.

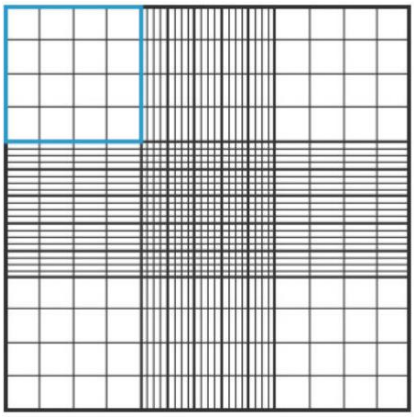
B. Hypotonic red blood cell lysis

1. Aspirate off the plasma and mononuclear layer as completely as possible from each tube, making sure to include any drops adhering to the wall of the tube.
2. To 5-10 ml of the centrifugation pellet, add 36 ml of pre-chilled water (ice or refrigerated water), mix gently and thoroughly for 30 seconds. Immediately add 4 ml of 10X PBS to restore normal tonicity and give a short mix.
3. Centrifuge the samples at 300 x g at 4 ^{0}C for 10 min and decant the red supernatant.
4. Repeat the lysis procedure and centrifuge at 4 ^{0}C for 5 min.
5. Repeat step 4 for one more time.
6. Usually, **three rounds of lysis** are enough to remove all red blood cells. Repeat step 4 and pellet cells when it is necessary.
7. Resuspend cell pellets in 50 ml of pre-chilled MACS running buffer and count cell using Hemocytometer.

C. CD16 micro immunomagnetic bead negative selection (labeling of neutrophils)

1. Pellet cells at 300 x g for 5 min at 4 ^{0}C
2. According to the kit protocol, resuspend cell pellet in 50 µl of pre-chilled MACS running buffer per 5 x 10^7 = 100 µl /10^8. Gently pipet up and down cell suspension to make single cell suspension.
3. Add 50 µl of CD16 MicroBeads per 5 x 10^7 cells.
4. Gently mix and incubate for 30 minutes at 4 ^{0}C.
5. After 30 minutes incubation, add 2 ml of buffer per 10^7 cells to the tube and gently mix. Centrifuge 300 x g at 4^{0}C for 5 minutes. Pipette off supernatant completely.
6. Add 500 µl of pre-chilled buffer per 10^8 cells and gently mix well.
7. Rinse LS column with 3 ml of pre-chilled buffer. Apply 500 µl of cell suspension to the column and wash the LS column 3 times with 3 ml of pre-chilled buffer each time. The neutrophils (labeled with the CD16 beads) should stick to the magnetized column while the eosinophils should flow right through into the elution. Collect all elution and spin for 5 min at 4 ^{0}C.
8. Resuspend cells in 5-10 ml of eosinophil culture medium.

9. Count cell: Clean glass hemocytometer and coverslip with alcohol before use. Moisten the coverslip with water and affix to the hemocytometer. The presence of Newton's refraction rings under the coverslip indicates proper adhesion. Gently swirl the flask to ensure the cells are evenly distributed. Take 10 µl of cell suspension and add to 90 µl of final concentration 0.08% of trypan blue that gets inside of dead cells and is excluded by live cells. Mix well and add 10 µl of mixture to hemocytometer. Using a microscope, focus on the grid lines of the hemocytometer with a 10X objective. Count unstained (live) cell within four of the grid line 16 squares including on the line at right-hand and bottom boundary line.



Viable cells = unstained cell x 10^4

Dead cells = stained cell x 10^4

Viability =(viable cell/ viable cell + dead cell) x 100. Typical viability is >95%.

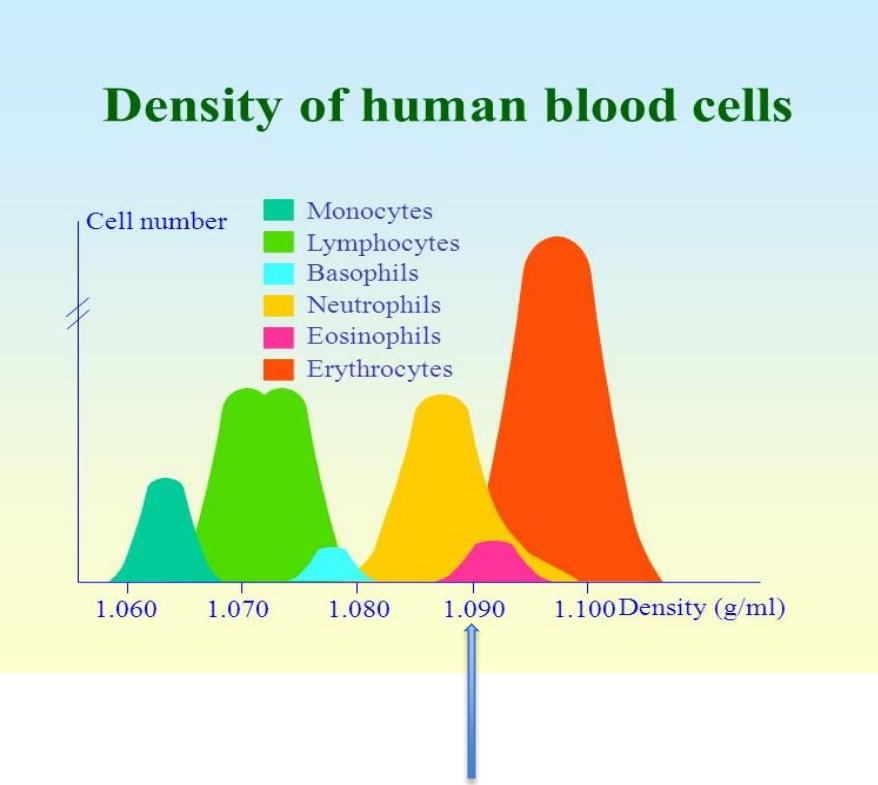
D. Cytospin and staining to assess eosinophil purity

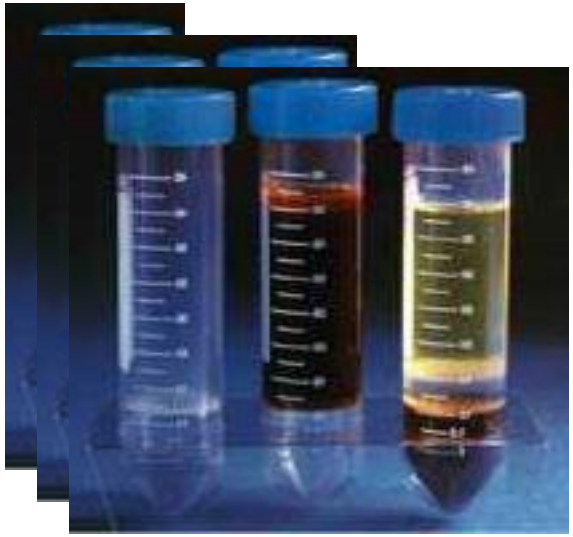
1. Take about 2 x 10^5 – 4 x 10^5 of the purified Eos. diluted to 150 ul with 1 x PBS. Spin at 350 rpm for 5 min. Air dry for >10 min.
2. KwikDiff stain

Purity is typically >95% with contaminating cells being neutrophils.

Human leukocyte isolation

Step 1: Density gradient centrifugation
Water 1 g/ml
Serum 1.024 g/ml
Percoll 1.125 - 1.135 g/ml
Ficoll-Paque: various densities
NOTE: density is temperature dependent!





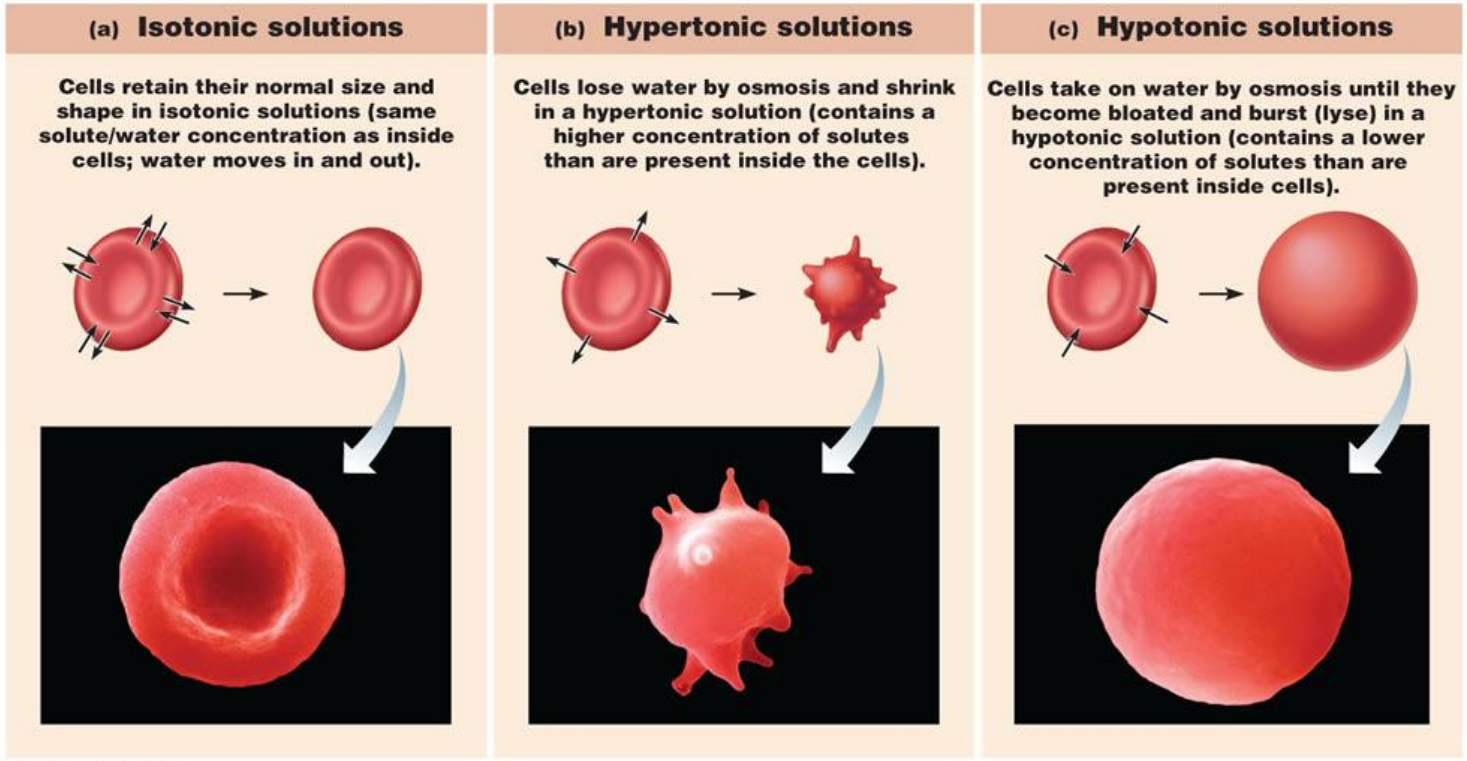
Plasma

Plt, mono, lymph, baso

Neut, Eos, RBC

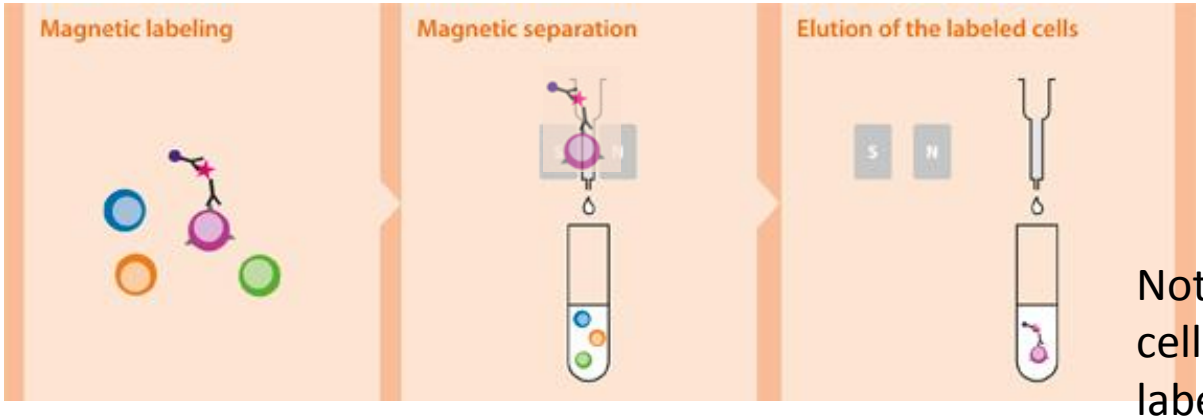
Human leukocyte isolation: removal of red blood cells

Step 2: Hypotonic RBC lysis



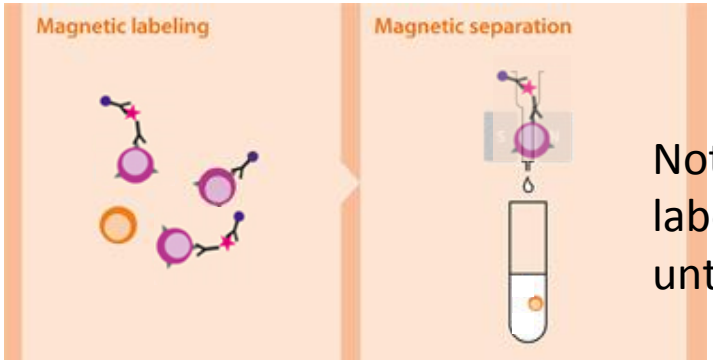
Human leukocyte isolation
Step 3: immunomagnetic labeling and separation

Positive selection and collection: tag the *cells you want, e.g. CD34+ stem cells*



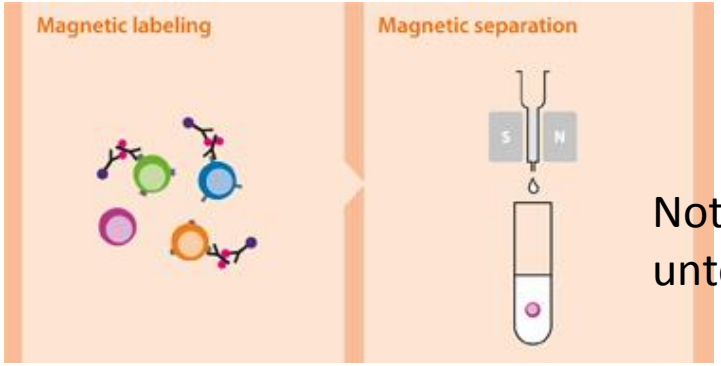
Note: wanted cells stay labeled

Negative selection: tag the *cells you want to get rid of, e.g. CD16+ neutrophils*



Note: neutrophils stay labeled, eosinophils are untouched

One step negative selection: tag the *mixed cells you want to get rid of, e.g. everything but eosinophils*



Note: eosinophils are untouched