Published in final edited form as:
Curr Protoc Immunol. 2001 May ; CHAPTER: Unit–7.31. doi:10.1002/0471142735.im0731s20.

Isolation of Human Eosinophils

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Highly purified eosinophils can be isolated from peripheral blood by negative selection using an antibody-based magnetic negative selection protocol. The Basic Protocol describes a sequential fractionation of peripheral blood in which granulocytes are enriched first from whole blood, followed by isolation of eosinophils. This technique is easy to use, fast, and highly reproducible. Support Protocol 1 describes a staining method to microscopically detect eosinophil using fast red and neutral red dyes. This allows one to evaluate the purity of eosinophils. Support Protocol 2 describes how to use a Wright-Giemsa-like stain (Hema 3) that allows for differentiation of granulocyte populations in cytospin slides of eosinophil preparations.

CAUTION: When working with human blood, cells, or infectious agents, biosafety practices should be followed (see Chapter 7 introduction).

BASIC PROTOCOL: ANTIBODY-BASED MAGNETIC NEGATIVE SELECTION OF EOSINOPHILS

Materials

- Human blood donor
- Sterile acidified sodium citrate (see recipe)
- 6% Dextran 70 in 0.9% NaCl (Pharmacosmos, Denmark)
- Ficoll-Paque Premium (GE Healthcare)
- HBSS without Ca2+, Mg2+, or phenol red (*APPENDIX 2*), 4°C
- 0.2% NaCl and 1.6% NaCl, 4°C
- Separation Medium: 0.5% (w/v) OVA in HBSS without Ca2+, Mg2+, or phenol red (*APPENDIX 2*), 4°C. (Alternatively, PBS with 2% FBS is available from StemCell Technologies as a separation medium and may be used.)
- Sterile 60-ml Luer-lock syringes
- 19-G butterfly needles
- 50-ml conical polypropylene centrifuge tubes
- Benchtop refrigerated centrifuge for 50 ml conical tubes (Eppendorf 5810R or equivalent)
- Sterile disposable transfer pipets
- Turk Blood Diluting Fluid (Ricca Chemical Company)

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- Magnetic separation apparatus capable of accommodating 0.5- or 0.6-inch magnetic columns (StemCell Technologies) kept in cold room
- 0.5- or 0.6-inch negative selection gravity-feed column (StemCell Technologies) with 3-way Luer stopcock assembly.
- 21-G needles
- Human eosinophil enrichment kit, (StemCell Technologies): negative selection kit with negative selection antibody cocktail. Monoclonal antibodies in cocktail include: anti-CD2 (*to deplete residual T cells*), anti-CD14 (*to deplete residual monocytes*), anti-CD16 (*to deplete residual neutrophils*), anti-CD19 (*to deplete residual B-cells*), anti-CD56 (*to deplete residual NK cells*), anti-glycophorin A (*to deplete residual red blood cells*), and anti-dextran.
- Magnetic colloid (included in the human eosinophil enrichment kit)

Additional reagents and equipment are required for obtaining peripheral blood by venipuncture (*APPENDIX 3F*), counting cells with a hemacytometer (*APPENDIX 3A*), and eosinophil staining with fast green/neutral red (or Hema 3 eosin-based staining, see Support Protocols).

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper sterile technique should be followed accordingly.

Isolate granulocytes

1 Draw 40 ml of blood from a human donor by venipuncture as in *APPENDIX 3F*, except use a 60-ml syringe equipped with a 19-G butterfly needle and prefilled with 10 ml of acidified sodium citrate. Retract the plunger with minimal traction until the syringe is filled to the 50-ml mark, then remove the syringe and (if desired) attach successive additional syringes (prefilled with the same solution) to the butterfly, repeating the drawing of blood until the desired amount of blood has been obtained. Remove the needle from the final syringe and place in a biosafety sharp container.

Activated platelets stick to eosinophils. The use of a large-bore needle and larger amounts of acidified sodium citrate prevents platelet activation. Aspirate blood with minimal traction to avoid platelet activation resulting from shear forces. Also see Critical Parameters.

From 320 ml of venous blood drawn from a nonatopic, nonallergic donor, 5–15 × 10^6 cells may be expected (numbers will vary from donor to donor).

2 Add 10 ml of 6% Dextran 70 in 0.9% NaCl to each syringe and gently invert (8 times) to mix the dextran with the blood. (Hetastarch, an alternative to Dextran, may also be used.) Let the syringes stand upright on their plungers for 45 to 60 min at room temperature inside a tissue culture hood.

3 Express the upper granulocyte-rich plasma layer from each upright syringe (~15–25 ml) by attaching a fresh 19-G butterfly needle (with the needle tip removed) to the syringe and pushing down on the plunger. Collect plasma in sterile 50-ml conical tubes.

4 Prepare an appropriate number of 50-ml conical polypropylene centrifuge tubes (usually one tube per 40 ml of blood drawn), each containing 23 ml room temperature Ficoll-Paque. Overlay with 20 to 25 ml of the expressed

granulocyte-rich plasma. Using a a benchtop refrigerated centrifuge for 50 ml conical tubes, centrifuge 20 min at 300 x *g*, room temperature, without using the centrifuge brake.

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 Ficoll-Paque (clear layer), whereas granulocytes (eosinophils and neutrophils) and erythrocytes are in the pellet. However, with blood from eosinophilic patients —i.e., patients with some hypereosinophilic syndromes—lower density eosinophils may also be found between the plasma/Ficoll-Paque interface (the mononuclear cell layer) and the pellet. In addition, some lower density eosinophils may also be present in the mononuclear layer itself.

5 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. Then change transfer pipets and aspirate the Ficoll-Paque layer, followed by an additional change of transfer pipets. In collecting the cells in the remaining pellet, bring the transfer pipet all the way down to the bottom of the conical tube and avoid splashing or contact with the tube wall to minimize contamination from any adherent mononuclear cells. Resuspend the pellet with 1 ml HBSS using a sterile disposable transfer pipet. If more than one conical tube has been loaded in the Ficoll-Paque step, pool the resuspended granulocytes from each tube in a 50-ml polypropylene centrifuge tube. Bring the volume up to 50 ml with 4 °C HBSS. If more than 4 syringes of blood have been drawn split the granulocyte yield into two 50 ml tubes

Cells should be kept on ice and wash solutions should be used at 4 °C from this point in the protocol forward in order to minimize eosinophil activation.

(For the collection of lower density eosinophils: For each tube, aspirate and discard only the mononuclear cell layer and the plasma above it. Resuspend the pellet in the Ficoll solution and transfer to a new tube, then dilute pellet and remaining Ficoll-Paque with at least an equal volume of 4°C HBSS to allow lower eosinophils to be pelleted.)

6 Centrifuge the tube(s) for 5 min at 300 x *g* and 4 °C, discard the supernatant(s), and dilute to 50 ml with 4°C HBSS.

The cells are washed twice to remove any adherent platelets and density-gradient medium.

7 *Optional:* Resuspend the erythrocyte-granulocyte pellet in the residual supernatant fluid with gentle agitation. Lyse the erythrocytes by adding 23 ml of 0.2% NaCl, as briefly as possible (< 30 sec), then restoring isotonicity by adding 23 ml of 1.6% NaCl. To minimize cell damage, avoid vortexing the cells vigorously. Centrifuge the tube(s) 5 min at 300 x *g* at 4 °C, and wash pellet twice with HBSS.

For functional eosinophil studies, we do not recommend erythrocyte lysis. The negative selection technique with anti-glycophorin A in the antibody cocktail is highly efficient at exclusion of erythrocytes (> 97–99% purity). However, for some applications, the elimination of all possibility of erythrocyte contamination is paramount, so lysis may be employed under these circumstances.

8 *Count granulocytes:* Dilute 10 microliters of cell suspension with 10 icroliters of Turk Blood Diluting Fluid (to lyse remaining red blood cells), and count granulocytes with a hemocytometer (*APPENDIX 3A*). Remember to account for dilution when calculating total number of granulocytes.

9 Centrifuge cell suspension for 5 min at 300 x *g*, 4 °C in order to pellet granulocytes. Aspirate and discard supernatant; loosen pellet by gentle manual agitation in residual fluid and keep on ice.

Isolate eosinophils with negative-selection antibody cocktail

10 Add 100 microliters of antibody cocktail for human eosinophil negative selection per 50×10^6 cells.

11 Incubate granulocyte suspension with antibody cocktail for 20 min at 4 °C.

12 For each 50×10^6 cells, add 60 microliter of magnetic colloid to the cell-antibody suspension and incubate for 20 min at 4°C. *Agitate tube periodically throughout incubation to ensure proper mixture with antibodies.*

13 In a cold room or 4°C refrigerated cabinet, attach a 21-G needle to the 3-way Luer stopcock assembly, place negative selection column into the slot of magnetic separation apparatus, and wash column with 20 ml of separation medium. Allow the fluid in the column to drop to the top level of the stainless steel mesh and close the three-way stopcock. Do not allow any portion of the stainless steel mesh to become dry. *A 21-G needle will allow a flow rate of 3.5 ml/min.*

Also see Critical Parameters. Avoid overloading the column by limiting the cell suspension to 3.0×10^8 magnetically bound cells per 0.5-inch column and 1.5×10^9 cells per 0.6-inch column.

14 Transfer the cell-antibody-colloid suspension to the top of the column. Open the 3-way stopcock and allow the cell suspension to enter the steel mesh. Once the top of the cell suspension has entered the steel mesh, begin to collect the effluent in a sterile 50-ml conical tube. Wash the column using 3 to 5 column volumes (12 to 20 ml) of separation medium. Keep column effluent at 4°C.

Non-eosinophil cells with bound immunomagnetic colloid will adhere to the magnetized column whereas eluted eosinophils will be in the wash effluent.

15 Determine the number of collected cells by counting in a hemocytometer (*APPENDIX 3A*). Determine purity of cells collected by staining a cytospin preparation of the cell suspension with fast green/neutral red staining or Hema 3 staining (see Support Protocols).

Alternatively, the purity of the eosinophil preparation can be ascertained by flow cytometry (see Chapter 5). Eosinophils stain positively with anti-Siglec-8 (Floyd et al., 2000) or anti-CCR3 antibody (Kitaura et al., 1996).

SUPPORT PROTOCOL: FAST GREEN AND NEUTRAL RED STAINING OF EOSINOPHILS

Fast green is a water-soluble, bluish-green, anionic triphenylmethane dye, which stains cytoplasmic granules of eosinophils green. Neutral red is a cationic azine dye, which stains

nucleoproteins red. When used together, these dyes are useful in performing differential staining of eosinophils in cytospin preparations.

Materials

- Purified eosinophil suspension (see Basic Protocol)
- Methanol
- 0.2% (w/v) fast green (Sigma) in 70% ethanol (store up to 1 month at room
- temperature in polypropylene tube)
- 0.5% (w/v) neutral red (Sigma) in distilled water (store up to 1 month at room temperature in polypropylene tube)
- Microscope slides
- Cytocentrifuge (Cytospin, Shandon/Lipshaw)

1. Label the slides and insert them into the carriage assembly of the Cytospin cytocentrifuge according to the manufacturer's instructions.
2. Load a volume of purified eosinophil suspension containing 5×10^4 cells on each slide.
3. Spin slides for 4 min at 350 rpm (16 x *g*) in the Cytospin centrifuge.
4. Air dry the slides and fix by immersing for 1 min in methanol.
5. Immerse slides for 10 min in 0.2% fast green solution, then wash with running tap water. (Staining may be done longer, including overnight. Overstaining is not an issue.)
6. Immerse slides for 5 min in 0.5% neutral red for 5 min (or longer), then wash with running tap water and air dry.

Slides are now ready for microscopic examination. Eosinophils are identified by their dark green–staining cytoplasmic granules and red-staining bilobed nuclei. Other leukocytes will exhibit only the red staining of their mononuclear or polylobular nuclei.

Support Protocol 2: HEMA 3 STAINING OF EOSINOPHILS

Hema 3 (Fischer Scientific) is a Wright-Giemsa-like stain that allows for differentiation of granulocyte populations in cytospin slides of eosinophil preparations.

Materials

- Purified eosinophil suspension (see Basic Protocol)
- Deionized water
- Fixative solution (contained in Hema 3 kit): 0.0002% (w/v) fast green in methanol
- Solution 1 (contained in Hema 3 kit): contains 0.125% (w/v) eosin Y, <0.55% (w/v) potassium phosphate monobasic, <0.4% (w/v) sodium phosphate dibasic, 0.01% sodium azide, in water
- Solution 2 (contained in Hema 3): contains 0.0625% (w/v) azure A, 0.0625% (w/v) methylene blue, <0.55% (w/v) potassium phosphate monobasic, <0.4% (w/v) sodium phosphate dibasic, 0.01% sodium azide, in water
- Microscope slides

- Cytocentrifuge (Cytospin, Shandon/Lipshaw)

1. Label the slides and insert them into the carriage assembly of the Cytospin cytocentrifuge according to the manufacturer's instructions.
2. Load a volume of purified eosinophil suspension containing 5×10^4 cells on each slide.
3. Spin slides for 4 min at 350 rpm (16 x *g*) in the Cytospin centrifuge.
4. Air dry the slides and immerse for 50 s in fixative solution.
5. Immerse slides for 30 s in Solution 1.
6. Immerse slides for 60 s in Solution 2, then wash slide with deionized water and air dry.

Slides are now ready for microscopic examination. Eosinophils are identified by their pink, granular cytoplasm and bilobed purple nuclei. The cytoplasm of contaminating neutrophils will not stain pink.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2;** for suppliers, see **APPENDIX 5.**

Sodium citrate, acidified (pH 5.2)

Acidified sodium citrate: Dissolve 37.3 g sodium citrate and 8 g citric acid in 450 ml distilled water. Adjust the pH to 5.2 with NaOH, then adjust volume to 500 ml with water. Sterilize by filtration through 0.22-μm filters. Store up to 2 weeks at 4°C.

COMMENTARY

Background Information

The application of negative selection techniques to the isolation of eosinophils has enabled researchers to consistently obtain eosinophils of high purity and number, even from non-hypereosinophilic donors (Hansel et al., 1990, 1991; Miltenyi et al., 1990). The system described in this unit is the StemSep system from StemCell Technologies. The previous version of this unit described use of the MACS system from Miltenyi Biotec (Lim and Weller, 2001); a commercially available negative selection system is available from R&D Systems as well. A non-column-based magnetic negative selection system is also available from StemCell Technologies.

The StemSep system employs binding of antibody to epitopes present on neutrophils, T cells, B cells, NK cells, and monocytes for the purpose of negative selection of these cell populations. While density gradient centrifugation isolates granulocytes, negative selection is necessary to eliminate neutrophils and other contaminating non-granulocyte populations. The negative selection cocktail contains antibody complexes against the following antigens: CD2 (*T cells*), CD14 (*monocytes*), CD16 (*neutrophils*), CD19 (*B-cells*), CD56 (*NK cells*), and glycophorin A (*red blood cells*). These tetrameric antibody complexes also bind to the dextran contained in the magnetic colloid (Lansdorp and Thomas, 1990), allowing for magnetic separation of the desired eosinophil population from the undesired, magnetically-bound populations by passing the cell suspension through a strong magnetic field (0.6 Tesla) in the presence of a stainless steel mesh column. The binding reaction is rapid and the quantity of antibody needed is relatively small (similar to that used for staining in flow

cytometry). For detailed background on the principles of immunomagnetic negative selection of cells from peripheral blood, see UNIT 7.4.

Separation of eosinophils by differential density centrifugation with metrizoate (Day, 1970), metrizamide (Vadas et al., 1979), and Percoll (Gartner, 1980) were procedures previously employed. These are technically more difficult and tedious, and require eosinophilic donors. Moreover, the yield of eosinophils from the Percoll gradient is low (38% to 56%) and the purity is variable (86% to 99%; Gartner, 1980).

Differences in some biological responses have been observed when eosinophils are isolated by Percoll density centrifugation, anti- CD16 beads, and *f*Met-Leu-Phe activation (Blom et al., 1995; Laviolette et al. 1993; Yazdanbakhsh et al., 1987a). This may be related, in part, to the fact that homogenous-density eosinophils are isolated by density centrifugation, whereas a heterogenous population of normodense and hypodense eosinophils are isolated by negative selection. Eosinophils may also be activated during the negative selection isolation procedure, given that human eosinophils can express CD16 (Hartnell et al., 1992; Sedgwick et al., 1996). This may be important in certain disease states. With these considerations in mind, the immunomagnetic purification procedure is nonetheless an easy and highly reproducible method of isolating eosinophils.

Critical Parameters

StemCell negative selection columns are available in different sizes, with varying optimal cell ranges. The 0.5-inch column is suitable for $5 \times 10^7 – 3 \times 10^8$ granulocytes, and the 0.6-inch column is suitable for $1 \times 10^8 – 1.5 \times 10^9$ granulocytes. A flow rate of <1 column volume/min for washing is optimal. A 21-G needle will have a flow rate of 3.5 ml/min; therefore use of this size needle will ensure efficient washing.

Solutions used through the density gradient separation step, including citrate, dextran, and Ficoll-Paque should be brought to room temperature prior to use. After isolation of granulocytes, all solutions must be kept at 4°C. It is imperative to use only polypropylene tubes. The donor should be advised not to engage in vigorous exercise prior to donating blood as this causes the number of circulating neutrophils to increase and necessitates the use of larger amounts of negative selection antibody cocktail. Activated platelets adhere readily to eosinophils and are thus a potential source of contamination. However, the measures adopted during blood drawing, the choice of acidified citrate as anticoagulant, and the extra washing during cell processing should help to eliminate this problem. To avoid mononuclear cell contamination, it is best to overlay rather than to underlay the granulocyte-rich plasma onto the Ficoll-Paque, taking care to form a sharp plasma–Ficoll-Paque interface. After centrifugation, the mononuclear layer should consist of a narrow band at the interface. Care should also be exercised in aspirating this band to ensure its complete removal. To remove mononuclear cells adherent to the wall of the conical tube, the latter can be swabbed with a sterile cotton-tip swab.

Ammonium chloride lysis of erythrocytes is not recommended because this reagent interferes with eosinophil antigen processing and cytokine responses and alters cellular morphology (Ide et al., 1994; Wang et al., 2007). In the previous version of this unit, we suggested red blood cell lysis with hypotonic saline, and another recently published protocol (Munoz and Leff, 2006) recommends red blood cell lysis with sterile water. However, we have adopted a protocol without red blood cell lysis given the potential for eosinophil granule abnormalities with red blood cell lysis (Malm-Erjefalt et al., 2004). High purity is observed (>97–99%) in most donors in the absence of red blood cell lysis with the combination of red blood cell sedimentation and the presence of anti-glycophorin A antibody in the negative selection cocktail. It should noted that occasionally greater red

blood cell contamination may be seen. We leave red blood cell lysis with hypotonic saline as an optional step for situations in which complete red blood cell elimination is required. It should be noted that elimination of red blood lysis from the protocol makes sedimentation of red blood cells an absolute requirement. We prefer the use of dextran, as opposed to the manufacturer's recommendation of hetastarch. This choice is due to observations that hetastarch may lead to more abnormalities in granule morphology than dextran (personal observations; Jackson et al., 1989.)

It is important not to overload the magnetic column; use a column size appropriate to the number of cells being processed or use multiple columns if necessary. Perform the differential count (Support Protocol) immediately after the cells come off the column.

Troubleshooting

See Table 7.31.1 for common problems in eosinophil isolation, along with possible causes and solutions.

Anticipated Results

With the StemCell negative selection system, eosinophils of > 97–99% purity can routinely be obtained. From 250 ml of venous blood from a nonatopic, nonallergic donor, 8–12 × 10^6 cells may be expected, though results are variable.

Time Considerations

The whole protocol takes 4 to 5 hr to perform.

Acknowledgments

Grant Support: This work was supported by National Institutes of Health grants R01 AI051645 and R01/R37 AI020241 (to PFW) and F32 AI081513 (to PA).

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Table 7.31.1

Troubleshooting Guide for Isolation by Negative Selection of Human Eosinophils

Problem	Possible cause	Solution
Mononuclear cell contamination	Poor layering on density solution	Check technique of overlaying onto Ficoll-Paque
	Density separation disrupted by braking	Make sure the centrifuge brake is off during density centrifugation
	Mononuclear layer incompletely aspirated	Check technique of aspirating off the mononuclear layer after Ficoll-Paque centrifugation
Neutrophil (or mononuclear cell) contamination	Not enough antibody added	Use proper amount of antibody
	Antibody cocktail and/or magnetic colloid are expired	Use fresh antibody cocktail and/or magnetic colloid
	The column was overloaded	Load fewer cells or use two columns
	Excessive flow rate through column applied (may result in a brown- tinged pellet due to the presence of magnetic colloid)	Reload the eluted cells onto the column and apply the recommended flow rate
Red blood cell contamination	Sedimentation and antibody cocktail are inadequate to eliminate red blood cells	Use red blood cell lysis protocol
Platelet contamination	Blood drawn with small- bore needle	Use 19-G needle
	Blood drawn with vigorous traction applied to syringe plunger	Draw blood more gently
	Not enough anticoagulant used	Increase anticoagulant
	Ice-cold HBSS was not calcium- and magnesium-free	Prepare HBSS properly
Poor cell viability	Improper solution pH or contaminated solutions or column.	Prepare new solutions; carefully wash the re-used column according to manufacture instructions.