BP_Neutrophil
Version: BluePrint

Reagents

Percoll 1.078 g/ml

Percoll	1000 ml	100 ml
10 x PBS	80 ml	8 ml
1 x PBS	720 ml	72ml
20% Albumin	46 ml	4.6 ml (0.5%)
1M TriNAcitrate	24.3 ml	2.4 ml (13mM)
Total	1870 ml	187 ml

Lysis Buffer
Add 4.15 gr NH_4Cl , 0.5 gr $KHCO_3$ and 18.5 mg EDTA (triplex III, 0.01%) to 500 ml of water.
Add 8.30 gr NH_4Cl , 1 gr $KHCO_3$ and 37 mg EDTA (triplex III, 0.01%) to 1,000 ml of water.

Keep on ice.
Make fresh each day

Buffer 1 - PBS, 13mM NaCitrate, 0.2% human serum albumin - room temperature
Buffer 3 - PBS, 13mM NaCitrate – ice cold

Sodium Citrate tribasic dehydrate, Sigma, S4641
1 x PBS, Sigma - D8537
10 x PBS, Sigma - P5493
human serum albumin 20% PAA, C11-096
Percoll, Fisher – 17089101
$KHCO_3$, Sigma - 60339
NH_4Cl, Sigma -A0171
EDTA (triplex III, 0.01%) – EDS
EDTA – Sigma – E7889

Materials

CD16 Microbeads Miltenyi
MACS LS column
Miltenyi cell filter (Miltenyi 130-041-407)

Procedure

Percoll

Dilute blood to double the original volume of blood, in a red top disposable bottle, using Buffer 1 at room temperature, i.e. if original blood volume was 400 ml, make up volume to 800 ml

Aliquot 12.5 ml of **1.078 g/ml** Percoll into 50 ml falcons

Layer diluted blood onto Percoll **1.078 g/ml**

Centrifuge 20 min, 800 g, acceleration 4, brake 0, at room temperature

Aspirate remaining supernatant from Percoll layer (once lymphocytes have been harvested).

Erythrocyte lysis

Using a pastette, take 3 ml of the red cell pellet from the bottom of the tube and transfer to a new 50ml falcon. Process 8 x 3ml to obtain approximately 60 million granulocytes.

Add up to 50 ml of ice cold lysis buffer. Incubate tubes on ice. Erythrocytes are lysed when suspension is 'transparent' and/or 'dark red'.
Mix every 3 minutes, do not let lysis sit for more than 5 minutes after complete. Lysis takes 2 minutes – 20 minutes dependent on donor.

Centrifuge directly when all tubes are lysed, at 500 g, 10 min, 4°C, 9 acc, 9 break

Resuspend pellets in 2ml of ice cold lysis buffer, use pastette to resuspend cells

Pool identical cell suspensions into one 50 ml tube, add up to 50 ml with lysis buffer, and leave on ice for 5 min.

Centrifuge at 500 x g, 10 min, 4°C, 9 acc, 9 break.

Resuspend pellets in 10 ml Buffer 3 (using pipette) → count cells

Add to 45ml of ice cold Buffer 3 and centrifuge 500 x g, 6 min, 4°C, 9 acc, 9 break.

Resuspend cells in ice cold Buffer 3 at a concentration of up to 50 million cells / 100 µl – any less than 50 million cells use same volumes of reagents, any more then scale up reagent volumes.

CD16 selection

Add 50 µl of CD16 microbeads per 50 million cells

Mix and incubate for 30 minutes at 4°C

Wash LS column with 3 ml of ice cold Buffer 3 – wash through a cell filter

Add ice cold Buffer 3 to sample tube - 2 ml per 50 million cells
Centrifuge 300 x g, 6 min, 4°C, 9 acc, 9 break.
Resuspend cells in 500 ul of ice cold Buffer 3

Apply cell suspension to the column through cell filter
Add 3ml of ice cold Buffer 3 to wash out tube – add to the column
Add 3 ml of ice cold Buffer 3 to column
Add 3 ml of ice cold Buffer 3 to column

Discard flow through (eosinophil rich product)
Add 5ml of ice cold Buffer 3 to column and plunge
Take aliquot of cells for cell count

Check purity using

CD66b	FITC	BIRMA 17C	NHS	9453	1 ul
CD16	PE	VEP13	Miltenyi	130-091-245	5 ul