Method
Indicate detailed steps of method.
Use SI units for quantities, xg plus rpm for named centrifuges, and indicate starting and final concentrations for use of reagents.

Equipment
Biosafety cabinet, Pipette-aid with variable speeds, Centrifuge with variable temperature, break and acceleration settings, Gilson pipettes, Cytospin centrifuge, Light microscope, 37 °C waterbath, Haemocytometer, Waste container, Glass waste container, Lab coat, Gloves, Racks for 50ml and 15ml tubes, Tourniquet, Sterile wipes, Butterfly needle and adaptor tubing, 50 ml Syringes, Gauze pads, Elastoplast, Sharps bin, Liquid waste container, Paper towels, 70% Alcohol spray bottle, Presept tablets, Alcohol-resistant marker pen, Pencil, Disposable sterile pipettes, Parafilm, Plastic pastettes, 50 ml BD Falcon tubes (BlueMax Polypropylene Conical Tube [35]2070), 15 ml BD conical tubes (BlueMax Polypropylene Conical Tube [35]2097), 10 ml narrow neck glass vials (Z10CN 10 ml N. Neck Glass Vial, #116096, Laboratory Sales UK Ltd 01706 356444), Barrier pipette tips, Microscopy slides, Cytospin filter papers (Shandon Filter Cards from Thermo (Electron Corporation) #5991022)

Solutions and reagents
3.8% Sodium Citrate
6% Dextran
0.9% Saline
Percoll
1 x Phosphate Buffered Saline (PBS) without Ca²⁺/Mg²⁺
10 x PBS without Ca²⁺/Mg²⁺
1M Calcium Chloride
Iscove’s Dulbecco’s modified Eagles medium (IMDM)
DiffQuik staining solution

Protocol
Safety considerations: Read and sign BA1-4 DD Blood Work, DDRA3, DDRA4
Workers are advised to have been vaccinated against Hepatitis B.
Laboratory safety policies for using human blood must be carefully observed.
Lab coats and gloves must be worn.
Double gloving is recommended.
Spills must be cleaned with Presept and disposed of appropriately.
Blood waste - yellow bags, pipettes - incinerator box, glass tubes - glass waste
General Points:
Mg and Ca affect the integrins that are responsible for neutrophil adherence, with adherence being Mg/Ca dependent.
We omit these divalent cations from the prep stage to avoid loss of cells sticking to plastic etc but should have it present in the assays to have a more physiological media.
All procedures are at room temperature unless otherwise stated.
All centrifugation steps require capped rotor buckets.
Use BD tubes not Grenier.
Label tubes before left in centrifuge or waterbath etc.

1) Freshly drawn venous blood is collected, adding 40 ml blood into 50 ml BD Falcon tubes containing 4 ml of 3.8% sodium citrate solution. Mix gently by inversion, cap, parafilm lid, and take to biosafety cabinet.

2) Centrifuge at 350 xg (1250 rpm in Sanyo Mistral 3000i Centrifuge in Rayne Lab TC room), for 20 minutes, at 20°C, with Brake 0, Acceleration 0.

3) Put 6% Dextran solution (approximately 25 ml for 4 tubes/160 ml blood) and 0.9% Saline to warm in 37°C waterbath.

4) Aspirate platelet-rich plasma (PRP), without disturbing the pelleted cells. Do this by removing all but last ~ 1 cm depth of upper layer gently with pipette, then remove last part down to final ~ 0.5 cm depth with a pastette.

5) To make autologous recalcified plasma (human serum) add 200 μl of 1M CaCl₂ (used straight from the fridge) to 10 ml PRP in a sterile 10 ml narrow neck glass vial, invert and incubate at 37°C for 1 hour. After incubation, remove serum from platelet plug with a pipette, transfer to a 15 ml BD conical tube, for later use (or storage of dated serum at -80°C). See if anybody wants the remaining PRP if not all used to make serum.

6) Leukocytes are now separated from erythrocytes by Dextran sedimentation. For every 10 ml of cells, add 2.5 ml of pre-warmed 6% Dextran (~ 6ml per initial tube/40 ml blood), then add pre-warmed 0.9% Saline to make up to total volume of 50ml. Gentle inversion. Loosen cap and allow to sediment at room temperature in the biosafety cabinet, without being disturbed, for maximum 30 minutes.

7) Prepare Percoll gradients – 2 gradients are required for 4 tubes/160 ml of starting blood volume.
27 ml of stock Percoll solution is made isotonic by adding 3 ml of 10 x PBS without Ca²⁺/Mg²⁺. This yields 30 ml of a 90% Percoll solution (enough for 2 gradients).
Bottom “81%” layer – add 1.9 ml of 1 x PBS without Ca\(^{2+}/\)Mg\(^{2+}\) to 8.1 ml of 90% Percoll solution.

Middle “70%” layer – add 3 ml of 1 x PBS without Ca\(^{2+}/\)Mg\(^{2+}\) to 7 ml of 90% Percoll solution.

Top “55%” layer – add 4.5 ml of 1 x PBS without Ca\(^{2+}/\)Mg\(^{2+}\) to 5.5 ml of 90% Percoll solution.

*Note the 81% layer is actually 72.9% Percoll etc.*

3ml of Bottom layer are placed in a 15 ml BD conical tube and 3 ml of the middle layer is then slowly layered on top using a pipette on the slowest setting, as carefully as possible to avoid mixing. The upper layer is not added at this stage. Gradients are stored at 4°C until use.

8) Once the Dextran sedimentation is complete, each leukocyte-rich upper layer is carefully removed and transferred to a fresh 50 ml BD Falcon tube. Collect the last part with a pastette, avoiding erythrocyte contamination. Top up to 50 ml with 0.9% Saline.

9) Centrifuge at 350 xg (1250 rpm in Sanyo Mistral 3000i Centrifuge in Rayne Lab TC room), for 6 minutes, at 20°C, with Brake 9, Acceleration 9.

10) Pour off the supernatant into a waste container with Presept tablet, gently tap the cells back into suspension and the pellets from 2 tubes of cells are resuspended in 3 ml of “55%” Percoll layer. Layer this carefully on top of the 81/68 layered gradient.

11) Centrifuge at 700 xg (1750 rpm in Sanyo Mistral 3000i Centrifuge in Rayne Lab TC room), for 20 minutes, at 20°C, with Brake 0, Acceleration 0.

12) Bring culture medias to Room temperature.

13) Cells are now harvested.

Remove the upper 2 thirds of the top supernatant layer with a pastette and discard. Collect the peripheral blood mononuclear cells (PBMC) from the 55/68 interface into a fresh 50 ml BD Falcon tube. Remove the upper 2 thirds of the next supernatant layer and collect the polymorphonuclear leukocytes (PMN) from the 68/81 interface into a fresh 50 ml BD Falcon tube. The erythrocytes should be pelleted at the bottom. If they have risen try to collect the PMN with minimal contamination. Pool the cell types from the different gradients. Be gentle with the cells.

14) The PBMC and PMN are both now made up to 50 ml in 1 x PBS without Ca\(^{2+}/\)Mg\(^{2+}\) to wash. Centrifuge at 250 xg (1100 rpm in Sanyo Mistral 3000i Centrifuge in Rayne Lab TC room), for 6 minutes, at 20°C, with Brake 9,
Acceleration 5. Pour off the upper supernatant, gently tap cells back into suspension.

15) Repeat wash step 14 above. Before centrifugation, sample each cell suspension on haemocytometer and get a cell count (NB count x 10⁴ = cells per ml). Count PMN first as they quickly spread on the glass.

16) Resuspend PMN to 1 x 10⁷ per ml in 1 x PBS without Ca²⁺/Mg²⁺, and PBMC to 4 x 10⁶ per ml in IMDM.

17) Before use take 100 μl of PMN preparation and cytospin onto a glass slide at 300 rpm for 3 minutes, air dry, DiffQuik stain, and check level of contamination. Should be ≥ 95% PMN (> 5% eosinophils, or > 0.5% PBMC should not be used for PMN studies).

18) Expected yields
   approximately 2 x 10⁸ PMN per 160 ml blood
   approximately 5 x 10⁷ PBMC per 160 ml blood

**SOLUTION DETAILS**

*Use full names, supplier, catalogue number and chemical formulae, use SI units for quantities, record MW of chemicals used, show molarity and wt/vol of solutions, record storage details and any other special requirements*

6% Dextran 500 (Amersham 17-0320-02) in 0.9% Saline (Baxter # UKF7124)
Dissolve at 37°C
Store 4°C

1M Calcium Chloride dehydrate
CaCl₂.2H₂O  MW 147.01
Sigma, Cat # C7902
Dissolve 73.5 g in 500 ml non-pyrogenic sterile water, filter sterilize.
Store aliquoted at 4°C

**SOURCE OF REAGENTS**

*Record supplier, catalog number, method to make ready for us, storage site, any special storage requirements*

3.8% Sodium Citrate solution
Phoenix Pharma Ltd. Cat # 1502/0022, 10 ampoules of 2ml,
Sore 4°C, protect from light

0.9% Saline
Baxter, Cat # UKF7124, 1000ml
Percoll
GE Healthcare, Cat" 17-0891-01, 1 litre

1 x PBS without Ca$^{2+}$/Mg$^{2+}$
Sigma, Cat# D8537, 500 ml

10 x PBS without Ca$^{2+}$/Mg$^{2+}$
Sigma, Cat# D1408, 500 ml

DiffQuik staining solution
Reastain QuickDiff
Gamidor# 102164

Iscove’s Dulbecco’s modified Eagles medium
(+ L-Glutamine, +25mM HEPES)
GIBCO, Cat# 21980-032, 500ml

REFERENCES
Indicate any literature source for the method

Authors, (Year), Journal, Volume, Page numbers

MODIFICATIONS
When any modification is made to this method, record the change here to keep a record of the old methodology with the date change made recorded here and top of protocol.

Modification 1
Date:
Start of June 2007
Changes:
Middle percoll layer changed from Middle “68%” layer (so previously added 3.2 ml of 1 x PBS without Ca$^{2+}$/Mg$^{2+}$ to 6.8 ml of 90% Percoll solution.)

Modification 2
Date:
Start of June 2007
Changes:
Each leukocyte-rich upper layer removed and transferred to a fresh 50 ml BD Falcon tube changed from 2 tubes of leukocyte-rich upper layer collected into one tube
Modification 3
Date:
11th June 2011
Changes
Mg and Ca affect the integrins that are responsible for neutrophil adherence, with adherence being Mg/Ca dependent.
We omit these divalent cations from the prep stage to avoid loss of cells sticking to plastic etc but should have it present in the assays to have a more physiological media.