

Materials and Methods

A Method for the Measurement of CD34+ Cells in Peripheral Blood Stem Cell Harvests and Bone Marrow Harvests

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Peripheral blood stem cell transplants are now a treatment option for a number of malignancies. The patient's haemopoietic system is stimulated to produce pluripotent cell precursors (stem cells) which escape from the bone-marrow into the peripheral blood circulation. These cells are then harvested and frozen. This procedure may be repeated a number of times. The patient is then treated with chemotherapy to destroy any diseased bone-marrow, after which the harvested stem cells are re-infused to repopulate the bone-marrow and allow haemopoietic recovery to take place.

The number of stem cells to be infused must be above a certain level to ensure marrow recovery. Accurate enumeration of the percentage of stem cells in each harvest is therefore very important, and can be measured by flow cytometry using CD34 and CD45 monoclonal antibodies. The weak CD45 expression of CD34 positive cells (haemopoietic progenitors) helps to define the percentage of CD34 positive cells.

The number of CD34 positive cells in harvested bone-marrow sometimes needs to be estimated if these cells are to be used for bone-marrow transplantation.

Specimen requirements

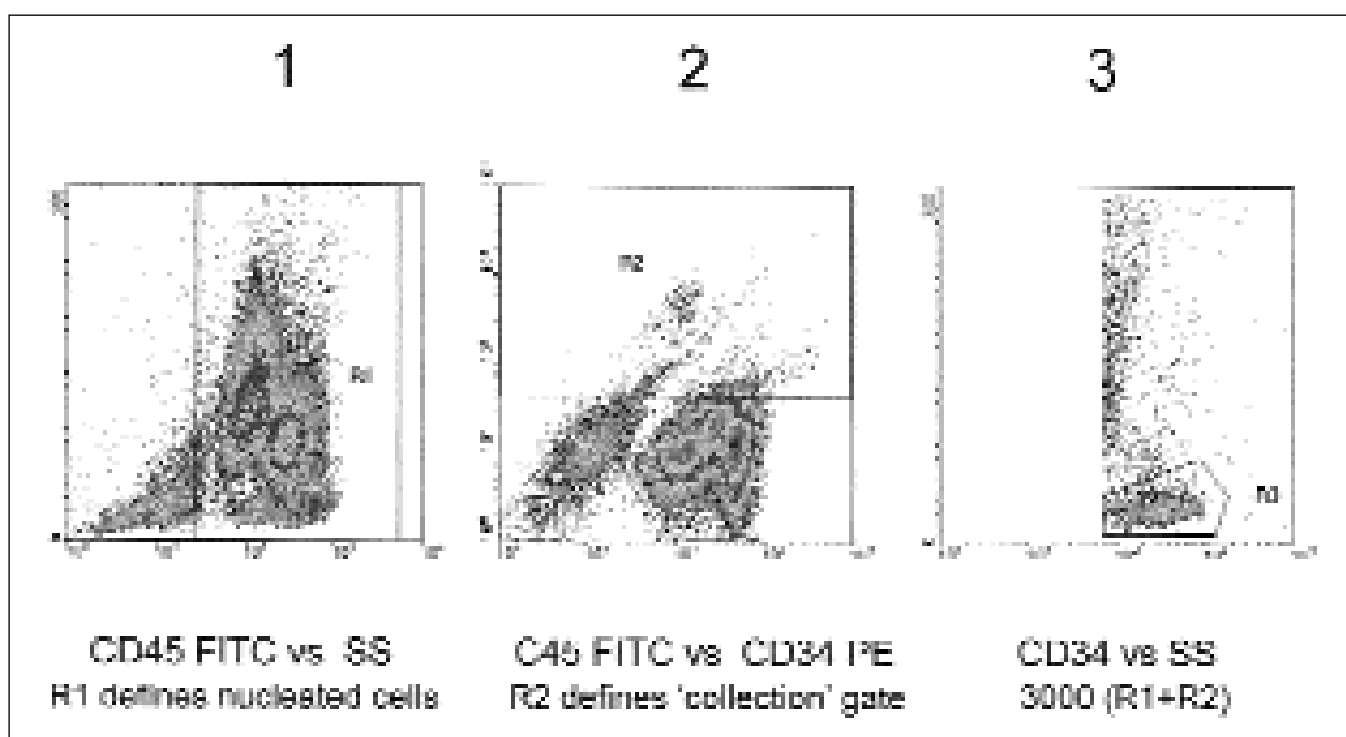
Peripheral blood or bone-marrow harvest is usually taken into acid citrate dextrose.

Reagents

- 1 Phosphate-buffered saline with BSA (PBSA)
- 2 Lysing solution
- 3 CD34-PE (phycoerythrin)
- 4 CD45-FITC (fluorescein isothiocyanate)
- 5 IgG1-PE control
- 6 2% Paraformaldehyde solution

Method

- 1 A white cell count may need to be done on the sample, if one has not been provided.
- 2 The white counts are usually very high, and the sample will usually need to be diluted. Mix the sample and remove an aliquot for diluting with PBSA to a cell count of $20 \times 10^9/l$. Make sure that you have at least 100 μ l of diluted sample for testing.
- 3 Label two tubes as follows: 1. CD45/IgG1 control. 2. CD45/CD34
- 4 Add CD45-FITC to both, IgG1-PE to tube 1 and CD34-PE to tube 2. Use appropriate amounts for your particular antibodies.
- 5 Add 50 μ l of the diluted cell suspension to each of the tubes. Mix gently and incubate on ice in the dark for 20 minutes, mixing after 10 minutes.
- 6 Add an appropriate amount of lysing solution to each of the tubes. Mix the tubes well.



- 7 Incubate the tubes as appropriate for the lysing method.
 - 8 Centrifuge at $300 \times g$ for 3 minutes.
 - 9 Discard the supernatant, flick the tubes to resuspend the cells, add about 3ml PBSA .
 - 10 Repeat step 8.
 - 11 Resuspend the cells after the final wash, and add 0.3ml of 2% paraformaldehyde solution.
 - 12 Samples are best left at 4°C for 10 minutes before running them on a flow cytometer.
- 6 Try to collect 3,000 events in **R2 only** for the control tube (CD45/IGg1), and also for the CD45/CD34 tube. After acquisition of each tube, record the number of cells you have processed in order to get the (3,000) R2 events. The total event count must be recorded for the calculation .
 - 7 Use the data from the R2 collected events for the analysis; this will provide reasonable counting statistics for most samples Observe the SS vs. PE plots from the R2 events for both the SS/CD34 and the SS/IGg1. Place a region R3 around the population of CD34 positives, usually clearly seen as a cluster of cells with low side scatter (Fig. 3). Calculate statistics for both the SS/CD34 and the Ss/IgG1 plots. If you are unhappy about defining the CD34 positives plot forward scatter vs. SS for R3. The scatter plot of R3 events should fall in a region of low to medium forward scatter and low side scatter for PBSCH, although bone-marrow CD34 cells do show a greater range of side scatter signal.

Acquiring data on the cytometer

- 1 As you will be looking for small populations it is important to minimize any carryover. Wipe the probe between tubes with a damp tissue, and aspirate through some PBS between samples.
- 2 Dilute the stained samples in PBS to approximately 0.5-1ml. Pre-run the tubes to check the instrument settings and adjust if necessary.
- 3 Collect 50,000 events for the CD45 / IGg1 tube followed by the CD45 / CD34 tube.
- 4 Set **R1**, a large rectangular gate drawn around the CD45 positives (nucleated white cells) on the CD45 vs. side scatter (SS) plot. (Fig. 1) Region **R2** plotted on the CD45 vs. CD34 plot (Fig. 2) is used to define the rare event collection gate - a generous region around the CD34+ve events. It is important that the CD45 threshold as defined by region R1 does not exclude CD34 positive cells which have low antigen density for CD45.
- 5 When you are satisfied with gates R1 and R2 the acquisition of data files can begin.

References

- Holyoake, T.L. & Alcorn, M.J. (1994) CD34+ Positive haemopoietic cells: biology and clinical applications. *Blood Reviews*, **8**, 113-124.
- Sutherland, D.R. *et al.* (1994). Sensitive detection and enumeration of CD34+ cells in peripheral and cord blood by flow cytometry. *Experimental Haematology*, **22**, 1003-1010.

This is the first of what we hope will be a regular series of 'how to ...' articles from the Cytometry Section.

We would welcome similar articles from other Sections and Microscopy Groups; please send your contribution to Sue Betteridge.

Wanted

I have been trying without success to find information about, and locate a supply of THOROTRAST, a thorium dioxide contrasting agent used in EM studies many years ago. Are there any readers who have used this product and who can suggest where I might obtain it? It is not available from any of the recognised EM consumables suppliers.

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