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Standardised protocol for circulating $CD34^+$ cell quantification by flow cytometry using the "Stem-Kit™ $CD34^+$ HPC Enumeration Kit" (Beckman Coulter)

Introduction

About 1-3% of mononuclear bone marrow (BM) cells expressed the CD34 antigen which is a highly glycosylated mucin-like structure. A part from the endothelial progenitors and stromal cells, the majority of the $CD34^+$ cells are haematopoietic stem cells and progenitors capable of reconstituting long-term, multilineage haematopoiesis after myeloablative therapy (ref). Their presence in low number (<5 $CD34^+/\mu l$) in the peripheral blood (PB) from normal unmobilized subjects demonstrates their continuous circulation under physiological conditions (1). This number could be significantly increased by mobilisation protocols including chemotherapy, G-CSF and CXCR4 antagonist or during some chronic myeloproliferative disorders (CMD). It has been recently shown by G. Barosi *(Blood, 2001)* that the absolute number of circulating $CD34^+$ cells could help to distinguish the Myelofibrosis with Myeloid Metaplasia (MMM) from other Ph^- CMD and could be considered as a marker of the disease. Furthermore, in this disease, the $CD34^+$ count is strongly associated with the myeloproliferation and predicts evolution toward blast transformation.
Definition of a standardise protocol for $CD34^+$ haematopoietic progenitor (HP) enumeration is mandatory to compare the $CD34^+$ cell count between different laboratories, to follow its evolution during the course of the disease or in multicenter trials. Several guidelines for it measurement by flow cytometry have been proposed (*Barnett et al., 1999; Brando et al., 2000; Gratama et al., 2001*).
The following protocol, accredited by the European Commission, is based on the ISHAGE (International Society of Hematotherapy and Graft Engineering) sequential gating strategy (*Sutherland et al, 1996*) recommended in these guidelines.

Principle of HPC quantification by the "Stem-Kit™ $CD34^+$ HPC Enumeration Kit" (Beckman Coulter)

The Stem-Kit Reagents are designed to identify the human HPC using the following criteria: $CD34^+$ cells exhibit low side-angle and low to intermediate forward angle light scatter characteristics of blast cells and express 1) the CD34 antigen, 2) the CD45 antigen with staining intensity characteristic of blast cells (detectable but at lower levels than lymphocytes and monocytes). Exclusion of dead cells from viable ones is achieved using 7-AAD viability dye.
The Stem-Kit™ Reagents from Beckman Coulter (Ref. IM3660) consist of a two-colour fluorescent (CD45-FITC/CD34-PE) murine monoclonal antibody reagent, a two-colour murine fluorescent (CD45-FITC/isoclonic control-PE) reagent to check the non-specific binding of the CD34-PE monoclonal antibody, a nucleic acid viability dye (7-AAD), a lysing reagent (NH_4Cl) to lyse red cells and Stem-Count fluorospheres to provide the user with the absolute count determination of the identified $CD34^+$ cells.

Specimen collection and preparation

Blood specimens, collected on anticoagulant, should be analysed as soon as possible and within 12-24 hours maximum. In that case, specimens should be stored at room temperature (18-25°C) before staining.
Prior to sample preparation, ensure that the white blood cell (WBC) concentration is no greater than 30 x 10^9 WBC/L; if necessary, dilute the samples with medium (HBSS) to reach an average value of 15 x 10^9 WBC/L. Don't forget to record the dilution factor for the calculation of the final $CD34^+$ absolute count.

Procedure for specimen processing and analysis

Perform the tests in duplicate as indicated in the Beckman Coulter instructions. Analysis of the $CD34^+$ rare events follows the sequential and cumulative gating strategy recommended in the "The ISHAGE guidelines for $CD34^+$ cell determination by flow cytometry" (*Sutherland et a.l., 1996*).
A manual gating allows the creation of a series of 8 histograms (see the following annexe as an analysis example of an apheresis sample stained with Stem-Kit Reagents): histograms 1-4 are intended to characterize $CD34^+$ HP, histogram 5-7 are intended to monitor parameters that are of importance during the acquisition step and histogram 8 is intended to discriminate and analyse viable events from the nonviable events.
Enumeration of total and viable $CD34^+$ cells is automatically performed by using the stemONE software also provided by Beckman Coulter (Ref. 6418306). The number of $CD34^+$ HP cells is calculated as the average of the region D statistical results for the two replicate samples.

References

Barosi G, Viarengo G, Pecci A, Rosti V, Piaggio G, Marchetti M, Frassoni F. Diagnostic and clinical relevance of the number of circulating CD34(+) cells in myelofibrosis with myeloid metaplasia. Blood. 2001 Dec 1;98(12):3249-55.

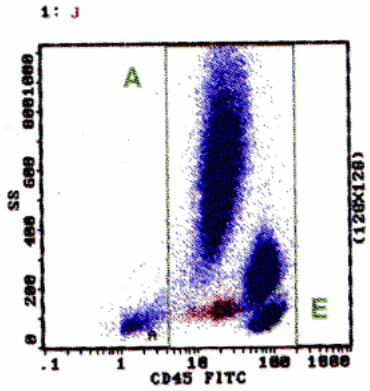
Barnett D, Janossy G, Lubenko A, Matutes E, Newland A, Reilly JT. Guideline for the flow cytometric enumeration of CD34+ haematopoietic stem cells. Prepared by the CD34+ haematopoietic stem cell working party. General Haematology Task Force of the British Committee for Standards in Haematology. Clin Lab Haematol. 1999 Oct;21(5):301-8.

Brando B, Barnett D, Janossy G, Mandy F, Autran B, Rothe G, Scarpati B, D'Avanzo G, D'Hautcourt JL, Lenkei R, Schmitz G, Kunkl A, Chianese R, Papa S, Gratama JW. Cytofluorometric methods for assessing absolute numbers of cell subsets in blood. European Working Group on Clinical Cell Analysis. Cytometry. 2000 Dec 15;42(6):327-46. Review.

Gratama JW, Sutherland DR, Keeney M, Papa S. Flow cytometric enumeration and immunophenotyping of hematopoietic stem and progenitor cells. J Biol Regul Homeost Agents. 2001 Jan-Mar;15(1):14-22. Review.

Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J Hematother. 1996 Jun;5(3):213-26.

ANNEXE (reproduced from StemKit™; Beckman Coulter)

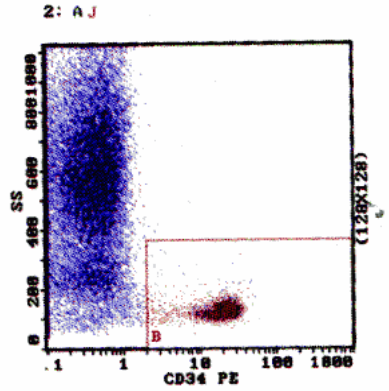


Histogram 1

- Histogram 1 displays viable events from region J (see Histogram 8). If not using 7-AAD, Histogram 1 displays all events minus all Stem-Count Fluorospheres (i.e. « -H » see Histogram 5).

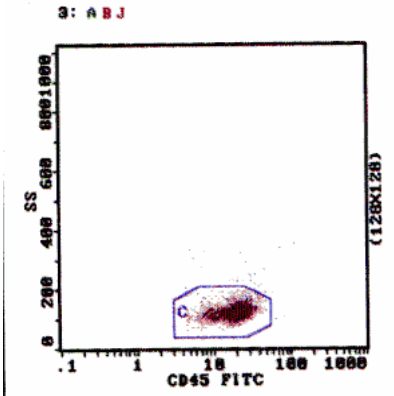
NOTE:

- Position Region A to include all $CD45^+$ events (leukocytes) while excluding $CD45^-$ events. Position Region E to include only lymphocytes (bright CD45, low Side Scatter).
- Region A is intended to serve as an appropriate denominator for viable WBC in the calculation of the percentage of $CD34^+$ HPC. Once the analysis is done, the absolute count of viable leukocytes is given on the statistic printout related to Region A.



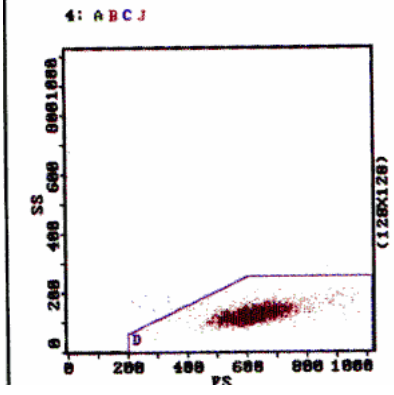
Histogram 2

- Histogram 2 displays events from region A and J. If not using 7-AAD, Histogram 2 displays events from region A.
- Adjust Region B to surround $CD34^+$ HPC, including $CD34^{dim}$ events. The upper limit along the y-axis may be set to include $CD34^+$ events with low to intermediate Side Scatter for $CD34^+$ HPC.



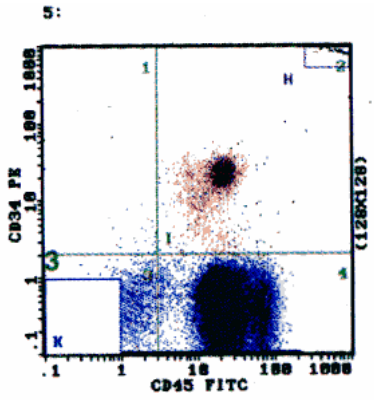
Histogram 3

- Histogram 3 displays events from A and B and J. If not using 7-AAD, Histogram 2 displays events from region A and B.
- Adjust Region C to include cells forming a cluster with characteristic $CD34^+$ HPC (i.e. low Side Scatter and low to intermediate CD45 staining). Note the characteristic shape of Region C.
  It is the cluster of events that determines where Region C is centered. Brightly FITC stained events (platelet aggregates and mature monomyeloid cells) must be excluded in the setting of this region.



Histogram 4

- Histogram 4 displays events from A and B and C and J. If not using 7-AAD, Histogram 2 displays events from region A and B and C.
- Lymph / Blast Region D identifies a cluster of events meeting all the fluorescence and light scatter criteria of ISHAGE Guidelines for $CD34^+$ HPC. Note the characteristic shape of Region D.
  If present, platelet aggregates that stain weakly must be excluded to the left of Region D.
- Once the analysis is complete, the absolute count of viable $CD34^+$ HPC is given on the statistic printout related to the $CD34^+$ Stem-Trol Control Cells or $CD34^+$ HPC (Region D).

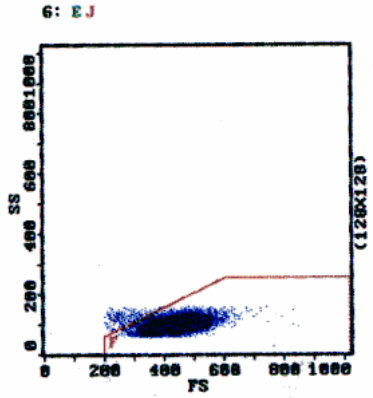


Histogram 5 displays all events.

NOTE:

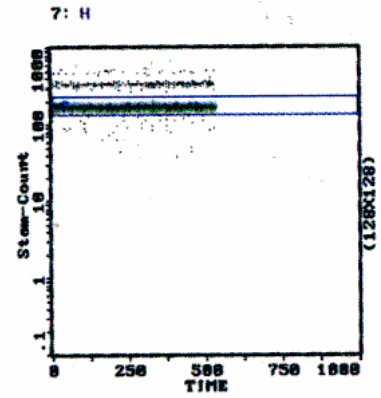
This histogram is useful to visualize the lower limit of CD45 expression within the $CD34^+$ events.

- Set Quadstat Region I2 to enclose all $CD45^+CD34^+$ events. Set the left boundary of Quadstat Region I2 to include al $lCD34^+CD45^{dim}$ events. Verify on Histogram 1 that the lower limit of Region A includes all $CD34^+$ events (use Histogram 5 as a guide).
- **Amorphous** Region H is drawn to include all Stem-Count Fluorospheres. Region H must include the last channel of the FL1 Log (on the right) and FL2 Log scales (on the top).
- Set Region K (named "listgate -") to exclude most of the double negative events from the acquisition.



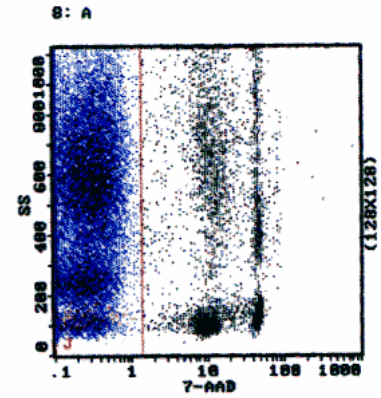
Histogram 6

- Histogram 6 displays events from E and J regions. If not using 7-AAD, Histogram 2 displays events from region E.
- Verify whether the Forward and Side Scatter gain parameters of the flow cytometer are optimally set for the processed sample. If necessary, adjust the Forward Scatter voltage / gain so that the smallest lymphocytes scatter in the middle-left part of the histogram. Adjust the Forward Scatter discriminator to ensure that even the smallest lymphocytes scatter above the discriminator.



Histogram 7

- Histogram 7 displays events from region H.
- Region G encloses the Stem-Count Fluorospheres singlet population. Check that the fluorosphere singlets accumulate homogeneously and constantly over time. Region G can be labeled as ≤CAL≤ to allow automatic calculation of absolute numbers of $CD34^+$ HPC. Type the correct assayed concentration of the current lot of Stem-Count Fluorospheres (refer to the next section of this insert or to the instrument manual for further details).



Histogram 8

- Histogram 8 displays events from region A.
- Viable events are included within Region J. The 7-AAD Viability Dye positive events are excluded from Region J.