ACS GUIDELINE FOR CD34 HAEMATOPOIETIC PROGENITOR CELL ENUMERATION

Second Edition 2017

Australasian Cytometry Society Guideline Document

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Second edition 2017 Reformatted to be read in conjunction with ACS '*Guidelines for Clinical Flow Cytometry Laboratory Practice*'.

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CONTENTS

The Australasian Cytometry Society (ACS) was established in 1979 and incorporated in 1992 with the aim of promoting research, development and applications in, and to disseminate knowledge of flow cytometry.
A function of the ACS is to assist with development and application of clinical flow cytometry applications for hospitals and laboratories in the diagnosis and treatment of disease. This includes the preparation of guidelines and education programs.
Guidelines produced by the ACS are issued as reference material to provide laboratories and accrediting agencies with minimum requirements for testing considered acceptable for good laboratory practice.
Failure to follow these guidelines may pose a risk to public health and patient safety.

SCOPE

The '*Guideline for CD34 Haematopoietic Progenitor Cell Enumeration'* is an ACS document to be read in conjunction with the ACS document '*Guidelines for Clinical Flow Cytometry Laboratory Practice*'. The latter overarching document broadly outlines guidelines for good pathology practices in Flow Cytometry laboratories.

This document is for use in flow cytometry laboratories performing assays for the enumeration of CD34+ hematopoietic stem cells, for the purpose of bone marrow transplantation. This document is intended to cover all sample types associated with bone marrow transplantation.

ABBREVIATIONS

7-AAD	7- Amino-actinomycin D. Nuclear binding dye used for indicating viability
ACS	Australasian Cytometry Society
CD34	Present on the majority of haematopoietic stem cells, and progenitor cells
CD45	Present on all white cells; Leukocyte Common Antigen
FSC	Forward Scatter
HPC	Haematopoietic progenitor cells [HPC-A: Apheresis, HPC-M: Marrow, HPC-C: Cord]
ISHAGE	International Society for Hematotherapy and Graft Engineering
PE	Phycoerythrin
SSC	Side Scatter

DEFINITIONS

Commentary	means comment(s) given to clarify Guidelines as well as to provide examples and guidance on interpretation. Prefaced with 'C'.
Dual Platform	means determination of absolute cell concentration using data derived from both flow cytometer and haematology analyser. For CD34 assays, the CD34+ events are expressed as a percentage of CD45+ events, then multiplied by the total white cell count.
Competent flow cytometrist	means a person who has been documented to be competent in clinical flow cytometry according to the Laboratory's Quality System
Guideline	means a consensus recommendation for best medical laboratory practice for a procedure, method, staffing resource or facility. Prefaced with 'G'.
Guidelines for Clinical Flow Cytometry Laboratory Practice	means the overarching document broadly outlining guidelines for good clinical flow cytometry practice in a pathology laboratory
Markers	means antigens on or in cells of interest used for diagnostic purposes

Single Platform	means utilizing a flow cytometer alone to determine the number of target cells by including a known concentration of microspheres.
Stain	means bind monoclonal antibodies to markers on cells of interest
Standard	means the minimum requirement for a procedure, method, staffing resource or laboratory facility that is required before a laboratory can attain accreditation.
Validate	The confirmation by examination and the procession of objective evidence that the particular requirements for a specific intended use are fulfilled. (Ref: NPAAC)
Verify	The application of the validation process only to a nonconforming aspect of an otherwise validated IVD. (Ref NPAAC) Verification can comprise activities such as: • performing alternative calculations • comparing design specifications • undertaking tests and demonstrations • reviewing document prior to issue.

INTRODUCTION

This ACS document, together with '*Guidelines for Clinical Flow Cytometry Laboratory Practice',* is intended to be used in clinical flow cytometry Laboratories to provide guidance on good practice in relation to flow cytometry and to assist assessors carrying out Laboratory accreditation assessments.

These Guidelines are intended to serve as consensus recommendations for best medical laboratory practice have been developed by ACS members and associates with reference to other guidelines as published in peer reviewed journals.

These are Guidelines and not Standards. These Guidelines should be read in conjunction with the current version of the ACS '*Guidelines for Clinical Flow Cytometry Laboratory Practice'.* For clarification Standards are described as:

• A Standard is the minimum requirement for a procedure, method, staffing resource or laboratory facility that is required before a laboratory can attain accreditation. The use of the verb 'must' in standards indicates mandatory requirements for pathology practice.

In each section of this document, points deemed important for practice are identified as either 'Guidelines' or 'Commentaries', as follows:

• A Guideline is a consensus recommendation for best medical laboratory practice for a procedure, method, staffing resource or facility. Guidelines are prefaced with a 'G' (e.g. G2.2**)**. The use of the word 'should' in each Guideline within this document indicates a recommendation for good pathology practice.

• A Commentary may be provided to give clarification to the Guidelines as well as to provide examples and guidance on interpretation. Commentaries are prefaced with a 'C' (e.g. C1.2) and are placed where they add the most value.

Appendices if attached to this document are informative**,** that is explanatory in nature and may provide examples or information of a clinical nature and should be considered to be an integral part of this document.

Note: ACS documents can be accessed at: www.cytometry.org.au

1. PRE ANALYTICAL PHASE

Refer to '*ACS Guidelines for Clinical Flow Cytometry Laboratory Practice'* for additional information regarding minimum specimen labeling requirements, request forms, collection and transport conditions.

1.0 Specimen Collection & Transport

G1.1 For venipuncture peripheral blood samples, EDTA, Sodium heparin or ACD-A may be used [(1)].

C1.1(i) A dry anticoagulant eg EDTA, should be used for Single Platform methods

C1.1(ii) EDTA anti-coagulated peripheral blood specimens is recommended if the specimen is to be processed within 24 hours of collection

C1.1(iii) ACD anti-coagulated peripheral blood specimens may be stable for up to72 hours at room temperature.

C1.1(iv) Each laboratory should validate sample stability for their own patient pool.

G1.2 Fresh HPC products should be tested as soon as possible after collection and processing. When this is not possible, the laboratory should verify that their storage temperature and preparation methods maintain specimen integrity comparable to freshly processed material.

G1.3 HPC samples already contain anticoagulant thus aliquots of these should not be put in tubes containing any anti-coagulant at all (i.e. 'neutral' tubes).

G1.4 Dual platform assays require a total white cell count. This should be performed at the laboratory initiating the request, within the time frame specified by the manufacturer of the haematology instrument used.

C1.4 When CD34 enumeration is performed at a referred laboratory, results of this total white cell count should accompany each specimen.

G1.5 Cryopreserved samples should be tested immediately after thawing.

2. ANALYTICAL PHASE

Refer to '*ACS Guidelines for Clinical Flow Cytometry Laboratory Practice'* for additional information regarding sample analysis and performance measures.

2.1 Sample Preparation and Assay Protocol

G2.1.1 Before processing a sample, a total white cell count is recommended to avoid excess cells being added. White cell counts less than 20 x 10^9/L are recommended[1;2], but are dependent on antisera volume used and manufacturer's guidelines.

C2.1.1 For HPC products, generally a dilution to the appropriate staining range should be performed in phosphate buffered saline with 1-2% protein added [1]. Dextran-Albumen Saline Buffer may also be used.

G2.1.2 Single platform is the preferred method and is strongly recommended for HPC harvest products.

C2.1.2 (i) For single platform assay methods, follow the bead/kit manufacturer's instructions and/or publications [2].

C2.1.2 (ii) Refer to publications for dual platform assay methods [3].

C2.1.2 (iii) More information covering all aspects of CD34 enumeration can be found in publications in the reference list below.

G2.1.3 Single platform methods are strongly recommended for frozen products.

G2.1.4 Accurate pipetting is essential for the single platform method. Reverse pipetting technique should be used, and use of an electronic pipette should be considered [(4)].

C2.1.4 (i) Single platform is based on having a known number or concentration of fluorescent microspheres to a specific volume of sample, enabling the determination of absolute CD34+ cells in a single tube (and percentage if required). See C2.3.6 for formulas.

C2.1.4(ii) Follow manufacturer's guidelines for pipette use.

G2.1.5 Methods employing washing steps are not recommended for any CD34 enumeration assays and cannot be used for single platform assays [(4)].

G2.1.6 For commercially available antibodies, follow the manufacturer's instructions or validate by individual laboratories. Where no recommendations are available, antibodies can be titrated and tested on appropriate cells.

C2.1.6(i) Consult appropriate regulatory guidelines eg NPAAC

C2.1.6(ii) Amounts (and concentration) of antibody per cell far below that required for saturation binding may result in weak staining, leading to

possible false negative results. Excess reagent may cause increased non-specific staining of negative cells.

G2.1.7 The use of class III antibodies for the detection of CD34+ cells is recommended[1;4]. The use of PE Conjugated CD34 is recommended [4].

C2.1.7 Monoclonal antibodies reacting with the CD34 molecule can be broadly subdivided into three classes on the basis of enzyme sensitivity of the epitopes recognised. The class III antibodies are considered to have the broadest cellular reactivity in normal haemopoietic populations[1;4].

G2.1.8 A monoclonal antibody to CD45 should be used in order to aid in recognition and enumeration of total leucocytes. The CD45 monoclonal antibody used should detect all isoforms, and glycoforms[1;4].

C2.1.8 CD14 may also be used to monitor for any non-specific CD34 labeling of monocytes, especially where alternative gating strategies are employed which do not include the simultaneous gating on CD45 intensity and light scatter properties.

G2.1.9 A stain for cell viability should be included for all thawed HPC harvest products, and on fresh samples if testing is delayed, or cells are manipulated. 7-AAD is recommended as a dye to exclude dead cells [1] .

G2.1.10 An isotype control is not required if using the ISHAGE sequential gating strategy [4]. An isotype control may be required if using an alternative gating strategy.

G2.1.11 Recommended antibody incubation temperature and times are room temperature (20-25°C) for 10 -30 minutes in the dark [(1)], or as recommended by the manufacturer's guidelines.

G2.1.12 When lysing is necessary (e.g. peripheral blood or bone marrow samples) a fixative free lysing agent should be used [(4)].

C2.1.12 Lysing reagents are not required for cryopreserved cells [(1)].

G2.1.13 Stained sample counts should be acquired within 1 hour or as per manufacturer's instructions [(1;4)]. Store at 4^{o}C until ready to acquire. Thawed samples should be acquired immediately after staining.

G2.1.14 Monoclonal antibodies to CD3 may be used to enumerate T cells in allogeneic HPC products including bone marrow, apheresis and cord blood collections.

C2.1.14 Laboratories performing T cell assays for preparation of products for therapeutic use should be enrolled in external QC/QAP programs where available. Otherwise inter-laboratory sample sharing arrangements should be conducted as a QC measure.

2.2 Sample Acquisition

G2.2.1 Where dual platform methods are used, it is essential that the acquisition includes all leucocytes and that thresholds or acquisition gates are only set to exclude non-cellular particles and platelets.

C2.2.1 Haematopoietic CD34+ cells are considered rare events (frequency may be <0.1%), therefore it is essential to try and reduce CD34+ background events that may occur in populations of non-stem cells and non-cellular particles. The contribution of these background events may vary between different types of tissues and different cell collection and processing protocols.

G2.2.2 For single platform methods, it is important that FSC threshold not be used, as this will exclude acquisition of the beads, which are smaller than cells.

G2.2.3 When the threshold is used on the CD45 channel, ensure that some debris is visible. The CD45 gate should be sufficiently wide to allow adjustment during analysis if required.

G2.2.4 Replicate samples though may be performed to enhance reliability of the final results[3;4] .

G2.2.5 The total number of events to be collected should be sufficiently large to obtain a statistically significant number of CD34+ cells. Where possible ISHAGE single platform recommendations of 75,000 CD45+ cells, and a minimum count of 100 CD34+ events are recommended [2].

C2.2.5(i) For paucicellular samples these criteria may not be possible to meet.

C2.2.5(ii) To achieve an intra-assay CV of 10% a minimum of 100 CD34+ events should be collected wherever possible**[3;4].**

C2.2.5(iii) Recommendation for single platform methods: While a minimum count of 1000 bead events is required for statistical robustness acquisition of greater than 2000 bead events is recommended [5].

2.3 Sample Analysis

G2.3.1 The ISHAGE sequential gating strategy is recommended[1;4].

C2.3.1(i) Refer to published protocols for dual platform[3], and single platform assay analysis[1;2;4].

C2.3.1(ii) Refer to common errors in applying the ISHAGE strategy described in the literature [6].

G2.3.2 CD34+ haematopoietic cells are characterised by low to medium forward scatter (comparable to small lymphocytes), low side scatter, high CD34 expression and medium levels of CD45 (less than that of lymphocytes and monocytes). Staining intensity may be reduced in cryopreserved samples.

G2.3.3 Some automated haematology analysers include nucleated red cells (NRC) in the total leucocyte count. When performing dual platform analysis, correct for NRC contamination if required.

G2.3.4 The volume of HPC product available for transplant must be recorded in order to calculate the absolute number of CD34+ cells in the original specimen.

G2.3.5 When a dilution step is performed in the assay, the dilution factor must be applied as a part of the CD34 absolute calculation.

G2.3.6 For single platform assessment, use fluorescent beads of a known concentration. Use beads according to manufacturer's recommendations.

C2.3.6(i) Commercially available products include bead suspensions and lyophilized in-tube bead pellets.

C2.3.6(ii) For bead suspension added to assay tubes:

$$\text{Absolute CD34/}\mu\text{L} = \frac{\text{No. CD34+events x Fluorescent bead concentration x dil factor}}{\text{No. of fluorescent bead events}}$$

C2.3.6(iii) For in-tube bead pellet assays:

$$\text{Absolute CD34/}\mu\text{L} = \frac{\text{No. CD34+events x bead count x dil factor}}{\text{No. of bead events x volume of sample}}$$

2.4 Performance Measures

G2.4.1 A process control should be used regularly to monitor reagent staining and lysis procedures.

C2.4.1(i) Various commercial quality control cells are available for this purpose. Note viability dyes cannot be used with these cells. Follow manufacturer's instructions.

C2.4.1(ii) If the control falls outside established pre-defined targets, the reason for this should be determined, and corrections made, prior to repeating the assay.

G2.4.2 Flow cytometer calibration and performance checks should be undertaken and recorded daily.

G2.4.3 Under good manufacturing practice, reagent qualification studies are recommended on all new lots and new deliveries.

G2.4.4 Each laboratory should define its own precision and intra-assay precision [7].

C2.4.4 This information can also be used to establish the allowable limits between replicate tubes

G2.4.5 Utilise internal control information.

C2.4.5(i) Correlate the absolute CD45/μL with the white count from the haematology analyser.

C2.4.5(ii) On fresh samples, dual platform estimation can be correlated with the single platform results, using the CD34% and an external white cell count.

G2.4.6 All quality assurance activities should be documented, including validation of any new reagents or procedural changes.

C2.4.6 Any changes to antisera, reagents or procedures should be validated with fresh and commercial cells, and if possible through the external CD34 Proficiency testing program.

G2.4.7 All laboratories performing testing for patient management should belong to and participate in an external CD34 Proficiency testing program eg RCPA, NEQAS.

G2.4.8 All laboratories performing testing for patient management should have pathology laboratory accreditation as appropriate eg Australia – NPAAC, New Zealand – IANZ.

3. POST ANALYTICAL PHASE

Refer to 'ACS *Guidelines for Clinical Flow Cytometry Laboratory* Practice' for additional information regarding reports, record keeping, result validation, follow up tests.

3.1 Reports

G3.1.1 A standardized laboratory worksheet should be developed for recording all data, calculations, final reported result and the laboratory personnel responsible for testing – this may be electronic.

G3.1.2 Laboratory data and calculations should be checked by a second competent person. These checks should be documented.

G3.1.3 Results and calculations should be reported according to local institution requirements.

C3.1.3(i) Peripheral blood CD34 quantification should be reported as the concentration of 'CD34 cells/μL'.

C3.1.3(ii) HPC products can be reported as CD34/uL, or when additional information is known, total cells per unit : '$x10^6$ CD34 cells' and/or as dose: '$x10^6$ CD34 cells/kg' (of recipient body weight).

C3.1.3(iii) Different transplantation centers may also have additional, specific requirements

G3.1.4 All sample specific issues should be recorded on the laboratory worksheet. All communication of results or issues with transplant unit staff or requesting physicians should be noted.

3.2 Interpretation

G3.2 Refer to appropriate international clinical guidelines regarding specific transplantation procedures and HPC product thresholds.

REFERENCES CITED

1 H42-A2 Vol. 27 No. 16 Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guidelines – Second Edition.2007; Clinical and Laboratory Standards Institute

2 Keeney M, Chin-Yee I, Weir K, et al: Single Platform Flow Cytometric Absolute CD34+ Cell Counts based on ISHAGE Guidelines. Cytometry (Communications in Clinical Cytometry). 1998; 34:61-70

3 Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. J Hematotherapy 1996; 5:213

4 Barnett D, Janossy G, Lubenko A et al: Guideline for the Flow Cytometric Enumeration of CD34+ Haematopoietic Progenitor cells. Prepared by the CD34+ Haematopoietic Stem Cell Working Party. General Haematology Task Force for British Committee for Standards in Haematology: Clin Lab Haem. 1999; 21:301-308

5 Brando B, Barnett D, Janossy et al: Cytofluorometric methods for assessing absolute numbers of cell subsets in blood. European Working Group on Clinical Cell Analysis. Cytometry 2000; 15:42(6) 327-46

6 Whitby A, Whitby L, Fletcher M et al. ISHAGE Protocol: Are we doing it correctly? Cytometry Part B (Clinical Cytometry) 2012; 82B:9-17

7 ICSH/ICCS Workgroup: Validation of Cell-based Fluorescence Assays: Practice

Guidelines from International Council for Standardization of Haematology and International Society for Clinical Cytometry. Cytometry Part B (Clinical Cytometry); 2013: 84(5): 279–357

PROCEDURAL REFERENCES

Recommended peer reviewed articles providing detailed methodologies for setting up assays, interpretation and reporting include:

Sutherland DR, Nayyar R, Acton E et al. *'Comparison of Two Single-Platform ISHAGE-based CD34 Enumeration Protocols on BD FACSCalibur and FACSCanto Flow Cytometers.'* Cytotherapy. 2009; 5:595-605

Sutherland DR, Marsh JCW, Davidson J, et al. *'Differential sensitivity of CD34 epitopes to cleavage by P.haemolytica glycoprotease: Implications for purification of CD34+ progenitor cells.*' Exp Hematol 1992; 20:590

Sowemimo-Coker S. '*Red Blood Haemolysis During Processing.*' Transfusion Medicine Reviews. 2002; 18(1): 46-60

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