ACS GUIDELINE FOR LYMPHOCYTE SUBSET IMMUNOPHENOTYPING

Third Edition 2025

Australasian Cytometry Society Guideline Document

First published 2005

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 Lymphocyte Subset Immunophenotyping'* 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 medical pathology practice where the primary consideration is patient welfare, and where the needs and expectations of patients, Laboratory staff and referrers (both for pathology requests and inter-Laboratory referrals) are safely and satisfactorily met in a timely manner.

References to specific guidelines in that document are provided for assistance under the headings in this document.

This document is for use in Laboratories providing clinical flow cytometry services.

ABBREVIATIONS

EDTA	Ethylene-diaminetetraacetic acid
ACD	Acid Citrate Dextrose
WBC	White Blood Cells
FS	Forward Scatter
SS	Side Scatter
MoU	Measurement of Uncertainty

DEFINITIONS

count	means to acquire data on a flow cytometer
Dual platform (DP)	A method where two instruments are used for the determination of absolute numbers (e.g. Haematology and Flow Cytometry analysers)
Guidelines for Clinical Flow Cytometry Laboratory Practice (GCFCLP)	means the overarching document broadly outlining standards for good clinical flow cytometry laboratory practice where the primary consideration is patient welfare, and where the needs and expectations of patients, Laboratory staff and referrers (both for pathology requests and inter-Laboratory referrals) are safely and satisfactorily met in a timely manner.
markers	means antigens on cells of interest used for diagnostic purposes
platform	Instrumentation or analyser on which test assays are performed
Single platform (SP)	A method where a single instrument, a flow cytometer, is used to determine absolute numbers of cells.

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 and 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 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 information regarding minimum specimen labelling requirements, request forms, collection, and transport conditions.

G1.1 Specimen Collection

G1.1.1 Samples should be tested soon after collection.

C1.1.1(i) EDTA, ACD, Sodium Heparin or Lithium Heparin anticoagulants may be used [(1)].

C1.1.1(ii) Lithium Heparin anticoagulated blood specimens are suitable if the specimen is to be processed preferably within 24 hours of collection[(2)]. Sodium Heparin, EDTA and ACD anticoagulated blood specimens are stable for 72 hours.

C1.1.1(iii) If the WBC and differential is obtained from the same sample used for flow cytometry then EDTA is recommended [(1)].

G1.1.2 Dual platform methodologies require a total white cell count and differential that should be performed within the time frame specified by the manufacturer of the haematology instrument used.

C.1.1.2(i) For fresh samples (<48 hours) the WBC can be used in calculating T cell absolute numbers. For older samples (> 48 hours) lymphocyte numbers are preferred.

C.1.1.2(ii) For distant laboratories and dispatch centers, a total white cell count and differential should accompany each specimen [(1)].

C.1.1.2(iii) Single platform methods are preferred where a laboratory can demonstrate improved assay performance[1].

C.1.1.2(iv) Where bead solutions are used, they require a blood-to-bead ratio of approximately 1:1 (vol/vol)[3].

G1.2 Specimen Transport

G1.2.1 Specimens should be maintained at 18-22 ºC in a leak proof container. Temperatures below 4 ºC and above 30 ºC must be avoided.

2. ANALYTICAL PHASE

Refer to ACS *'Guidelines for Clinical Flow Cytometry Laboratory Practice'* for information regarding sample analysis and performance measures in addition to that given below.

G2.1 Sample analysis

G2.1.1 Cell concentration needs to be considered where total white cells numbers are high.

C2.1.1(i) Refer to manufacturers cell count ranges at which assays are validated. Otherwise, leukocyte numbers up to 1 x 10^6 cells/tube may be acceptable.

C.2.1.1(ii) Example: if WBC concentration is 10 x 10^9/L (or 20 x 10^9/L) and test volume is 100 µL (or 50 µL) cell number in tube would be 1 x 10^6 cells.

G2.1.2 All tubes need to include CD45. CD45 vs SS is recommended for gating lymphocytes, excluding monocytes and other non-lymphoid populations. To

improve exclusion of monocytes, consider the addition of CD14, exclusion of CD3 negative, CD4 positive (low) cells, and/or usage of FS properties.

G2.1.3 A suitable panel for assessing lymphocyte subsets must include markers for T cells (CD3, CD4, CD8), B cells (CD19), and NK cells (CD3 negative, CD16 and/or CD56)[1].

C.2.1.3(i) CD20 is not recommended as a B cell marker because it is expressed by subset of T cells and because it may be blocked during the assay if it is the target of monoclonal immunotherapy.

C.2.1.3(ii) CD56 and CD16 may be used on the same fluorophore.

G2.1.4 To reduce test error sample manipulation should be minimised. SP methods must not have a wash step. Wash steps should be avoided for DP methods[1,4].

G2.1.5 Preferred method for sample preparation is stain, lyse, fix.

G2.1.6 Thoroughly mixing specimens and counting beads prior to pipetting is important to ensure the sample transferred contains a homogenous mix.

C2.1.6(i) It is recommended to fully invert the blood specimen by 180 degrees at least 5 times before pipetting. For accurate enumeration of cells and beads the reverse pipetting technique is recommended using air displacement pipettes.

G2.2 Performance Measures

G2.2.1 Count at least 5000 lymphocytes in each tube to ensure that enough cells have been counted when lymphocyte subsets are analysed[1].

C.2.2.1(i) For B cell depleted samples, at least 10,000 lymphocytes are required. For example, an assay performing at a 10% CV, the estimated number of lymphocyte events to be acquired when the expected frequency of the B cells is 1% requires a total number of 10,000 acquired lymphocyte events, 100 events of which are expected to be B cells[5].

G2.2.2 Control material should be used and have validated ranges for the analytes measured.

C.2.2.2(i) A control should be run either daily or with each assay performed. A CD4 depleted control or a normal control should be run.

G2.2.3 Analysis should include internal reliability checks of results.

C.2.2.3(i) The sum of T, B and NK cell percentages (the Lymphosum) should be between 95 and 105% (minimally 90-110%) [1].

C.2.2.3(ii) The sum of the CD4 and CD8 cell percentages should equal the total T-cell % and be within the range of ±5% to a maximum of ±10% variability [1]. Values outside these ranges warrant further investigation. For example, co-expression of CD4+ and CD8+ may cause high values; the presence of increased gamma-delta T cells, which are usually CD4- and CD8-, may cause low values.

C.2.2.3(iii) The parameter 'Time' may be used to monitor instrument fluidics. Monitoring Bead Count Rate with time/fluorescence histograms can be used [3,6,7].

3. POST ANALYTICAL PHASE

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

G3 Reports

G3.1 For each of the lymphocyte populations and for the T cell subpopulation populations (CD3/4, CD3/8) absolute numbers should be reported.

C.3.1(i) Percentages may be reported in addition to absolute numbers.

C.3.1(ii) Refer to clinician requirements for units of measure for reporting results. There are currently no consensus units of measure for lymphocyte subset enumeration.

G3.2 Report data with corresponding reference limits of expected normal values.

C.3.2(i) Each laboratory should establish reference limits for antigens being tested where possible[1,2,8].

C.3.2(ii) Therapeutic ranges may be determined by clinicians for their own interpretation according to treatment.

G3.3 Measurement of uncertainty (MoU) should be calculated for lymphocyte subsets.

C.3.3(i) Using QC data or the error estimate from control donor bleeds, the standard uncertainty for each subset is calculated, this is multiplied by 2 (coverage factor for 95% confidence) to give the expanded

measurement of uncertainty[9,10]. MoU should be evaluated at regular intervals (e.g. 1 year).

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PROCEDURAL REFERENCES

The majority of laboratories performing Lymphocyte subsets use commercial kits which have widespread availability with detailed descriptions of methodology. For background on the methods, interpretation and publications refer to:

WHO publication, Laboratory Guidelines for Enumerating CD4 T Lymphocytes in the context of HIV/AIDS (revised version 2009), World Health Organisation Library Cataloging-in-Publication Data, SEA-HLM-392 (Revision), Jan 2009.

EDITORIAL COMMITTEE

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Revision	Change Summary	Active Date	Review Committee Chair
First Edition	• First Edition	2005	Beth Rees
Second Edition	• Full review, reformatted	2017	Neil McNamara
Third Edition	• Abbreviations: FS, MoU • Definitions: Single Platform • C2.1.3 : CD20, CD16, CD56 edits • C2.1.5, C.21.6: sample preparation edits • C2.2.1 : B cell acquisition comments • C2.2.2 : CD4 depleted control • C2.2.3 : CD4+8+ coexpression • C3.3.1 : MuO calculation	2025	Paula Keating