DRAFT

ACS GUIDELINE FOR

HAEMATOLOGY ONCOLOGY

IMMUNOPHENOTYPING

Fourth Edition 2025

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 ACS *'Guideline for Haematology Oncology 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.

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

ABBREVIATIONS

ACD	Acid Citrate Dextrose
CSF	Cerebrospinal fluid
EDTA	Ethylene-diaminetetraacetic acid
WBC	White Blood Cells
RPMI	Roswell park Memorial institute medium, a sample preservative
Hanks	Hank's balanced Salt Solution, a sample preservative

DEFINITIONS

CD	Cluster of differentiation number used to classify cell surface molecular targets for immunophenotypic identification of cells e.g. CD3 for the pan T cell antigen
Competent clinical flow cytometrist	A person who has a minimum of two years' clinical flow cytometry experience, and who has been documented to be competent in clinical flow cytometry according to the Laboratory's Quality System
Count	*v*. To acquire data on a flow cytometer *n*. number of events or cells
Guideline	A consensus recommendation for best medical laboratory practice for a procedure, method, staffing resource or facility
Guidelines for Clinical Flow Cytometry Laboratory Practice (GCFCLP)	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.
Marker	An antibody directed to an antigen of interest in or on a cell used for diagnostic purposes

Process/ed/ing (e.g., sample processing)	Washing, staining and acquisition of cells by the flow cytometer
Screen (e.g., lymphoma screen)	A set of reagents suitable for the initial evaluation of a cell lineage based on the needs of a laboratory taking into consideration the patient population within the laboratory's catchment, clinical indication and estimated pre-test probability of the individual sample.

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 labelling requirements, request forms, collection and transport conditions.

G1.1 Specimen Collection and Storage

For flow cytometry processing appropriate anticoagulant and storage should be used according to sample type and disease investigation.

G1.1.1 For peripheral blood, bone marrow aspirates EDTA, lithium/sodium heparin or ACD solution may be used according to test procedures [1; 2].

C1.1.1(i) EDTA samples are better tested up to 48 hours, lithium heparin 48 to 72 hours [1; 2; 3], ACD up to 72 hours [1].

C1.1.1(ii) Lithium/sodium Heparin is preferred as sample integrity is preserved for a longer period. EDTA is useful as it allows morphologic assessment and FBC differential testing. ACD is not recommended for bone marrow aspirate samples in which incorrect sample to anticoagulant ratio can alter pH and reduce cell viability [1].

G1.1.2 Peripheral blood, bone marrow aspirates can be stored at 18-25ºC [1; 2].

C1.1.2 It is recommended PB and BM be tested within 48 hours when stored at 18-25ºC. B cell clonality studies may be valid up to 72 hours when stored at 4°C. Myeloid/monocyte expression is better retained when stored at 4°C[2].

G1.1.3 Tissue biopsies in isotonic medium (such as phosphate buffered saline, Hanks, or RPMI) usually do not require anticoagulant. Specimens should be immersed in appropriate medium as soon as practicable following collection

and refrigerated (+4ºC) on arrival if delay in sample processing is anticipated [1, 2]. Sample should be processed as soon as possible, ideally within 24 – 48 hours. A live/dead stain is recommended to assess viability and a disclaimer included in the diagnostic report if sample integrity is considered compromised.

G1.1.4 **CSF should be processed as soon as possible, ideally within 60 minutes from collection if performed locally. However, as this is often impractical, the addition of transport medium such as RPMI will mitigate cell loss for samples processed on the same day as collection, while adding a stabilizing reagent such as Transfix should be considered if further delay is anticipated noting that while the latter compromises morphological assessment, flow cytometric analysis has been validated for up to at least 72 hours [1] [2] [3] [14].**

G1.1.5 **Other fluids (e.g. pleural, fine needle aspirate): Anticoagulants are not necessary. EDTA, Lithium/sodium heparin may be used. ACD is not recommended [3]. Fluids should be processed within 24 hours although possibly up to 72 hours if unavoidably delayed and stored at 4°C. Tissue culture medium may also be added.**

G1.1.6 **The sample type and site and time of collection should be provided on the sample tube/container along with patient identifiers. This information should be included in the final report.**

G1.1.7 **A total white cell count and differential on all peripheral blood samples should be performed at the laboratory initiating the request. For distant laboratories and dispatch centers a white cell count, differential and unstained blood film should accompany each specimen.**

G1.2 Specimen Transport

G1.2.1 Samples must be delivered to the laboratory as soon as possible to minimise loss of cell viability.

C1.2(i) Tissue samples should be kept moist during transport to the laboratory, immersed in tissue culture media, sterile saline, or saline soaked gauze for transport at 18-25ºC [3]. When culture media has not been used for transport to the laboratory, this should be added as soon as possible upon receipt if delay in processing is anticipated. The sample should be stored at 4°C and processed within 24 hours, or no more than 48 hours without extenuating circumstances [3].

C1.2(ii) All other specimens, such as blood and bone marrow, should be maintained at 18 to 25º C in a leak proof container and processed within 72 hours depending on anticoagulant (C1.1.1). Temperatures below 4ºC and above 30ºC must be avoided [1].

C1.2(iii) Non-viable cells are a significant source of false positive staining. Light scatter properties can be useful indicators for cell viability but are not fool proof. Viability testing is recommended for all tissue biopsies and fluids (except CSF. Refer to specific requirements) and for blood and bone marrow if >48 hours old.
Other situations to consider viability testing include suspected high-grade lymphoma (high proliferation), and patients receiving chemotherapy or radiation therapy.

C1.2(iv) Delay in sample testing should be indicated in the final report.

G1.2.2 Laboratories should have a procedure in place to optimise the integrity of samples that arrive after hours, particularly irreplaceable specimens.

C1.2.2(i) After hours procedures should address result urgency, sample type, sample stability, storage, and staff availability.

C1.2.2(ii) Laboratories should provide resources to support a 24 - 48-hour

window for processing clinically important, irreplaceable samples over weekends and public holidays.

G1.3 Test Requests

Requests for leukaemia/lymphoma testing should include any relevant clinical observations and history to assist with selection of appropriate screening +/- secondary panels to be performed.

C1.3(i) It is critical the correct screen +/- secondary panel are performed particularly where rare or unusual diagnoses are to be considered.

C1.3(ii) Previous abnormal immunophenotypes should be noted either on the test request or by search of laboratory records. This is to ensure relevant markers are investigated for residual disease/relapse.

G1.4 Antibody Reagents

Fluorescent marker antibodies used in panels should be validated by clinical correlation following IVD standards.

C1.4 Antibody reagent/panel selection will be influenced by the medical indication, sample type, quality, and downstream considerations (e.g. additional markers required for gating a population subjected to targeted immunotherapy or for contributing to a definitive diagnosis, classification, prognosis or later MRD assessment) [4, 5, 7-11].

C1.4(i) Leukaemia/lymphoma ***screens*** should include CD45 [4].

C1.4(ii) Lymphoma ***screens*** should include as a minimum, markers for

- B cells: CD19, CD20, CD10, CD5, kappa/lambda light chains. CD5 and/or CD10 status may facilitate further panel selection [11]. See C 1.4(v)

- T/NK cells: CD3, CD4, CD5, CD7, CD8, CD16/CD56 [4].

C1.4(iii) Leukaemia ***screens*** should include as a minimum, markers for:

- Progenitors: CD45, CD34, CD10, CD19, CD117, HLA-DR
- Myeloid/monocytic lineage: CD11b, CD13, CD14, CD16, CD33, CD64
- Lymphoid lineage: CD2, CD3, CD4, CD5, CD7, CD8, CD19, CD20, CD56, kappa/lambda light chains [4]

C1.4(iv) Intracytoplasmic markers: MPO, CD79a, CD22, CD3 are required for lineage definition for leukaemia screens (see 'Acute leukaemia of ambiguous lineage' in WHO) [5].

C1.4(v) Additional markers should be tested to assist diagnosis following the current WHO Leukaemia/Lymphoma Classification [5][12][13]. This may be performed in a two-tiered approach depending on the circumstances.

Example 1: Chronic lymphocytic leukaemia. Consensus "required" diagnostic markers: CD19, CD5, CD20, CD23, Kappa, and Lambda; "recommended" markers: CD43, CD79b, CD81, CD200, CD10, and ROR1 [16].

Example 2: Hairy cell leukaemia: CD11c, CD25, CD103, CD123, CD200 [17].

Example 3: Suspected monoclonal T-cell population: TRBC1, TRBC2, T-cell receptor gamma-delta, CD26 (Sezary), CD57 (T-LGL) [18].

Example 4: Plasmacytoid dendritic cell neoplasm: CD123, CD303 [19].

Example 5: Suspected plasma cell neoplasm: CD19, CD27, CD38, CD45, CD56, CD81, CD117, CD138, cyt-Kappa, cyt-Lambda [20].

G1.5 Sample Preparation

Samples should be prepared with total white cell count ≤ 10 x 10^9/L [6]. If

higher consider diluting the sample to a cell concentration to this range in isotonic medium.

C2.1.1(i) The sample preparation total white cell count target is assuming antibodies for lymphocytes were titred to saturate target antigens usually at 1 x10^6 cells in 100 uL of sample [6]. Refer to manufacturers cell count ranges at which assays are validated. However, each laboratory will need to determine appropriate ranges if this information is not provided.

C2.1.1(ii) For paucicellular samples, bulk lysis or buffy coat preparations may be used to increase the leukocyte concentration and should be validated by the laboratory for each specific application [15].

2. ANALYTICAL PHASE

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

G2.1 Data analysis

G2.1.1 A minimum of 5,000 cellular events in the target gate (e.g. lymphocytes, blasts) should be acquired where possible.

C2.1.1 Acquisition of a minimum of 5,000 events of any cell type is required to achieve a sensitivity of 2% (for 100 target events). To achieve an intra-assay CV of 10% a minimum of 100 target events would be required**.** Laboratories need to assess the desired sensitivity of assays performed [2]. For rare event analysis requiring the acquisition of a higher number of denominator cells, refer to ACS Guideline for Minimal Residual Disease Testing [21].

G2.1.2 Laboratory should have a process to ensure identification and traceability

of secondary sample/assay tubes.

G2.1.3 **Analysis of acquired data should include reference to CD45, FSC and SSC parameters [7].**

C2.1.3 It is recommended event count versus time be recorded to monitor fluidics, and cautious gating of FSC Area versus Height performed to exclude doublets [2].

C2.1.4 **Analysis should be performed by a competent clinical laboratory staff member with documented competency-based assessment of leukaemia and lymphoma cases.**

C2.1.4(i) A sufficient number of abnormal and normal cases should be studied by laboratory and medical staff over a given period to maintain proficiency [1]. Exposure to a minimum of 100 new cases per year of a cross section of haematological neoplasms is recommended [2]. Interpretation of flow cytometric data by laboratory personnel dislocated from the technical and quality aspects of sample processing and access to the primary list-mode files should be discouraged [3].

C2.1.4(ii) Flow cytometry scatter plots and data generated from all leukaemia /lymphoma cases should be reviewed by two competent staff members.

G2.2 **Performance Measures**

G2.2.1 **For Lymphoma and Leukaemia screening, it may be necessary to process a normal control sample on a daily or weekly basis, as appropriate, unless the laboratory is active and within-run positive and negative control results demonstrate appropriate reactivity.**

C2.2.1 Where absolute numbers (e.g. cells/uL for blood samples) are reported, a control reagent should be periodically tested, have specified ranges for the analytes measured, and reasons for deviations determined. See ACS Guideline for Lymphocyte Subset Testing.

C2.2.2 A normal control sample should also be processed following major instrument maintenance or major changes to fluorescence spillover compensation data.

G2.2.2 Account should be made for all populations tested.

C2.2.2(i) Lymphoma screens: A *lymphosum* of B, T and NK cell populations can be calculated for blood and bone marrow samples**.** Lymphocyte gated populations unaccounted for >5% for blood and >10% for bone marrow may require further investigation. Note that for bone marrow samples, a summary of *all* major populations as a percentage of total cells or total leukocytes is less accurate and subject to sample quality, which nevertheless may be helpful to illustrate an under representative sample [(2)].

C2.2.2(ii) Leukaemia screens: All haematopoietic cell populations need to be examined. The absence of CD34, CD117, TdT does not preclude malignancy from blast gated populations.

G2.2.3 Viability Assessment

C2.2.3 Results should not be rejected based solely on low viability. The FSC/SSC plot should be used to exclude non-viable cells from analysis to reduce non-specific antibody binding from the data. If viability is <75% consider rejecting the sample if replaceable. If irreplaceable, a disclaimer statement regarding suboptimal specimen viability should be included in the report [(3)].

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.

G3.1 Reports

G3.1.1 Reports should identify any abnormal population gated, its size, markers tested, relevant staining reactivities, and a diagnosis where possible [2].

There is no universal standard for defining antigen expression based on a specific cutoff of percentage of a cell population expressing an antigen above a negative control. While a 10% cutoff has been applied in specific circumstances, it is more important to describe less frequent populations in an appropriate context [2] [4] [5] [9].

C3.1.1(i) CD number listing should be in numeric then alphabetic listing order notwithstanding C3.1.1(ii).

C3.1.1(ii) CD numbers may be accompanied by descriptive and grouped by cell types/function.

C3.1.1(iii) Staining intensity should be reported if relevant e.g. dim, bright in relation to appropriate intrinsic reference control cells within the same sample (if present) or known normal expression if not present. It is not necessary to report percentage positivity for individual markers [2] [4] [9]. Reporting tables of percentage positive results is discouraged.

C3.1.1(iv) Issues related to sample quality should be described (e.g. poor viability, low cell numbers, clotted, delayed transport, other).

C3.1.1(v) Report should include a comment to reflect that all flow cytometry results should be correlated/interpreted in the context of clinical, morphologic, and cytogenetic/molecular findings [5].

C3.1.1(vi) Report should include name of the validating pathologist.

G3.1.2 Reports should be completed in a timely manner in alignment with the urgency of the clinical scenario and local laboratory practice.

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PROCEDURAL REFERENCES

For background on the methods, interpretation and publications refer to the references cited in this guideline.

Editorial Committee

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Further ACS clinical flow cytometry guidelines documents are available on the website: www.cytometry.org.au

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Revision	Change Summary	Active Date	Author of Change
First Edition	• First Edition	2006	Lyndsay Peters
Second Edition	• Second Edition	2017	Neil McNamara
Third Edition	• Minor amendments to G1.2 Specimen transport • Addition of G1.2.2 Friday evening / long weekend samples • Minor amendments to G1.4 Antibody reagents. Additional emphasis on the use of screening markers in C1.4 • Additonal commentary C3.1.1(iv) • Updated reference format • Additional references to guidelines (references 7 – 11) • Addition of Review Committee	2020	Sheree Bailey (representing the 2020 Editorial and Review Committees)

Fourth Edition	• Added definitions "sample processing", "screen". • Harmonized the use of the word "processing" instead of "analysed" and "tested" throughout the document • Amendments to numerous sections as follows: • G1.1.2 • G1.1.3 • G1.1.4 • G1.1.5 • G1.2.1 • G1.2.2 • G1.3 • G1.4 [C1.4(v)] • G1.5 • G2.1 • G2.2 • G3.1 • Updated reference format • Additional references • Addition of Review Committee	2025	Editorial and Review Committees for Haematology Oncology Immunophenotyping