ACS GUIDELINE FOR FLOW CYTOMETRIC ASSESSMENT OF HLA ALLOANTIBODIES

First Edition 2017

Australasian Cytometry Society Guideline Document

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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 Testing* 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 cytometric crossmatching services developed in-house. It does not cover commercial solid-phase assays for use on flow cytometers or Luminex testing platforms.

For details on procedural matters and methodologies readers are directed to the Procedural Reference list at the end of this document for ACS recommended published peer reviewed texts, articles and guidelines.

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ABBREVIATIONS

^{o}C	degrees Celsius
ACD	acid citrate dextrose, an anticoagulant
APHIA	Asia-Pacific Histocompatibility and Immunogenetics Association
ASHI	American Society for Histocompatibility and Immunogenetics
CD	Cluster of Differentiation
CDC	Complement Dependent Cytotoxicity
CO_2	Carbon Dioxide
DSA	Donor specific antibodies
FCXM	Flow Cytometric Crossmatch
g	Relative centrifugal force (RCF)
HLA	Human Leucocyte Antigen
IgG	Immunoglobulin G
IVD	in-vitro diagnostic
MCS	Mean Channel Shift
µl	microlitre
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
RBC	Red Blood Cell
XM	Crossmatch

DEFINITIONS

Acceptable mismatch	A non-self HLA antigen to which a recipient has no antibody reactivity, prior to transplantation.
Antibody cocktail	A mixture of more than one antibody.
B-cells	Bone marrow matured lymphocytes that express membrane bound immunoglobulin. In response to antigen contact these differentiate into antibody secreting plasma cells or memory B cells.
Bw4, Bw6	Public serologically detectable antigenic determinants that co-migrate with HLA-B antigens (and some HLA-A).
Complement dependent cytotoxicity (CDC)	A laboratory test to identify presence of antibodies in a serum sample using lymphocytes as targets and cell viability as the read-out.
CD	Cluster definition number used to identify individual cell markers e.g. CD3 for the pan T cell antigen
Crossmatch test (XM)	A test to identify antibody mediated reactivity to target antigens in a potential organ donor. The test report must be either positive or negative.
DR52, DR53	Serologically defined class II surface specificities encoded by DRB3* and DRB4* genes respectively
Competent clinical flow cytometrist	means 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
$F(ab')_2$	Divalent fragment of IgG generated following pepsin digestion of whole IgG
Fc receptor	A cell surface molecule specific for the heavy chain of certain immunoglobulin classes. Various forms found on lymphocytes, macrophages, natural killer cells and mast cells.
Guideline	means a consensus recommendation for best medical laboratory practice for a procedure, method, staffing resource or facility
Guidelines for Clinical Flow Cytometry Laboratory Practice	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.
Human Leucocyte Antigens (HLA)	Cell surface molecules determined by highly polymorphic linked genes on chromosome 6 (HLA-A, -B, -Cw, -DR, -DQ, -DP: the major histocompatibility complex or MHC).
HLA class I molecules	HLA-A, -B and –Cw molecules with structural and functional similarity. Expressed by almost all nucleated cells and platelets.

HLA class II molecules	HLA-DR, -DQ and –DP molecules with structural and functional similarity. Constitutively expressed only on specialised antigen presenting cells but may be inducible.
In-house IVD	means an IVD that is developed de novo, or developed or modified from a published source, or developed or modified from any other source, or its intended purpose, within the confines or scope of a Laboratory or Laboratory network, and is not supplied for use outside the Laboratory or Laboratory network. Commercial IVDs being used clinically for a purpose other than that originally intended by the manufacturer are also classed as in-house IVDs and are subject to the requirements of this standard.
In vitro diagnostic medical device (IVD)	means a medical device test if it is a reagent, calibrator, control material, kit, Specimen receptacle, software, instrument, apparatus, equipment or system, whether used alone or in combination with other diagnostic goods for in vitro use. It must be intended by the manufacturer to be used in vitro for the examination of Specimens derived from the human body, solely or principally for the purpose of giving information about a physiological or pathological state, a congenital abnormality or to determine safety and compatibility with a potential recipient, or to monitor therapeutic measures. The definition of an IVD does not encompass products that are intended for general Laboratory use that are not manufactured, sold or presented for use specifically as an IVD.
Lymphocyte	Mononuclear leukocytes of various lineages (B cell, T cell, NK cell).
Median Fluorescent Intensity (MFI)	A semi-quantitative readout of the degree of antibody binding, indirectly measured by a fluorescent label.
Platelets	Small, irregular blood borne anucleate cells which are an important component of a thrombus (clot).
Quality Assessment	means a measurement and monitoring function of quality assurance for determining how well health care is delivered in comparison with applicable standards or acceptable bounds of care.
Quality Assurance	means part of quality management focused on providing confidence that quality requirements will be fulfilled.
Quality control	means the study of those errors that are the responsibility of the Laboratory, and the procedures used to recognise and minimise them. This study includes all errors arising within the Laboratory between the receipt of the Specimen and the dispatch of the report. On some occasions, the responsibility

	of the Laboratory may extend from the collection of the Specimen from the patient and the provision of a suitable container, to the dispatch and delivery of the report. Internal quality control: means processes and activities that are used within the Laboratory to monitor the day-to-day operational and analytical performance of test procedures. These activities may include on-going instrument standardisation checks, instrument maintenance, analysis of control material, statistical or graphical assessment of results from control material.
Rituximab	Brand of therapeutic chimeric CD20-specific monoclonal antibody.
Sensitisation	An immune response to an antigen resulting in T and/or B cell memory.
Stain	means bind monoclonal antibodies to markers on cells of interest to
Standard	Means a minimum requirement for a procedure, method, staffing resource or laboratory facility that is required before a laboratory can attain accreditation
T cell	Mononuclear leukocyte having developed in the thymus.

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

BACKGROUND

Failure to detect the presence in a recipient of preformed cytotoxic antibodies to donor Human Leukocyte Antigens (HLA) can have a devastating effect on a transplanted organ, particularly when it results in hyperacute rejection of the graft [1; 2]. These donor specific antibodies (DSA) typically develop in response to exposure to foreign HLA as a result of pregnancy, blood transfusion or transplant.

High concentrations of these antibodies are readily detectable with the complement dependent cytotoxicity (CDC) assay. However the flow cytometric crossmatch (FCXM) will also detect non-complement fixing antibodies [3], is 10 to 100 fold more sensitive [4; 5] and has been very effective in identifying clinically relevant lower levels of DSA that contribute to accelerated graft failure and early graft loss, not detectable by CDC [6].

HLA ALLOANTIBODY DETECTION - THE FLOW CYTOMETRIC CROSSMATCH.

The FCXM assay is performed by incubating lymphocytes from the potential donor with serum from the recipient. Any DSA in the patient serum will bind to the donor lymphocytes and will be detected following a second incubation with a polyclonal anti-human immunoglobulin antibody (typically conjugated to fluorescein). Antibodies directed against class I HLA will bind to all lymphocytes while antibodies to class II HLA will only bind to B cells. A multicolour approach is used with the inclusion of two additional fluorochrome labelled monoclonal antibodies to allow identification of B and T cells (typically CD19 and CD3) to assist with antibody specificity. A significant increase in fluorescence of the cell in the patient serum relative to the negative control serum indicates the presence of DSA.

Pronase digestion of the lymphocytes prior to crossmatching, cleaves the Fc receptors, reducing nonspecific binding of antibody by the Fc receptor [7; 8; 9] , particularly by B cells (which have relatively high numbers of Fc receptors on their cell surface). CD20 is also cleaved by pronase - digestion of B cells by pronase is a useful additional step in patients treated with Rituximab [10].

These assays are performed on lymphocytes recovered from anti-coagulated blood, and serum.

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 in addition to those shown following.

G1.1 Specimen Collection
Universal precautions should be strictly observed when collecting and handling samples.

G1.1.1 All samples and their accompanying request forms must contain at least three unique identifiers for the individuals being tested.

C1.1.1 Typically this will be the individual's name and date of birth, but could be another unique code or identifier. It should also allow identification of the individual being tested to the medical practitioner originating the request.

G1.1.2 All specimen tubes must be individually labelled and include the date each sample was collected

G1.1.3 PBMCs can be recovered from peripheral blood (anti-coagulant present e.g. acid citrate dextrose (ACD), sodium heparin) Lymph node, Spleen, and thawed cryopreserved mononuclear cells.

G1.1.4 Serum samples: Blood must be clotted prior to serum being recovered for testing.

C1.1.4 (i) Whole blood should be kept at room temperature for 1 hour to allow it to clot.

C1.1.4 (ii) Serum can be stored at 4^{o}C for up to 72 hours until testing **(11).**

C1.1.4 (iii) Long term serum storage should occur at or below a temperature of -70^{o}C (some IgG is always lost during freezing/thawing).

G1.1.5 There are no minimum volume requirements. Suggested are given below for Flow Cytometric Cross Match (FCXM) samples.

C1.1.5 (i) Fresh blood specimens:

a) 20 mls anti-coagulated blood (ACD, heparin or equivalent) from the potential donor
b) 10 mls clotted blood from the potential recipient
c) 20 mls anti-coagulated blood (ACD, heparin or equivalent) from the potential recipient. (An auto patient crossmatch is preferable but if blood is not obtainable, omission will not invalidate the test.)

C1.1.5 (ii) Cryopreserved specimens:

a) 10^{7} cryopreserved PBMC
b) 0.5 mls serum

G1.1.6 Specimens to be tested >48 hours after collection should be treated with caution. Use of a viability stain may be used eg 7AAD, Tryptophan Blue.

C1.1.6 (i) Use of older blood must be noted on the worksheet and the report. The report should include a comment reflecting the effect this may have on the results.

C1.1.6 (ii) PBMCs should be > 80% viability. Nonviable cells may non-specifically bind to many antibodies and interfere with accurate detection of the presence of DSA. If the sample is irreplaceable and <75% viable cells are present, issue a disclaimer statement about suboptimal viability. If significant DSA are detected, a repeat specimen may not be required.

G1.2 Specimen Transport

G1.2.1 Packaging, labelling and transport of specimens should comply with the requirements for shipping of Biological Samples as Dangerous Goods[15; 16] along with any relevant local state based legislation.

G1.2.2 Specimens should be maintained at 16-25^{o}C using a leak-proof triple packaging system.

C1.2.2 Temperatures below 4^{o}C, and above 30^{o}C must be avoided.

G1.2.3 Serum shipped frozen on dry ice must be protected from CO_2 and conform with the Dangerous Goods requirements for Infectious substances and Dry Ice.

G1.3 Flow Cytometric Cross Match Reagents

G1.3.1 Reagents must be stored according to manufacturer's instructions or in a manner documented to allow them to perform accurately and reliably when used in the assay.

G1.3.2 Manufacturer's instructions must be followed unless the laboratory has validated their modified use.

G1.3.3 All reagents and critical material must not be used past their expiry date unless they have been validated against in date reagents to demonstrate their performance remains equivalent and has not deteriorated.

C1.3.3 Reagents received without an expiration date must be given an appropriate expiry date using evidence demonstrating optimum performance is maintained for the duration of its shelf life.

G1.3.4 Each assay must document which reagent lot was used in that assay.

G1.4 Fluorescently Labelled Antibodies

G1.4.1 FCXM antibody fluorochrome combinations should be selected to suit the local laboratory and their cytometer

G1.4.2 All antibodies should be titrated where necessary to determine optimal sensitivity (signal to noise) or at a minimum, adequate separation of positive and negative peaks.

C1.4.2 Newly delivered antibodies may be tested at titrated levels in parallel with antibodies in use to verify their performance .

G1.4.3 A $F(ab')_2$ anti-human IgG antibody specific for the Fc region should be used to assess binding of human IgG

G1.4.4 All antibodies should not cross react with any other multicolour reagent or foetal calf serum.

G1.4.5 Cocktail antibodies should (individually) undergo testing prior to use to demonstrate the performance of each new antibody Lot and /or delivery is equivalent to the current antibody.

G1.4.6 Expired reagents can be used where their performance remains equivalent to that demonstrated in pre-release testing. Data must be available to support this.

G1.5 Computer Software

G1.5.1 All computer software programs (and version upgrades) used to transfer or analyse data, perform calculations and generate results and reports must undergo documented validation prior to use.

G1.5.2 Where existing software is revised or corrected, a documented review of historic results must be performed to determine whether corrective action is required for any previously issued results or reports.

2. ANALYTICAL PHASE

Refer to ACS *'Guidelines for Clinical Flow Cytometry Laboratory Practice'* for information regarding sample analysis and performance measures in addition to those shown following.

G2.1 PBMC Isolation

G2.1.1 Fresh PBMCs should be isolated from the sample according to the laboratory's standard protocols: Ficoll hypaque, a commercial equivalent e.g. Lymphoprep™ or a validated bead based method.

C2.1.1 Avoid using Lympho-Kwik and Percoll containing reagents. These products increase background binding thereby reducing the sensitivity of the assay and may result in reporting a false negative result.

G2.1.2 Removal of platelets is essential. Platelets are rich in HLA class 1 and if present in detectable numbers may cause a false negative result by competing with lymphocytes for antibody binding sites.

G2.1.3 Overall purity of the cell preparation should be > 90% lymphocytes. The total numbers of contaminating cells such as platelets, RBCs, and granulocytes should be < 10%.

G2.2 Pronase Treatment

The proteolytic enzyme pronase can be used to treat PBMC prior to crossmatching. Pronase will cleave Fc receptors on the surface of B cells, and to a lesser extent, on T cells [(8).] This decreases non-specific background fluorescence enhancing the signal to noise ratio improving specificity and sensitivity for B cells.

G2.2.1 Ensure pronase treated cells are counted after digestion (some cells are lost during digestion).

G2.2.2 Pronase treated cells should be crossmatched within a couple of hours after digestion before Fc receptors are re-generated and re-appear on the cell surface.

G2.2.3 It is recommended that a control sample be included to monitor the performance of pronase. (e.g. measuring expression of CD20, which is cleaved by pronase)

C2.2.3 Care must be taken with interpretation of pronase treated T cell results as pronase can result in a false positive result [(12)]. (Other testing and results e.g. B cell, Luminex, CDC etc. should be considered to help interpretation.)

G2.2.4 The laboratory must establish an appropriate concentration of pronase for use so that it does not reduce HLA expression [(13)].

G2.3 Serum Sample Assay

G2.3.1 Serum from the potential recipient must be included in the assay. A serum sample collected immediately prior to the crossmatch is preferred.

C2.3.1 (i) An older serum may be used when a patient has been undergoing screening for some time and has had no sensitising events since serum collection. However, this must be noted on the report.

C2.3.1 (ii) As an option, the peak and/or other historic sera of interest from the patient may be used. Serum samples of any age can be used. These are likely to be frozen.

C2.3.1 (iii) current serum from potential donor(s) may be used as an autologous control

G2.3.2 Frozen sera thawed for use, including positive and negative control sera, should be centrifuged (10000g, 10 mins) prior to use to remove aggregated immunoglobulin and immune complexes

C2.3.2 Aggregates can increase non-specific background staining, reducing sensitivity resulting in reporting of false negative results

G 2.4 Flow Cytometric Cross Match Assay

G2.4.1 An optimum serum to target ratio must be established. The cell number to serum ratio should remain constant e.g. 100,000 cells for every 10µl serum.

G2.4.2 Assays must include antibodies to identify T cell and B cell populations, and detect clinically relevant IgG capable of binding to donor specific HLA class I and class II (and patient HLA where required).

G2.4.3 Cells may require fixation post staining with the Ab cocktail unless they are run on the cytometer within 2 hours of completion of the antibody staining step.

C2.4.3 Fixed samples should be kept at 4^{o}C in the dark and should be acquired within 24 hours. This needs to be validated.

G2.4.4 Adequate washing is required post crossmatch incubation to ensure all unbound IgG is removed prior to addition of the antibody cocktail.

G 2.5 Instrument Calibration

G2.5.1 Calibration beads/fluorescent standards need to be run each time the instrument is turned on and following maintenance, adjustments or problems where instrument performance may be affected.

G2.5.2 A fluorescent standard for each fluorochrome used in the assay should be included.

G2.5.3 Results for each run should be recorded along with an indication that all relevant parameters were accepted as satisfactory for that run.

G2.5.4 Where more than one fluorochrome is in use, appropriate compensation of spectral overlap must be applied.

G 2.6 Sample Acquisition

G2.6.1 Gating strategies need to ensure that the population of interest is being selected without significant contamination.

C2.6.1 (i) B cells – acquire CD19 or CD20 positive cellular events. CD19 must be used to identify pronase treated B cells (pronase cleaves CD20).

C2.6.1 (ii) T cells – acquire CD3 positive cellular events.

G2.6.2 A minimum stop condition must be defined for routine assays e.g. 200 B cellular events.

G2.7 Performance Measures

G2.7.1 Replicate tubes should agree within a defined range (e.g. ± 5.0%CV).

G2.7.2 Negative and Positive control sera should be tested and fall within defined ranges.

C2.7.2 (i) The positive control value for a given assay can exceed the upper limit.

C2.7.2 (ii) If the positive control value is below the lower limit, the assay must be repeated if the result appears negative. However, a positive result may still be reported but a comment must be included about the reduced sensitivity.

G2.7.3 The MCS or ratio between the Negative and Positive controls must have a defined minimal value.

G2.7.4 The Negative FCXM control may be pooled human serum or from a single donor. It must be negative by both solid phase antibody screen and FCXM screen.

C2.7.4 (i) The FCXM screen should demonstrate low background staining for a panel of cells. A good negative control will show a fluorescence shift only slightly above that observed for the same cells suspended in PBS.

C2.7.4 (ii) Aliquots for use should be stored at -70^{o}C until required.

C2.7.4 (iii) Every time a new lot of negative control is selected, background staining and fluorescence should be comparable to the existing one otherwise a new cut-off value needs to be determined. This should be done using a statistically significant sampling of flow crossmatch results on normal cells.

G2.7.5 The positive control should consist of pooled positive sera from sensitised patients with broad HLA specificity and high anti-HLA IgG titre.

C2.7.5 (i) The pool must include IgG specific for the full range of HLA specificities in the local population.

C2.7.5 (ii) Inclusion of anti-Bw4 and Bw6 would guarantee reactivity with most donor T and B cells.

C2.7.5 (iii) Adding sera with anti-DRw52 and DRw53 reactivity will help for B cells, however all B cells tend to react nicely with Class I antibody pools.

C2.7.5 (iv) Aliquots for use should be stored at -70^{o}C until required.

C2.7.5 (v) The pooled positive control may be assayed neat and at a dilution. The dilution is usually set above the upper threshold of the negative

control, i.e. a “borderline” or weakly positive reaction. This is to ensure consistency in determining the lower limits of a positive test.

G2.7.6 Each laboratory must participate in an external proficiency testing programs.

C2.7.6 External QAP providers include, but are not restricted to

a. APHIA FCXM QAP
b. ASHI AC QAP (Serum Antibody Screening/Identification and Crossmatching)
c. UCLA FCXM QAP (Flow and Virtual Crossmatch Exchange)

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 those shown following.

3.1 Results

G3.1.1 Results should be reported for each untreated and pronase treated B and T cell population.

G3.1.2 Each result should include the raw linear data, calculated channel shift or ratio and the positive/negative result.

G3.1.3 An interpretive comment must be made involving a competent tissue typing flow cytometrist.

C3.1.3 Result interpretation should take into account HLA antibody results acquired by solid phase assay (Luminex technology) where available.

3.2 Reports

G3.2.1 The report must contain the sample name (including a second unique identifier), collection date(s) of each sample(s), specimen source/type and the date tested.

G3.2.2 The report must include appropriate advice on the crossmatch results in the context of the patient's antibody profile.

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PROCEDURAL REFERENCES

For background on the methods, descriptions of methodology, interpretation and publications recommend:

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