ACS GUIDELINE FOR

FETOMATERNAL HAEMORRHAGE

TESTING

First Edition 2022

Australasian Cytometry Society Guideline Document

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To be read in conjunction with ACS '*Guidelines for Clinical Flow Cytometry Laboratory Practice*'.

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 Fetomaternal Haemorrhage 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 clinical flow cytometry laboratories performing assays for the detection and enumeration of fetomaternal haemorrhage. The document is intended to cover flow cytometric methods using fluorescent labelled anti-RhD and anti-HbF reagents either independently or together. The choice of method may depend on the clinical question and knowledge of the RhD status of the mother and baby.

ABBREVIATIONS

ACS	Australasian Cytometry Society
ANZSBT	Australian and New Zealand Society of Blood Transfusion
CA	Carbonic Anhydrase
CV	Coefficient of variation
EDTA	Ethylenediaminetetraacetic Acid
FMH	Fetomaternal Haemorrhage
FS	Forward Scatter
HbF	Haemoglobin F/Fetal Haemoglobin
IVD	In Vitro Diagnostic Medical Device
MU	Measurement Uncertainty
NBA	National Blood Authority
RhD	Rhesus D antigen
RBC	Red Blood Cell
SS	Side Scatter
WBC	White Blood Cell

DEFINITIONS

Adult F-cells	HbF-containing adult RBCs[1]
RhD immunoglobulin	means RhD immunoglobulin given to RhD negative pregnant and post-partum women to prevent allo-immunisation.
Count	means to acquire data on a flow cytometer
Fetomaternal haemorrhage	means physical disruption of the placental cellular barrier between fetal and maternal blood, leading to entry of fetal blood into the maternal circulation.[1]
FL1, FL2, FL3 etc.	refers to the fluorescent light detectors of a flow cytometer.
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.
Method validation	means the process of defining an analytical requirement, then confirming that the method under consideration has the performance capabilities to fulfil the stated requirement.[2]
Method verification	means procedures to test to what extent the performance data obtained by manufacturers of IVD products during method validation can be reproduced in the environments of end-users.[2]
Serological weak D phenotypes	means RBCs with no or weak reactivity with an anti-RhD reagent in initial testing, but which agglutinate moderately or strongly with anti-human globulin (a weak D test).[3]

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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.

1.1 Specimen Collection

G1.1 Samples should be peripheral blood collected into anticoagulant.[1,4]

C1.1 (i) EDTA, citrate and heparin have all been cited as suitable anticoagulants for this test.[5] Clotted samples are not suitable for testing.

C1.1 (ii) A delay of 30-45 minutes post-delivery, prior to taking the blood sample, may allow adequate mixing of maternal and fetal blood.[6]

1.2 Specimen Storage and Transport

G1.2 If there is a delay between specimen collection and analysis, transport and storage conditions must ensure the specimen's viability is maintained. Specimens should be kept between 2°C and 8°C during transit and storage.[7]

C1.2 Individual institutions may undertake sample stability investigations to demonstrate satisfactory sample stability and integrity after desired storage interval.

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 Analysis

G2.1.1 FMH assessment should be based on the expression of the RhD antigen on the RBC cell surface and/or the level of HbF in the RBCs.[1]

C2.1.1 (i) For anti-HbF methods choose a reagent which enables clear separation of fetal red cells from expected level of adult F-cells – some reagents may give a brighter signal, and therefore allow better separation, than others.

C2.1.1 (ii) For anti-RhD methods choose a reagent which enables clear separation of RhD positive fetal cells and RhD negative cells. Note results from patients with a serological weak D phenotype (0.2% - 1% of routine RhD blood typings) may be difficult to interpret using anti-RhD alone.[3]

C2.1.1 (iii) Methods using anti-RhD may not be suitable in pregnant patients where the Rh phenotype of the fetus is unknown. In these cases methods using anti-HbF are indicated.[1]

C2.1.1 (iv) Pregnant patients who have very high levels of HbF (e.g. hereditary persistence of fetal haemoglobin, some thalassaemias and haemoglobinopathies) may be difficult to interpret with assays using anti-HbF alone.[1]

C2.1.1 (v) A commercial kit is available which contains anti-carbonic anhydrase (anti-CA) and anti-HbF.[8] The anti-CA is mostly non-reactive with fetal cells and when combined with anti-HbF may assist with enumerating fetal cells in the presence of very high adult F-cells. Anti-CA is currently not commercially available as an independent reagent.

C2.1.1 (vi) A commercial IVD assay kit using anti-HbF alone is also available.[9]

G2.1.2 Assays using HbF to detect FMH should include fixation and permeabilisation steps to allow intracellular staining of HbF molecules.[1,4]

C2.1.2 Weak Glutaraldehyde solutions (e.g. 0.05%[1,4]) can be used for fixation, followed by permeabilisation with a weak detergent solution (e.g. 0.1% TritonX[1,4]). Volumes, concentrations, incubation times and temperatures, and washing steps used should follow manufacturer's instructions and/or be verified/validated (as appropriate) in-house.

G2.1.3 Analysis should be based on assessment of single red blood cells.[1]

C2.1.3 (i) FS/SS properties can be used to isolate singlets – suggest using logarithmic mode for both as this will create a tighter RBC cluster.[1]

C2.1.3 (ii) Vigorous mixing of the sample - e.g. vortexing, racking, mixing with a pipette - during processing and prior to acquisition may help to minimize aggregate formation.[1]

C2.1.3 (iii) Samples showing significant RBC aggregation (>1% of RBCs[1]) may not be suitable for analysis because of an artifactual lowering of the

numerator for percentage of fetal RBCs. Each laboratory should determine the acceptable level of RBC aggregation in-house.

C2.1.3 (iv) Exclude debris from gating to ensure accurate results. This can be achieved using a combination of a threshold/discriminator on the FS detector to exclude smaller debris particles, and tight gating of the red cell singlet population using log FS and SS (as in C2.1.3 (i)).

G2.1.4 Appropriate compensation values should be determined and applied to ensure accurate results

C2.1.4 Compensation may need to be applied to all active fluorescent detectors, due to the complicating influence of auto fluorescent events (WBCs, free neutrophilic/eosinophilic granules).

G2.1.5 The gating strategy used should ensure that true fetal cells can be identified from adult F-cells (if using anti-HbF) and contaminant particles (all methods).

C2.1.5 (i) When assessing the intensity of HbF expression, dotplots may provide better visualisation of the separation point between fetal cells and adult-F cells than histograms.

C2.1.5 (ii) WBCs and other fluorescent contaminants can be gated out of the analysis by exploiting their auto-fluorescent properties e.g. if HbF is being assessed in FL1, and FL2 is blank, WBC/WBC particles will appear positive in FL2 and can be excluded from the analysis.[1]

G2.1.6 A minimum of 100,000 red blood cells and 100 target cells (fetal cells, when present) should be acquired for an estimated CV of 10% at a limit of detection of 0.10%[1]

2.2 Performance Measures

G2.2.1 Each analytical run should include quality control material. Two levels of positive controls, and a negative control, should be run with each assay. One positive should be a low control at approximately the decision-making level for additional RhD immunoglobulin (6mL[10], approximately 0.27% fetal cells), the other a high control.[1]

C2.2.1 IVD/CE marked quality control material is available and recommended.

G2.2.2 Measurement Uncertainty (MU) of the assay should be determined by each laboratory during the verification/validation of the test, prior to implementation, and reviewed at regular intervals.

C2.2.2 Routine QC data may be used for ongoing calculation of MU.

G2.2.3 The laboratory should participate in an external quality control program.

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 Reports

G3.1 The calculation provided in the ANZSBT guidelines should be used to determine the size of the FMH in mL.[7]

G3.2 The report should clearly state the size of the FMH in mL, and may include the recommended dose of RhD immunoglobulin.[7,10]

C3.2 (i) Reporting the % of fetal cells is not recommended, as this is a possible source of confusion when interpreting the results.[7,10]

C3.2 (ii) Very low or negative results should be reported according to the sensitivity limit of the assay, as determined in-house, for example <2.2mL (equivalent to <0.1% fetal cells).

C3.1 (iii) Positive results may be phoned to ensure timely administration of RhD immunoglobulin and/or appropriate clinical care. The level at which this occurs should be determined by each institution.

G3.3 The administration of RhD immunoglobulin should be guided by the RhD status of the mother.

C3.3 Consult the NBA guidelines for the administration of RhD immunoglobulin.[10]

G3.4 **If the RhD status of the mother is not known this should be stated in the report to ensure clinical follow-up.**

C3.4 Tables and/or statements may be included in the report as guidance for different clinical scenarios.

G3.5 **Reports should be completed and released within 72 hours of delivery or sensitising event, as per the ANZSBT guidelines.[7]**

C3.5 Different clinical scenarios, e.g. large FMH, may require more urgent results. Situations which require a more urgent result should be determined by each institution and procedures documented accordingly.

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ACS guideline documents are available on the website: www.cytometry.org.au

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