ACS GUIDELINE FOR

EOSIN-5-MALEIMIDE TESTING OF RED BLOOD CELLS

Second Edition 2019

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*'.

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 Staining for Abnormalities in Red Cell Membranes Using Eosin-5-Maeimide* 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

ACS	Australasian Cytometry Society
CDA Type II	Congenital Dyserythropietic Anaemia Type II
CHC	Cryohydrocytosis
E5M/EMA	Eosin-5-Maleimide
EDTA	Ethylenediaminetetraacidic acid
FSC	Forward scatter
HS	Hereditary spherocytosis
MCF	Mean channel fluorescence
RBC	Red blood cell
SAO	South East Asian Ovalocytosis
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SS	Side scatter
WBC	White blood cell

DEFINITIONS

Count	means to acquire data on a flow cytometer
Doublet	two cells in the sample core stream seen as one by the flow cytometer
Competent 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
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
Mean Channel Fluorescence	calculated mean of the fluorescence channels measured for a sample or group of samples (e.g., the 6 x normal controls).
Side Scatter (SS)	measurement of light at right angles to the incident light source. Note: This measurement is related to the internal and surface complexity of a cell or particle, i.e., cytoplasmic granularity, membrane irregularity, and/or nuclear shape1
Threshold	discriminator set to a defined level and only signals with an intensity greater than or equal to the threshold channel value will be processed and sent to the computer.

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

A reduction in fluorescence intensity of Eosin-5-Maleimide (EMA) binding to red blood cells (RBC) indicates a membrane protein-associated disorder highly predictive for HS, the RBCs of SAO and CHC. Some CDA Type II can also show decreased fluorescence [(1)].

The Eosin-5-Maleimide (EMA) binding test validated by SDS-PAGE methodology demonstrates membrane deficiency is present when patient RBCs showed a reduced MCF of EMA [(2; 3)]. The EMA binding test gave reduced fluorescence readings for RBCs with a combined deficiency of ankyrin/spectrin. There are discrepant findings for isolated ankyrin only deficiencies using the EMA binding assay [(2; 4)].

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.

G1.1 Specimen Collection

G1.1 The anticoagulant of choice for the EMA binding test is EDTA with a collection of 0.5-1.0 mL of peripheral blood [(1)].

C1.1. (i) Other anticoagulants should be validated for comparative performance [(1)].

C1.1 (ii) Patients with red cell abnormalities are sometimes transfused. Pretransfusion samples are preferred.

G1.2 Specimen Transport and Storage

G1.2.1 Specimens should be maintained at 4°C from through collection to transport, analysis and subsequent storage [(1).]

C1.2.1 (i) Low temperature minimizes protease activity from the WBC fraction as well as RBC protease.

. C1.2.1 (ii) Patients with haemolytic anaemias have elevated levels of reticulocytes, thus higher levels of protease activity and should be tested more quickly.

G1.2.2 Samples should be tested within 4 days provided storage conditions have been adhered to [(1)].

C1.2.2 (i) RBC lysis should be noted. Once red cell lysis has commenced not only are proteases in RBCs activated, but the fragile population of interest will be significantly reduced giving a higher probability of a false-negative result [(1)].

. C1.2.2 (ii) Some rare RBC disorders may undergo lysis with storage at 4^{o}C e.g. CHC variant of Hereditary Stomatocytosis.

. C1.2.2 (iii) Testing on samples after 5 days should be avoided [(4)] .

2. ANALYTICAL PHASE

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

G2.1 Reagents

G2.1.1 EMA is light sensitive and unstable above minus 20^{0}C and must be stored frozen until use [(1)].

C2.1.1 Refer to manufacturer's instructions for handling and storage. Alternatively once reconstituted it is recommended aliquots be stored at minus 80^{o}C. After thawing for testing it should be discarded at end of the day [(1)].

G2.2 Sample analysis

G2.2 Whole blood should be washed with isotonic solution, spun and washed packed RBCs used for testing.

C2.2 Forming a pellet maintains RBC concentration consistency.

G2.3 Performance Measures

G2.31 A minimum of 15,000 red cell events should be acquired for each tube assayed to achieve adequate test sensitivity [(1)].

C2.3.1 Acquisition threshold/discriminator is reduced to ensure RBC with low MCV are captured. Doublets are reduced by using low flow rates.

G2.3.2 Six normal date-matched controls should be setup with the sample [(1)].

C2.3.2(i) Healthy adult RBCs are used as normal controls as well as for checking the performance of the flow cytometer. They must have normal RBC indices, no haematological malignancies and a normal biochemistry profile.

C2.3.2(ii) The patients Mean Channel Fluorescence (MCF) staining of EMA on RBCs is compared with the normal controls, expressed as a ratio to exclude analyser laser drift.

C2.3.2(iii) A positive control for HS is not essential because the defect is always associated with loss of fluorescence [(1)].

C2.3.2(iv) A minimum of four normal controls are recommended for valid reporting after outliers excluded.

G2.3.3 The ratio of Mean Channel Fluorescence (MCF) staining of EMA on RBCs for patient against normal controls should be calculated.

C2.3.3(i) Using results expressed as a ratio minimises the effect of analyser laser drift and variation in EMA aliquots.

C2.3.3(ii) Laboratories need to determine in-house reference ranges for ratios reported on EMA staining.

G2.3.4 In the absence of external QAP/QC programs laboratories should participate in inter laboratory testing of shared samples if possible.

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 Reports should provide a ratio of the test/average of normal controls [(1)].

C3.1 MCF of the test sample and control average may be included

G3.2 Reports should include reference interval for ratios of normal and abnormal populations.

C3.2 A reference range including abnormal samples should be calculated preferably over multiple assays and include 20 abnormal samples [(1)].

G3.3 Sample condition should be included on reports where relevant e.g. haemolysis, history of recent transfusion

REFERENCES CITED

1.H52-A2 Red Blood Cell Diagnostic Testing Using Flow Cytometry; Approved Guideline – Second Edition. Clinical and Laboratory Standards Institute

2. King MJ, Behrens J, Rogers C, Flynn C, Greenwood D, Chambers K. Rapid flow cytometric test for the diagnosis of membrane cytoskeleton-associated haemolytic anaemia. Br J Haematol. 2000;111(3):924-933.

3. Bianchi P, Fermo E, Vercellati C, et al. Diagnostic power of laboratory tests for hereditary spherocytosis: a comparison study in 150 patients grouped according to molecular and clinical characteristics. Haematologica. 2012;97(4):516-523.

4. Giroden f, Garcon L, Bergoin E. et al. Usefulness of the eosin-5'-maleimide cytometric method as a first-time screening test for the diagnosis of hereditary spherocytosis: comparison with ektacytometry and protein electropherosis. *Br J Haematol*, 2008;140(4):468-470.

PROCEDURAL REFERENCES

For setting up the EMA assay and the following reference is highly recommended:

H52-A2 Red Blood Cell Diagnostic Testing Using Flow Cytometry; Approved Guideline – Second Edition. Clinical and Laboratory Standards Institute

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

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