

AUSTRALASIAN FLOW

CYTOMETRY GROUP

July 2005

RECOMMENDED GUIDELINE

STANDARDS FOR SCREENING OF HEREDITARY

SPHEROCYTOSIS

2005 COMMITTEE MEMBERS

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14. SCEENING FOR HEREDITARY SPHEROCYTOSIS

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14.1 INTRODUCTION

The aim is to differentiate between hereditary spherocytosis and immune and non-membrane-associated haemolytic anaemias. Measuring the fluorescence intensity of eosin-5-maleimide (EMA) labelled red cells has been shown to be an effective screen for the detection of hereditary spherocytosis. EMA predominantly binds covalently to the ϵ -NH₂ group of the lysine-430 in the band 3 protein of the red cell cytoskeleton. The red cells of patients with hereditary spherocytosis have a lower EMA mean channel fluorescence than normals and other haemolytic anaemias. The analysis is performed on peripheral blood samples.

14.2 SPECIMEN COLLECTION, TRANSPORT AND INTEGRITY

14.2.1 Specimen Collection

14.2.1.1 Universal precautions should be strictly observed when collecting blood samples (see 1.1 Safety Guidelines).

14.2.1.2 All samples submitted for testing must be immediately labeled with the date and time of collection and at least one unique patient identifier (complying with local regulations). A request form with a unique patient identifier, presumptive diagnosis, age, sex, date and time of specimen collection, name of physician and test required should accompany all samples⁷.

14.2.1.3 EDTA anticoagulated peripheral blood kept at 4 °C is recommended. Samples should be tested within 24 hours of collection. This time may be extended if the laboratory can demonstrate that the results are reproducible over the extended period.

14.2.1.4 Any specimen that is over 24 hours old (or over the above established time period), unlabelled, incorrectly labeled or of insufficient volume should be recollected.

14.2.2 Specimen Transport

14.2.2.1 Packaging, labeling and transport of specimens should comply with all current local, state, national and international regulations for the regions through which the specimens will pass.

14.2.2.2 It is recommended specimens are maintained at 4 °C in a waterproof container.

14.2.3 Specimen Integrity

14.2.3.1 Visually inspect the specimen for clots, haemolysis or container defects. Where appropriate, recollect the sample if the specimen shows any visual signs of deterioration.

14.2.3.2 Specimens that are collected or transported outside of these guidelines should be treated with caution. The deficiencies in the sample should be noted and the report should reflect the effect that these deficiencies may have on the results.

14.3 SPECIMEN PROCESSING

14.3.1 The red cells should be washed with Dulbecco's A phosphate buffered saline **prior to labeling with** Eosin-5-maleimide (dye).

14.3.2 EMA is light sensitive and hydrolyses in aqueous buffer. Once labeled it is recommended the **red** cells are incubated in the dark for 1 hour at room temperature with intermittent mixing. This time may be **shortened** if the laboratory can demonstrate that the results are reproducible over the **shortened** period.

14.4 CONTROLS

14.4.1 Unlabelled cells may be used to optimise the red cell light scatter settings, set the acquisition region around the red cells and to establish a positive analysis region for the EMA/count histogram.

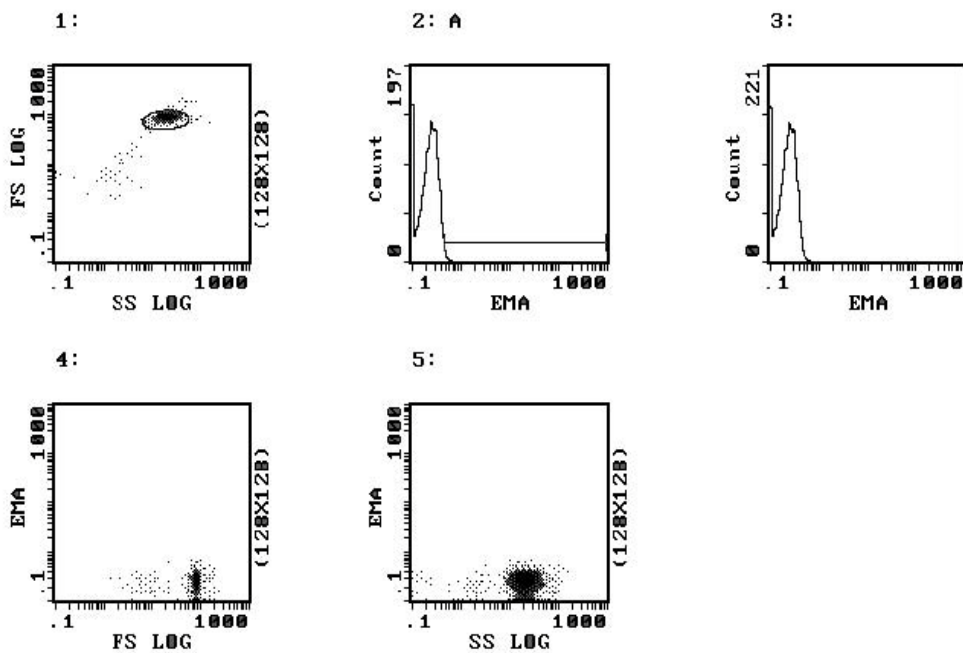
14.4.2 **Red** cells labeled with Glycophorin A (CD235a) may be used to check the efficiency of the gating.

14.4.3 **It is recommended** six normal controls **are** set up per assay.

14.5 SAMPLE ANALYSIS

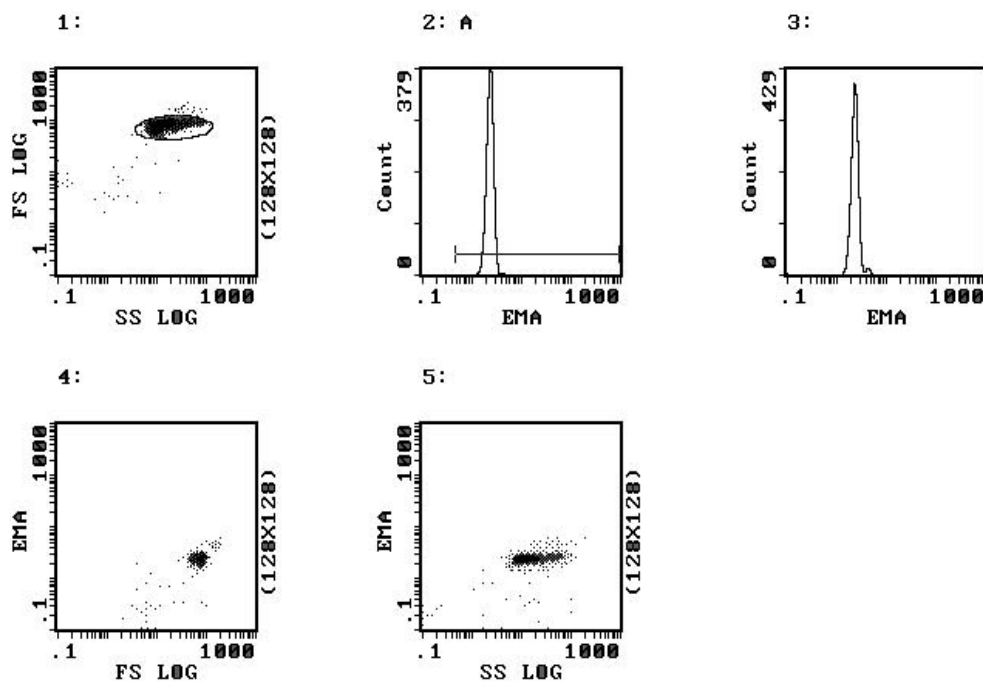
14.5.1. Red cells **are preferably** gated using log forward scatter/log side scatter

14.5.2 The first tube should contain unlabelled cells to maximise the cells of interest,



minimise contamination and set the positive analysis region⁸.

14.5.3 Red cells labeled with CD235a may then be run to determine the percent of red cells within the gate followed by the subsequent test panel.



14.5.4 This is an example of a normal EMA result⁸.

14.5.5 There should be good resolution between the unstained and positive cell populations.

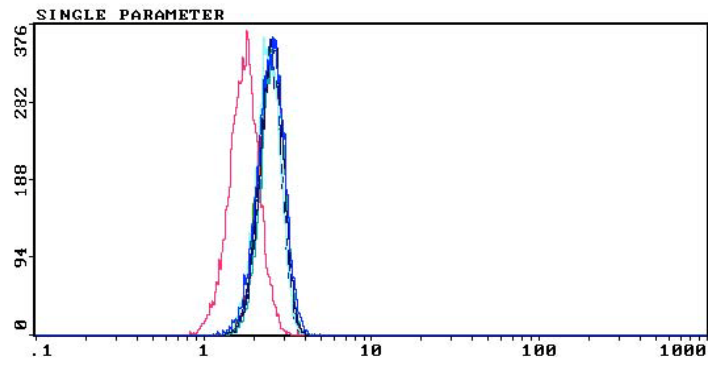
14.5.6 At least 15,000 gated events should be counted for each tube in the FL1 channel (green fluorescence).

14.5.7 The mean channel fluorescence (MCF) for the patient and normal controls should be measured.

14.5.8 The mean and the range for the normal controls samples should be calculated.

14.5.9 The patient's results should be compared to the mean and range of the normal controls.

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HS Patient coloured Pink and the Controls Blue⁸

14.5.10. Some groups look at calculating a ratio comparing the fluorescence intensity of the normal controls against HS patients to determine whether a patient has Hereditary Spherocytosis⁹.

14.6 DATA REPORTING

14.6.1 Report all unique patient identifiers including name/code, medical record number, laboratory ID/accession number and collection date/time as well as print date/time.

14.6.2 Report the patient's MCF.

14.6.3 Report the mean of the MCF and range of the MCF for the normal controls.

14.6.4 Each laboratory should establish reference limits to differentiate between the normal, **equivocal** and hereditary spherocytosis.

14.7. DATA STORAGE⁷

14.1. Both the Data stored and the methods used to obtain the data must be thoroughly documented.

14.2 The variability observed in LS patterns in patient samples requires that data be stored in list-mode form because gating may not be assignable *a priori*.

14.3. All primary files, worksheets and report forms must be retained for 2 years or as required by state or local regulations which ever is longer.

14.4. After the retention period disposal is at the discretion of the Laboratory Director.

14.5. QA and QC data files should include all parameters, analysis regions and analytical results used to verify performance (instrument and method).

14.6. Data may be stored as paper hard copy and/or archival files.⁹

14.8 NOTES

14.8.1 Red cells tend to agglutinate in the presence of albumin or other proteins that may be present in wash solutions.

14.8.2 Reproducibility of results is partially dependent on EMA stability and concentration. The dye should be stored at -20°C for no more than 4 months. This time may be extended if the laboratory can demonstrate that the results are reproducible over the extended period.

14.8.3 A reduction in EMA binding is not totally specific for hereditary spherocytosis. The mean channel fluorescence is also reduced in Congenital Dyserythropietic Anaemia Type II (CDAII), South-east Asian Ovalocytosis (SAO) and cryohydrocytosis. These can be differentiated from HS by their distinct clinical features. Hereditary Pyropiokilocytosis (HPP) has a distinctive reduced MCF.

14.9 QUALITY ASSURANCE

14.9.1 Analysis should include internal reliability checks of results, such as unlabelled red cells to determine the positive analysis region and CD235a to verify the presence of red cells.

14.9.2 Regulatory bodies currently require that a laboratory keeps all equipment maintenance and calibration records, staff training records, up-to-date method protocols,

daily operator/reagent records, verification of transcription of results from machine printouts, procedures for amendment of results and checks by supervisors/pathologists.

14.9.3 Where possible, the laboratory should belong to and participate in a recognised external quality assurance program with regular review of the results.

14.10 NORMAL RANGES

14.10.1 Each laboratory should determine its own reference limits because significant laboratory to laboratory differences related to these variables have been reported.

14.10.2 Data should be collected on a randomly chosen set of representative individuals.

14.10.3 Accumulate and analyse reference data in smaller sets (e.g. 30 individuals), which can then also be pooled and analysed. If the last two sets of pooled data are found to give the same reference range within experimental error, this gives increased confidence that the reference range selected is not unduly affected by the small sample size.

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