

ACS GUIDELINE FOR PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA IMMUNOPHENOTYPING

Third Edition 2026

Paper-based publications

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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 Paroxysmal Nocturnal Haemoglobinuria (PNH) 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.

Reference 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

ACD	Acid citrate dextrose, anticoagulant
EDTA	Ethylendiamineteraacetic acid, anticoagulant
FITC	Fluorescein isothiocyanate, fluorochrome
FLAER	Fluorochrome-conjugated (Alexa 488) non-lysing mutated form of proaerolysin
FSC	Forward Scatter
PE	Phycoerythrin, fluorochrome
SSC	Side Scatter

DEFINITIONS

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
Markers	means antigens on cells of interest used for diagnostic purposes
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.

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 and 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 Peripheral blood is required as expression of GPI-linked proteins varies during maturation. Thus, bone marrow samples are not acceptable.

C1.1 Collect venous blood into a vacutainer tube containing an anticoagulant such as ethylenediamine tetra-acetic acid (EDTA). Heparin and ACD are also acceptable ^(1,2).

1.2 Specimen Storage

G1.2 Store at room temperature and at 4 degrees Celsius for prolonged storage.

C1.2(i) The test should be performed within 48 hours of collection⁽³⁾ for white blood cells (WBC) but up to 72 hours (EDTA) may be acceptable. ACD samples are stable for 72 hours at Room Temperature.

C1.2(ii) For red blood cells (RBC), it is preferable that samples are tested within 48 hours but samples stored for up to one week at 4°C may be adequate ⁽³⁾.

C1.2(iii) Note: Type III PNH cells may haemolyse in vitro during storage.

1.3 Specimen Preparation

G1.3.1 Testing of WBC neutrophils and monocytes is essential. Testing of RBCs is required to determine PNH subtypes. RBC testing alone is not adequate as it may result in underestimation of the size of PNH clones in the presence haemolysis or transfusion ⁽³⁾.

C1.3.1 It is noted there may be insufficient neutrophils or monocyte depending on disease presentation in which case diagnosis can be based upon red cell populations and neutrophils or monocytes.

G1.3.2 RBCs may be washed in clean PBS.

C1.3.2 For RBCs the presence of protein may promote agglutination of RBCs, particularly when there are numerous antibodies present.

G1.3.3 For WBC the use of a stain-lyse-wash procedure is preferable ^(3,11). Samples do not need to be washed prior to WBC testing alone.

C1.3.3(i) The mean fluorescence index (MFI) may be higher when the prelysing methods are used as it avoids the difficulties introduced by the presence of large numbers of RBCs and all major sub-populations of WBCs are less heterogeneous ⁽⁴⁾. This may be beneficial in the context of leucopenia.

C1.3.3(ii) To increase the number of leucocyte acquisition in leucopenic samples, it may be necessary to stain a larger volume of sample, using an increased volume of reagents, or staining multiple tubes with standard volume and combining acquisition data.

2. ANALYTICAL PHASE

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

2.1 Reagents

G2.1.1 Markers are required for lineage gating (non GPI-linked, lineage specific), and for assessing PNH status (GPI-linked). Deficiency of two or more GPI-antigens is required on more than one lineage, typically RBCs and neutrophils, to diagnose PNH. Monocytes should always be assessed and may be helpful where neutropenia is severe.

C2.1.1(i) Testing for an RBC clone may not be necessary if screening of neutrophils and monocytes does exhibit a PNH clone.

G2.1.2 Erythrocyte markers: CD235a is recommended for RBC gating ⁽¹²⁾. At least one GPI-linked marker shall be used for the detection of GPI-deficient (PNH) RBC clones ⁽³⁾. CD59 is recommended using monoclonal antibody clones MEM-43 and OVA9A2 ⁽⁵⁾.

C2.1.2(i) CD55 is less abundantly expressed on RBCs and is not recommended as a sole agent as it does not provide adequate separation of class II and class III RBCs.

C2.1.2(ii) PE flouochrome is recommended for CD59. FITC may cause agglutination ⁽⁵⁾.

C2.1.2(iii) Glycophorin A (CD235a) is optional as a RBC lineage marker for routine screening but recommended for high sensitivity testing. CD235a has a very high surface density on RBCs. Correct titre of antibody is required to avoid RBC aggregation ⁽³⁾.

G2.1.3 For high sensitivity testing for minor populations of RBCs with a deficiency of GPI-linked proteins, dual staining is preferable with CD235a and CD59.

G2.1.4 WBC markers: A minimum of two GPI-linked markers shall be used for the detection of PNH in each WBC (CD45+) lineage assayed ⁽³⁾.

G2.1.5 Granulocytes: FLAER with CD24-PE or CD157-PE ⁽¹¹⁾ is recommended to detect GPI-deficient neutrophils.

C2.1.5(i) CD59 is not recommended as it can give false positive neutrophil clones ⁽⁶⁾.

C2.1.5(ii) CD16 is not recommended as polymorphisms may cause false positive neutrophil clones in MDS ⁽³⁾.

C2.1.5(iii) Lack of CD157 in non-PNH cases has been reported, so results should be interpreted with caution, in conjunction with other results and clinical picture.

G2.1.6 Monocytes: FLAER with CD14-PE or CD157-PE is recommended to detect GPI-linked monocytes⁽¹¹⁾.

C2.1.6(i) Lack of CD157 in non-PNH cases has been reported, so results should be interpreted with caution, in conjunction with other results and clinical picture.

G2.1.7 WBC Lineage markers:
For gating neutrophils, CD15 is recommended ^(3,11).
For gating monocytes, CD64 is recommended, as this is beneficial in MDS/CMML ^(6,7,11).

C2.1.7 CD33 may be used for gating neutrophils or monocytes but is less effective with multi-lineage dysplasia, MDS, and CMML. Note that contamination from cells of other lineages (e.g.

lymphocytes in the monocyte gate) may not express the same markers, thus may give false positive clones.

2.2 Data Analysis

G2.2 Analysis Gating: At least two lineage antibodies which are not GPI-linked shall be used to gate each WBC lineage to permit accurate identification.

- C2.2(i) Granulocyte gating: CD45/CD15 plus CD45/SS are recommended ^(3,11).
- C2.2(ii) Monocyte gating: CD45/CD64 plus CD45/SS are recommended ^(3,11).
- C2.2(iii) RBCs can be identified by gating by their light scatter properties. Use of logarithmic scale FSC/SSC helps to exclude debris including platelets and aggregates ⁽³⁾.
- C2.2(iv) For high sensitivity testing, RBCs should be gated by their light scatter properties and CD235a. This permits accurate lineage definition and permits reproducible detection of GP-deficient populations of less than 1.0% of erythrocytes.

2.3 Performance Measures

G2.3.1 **Minimum events: For routine purposes acquisition of 5,000 RBC events and 50,000 total events for neutrophils is recommended.**

C2.3.1(i) The identification of a PNH clone should be achieved with the detection of 50 -100 events in the negative gate. With the acquisition of the minimum events stated above, a minimum of 5,000 events of the target cell type is required to achieve a sensitivity of 1% (50 events) ⁽³⁾.

C2.3.1(ii) If a lesser number of clonal events is obtained consider additional testing to obtain required number of events for statistical validity (eg longer acquisition, increased number of target cell population, running multiple tubes and merging files).

G2.3.2 **For higher sensitivity testing⁽³⁾, acquisition of at least 500,000 events of the target population is required to achieve a sensitivity of 0.01% (50 events).**

C2.3.2(i) High sensitivity assays are not needed for the diagnosis of classic PNH but are much more useful for the detection of small PNH populations in patients with BM failure disorders (AA, MDS, RCUD). High sensitivity testing aims at detecting very small populations for the use of emerging therapies and is useful in long term management of AA/MDS patients ⁽³⁾.

C2.3.2(ii) Higher sensitivity results may not be achievable in neutrophil and monocyte assays. Sensitivities on 0.1% in neutrophils and 0.5% in monocytes is likely adequate for diagnostic purposes.

G2.3.3 Controls: The cells from a normal subject can be tested to verify reagent integrity (e.g. on receipt of new reagents).

C2.3.3 A normal sample within a run is sufficient as a control for antibodies tested. Normal cells within the sample may serve as internal controls ⁽³⁾.

G2.3.4 Each laboratory should establish the sensitivity of the test for both erythrocytes and leucocytes.

C2.3.4 Backgrounds can be tested using normal samples recording (e.g. <0.5%, <0.01%).

G2.3.5 The laboratory should participate in an external Quality Assurance Program for PNH Immunophenotyping.

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. Reporting

G3.1 The size of the PNH clone should be reported for all of the cell lineages tested. Subtyping of RBC clonal populations is recommended ⁽³⁾.

C3.1(i) In the majority of the patients with classic PNH, the size of the neutrophil and monocyte PNH clone will be larger than PNH RBC clone. This is due to the shortened RBC survival of PNH RBCs when compared to normal RBCs and to the effect of dilution of the abnormal population by the transfusion of RBCs in some patients.

C3.1(ii) Classic PNH will usually have >10% of cells from at least two lineages in the abnormal RBC clone, unless the patient had been recently transfused. For RBCs, the percentage of Type II and Type III cells should be reported both at diagnosis and for follow-up.

C3.1(iii) The level of significance for reporting of figures should reflect the level of test sensitivity (e.g. for routine testing rounded figures, without decimal places).

G3.2 For high sensitivity testing, limits of detection should be included on reports.

APPENDIX A

Principle of PNH Testing

Flow cytometry evaluation of erythrocytes and neutrophils provides a rapid, sensitive and specific test for screening and identification of PNH clones. Testing for the absence of GPI-linked proteins on both RBCs and neutrophils is recommended for the diagnosis of PNH. It is recommended that two GPI-linked proteins are assessed to confirm PNH, as rare hereditary disorders may lack a specific cell surface antigen causing negativity with one antibody and the sensitivity of the test is increased by assessing negativity for two GPI-linked proteins.

- The percentage of neutrophils with the PNH clone most accurately reflects the size of the PNH clone and can be correlated with haemolysis. A wide variety of different altered expression profiles are found between patients.

The size of the PNH clone can be underestimated by analysis of the red cell clone which may be relatively reduced due to haemolysis and/or possible recent transfusions. For the testing of WBC, it is preferable to analyse neutrophils and confirm the findings on the monocytes. If both the neutrophils and monocytes are low in number, it may be necessary to enrich the leukocytes e.g. test a larger sample volume or pre-lyse.

Testing of lymphocytes is not recommended as the prevalence of GPI negative cells may be underestimated in lymphocytes due to the long life of the normal clones of B and T-cells.

- Patients with established PNH should have their clone size measured at regular intervals. Annual monitoring is sufficient if the disease is stable. In patients on Eculizumab, regular monitoring is suggested until disease stability is achieved.
- A working classification has been proposed that classifies patients into three subgroups⁽¹⁾:
 1. **Classic PNH**: characterised by overt episodes of intravascular haemolysis.
 2. **Hypoplastic PNH**: in the setting of another marrow disorder, with no overt haemolysis (e.g. PNH/aplastic anaemia or PNH /refractory anaemia/ myelodysplasia)
 3. **PNH subclinical (PNH-sc)**: PNH cells present in the setting of another marrow disorder (e.g. PNH-sc/aplastic anaemia). Overt symptoms of PNH are not present.

Clinical Significance

On erythrocytes, GPI deficiency can be partial (type II cells) or complete (type III cells).

Cells with normal levels of GPI are referred to as type I cells. The classification of PNH RBCs derives from different lysis sensitivities with type III and type II cells being 15-25 times and 3-5 times respectively more sensitive to complement than type I cells. About 40% of PNH patients have a combination of the types I, II and III PNH cells. PNH type II and type III cells together are considered representative of PNH clones as they represent cells more sensitive to complement than are normal type I cells.

- Using multicolour flow cytometry of both erythrocytes and leukocytes, small PNH clones may be detected in aplastic anaemia and myelodysplastic/myeloproliferative disease. See review articles by Richards ^(7,8,9). Such patients rarely present with overt haemolysis as is seen in classic PNH.

APPENDIX B

GPI-linked antibodies

Suggested GPI-linked antibodies may be chosen from those mentioned in this document. The expression of GPI-linked antibodies on normal cells has been detailed ⁽⁴⁾:

- On neutrophils: The optimal markers are FLAER and CD24 or CD157.
- On monocytes: Either FLAER and CD14 or CD157 combinations are preferable. It has been suggested CD157 may be very good on both neutrophils and monocytes, and much better than CD14 on monocytes ⁽⁶⁾.
- Granulocytes and monocytes may be analysed in the same tube containing at least: one neutrophil lineage specific marker plus one monocyte lineage specific marker (e.g. CD15/CD64); at least two GPI-linked protein markers for neutrophils (e.g. FLAER and CD24 or CD157) plus two for monocytes (e.g. FLAER and CD14 or CD157); CD45 in addition improves lineage discrimination.

Evaluation of the amount of expression which can be expected has been described in publications ^(4,7,8).

APPENDIX C

FLAER

Based on current literature, the FLAER (fluorochrome-conjugated (Alexa 488) non-lysing mutated form of proaerolysin) antibody provides the strongest discrimination of the normal population from the GPI-deficient myeloid populations. This is a non-lysing inactive form of the bacterial toxin *aerolysin* which binds specifically to GPI-anchors and is less sensitive to the maturational stage of the cells than other GPI antibodies ⁽³⁾.

FLAER cannot be used for detecting PNH RBCs as latter do not possess surface-bound proteolytic enzymes required to process the pro-aerolysin.

NB. Protect from light and from prolonged exposure to temperatures above 2-8 degrees Celsius.

Recommended Clones

Table 1. Recommended clones/conjugates for high sensitivity PNH assay on Beckman Coulter Cytometers ⁽¹¹⁾.

Target	Antibody Conjugates	Purpose	Clone (Vendor)
WBC	FLAER-Alexa488	GPI-linked	NA (Cedarlane)
	CD24-PE	GPI-linked (neuts)	SN3(eBio), ALB9(BC)
	CD14-PE	GPI-linked (monos)	61D3(eBio), RMO52(BC), Tuk4(Invitrogen)
	CD14-APC700	GPI-linked (monos)	RMO52(BC)
	CD157-PE	GPI-linked (neuts & monos)	SY11B5(eBio, BC, EXBIO, BD, Sysmex)
	CD64-PC5	Gating on monocytes	Clone 22(BC)
	CD64-ECD		Clone 22(BC)
	CD64-PC7		Clone 22(BC), 10.1(EXBIO)
	CD15 PC5	Gating on monocytes	80H5(BC)
	CD45-PC7	Debris & Unlysed RBC exclusion, pattern recognition	J33(BC)
	CD45-KO		J33(BC)

Table 2. Recommended clones/conjugates for high sensitivity PNH assay on Becton Dickinson Biosciences Cytometers ⁽¹¹⁾.

Target	Antibody Conjugates	Purpose	Clone (Vendor)
WBC	FLAER-Alexa488	GPI-linked	NA (Cedarlane)
	CD24-PE	GPI-linked (neuts)	SN3(eBio), MLB(BD)
	CD14-PE	GPI-linked (monos)	61D3(eBio), MoP9(BD)
	CD14-APC	GPI-linked (monos)	Tuk4(Invitrogen)
	CD157-PE	GPI-linked (neuts & monos)	SY11B5(eBio, BC, EXBIO, BD, Sysmex, BC)
	CD64-APC	Gating on monocytes	10.1(BD, eBio)
	CD64-PECy7		10.1(EXBIO), 22(BC)
	CD15-APC	Gating on neutrophils	HI98(BD)
	CD15-PerCPCy5.5		MEM-158(EXBIO)
	CD15-PerCPeFlour710 ^B _c		MMA(eBio)
	CD45-PerCP	Debris & Unlysed RBC exclusion, pattern recognition	2D1(eBio)
	CD45-eF450		2D1(eBio)
	CD45-APCH7		2D1(eBio)

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Editorial committee (2026)

Susan Wright (Chair) and in alphabetical order: Deni Cupit, Tania Jobey, Na Kang, Kate Marson, Eugene Ng

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First Edition	First Edition	2005	
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Third Edition	- G1.3.2, C1.3.2 – minor amendments - C1.3.3(ii) – add guidance for leucopenic samples - G2.1.2 – minor updates - G2.1.5 – minor updates. Removed CD66b from recommended - G2.2 – minor updates - G2.3.2(ii) – additional comments regarding sensitivity of WBC clones - Appendix C – addition of table of recommended clones - Updates to citations	2026	Susan Wright