

# PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA (PNH): GUIDELINES

## 1.0 Principle:

1.1 Paroxysmal Nocturnal Haemoglobinuria (PNH) is an acquired haematopoietic stem cell disorder characterised by bone marrow failure, chronic haemolytic anaemia with acute episodes and thrombosis. PNH is so-named for the dramatic manifestation of nocturnal haemolysis and morning haemoglobinuria, and was initially considered a haemolytic anaemia.

1.2 It is caused by a single mutation in the **PIG-A** gene of a haemopoietic stem cell that results in the disruption of glycosylphosphatidylinositol (GPI) synthesis and a deficiency in all GPI-anchored proteins. The **PIG-A** gene is located on the X-chromosome and hence a single mutation can result in a GPI-deficient phenotype (including females due to X-inactivation). The consequence is the absence of the GPI linked complement inhibitors CD55 and CD59 which results in blood cells susceptible to complement mediated hemolysis. Classic haemolytic PNH occurs when the majority of the stem cells harbour the PIG-A mutation.

1.3 Flow cytometry evaluation of erythrocytes and granulocytes provides a rapid, sensitive and specific test for screening and identification of PNH clones. Testing for the absence of GPI-linked antigens on both red cells and granulocytes is recommended for the diagnosis of PNH. It is recommended that two GPI-linked antigens 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 dual negativity for two GPI-linked antigens. The percentage of granulocytes 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.

1.4 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 white cells, 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 eg test a larger sample volume or prepare a buffy coat.

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.

1.5 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.

1.6 A working classification has been proposed that classifies patients into three subgroups<sup>1</sup>:

1. Classic PNH : characterised by overt episodes of intravascular haemolysis.
2. Hypoplastic PNH: in the setting of another marrow disorder, with no overt haemolysis (eg. PNH/aplastic anaemia or PNH /refractory anaemia/myelodysplasia)
3. PNH subclinical (PNH-sc): PNH cells present in the setting of another marrow disorder (eg. PNH-sc/aplastic anaemia). Overt symptoms of PNH are not present.

## **2.0 Clinical Significance:**

2.1 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 red cells 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.

2.2 Using multicolor 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<sup>2,4</sup> and Wang<sup>6</sup>. Such patients rarely present with overt haemolysis as is seen in classic PNH.

**The technical recommendations detailed below have been compiled with reference to the recent *Guidelines* published on behalf of the Clinical Cytometry Society<sup>9</sup>.**

## **3.0 Specimen Collection:**

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

Collect venous blood into a vacutainer tube containing an anticoagulant such as ethylenediamine tetra-acetic acid (EDTA) (see published methods<sup>4,8</sup>); heparin and ACD are also acceptable

Store at room temperature initially and at 4<sup>0</sup>C for prolonged storage.

**3.2 The test should be performed within 48 hours of collection for leucocytes but up to 3 days may be acceptable. For red cells, it is preferable that samples are tested within 48 hours but samples**

stored for up to one week at 4°C may be adequate.

**NB Type III PNH cells may haemolyse in vitro during storage.**

## 4.0 Specimen Preparation:

4.1 For red cells, it is important to avoid the presence of protein support in both the incubation and the washing process as this promotes agglutination of red cells, particularly when there are numerous antibodies present.

4.2 For granulocytes, the MFI is higher when the prelysing methods are used as it avoids the difficulties introduced by the presence of large numbers of red cells and all major sub-populations of leucocytes are less heterogeneous<sup>3</sup>. If the leucocyte count is low, prelysing with NH<sub>4</sub>CL may be preferable.

However, Borowitz<sup>9</sup> suggests that the stain-then-lyse method may better preserve the light scatter characteristics for clearer gating.

## 5.0 Reagents:

5.1 Erythrocyte evaluation:

At least one GPI-linked marker shall be used for the detection of PNH red cell clones :

- CD59 is recommended. CD55 is less abundantly expressed on RBC and is not recommended as a sole agent as it does not provide adequate separation of class II and class III RBC.
- The clone CD59 MEM43 PE is suggested. The FITC clone may cause agglutination
- Routinely, the RBC can be identified by their light scatter properties. Additional CD235a gating may be required for higher sensitivity assays in aplastic anaemia/MDS.

5.2 Granulocyte evaluation:

A minimum of two GPI-linked markers shall be used for the detection of PNH neutrophils or monocytes:

- Neutrophils: FLAER with CD24 is recommended and/or CD16 with CD66b or CD55. CD59 is not recommended as it can give false positives on the granulocytes. NB CD16 is absent from eosinophils and may be lost from granulocytes in MDS.
- Monocytes: FLAER with CD14 is recommended. CD14 with CD59 is also acceptable.

Refining the neutrophil and monocyte gates using CD15 or CD33 is recommended. Use of CD64 is optional for monocytes.

5.3 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 form of the bacterial toxin *aerolysin*. However, FLAER cannot be used for detecting PNH red cells 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°C.

For routine purposes. 5000 -10000 cells of interest is adequate to achieve a sensitivity of 1%.

For higher sensitivity testing, at least 50,000 events of the target population should be collected. The identification of a PNH clone can be achieved with the detection of 50 -100 events in the negative gate. If a lesser number of "negative" events is obtained, the additional event numbers should be obtained by additional testing in order to achieve statistical validity. This precision in measurement is only required to detect very small populations for the use of emerging therapies and is useful in long term management of AA/MDS patients

## **6.1 Routine Red cell analysis**

6.1.1 Single colour labelling is satisfactory for the accurate detection of GPI-deficient red cells using CD59.

6.1.2 It is critical to avoid the presence of protein both in the incubation and washing procedures as this promotes agglutination of the red cells.

NB For high sensitivity testing for minor populations of red cells with a deficiency of GPI-linked proteins, dual staining is required with CD235 and CD59. This permits accurate lineage definition and permits reproducible detection of GPI deficient populations of less than 1.0% of erythrocytes.

## **6.2 Routine Granulocytes analysis:**

6.2.1 Deficiency of two or more GPI-antigens is required on one lineage, typically neutrophils, but simultaneous analysis of the monocytes is useful for confirmation as the percentage of PNH cells will be similar for both cell types. Monocytes can also be tested if the neutropenia is severe. For these assays a minimum of 3 colours is recommended.

The use of the FLAER assay is particularly useful as it provides the strongest discrimination between PNH cells and cells with normal GPI expression.

One lineage antibody which is not GPI-linked shall be used to permit accurate lineage identification : CD33/SS or CD15/SS for granulocytes; CD64 for monocytes. CD45/SS adds further lineage definition.

6.2.2 It is important to review a blood film especially if results are discordant, as the presence of immature granulocytes can erroneously suggest antigen loss<sup>6</sup>. It is noted that immature myeloid cells show low or absent expression of many GPI-linked antigens. CD16 expression may appear to be lost on some normal neutrophils due to CD16 polymorphisms.

6.2.3 Suggested antibody combinations should be trialled and selected by the laboratory. For these antibody combinations, each laboratory shall determine their sensitivity on at least 20 normal samples.

The expression of GPI linked antibodies on normal and PNH cells has been detailed by Hernandez-Campo<sup>3</sup>.

- On neutrophils, The clone size may appear greater with FLAER/CD24 or CD16/CD66b than with CD55/CD59<sup>6</sup>. The CD55/CD59 combination is not optimal on granulocytes (as they can give rise to false positives).
- On monocytes: Either the FLAER/CD14 or CD59/CD14 combination is preferable for monocytes. It is noted that CD59 expression is low on normal monocytes and other antibodies could be trialled eg CD48, CD157.

Evaluation of the amount of expression which can be expected may be assisted by reference to the article by Hernandez Campo et al<sup>3</sup> or by Richards<sup>2,4</sup>.

## **7.0 Quality Control:**

7.1 The cells from a normal subject shall be tested to provide a normal control

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

7.3 The laboratory should participate in an external Quality Assurance program for PNH. As the condition is rare, an exchange of samples between laboratories is encouraged.

## **8.0 Reporting:**

8.1. The size of the PNH clone is reported as the higher number of either the PNH granulocytes or erythroid cells.

In the majority of the patients with classic PNH, the size of the granulocyte and monocyte PNH clone will be larger than PNH red cell clone. This is due to the shortened red cell survival of PNH red cells when compared to normal red cells and/or the effect of dilution of the abnormal population by the transfusion of red cells in some patients

8.2. In addition, the results shall be reported for the percentage of PNH cells lacking GPI-linked antigens for each cell population analysed. For erythrocytes, the percentage of Type II and Type III cells shall be reported both at diagnosis and for follow-up. Classic PNH will usually have >10% of cells from at least 2 lineages in the abnormal clone, unless the patient had been recently transfused. See appendix: report format.

## 9.0 Reference Limits:

8.4 Related to the assay specificity of each laboratory.

## 10.0 Limitations on Use:

10.1 Interpretation of results may be limited if :

- blood is old ( > 48 hours for leucocytes; > 1 week old stored at 4°C for RBC)
- samples showing signs of deterioration
- very low numbers of neutrophils and monocytes. The number of events for the target population (negative for both markers) should be 50 -100 in order that a desirable specificity is achieved.

10.2 An increase in immature myeloid cells requires careful analysis and possible confirmatory use of FLAER. Alternatively the deficiency should be studied on other lineages.

## 11.0 References:

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