

# Standardising Leucocyte PNH Clone Detection: An International Study

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## INTRODUCTION

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a haematopoietic stem cell disorder, characterised by somatic mutations within the PIG-A gene that leads to absence or reduced expression of GPI linked antigens by all blood cells derived from this mutated stem cell. PNH patients have underlying bone marrow failure often accompanied by haemolytic anaemia and an increased risk of thrombosis. Clinical management can be challenging therefore accurate and rapid diagnosis of PNH is essential. Flow cytometry is now recognised as the method of choice to detect/quantify PNH RBC and WBC clone size (1). However, the UK NEQAS proficiency testing (PT) programme has identified large variance in testing protocols and reagent selection in PNH leucocyte clone detection (2). Thus, in an attempt to achieve standardisation, UK NEQAS LI studied the use of FLAER as a single colour reagent with a standardised protocol and found it produced comparable results to those obtained using multi colour techniques (3). This follow up study further examines the use of FLAER by including it in a standardised reagent cocktail, integrating this into an internationally standardised PNH testing protocol (4) and comparing this to in-house methods.

## METHOD

UK NEQAS LI issued 3 stabilised samples (2) to 19 international centres. These were selected based on their UK NEQAS LI PNH PT programme performance and associated laboratory experience.

- Samples were manufactured to contain 0%, 0.1% and 8% PNH leucocyte clone populations
- Centres were issued with standardised, platform specific protocols and pre-titred antibody cocktails that had been optimised for use on their flow cytometer. Reagent combinations and details of fluorochromes and clones used are shown in table 1
- Centres tested all samples using both the centrally supplied standardised reagents/protocol (figure 1) and their in-house methods and local reagents
- All results were returned to UK NEQAS LI for data analysis

### Standardised Reagent Panels

Beckman Coulter Cytometers		Becton Dickinson Cytometers	
Neutrophils	Monocytes	Neutrophils	Monocytes
FLAER (Alexa488)	FLAER (Alexa488)	FLAER (Alexa488)	FLAER (Alexa488)
CD24 (PE/SN3)	CD14 (PE/61D3)	CD24 (PE/SN3)	CD14 (PE/61D3)
CD15 (PC5/80H5)	CD64 (PC5/22)	CD15 (APC/HI98)	CD64 (APC/10.1)
CD45 (PC7/J33)	CD45 (PC7/J33)	CD45 (PerCP/D2)	CD45 (PerCP/D2)

**Table 1:** Standardised panels for each flow cytometer type with details of fluorochrome and clone of each reagent shown in brackets.

### Normal Sample

	Neutrophils		Monocytes	
	In-house	Study	In-house	Study
Median	0.00	0.00	0.00	0.00
Range	0.01	0.01	0.04	0.00
Interquartile Range	0.00	0.00	0.01	0.00

### Classical PNH Sample

	Neutrophils		Monocytes	
	In-house	Study	In-house	Study
Median	8.10	8.05	8.80	8.57
Range	2.53	1.69	4.57	3.95
Interquartile Range	1.29	0.48	1.53	0.91

### High Resolution PNH Sample

	Neutrophils		Monocytes	
	In-house	Study	In-house	Study
Median	0.1	0.09	0.11	0.13
Range	0.33	0.15	0.26	0.20
Interquartile Range	0.03	0.02	0.08	0.08

**Table 2:** Comparison of range of results obtained for in-house and standardised testing

## RESULTS

- Both approaches were in consensus when reporting the presence or absence of PNH clones
- Average leucocyte PNH clone size was similar for both approaches
- For both the PNH sample with 8% clone and the High resolution PNH sample with 0.1% clone there was lower variation around the median for the standardised approach when compared to 'in-house' methods for both the neutrophil and monocyte populations (see table 2)

## DISCUSSION

Whilst the overall medians between the two approaches for leucocyte PNH clone detection (neutrophils and monocytes) were similar, the standardised approach had lower variation around the median compared to 'in-house' methods for these populations.

Our results highlight the importance of reagent choice and a standardised approach in performing PNH analysis, even amongst experienced laboratories.

Whilst this study used highly experienced laboratories additional studies and panel optimization are planned to evaluate if greater concordance can be achieved among less experienced laboratories and therefore further underline the benefits of standardisation.

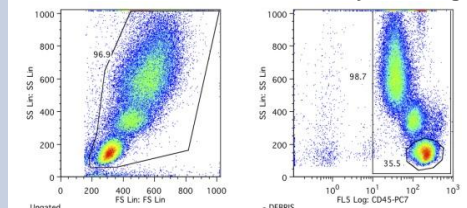
## ACKNOWLEDGEMENTS

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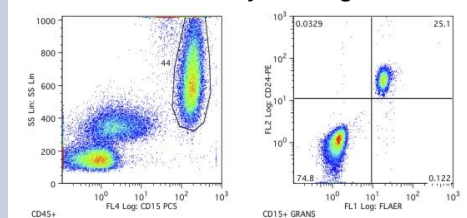
## References

1. Borowitz MJ, Craig FE, DiGiuseppe JA, Illingworth AJ, Rosse W, Sutherland DR, Wittwer CT and Richards SJ. Guidelines for the diagnosis and monitoring of Paroxysmal Nocturnal Hemoglobinuria and related disorders by flow cytometry. Clinical Cytometry 2010; 78B: 211-230.
2. Richards SJ, Whitby L, Cullen MJ, Dickinson AJ, Granger V, Reilly JT, Hillmen P and Barnett D. Development and evaluation of a stabilized whole blood preparation as a process control material for screening of paroxysmal nocturnal hemoglobinuria by flow cytometry. Clinical Cytometry 2009; 76B: 47-55
3. Whitby L, Fletcher M, Whitby A, Reilly JT, Richards SJ, Sutherland R and Barnett D. Assessment Of Fluorescently Labeled Aeryolin S And A Standard Protocol For The Detection Of Paroxysmal Nocturnal Haemoglobinuria (PNH) Cell Populations. Clinical Cytometry 2011; 80B: 410
4. Sutherland R, Keeney M and Illingworth A. Practical guidelines for the High Sensitivity Detection and Monitoring of Paroxysmal Nocturnal Haemoglobinuria Clones by Flow Cytometry. Clinical Cytometry 2012; 82B: 195-208

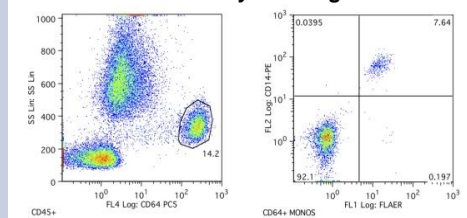
### Debris Exclusion and Leucocyte Gating



### Granulocyte Gating



### Monocyte Gating



**Figure 1:** Example of the gating strategy for granulocyte and monocyte populations in the standardised protocol.