VALIDATION OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA DETECTION BY FLOW CYTOMETRY

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Background:

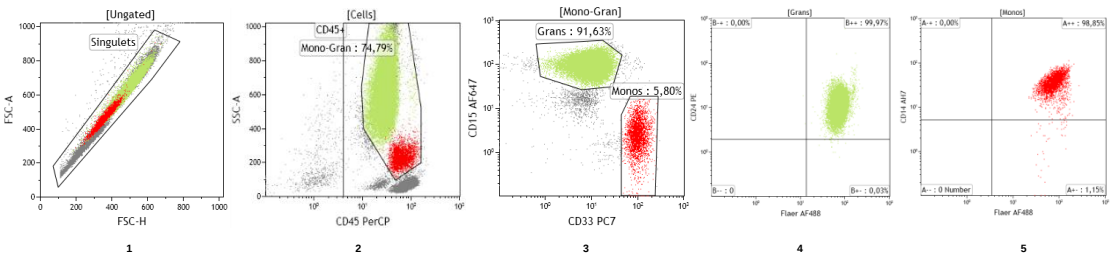
Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare and acquired disease due to a mutation, in hematopoietic stem cells, of phosphatidylinositol glycan complementation class A (PIG-A) gene leading to a partial or total deficiency of the enzyme involved in the synthesis of glycosylphosphatidylinositol (GPI). This prevents the binding of many proteins linked to the cell membrane of erythrocytes and leucocytes, such as CD 55 and CD 59. Without these 2 proteins on the red blood cells (RBC) surface, RBC are more sensitive to the lytic action of the complement system, which leads to intravascular haemolysis.

Diagnostic of PNH is carried out by flow cytometry (BD FACSCANTO II). In this study, we have followed the guidelines established by *Borowitz et al. (Cytometry B, 78B-211, 2010).* The guidelines include a first panel of antibodies to identify neutrophils and monocytes and a second panel directed against GPI-linked proteins (CD 24 and CD 14). Besides antibodies, the protocol uses an inactive variant of aerolysin (FLAER) which is directly linked to GPI anchor. When GPI-deficient leucocytes are detected, an additional panel is used to quantify GPI-deficient RBC, using glycophorin A gating and CD59. The validation of PNH detection was undertaken in accordance to ISO15189 standard and included the following parameters: intra-assay precision, bias, uncertainty of measurement, limits of detection and quantification (sensitivity), interference analysis, overall linearity of flow-cytometric measures and sample stability.

Method:

The panel used by Borowitz et al. includes Flaer AF488 - CD45 PerCP - CD33 PC7 - CD15 AF647 - CD 24PE - CD 14 AH7 provides us a sequence of analysis that discriminates neutrophils from monocytes and highlights GPI-deficient cells with **2 parameters**:

- Plot 4: GPI-deficient neutrophils are in the lower left corner (CD 24 - Flaer -)
- Plot 5 : GPI-deficient monocytes are in the left lower corner (CD 14 - Flaer -)



Results:

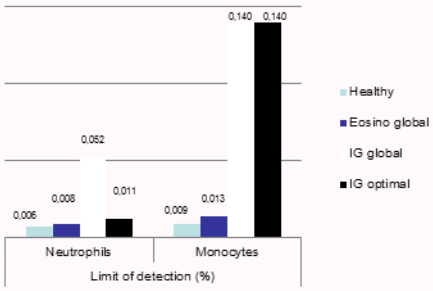
- **Sensitivity** (*N* = 30 normal samples)
  D = Limit of detection (%) = 3 * σ
  Q = Limit of quantification (%) = 10 * σ

The background of 30 normal samples allowed us to define the sensitivity of the method. As shown in the table, the method can detect GPI deficient clones as low as 0,01 %.

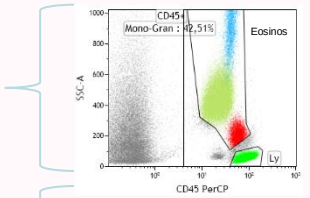
Neutrophils	Monocytes	Red Blood Cells
D = 0,006	D = 0,009	D = 0,004
Q = 0,021	Q = 0,028	Q = 0,012

- **Interferences by eosinophils and immatures granulocytes**

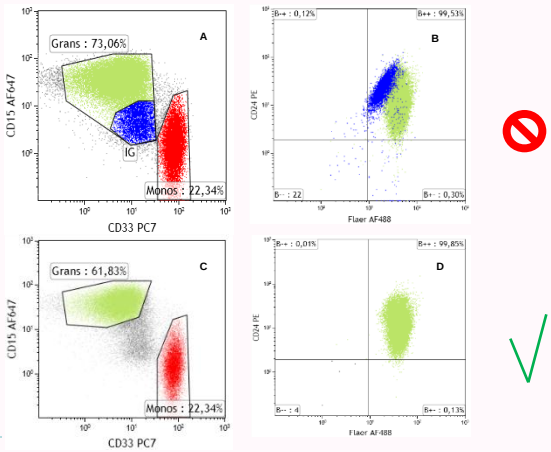
Samples with eosinophilia or immature granulocytes were tested (N=30 for each category)



The estimation of detection limit (left) and analysis of cytograms (right) show that eosinophils don't cause interference in the PNH assay.



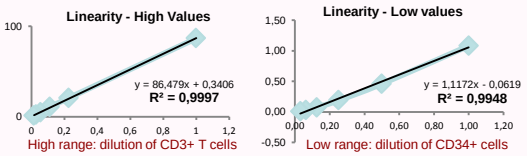
Immature Granulocytes (IG) can induce many interferences if gating is not precise enough (plot A & B). However, even after gating off IG, some false GPI deficient events are detected (plot C & D).



- **Intra-assay precision**

GPI deficient events (%)	Neutrophils	Monocytes	RBC
Mean	10,59	9,75	11,24
Standard deviation	0,15	0,42	0,11
Coefficient of variation (%)	1,38	4,28	1,02

- **Overall linearity of cytometric measures**



- **Combined uncertainty of measurement u_c (neutrophil analysis)**: based on 15 external quality controls (UKNEQAS), and computed from our average bias and the group dispersion with regards to the median.

	CD 24	Flaer
u_c (%)	9,50	9,38

- **Stability**

Typing must be done within 2 days for WBC analysis and within 7 days for RBC, to avoid antigen alteration. Samples must be stored at 4°C. Stained samples must be analyzed at 1 to 3 hours after fixation.

Conclusion:

The detection of PNH by flow cytometry may be validated according to ISO15189 standard, when using published international guidelines. The estimation of linearity should be completed with dilutions of a sample with a large PNH clone.

References:

- Borowitz *et al. Guidelines for the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria and related disorders*. Cytometry Part B 2010; 78B: 211-230.
- Sutherland DR *et al. Praticcal guidelines for the high-sensitivity detection and monitoring of paroxysmal nocturnal hemoglobinuria clones by flow cytometry*. Cytometry Part B 2012; 82B: 195-208.
- COFRAC. *Guide de vérification/validation des méthodes en biologie médicale*. SH GTA 04 – rév. 00 – Avril 2011.