Best Practices for High-Sensitive PNH WBC and RBC assays

Andrea Illingworth MS  
Dahl Chase Diagnostic Services

Rob Sutherland MS  
University of Toronto

ESCCA 2016  
Edinburgh, Scotland
Guidelines for the Diagnosis and Monitoring of Paroxysmal Nocturnal Hemoglobinuria and Related Disorders by Flow Cytometry


on behalf of the Clinical Cytometry Society

### Possible WBC reagent combinations: ICCS guidelines 2010

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<td>CD33</td>
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<tr>
<td>6 color</td>
<td>G+M</td>
<td>FLAER</td>
<td>CD24</td>
<td>CD14</td>
<td>CD15</td>
<td>CD33</td>
<td>CD45</td>
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Slide courtesy A Illingworth and DR Sutherland


Current status of PNH Testing

- Review of current status of previously available panels based on previous 2010 and 2012 Guidelines
  - 2C RBC panel
  - 4C granulocyte and monocyte reflex tube
  - 5C CD157 WBC tube
  - New 6C and 7C Navios and Canto panels

- Review of currently used antibodies
  - GPI markers
  - Gating antibodies
High-Sensitivity Flow Cytometry Is Needed for Accurate Diagnosis and Monitoring

41% of PNH+ Samples Show a Clone of <1% \(^1\)

Dahl-Chase data Dec 2007 - Aug 30, 2014

Slide courtesy of A Illingworth
High-Sensitivity Detection & Monitoring of PNH

- ICCS provided generic guidelines for the diagnosis and monitoring of PNH by flow cytometry\(^1\)

However, the following were not addressed:
  - Specific reagents/reagent cocktails (RBCs and WBCs)\(^2\)
  - Detailed standardized staining/processing protocols\(^2\)
  - Detailed instrument set-up/compensation strategies\(^2\)
  - Associated detailed analytic strategies\(^2\)
  - Validation of sensitive, standardized methodologies\(^2\)

- The development and validation of sensitive standardized methodologies are necessary to reliably detect PNH clones <1%\(^2\)

2. Sutherland DR et al. Cytometry Part B. 2012;82B:195-208

Slide courtesy of A. Illingworth and D.R. Sutherland
Practical Guidelines for the High-Sensitivity Detection and Monitoring of Paroxysmal Nocturnal Hemoglobinuria Clones by Flow Cytometry

D. Robert Sutherland, Michael Keeney, and Andrea Illingworth

RBC panel and sample requirements

- **Specimen**
  - Peripheral Blood in EDTA most tested but Heparin or ACD acceptable
  - Bone marrow not recommended

- **GPI-linked antibody**
  - CD59 best delineates PNH Type III, Type II and normal Type I RBCs
  - Some PE conjugates perform poorly – test and validate!
  - PE-conjugated MEM43 and OV9A2 most tested / validated clones
  - CD55 too dim to delineate PNH Type III, Type II and normal RBCs

- **Gating antibody**
  - CD235a is the only available RBC-specific reagent
  - Most CD235a conjugates perform poorly even after titration!!
  - KC16 and 10F7MN most tested / validated clones
  - Negatively-charged FITC conjugates minimize aggregation
RBC Assay – Instrument Setup

- **Forward and Side Scatter Adjustment**
  - Use Log FS versus Log SS (not linear)
  - Place RBC population in middle of plot so if aggregates are present, they are clearly visible
  - Ensure that discriminator does not exclude *bona fide* RBCs

- **Voltage Adjustment of PMTs**
  - Adjust voltage of PMTs specific for the RBC assay
  - Unstained RBCs should be ‘on-scale’ in both FL1 (CD235a-FITC) and FL2 (CD59-PE) parameters*

- **Collect ‘TIME’ parameter**

- **Compensation**
  - Set two-color compensation specific for the RBC assay
  - Validate with stained and unstained normal samples

*If FC500 or later in use DO NOT use “baseline offset”

Slide courtesy of A. Illingworth and D.R. Sutherland
Manual Voltage and Compensation Setup for 2C PNH Testing in RBCs

Step 1

- Clear all compensation settings
- Run unstained normal RBCs
- Adjust voltages of FL-1 and FL-2 to bring the population on scale
Manual Voltage and Compensation Setup for 2C PNH Testing in RBCs

Step 2

• Run normal RBCs at this voltage with CD59-PE only and check for FL-2 “bleeding” into FL-1

• Adjust compensation (FL-1 minus FL-2)

Slide courtesy of A. Illingworth
Run normal RBCs at this voltage with CD235a-FITC only to check for bleeding of FL-1 into FL-2

Adjust compensation (FL-2 minus FL-1)

Slide courtesy of A. Illingworth
Verification for 2C RBC PNH Assay
Normal vs PNH Sample

**Step 4**
Run normal blood with CD235a and CD59 to verify the position of CD235a+/CD59+ RBC (Type I cells)

**Step 5**
Run suspected PNH Patient with CD235a and CD59 to determine the % RBCs with CD59 Deficiency (Type II and/or Type III cells)

Presence of **31.6% PNH Type III** and **43.1% Type II** PNH RBCs

Slide courtesy of A. Illingworth
1.5 μL CD235a-FITC (clone KC16) + 0.5 μL CD59-PE (clone MEM43)

Cocktail: 15 μL CD235a + 5 μL CD59 +180 μL PBS; use 10 μL/test
High-sensitivity RBC assay: Normal Sample

1.5 uL CD235aFITC (clone KC16) + 0.5 uL CD59PE (clone MEM43)
Cocktail: 15uL CD235a + 5uL CD59 +180uL PBS. Use 10uL/test

Sutherland DR et al. Cytometry Part B. 2012;82B:195-208
**Recommended RBC panel for Both BC and BD platforms**

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Antibodies and Conjugates</th>
<th>Purpose</th>
<th>Clone and Vendor</th>
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<tbody>
<tr>
<td>RBC</td>
<td>CD59-PE</td>
<td>GPI-linked for RBC</td>
<td>OV9A2 (eBioscience) MEM-43 (Invitrogen)</td>
</tr>
<tr>
<td></td>
<td>CD235a-FITC</td>
<td>Gating on RBC</td>
<td>KC16 (BC) 10F7MN (eBio) JC159 (DAKO)</td>
</tr>
</tbody>
</table>
Accurate Detection of PNH WBC Clones

- **Granulocytes**
  - PNH clone in Granulocytes has been reported to provide most accurate estimate of PNH clone size

- **Monocyes**
  - PNH clones in Monocytes should also be determined in a second tube or if instrumentation permits, the same tube
  - PNH clone in Monocytes may be different (often higher) than Granulocyte PNH Clone

- **Type II WBCs** (Granulocytes and Monocytes) can occasionally be recognized and should be included in the total WBC clone

- **Lymphocytes** are not a suitable target for PNH assessment but are useful “internal controls” to confirm optimal instrument setup/compensation and monitor antibody conjugate performance

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Comparison of PNH Clones Sizes
Granulocytes vs Monocytes

PNH clone size in Monos and Grans is similar

PNH Clone size in Grans > than in Monos

PNH Clone size in Monos > than in Grans

Data on file at Dahl-Chase Diagnostic Services

Graphic courtesy of A. Illingworth
Unusual case – need to retest all 3 cell lineages RBC, WBC (Granulocytes and Monocytes)

**DIAGNOSIS:** PNH Clone Identified in WBC and RBC

**Comment:** Flow cytometric analysis shows a PNH clone within the monocytes (20.2%) and RBCs (3.0%) as well as a minor PNH clone in the granulocytes (0.5%). These findings indicate an increase in the PNH monocyte clone size. Clinical correlation with other laboratory data and follow-up is recommended.

**Reference:**

**Flow Results:** Immunophenotypic analysis was performed using gating antibodies CD45, CD15, CD64, CD235a, GPI-linked antibodies CD59, CD14, CD24, as well as fluorescent Aerolysin (FLAER).

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<thead>
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<th>Cell Type and Deficiency</th>
<th>2-19-15</th>
<th>11-13-14</th>
<th>10-06-14</th>
<th>08-07-14</th>
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<td>RBCs with CD59 deficiency</td>
<td>3.0%</td>
<td>1.2%</td>
<td>0.9%</td>
<td>0.7%</td>
<td>0.4%</td>
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<td>Monocytes with FLAER/CD14 Deficiency</td>
<td>20.2%</td>
<td>14.9%</td>
<td>7.9%</td>
<td>3.2%</td>
<td>2.1%</td>
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<td>Granulocytes with FLAER/CD24 Deficiency</td>
<td>0.5%</td>
<td>0.8%</td>
<td>0.5%</td>
<td>0.00%</td>
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WBC Assay – Instrument Setup

- **Forward and Side Scatter Adjustment**
  - Use FS and SS in linear mode (not log)
  - Set voltages to ensure all WBC populations “on scale”
  - Ensure FSC threshold does not exclude lymphocytes (used as internal controls)

- **Voltage Adjustment of PMTs**
  - Optimize PMTs to best separate antigen-positive and antigen-negative cells
  - Ideally:
    - use PNH case (showing FLAER/CD24-negative and positive cells)*
  - Other option:
    - use antibodies which show negative and positive staining (e.g. CD3)*

- **Compensation (set up specific matrix for PNH assay)**
  - Set compensation specifically for this WBC assay (FLAER-Alexa488 has different compensation requirement than FITC-conjugate)
  - Set up “control dot plots”
    - check that all populations (including internal control cells) are “on-scale” after setting compensation

- **Verification of settings** with normal sample and/or PNH+ sample

*If FC500: DO NOT use “Baseline offset”
Voltage Adjustment of PMTs

- Good for initial setup
  - Use unstained cells to get populations “on scale”

- Better:
  - Use cells which show positive and negative staining for an antibody (e.g. CD3)
  - Optimize voltage of PMTs so that there is good separation between CD3+ T-cells and CD3-negative B-cells

*If FC500: DO NOT use “Baseline offset”

Repeat for all PMTs (ideally with same antibody in other fluorochromes)

Slide courtesy of A. Illingworth and D.R. Sutherland
PNH granulocytes with CD15 gating

4-C Granulocyte cocktail: FLAER, CD24-PE, CD15-PECy5, CD45-PECy7

Assay sensitivity 0.02%

PNH monocytes with CD64 gating

4-C Monocyte cocktail: FLAER, CD14-PE, CD64-PECy5, CD45-PECy7

Assay sensitivity 0.04%
Validation of High-sensitivity WBC assay - 1 Establish “Background Frequency” of PNH WBC Phenotypes in Normal Samples

Granulocytes CD15 gating

Monocytes CD64 gating

Frequency = 0.0013% (n=10)  
Frequency = 0.0033% (n=10)
5-color CD157-based assay for high sensitivity granulocytes and monocytes (BC)

PNH SAMPLE

FLAER CD157-PE CD64-ECD CD15-PC5 CD45-PC7

Sutherland DR, Acton E, Keeney M, Davis BH, Illingworth A. Cytometry Part B 2014;86B: 44-55.
5-C CD157-based assay on 3-laser BD Canto II

FLAER-Alexa488
CD157-PE
CD45-PerCP-Cy5.5
CD64-APC
CD15-eFluor450

3-laser Canto II
6-C PNH Assay on BC Navios

FLAER-Alexa488, CD24-PE, CD15-PC5, CD64-PC7, CD14-APC700, CD45-KO

PNH+ Sample

Slide courtesy of A Illingworth
**Recommended antibody panels for PNH WBC testing**

**Beckman coulter instruments**

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Antibodies and Conjugates</th>
<th>Purpose</th>
<th>Clone (Vendor)</th>
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<tr>
<td>4C Granulocytes</td>
<td>FLAER CD24-PE</td>
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<td>NA (Cedarlane)</td>
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<tr>
<td>4C Monocytes (Reflex)</td>
<td>FLAER CD14-PE</td>
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<td>SN3 (eBio), ALB9 (BC)</td>
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<tr>
<td>5C Grans and Monos</td>
<td>FLAER CD157-PE CD64-ECD</td>
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<td>61D3 (eBio), RMO52 (BC), Tuk4 (Invitrogen), RMO52 (BC)</td>
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<tr>
<td>6C Grans and Monos</td>
<td>FLAER CD24-PE</td>
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<td>SY11B5 (eBio)</td>
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<td>CD64-PC5 (4C) CD64-ECD (5C) CD64-PC7 (6C)</td>
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<td>CD45-PC7 (4C,5C) CD45-KrO (6C)</td>
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<td>J33 (BC) J33 (BC)</td>
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Slide courtesy A Illingworth and DR Sutherland
# Recommended WBC Antibody Panels: BD Cytometers

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<td><strong>CD24-PE (4C 6C)</strong></td>
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<td><strong>CD45APC-H7 (5C 6C)</strong></td>
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<td>2D1 (BD)</td>
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A. For 3-laser Canto  
B. For 2-laser Canto  

Slide courtesy  
DR Sutherland  
PW Wallace and A Illingworth
Current Practices for PNH Testing
PNH Findings

• Only 14/105 laboratories (13%) using a method that followed the international consensus guidelines

• No 2 laboratories using identical methods

• 5 Laboratories only tested 1 population of cells (either RBCs or granulocytes)
  – Of these 3 tested only Red Blood Cells (RBCs) despite 31% of PNH cases not having a RBC clone of >3%*

• 37/84 (44%) of centres testing for red cell clones did not perform the recommended two washing steps

Labs are still producing incorrect Results

- **False positives:**
  - High background vs low sensitivity
  - Suboptimal gating antibody/strategy
  - Poor quality of plots creating “clones”

- **False negatives:**
  - Gating on GPI marker instead of lineage-specific marker
  - No washing of RBCs

- **Falsely decreased clone size:**
  - Clone size based on gate set on total WBCs instead of gated target population (monos and grans)
  - Compensation and voltage issues
  - Setting PNH gate

- **Misleading reports**
Why do we need to have revised ICCS/ESCCA PNH Consensus Guidelines

- Continued issues with:
  - Instrument optimization
  - Reagent clone and fluorochrome selection
  - Moving from validated 4C assays to 5C and 6C assays
  - Sample preparation
  - Interpretation of minor PNH populations, technical artifacts etc.
  - Reporting

- No standardized validation protocol available

- No control material available

Need evidence-based consensus guidelines which all labs can follow
Working group 1 – Instrument and Reagents

- **Chair:** Rob Sutherland
- **Participants:** Daniel Payne, Andrea Illingworth, Iuri Marinov, Jolene Cardinali, Paul Wallace, Bruce Greig, Gabriel Luchetta, John Andreasen
- **Current Outline:**
  - Recommended Instrument setup for both platforms (BC and BD)
  - Selection of optimal Reagent conjugates, titrations and validation
    - For RBC assay
    - For 4C, 5C and 6C WBC assays
  - Sample preparation and staining for RBC and WBC assay
  - Other approaches
    - Reticulocytes
    - C3d assay
    - Viability staining
    - Non-FLAER based assays
Working Group 2 – Analysis and Reporting

- **Chair:** Andrea Illingworth
- **Participants:** Iuri Marinov, Orianne Wagner Ballon, Rob Sutherland, Luigi Delvecchio
- **Current Outline:**
  - Gating strategies
    - RBC assay (2C)
    - WBC assays (4C, 5C and 6C)
  - PNH Templates (gating, diagnostic and internal control plots)
  - PNH gate setting (Type II and Type III)
  - Sensitivity versus LLOD
  - Difference in PNH clones sizes between granulocytes and monocytes
  - Reporting terminology and minor PNH clones (less than 1%)
  - PNH+ patient Case Library (plots, interpretation and reporting)
Working Group 3 – Validation of PNH Assay

- **Chair:** Teri Oldaker
- **Participants:** Paul Wallace, Virginia Litwin, Jeannine Holden, Maryam Saber, Liam Whitby
- **Current Outline:**
  - Validation Documentation
  - Performance specifications
  - Samples
    - Experimental design based on ICSH/ICCS Guidelines
    - Tabular examples
  - Quality Control (assay and instrument)
  - Proficiency testing
  - Ensuring Staff Competency