Step 1 – Set up (4-colour protocols)

Instrument optimisation

For accurate and reliable analysis of paroxysmal nocturnal haemoglobinuria (PNH), it is recommended to perform the following:

1. Daily performance check
   - Use standard beads (Flow Check® beads, calibration beads, Cytometer Setup and Tracking [CS&T] beads).

2. Application setting
   - Photomultiplier tube (PMT) setting, time delay, colour compensation (needs to be adjusted each time the voltage is changed).

For PNH analysis we target specific cell populations (granulocytes/neutrophils, monocytes, red blood cells [RBCs]) and use optimally titrated monoclonal antibodies, which require specific application settings (PMT setting and colour compensation). Therefore, establish appropriate cytometer settings to see negative and positive cell populations ‘on scale’ and optimise for PNH analysis.

**NOTE:** Ideally, application setting should be done with labelled and non-labelled target cells (granulocytes, monocytes, RBCs) from a PNH patient; however, in the majority of cases application setting is performed using calibration beads or lymphocytes (eg CYTO-COMP™ cells).

Set up and quality control for white blood cell (WBC [granulocyte]) assay

Prepare 3 tubes:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>100 µl peripheral blood + CD15 + CD45</td>
</tr>
<tr>
<td>2</td>
<td>100 µl peripheral blood + CD15 + CD45 + CD24 + FLAER</td>
</tr>
<tr>
<td>3</td>
<td>100 µl PNH blood or mix tubes 1 and 2 (prepare this tube after acquisition of tubes 1–2 on flow cytometer)</td>
</tr>
</tbody>
</table>

FLAER, fluorescent aerolysin

Step 1: Forward scatter (FS) and side scatter (SS) adjustment for WBCs

- Run tube 1 and set lightscatter volatges in lin:lin light scatter to bring all WBCs on scale.
- Ensure FS threshold is not excluding lymphocytes (lymphocytes can be used as internal controls)

FS, forward scatter; SS, side scatter
Step 2: Voltage adjustment of PMTs for WBCs

- Follow vendor-specific protocol for setting up voltages and compensation settings to ensure that negative and positive populations are ‘on scale’ and properly compensated.
  - If FC500 or later in use, DO NOT use ‘negative offset’.
- Run tube 1 to verify that the CD45+/CD15++ granulocytes (no FLAER or CD24 added to create ‘bona fide’ PNH cells) are clearly visible and on scale.

FITC, fluorescein isothiocyanate; FLAER, fluorescent aerolysin; GPI, glycosylphosphatidylinositol; PE, phycoerythrin
• Run **tube 2** to verify the presence and location of normal CD45+/CD15++ granulocytes showing CD24 and FLAER expression.

In this tube, all markers are added, and the normal granulocytes should show bright expression for FLAER (~2 decades higher than negative population) and moderate expression for CD24 (~1.5 decades higher than negative population). CD24 should not be titrated too much and special attention should be paid to the compensation. Although the FLAER signal is detected in the same PMT as FITC, the compensation for FLAER is different (usually less) than that for FITC. This means that the compensation set-up process should include a FLAER-stained sample rather than an FITC-stained sample, otherwise there may be overcompensation.

**FITC, fluorescein isothiocyanate; FLAER, fluorescent aerolysin; PE, phycoerythrin; PMT, photomultiplier tube**

**Step 3: Verify the location of PNH and normal populations**

• Run **tube 3** to verify the position of normal FLAER+/CD24+ granulocytes, and FLAER- and CD24-deficient PNH granulocytes.

This test sample can either be a PNH-positive case (as in this example) or a mixture of tubes 1 and 2 (if no PNH-positive sample is available).

**FLAER, fluorescent aerolysin; PE, phycoerythrin**
Set-up and quality control for RBC assay

Prepare 6 tubes:

<p>| | | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Unstained RBCs</td>
<td>Make 1:100 dilution of normal RBCs (eg add 10 µL peripheral blood to 990 µL PBS)</td>
</tr>
<tr>
<td>2</td>
<td>RBCs only</td>
<td>Use 50 µL of 1:100 dilution of peripheral blood</td>
</tr>
<tr>
<td>3</td>
<td>RBCs + CD59-PE</td>
<td>Use 50 µL of 1:100 dilution of peripheral blood and stain with appropriate amount of CD59-PE only</td>
</tr>
<tr>
<td>4</td>
<td>RBCs + CD235a-FITC</td>
<td>Use 50 µL of 1:100 dilution of peripheral blood and stain with appropriate amount of CD235a-FITC only</td>
</tr>
<tr>
<td>5</td>
<td>RBCs + CD59-PE + CD235a-FITC</td>
<td>Use 50 µL of 1:100 dilution of peripheral blood and stain with appropriate amount of CD59-PE and CD235a-FITC</td>
</tr>
<tr>
<td>6</td>
<td>PNH blood + CD59-PE + CD235a-FITC, or mix tubes 2 and 5</td>
<td>Prepare this tube after acquisition of tubes 1–5 on flow cytometer</td>
</tr>
</tbody>
</table>

It is important to have an RBC-specific voltage set-up (FS log, SS log) and compensation to bring the CD59-negative RBCs on scale. The typical leukaemia and lymphoma WBC voltage/compensation set-up should not be used for the RBC assay. Voltages are typically higher for the PNH RBC assay.

Step 1: Light-scatter and PMT setting for RBCs

- Run tube 2 and check light scatter voltages in log:log light scatter to bring RBCs on scale. Make sure the discriminator does not ‘cut off’ cells.
- Clear all compensation settings (as the RBC assay set-up involves only two colours, manual compensation is generally sufficient).
  - If FC500 or later in use, DO NOT use ‘negative offset’.
- Adjust voltages of FL1 and FL2 to bring the population on scale.
The intersection point of the two red lines (on the right) provides a rough estimate for the x and y mean channels for the negative population. The red lines can also be used as a guide for the expected locations of the CD59+ only population and the CD235a+ only population.

FITC, fluorescein isothiocyanate; RBC, red blood cell; SS, side scatter

Example of PMT setting for RBC analysis with the BD FACSCanto machine (compensation is set to 0); step 1.

BD, Becton Dickinson; FITC, fluorescein isothiocyanate; FS, forward scatter; PE, phycoerythrin; PMT, photomultiplier tube; RBC, red blood cell; SS, side scatter
Step 2: Manual compensation settings for RBCs (FL1 – FL2)

- Run tube 3 and adjust compensation: FL1 minus FL2. Set-up and quality control for RBC assay

Example of PMT setting for RBC analysis with the BD FACS Canto machine (compensation is set to 0); step 1.

FITC, fluorescein isothiocyanate; PE, phycoerythrin

Step 3: Manual compensation settings for RBCs (FL2 – FL1)

- Run tube 4 and adjust compensation: FL2 minus FL1.

Typically the population shows some bleeding (spillover) into the FL2 channels. This needs to be compensated by ‘moving’ the population (see arrow) into the location where it shows CD235a staining only and is aligned using the x mean channel guide (blue line) of the negative unstained population from the first tube. Keep in mind that this CD235a brightly positive population may not be as tightly clustered as the negative population and may ‘fan out’ in the higher decades (‘trumpet effect’). Caution should be exercised to avoid overcompensation where most of the cells are ‘crushed’ against the axis.

FITC, fluorescein isothiocyanate; PE, phycoerythrin
Step 4: Verify and fine-tune the RBC setting

- Run **tube 5** to verify the position of normal Type I RBCs.
- Run **tube 6** to verify the position of PNH Type III, PNH Type II and normal Type I RBCs.

Running a PNH-positive patient should show the CD59-deficient Type III RBCs in the correct location (CD235a+ and CD59–). The dark blue line represents a rough guide for the mean channel of the CD235a+/CD59– Type III cells. Overcompensation will cause the Type III cells to be ‘crushed’ and not be visible, and undercompensation will cause this population to move into the Type II region and cause underestimation of the Type III cells. Both the dual-parameter dot plot (CD235a/CD59) and the single-parameter (CD59) histogram may be used to determine the Type III and Type II clone sizes. The dot plots (left) typically show a better separation of the cell clusters and also identify staining and compensation artefacts. The single-parameter histograms may be used in addition to help with cursor settings, as with the light blue lines (usually with larger clone sizes).

FITC, fluorescein isothiocyanate; PE, phycoerythrin; RBC, red blood cell

*These protocols were developed in close collaboration with Mrs Andrea Illingworth of Dahl-Chase Diagnostic Services in Bangor, ME, USA, Drs Thomas Matthes and Mathieu Hauwel of the Swiss Flow Cytometry School at the University Hospital of Geneva, Switzerland, and Dr Iuri Marinov of Hematology and Blood Transfusion in Prague, Czech Republic. Images were provided with permission from the netflow Steering Committee and Swiss Flow Cytometry School.*