

Step 2 – Stain and acquire (4-colour protocols)*

Principle

Cells are stained with lineage-specific antibodies to clearly identify populations of interest. Glycosylphosphatidylinositol (GPI)-anchored protein deficiency is tested using specific antibodies (eg CD59, CD24) and a GPI-binding reagent (fluorescent aerolysin [FLAER]). Negative controls are included in the following protocol, which is optional for routine day-to-day testing. Additionally, normal blood from healthy donors can be used as a positive control.

It is recommended for each antibody to be titred to determine the optimal working concentration (see “Antibody titration”)

Method

White blood cells (WBCs)

To screen for the presence of paroxysmal nocturnal haemoglobinuria (PNH) clones, it is usually sufficient to perform the granulocyte assay only. If a PNH clone is identified in the granulocytes, the monocyte assay should also be performed.

- 1) Invert the tube with the whole blood several times to mix the sample thoroughly.
- 2) Take four 100 µL aliquots of blood and transfer them to the bottom of four 5-mL fluorescence-activated cell sorting (FACS) tubes.
 - **Be careful not to leave any blood on the edges of the tube.**
- 3) Add the antibody cocktail and mix by gentle vortexing.

	Fluorochrome	A488	PE	PC5 (BC) APC (BD)	PC7 (BC) PerCP (BD)
Granulocyte cocktail	Test	FLAER	CD24	CD15	CD45
	Control ^a	x	x	CD15	CD45
Monocyte cocktail	Test	FLAER	CD14	CD64	CD45
	Control ^a	x	x	CD64	CD45

^aThis control is not required for everyday testing

BC, Beckman Coulter; BD, Becton Dickinson; FLAER, fluorescent aerolysin; PE, phycoerythrin

- 6) Incubate at room temperature in the dark for 30 minutes.
 - **Be careful not to expose the antibodies to light.**
- 7) Add appropriate amount of vendor-specific lysing solution (Optilyse® C or any other lysing solution) and follow specific protocol.
- 8) Add 2 mL phosphate-buffered saline (PBS) with albumin (PBA) and spin for 3 minutes at 300 g.
- 9) Discard supernatant.
- 10) Resuspend in 0.5 mL PBA.
- 11) Vortex to dissociate aggregates.
- 12) Acquire at least 50,000 CD15+ granulocytes and as many as possible CD64+ monocytes, using an appropriate acquisition template (see “Step 3 - Analyse”).

- 13) If a PNH clone is identified in the granulocytes, set up two more tubes for the monocyte assay and follow steps 2–11.

Red blood cells (RBCs)

- 1) Invert the tube with the whole blood several times to mix the sample thoroughly.
- 2) Add 1 mL PBS to the tube.
- 3) Add 10 µL peripheral blood (1:100 dilution).
- 4) Take two 100 µL aliquots of 1:100-diluted blood and transfer to the bottom of two 5-mL FACS tubes.
 - **Be careful not to leave any blood on the edges of the tube.**
- 5) Add the appropriately titred antibody cocktail and mix by gentle vortexing.

	Fluorochrome	FITC	PE
Erythrocyte cocktail	Test	CD235a	CD59
	Control ^a	CD235a	x

^aThis control is not required for everyday testing
 FITC, fluorescein isothiocyanate; PE, phycoerythrin

- 6) Incubate at room temperature in the dark for at least 20 minutes (up to 60 minutes).
 - **Be careful not to expose the antibodies to light.**
- 7) Add 2 mL PBS and spin for 3 minutes at 300 g.
- 8) Discard supernatant.
- 9) Add 2 mL PBS and spin for 3 minutes at 300 g.
- 10) Discard supernatant.
- 11) Resuspend the pellet in 500 µL PBS.
- 12) 'Rack' the tube vigorously 3–4 times to dissociate aggregates.
- 13) Acquire at least 50,000 CD235a+ RBCs using an appropriate RBC acquisition template (see "Step 3 – Analyse"). If a small PNH clone is identified, acquire more events to increase confidence in the analysis, as 100 clustered PNH cells are typically required to verify the presence of a true PNH clone.
 - **Acquire within 15 minutes, as the staining intensity of CD235a fades rapidly.**

*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.