

Step 2 – Stain and acquire (6-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 CD14, CD24, CD59, CD157) and a GPI-binding reagent (fluorescent aerolysin [FLAER]).

Method

White blood cells (WBCs)

Single tube, 6-color panel for simultaneous analysis of paroxysmal nocturnal haemoglobinuria (PNH) granulocytes and monocytes.

The table below summarises the recommended reagents (monoclonal antibody clones) for WBC analysis on Beckman Coulter and Becton Dickinson instruments. Each antibody should be titrated to determine optimal working concentration (see “Antibody titration”). Different fluorochrome conjugates are possible, depending on laser and detector configuration of each instrument.

Recommended reagents/monoclonal antibody clones for 6-color WBCs analysis:

| Antibodies/reagents | Purpose | Beckman Coulter | Becton Dickinson |
|---------------------|-------------------------|-----------------|------------------|
| FLAER | GPI-linked | NA (Cedarlane) | NA (Cedarlane) |
| CD14 | GPI-linked | RMO52 61D3 | MφP9 61D3 |
| CD15 | Gating for granulocytes | 80H5 | HI98, MMA |
| CD24 | GPI-linked | ALB9 SN3 | ML5 SN3 |
| CD45 | Backgating | J33 | 2D1 |
| CD64 | Gating for monocytes | 22 | 10.1 |

FLAER, fluorescent aerolysin; GPI, glycosylphosphatidylinositol

- 1) Invert the tube with the whole blood several times to mix the sample thoroughly.
- 2) Take 100 µL of blood and place it in the bottom of a 5-mL tube.
 - **Be careful not to leave any blood on the edges of the tube.**
- 3) Add the corresponding amount of previously titrated antibodies (antibody cocktail) and mix by gentle vortexing.
- 4) Incubate at room temperature in the dark for 30 minutes.
 - **Be careful not to expose the antibodies to light.**
- 5) Add an appropriate amount of vendor-specific or any other lysing solution and follow the specific protocol.
- 6) Add 2 mL phosphate-buffered saline (PBS) with albumin (PBA) and spin for 3 minutes at 300 g.
- 7) Discard supernatant.
- 8) Resuspend in 0.5 mL PBA.
- 9) Vortex to dissociate aggregates.
- 10) Acquire at least 50,000 CD15+ granulocytes, using an appropriate acquisition template (see “Step 3 – Analyse”).

Red blood cells (RBCs)

Invert the tube with the whole blood several times in order to mix the sample thoroughly.

- 1) Add 1 mL PBS to the tube.
- 2) Add 10 µL peripheral blood (1:100 dilution).
- 3) Take 100 µL 1:100 diluted blood twice and place it in the bottom of two 5-mL fluorescence-activated cell sorting (FACS) tubes.
 - **Be careful not to leave any blood on the edges of the tube.**
- 4) Add the appropriately titrated antibody cocktail and mix by gentle vortexing.

| Antibodies/reagents | 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

- 5) 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.**
- 6) Add 2 mL PBS and spin for 3 minutes at 300 g.
- 7) Discard supernatant.
- 8) Add 2 mL PBS and spin for 3 minutes at 300 g.
- 9) Discard supernatant.
- 10) Resuspend the pellet in 500 µL PBS.
- 11) 'Rack' the tube vigorously 3–4 times to dissociate aggregates.
- 12) 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.