# Red blood cell (RBC) spiking assay*

**Every laboratory should perform a 'spiking' assay to assess the sensitivity of the paroxysmal nocturnal haemoglobinuria (PNH) test**

Sensitivity refers to the smallest number of cells that can specifically be detected with a particular assay. The typical acquisition number for granulocytes is 50,000 cells. The number of monocytes is typically lower and thus results in lower sensitivity than that achieved with granulocytes.

A 'spiking' assay, which is basically a serial dilution of PNH blood with normal blood, can verify the sensitivity of detecting PNH clones at lower dilutions.

In case PNH blood is not available, PNH clones can be mimicked by using only gating markers and not glycosylphosphatidylinositol (GPI)-linked markers in the staining solution. For example:

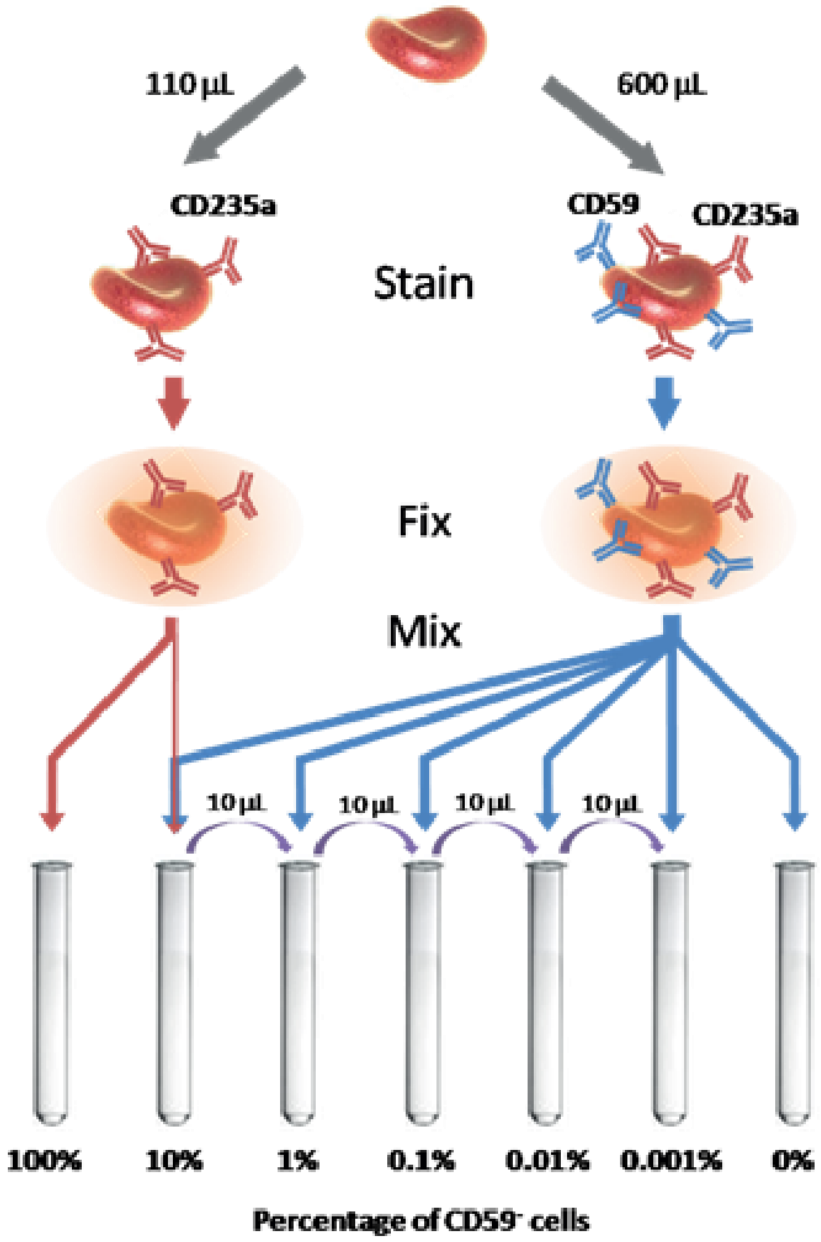
- RBCs, 2-colour panel: X/CD235a will show location of CD59-negative RBCs
- Granulocytes, 4-colour panel: X/X/CD15/CD45 will show the location for fluorescent aerolysin (FLAER)/CD24-negative granulocytes
- Monocytes, 4-colour panel: X/X/CD64/CD45 will show the location of FLAER/CD24-negative monocytes

The detailed protocol below shows how to establish RBC assay sensitivity based on a normal blood sample. In this case, sensitivity is determined by diluting RBCs stained only for GPA [therefore negative for CD59] into RBCs stained for both GPA and CD59, thus mimicking PNH clones of increasing size.

## Protocol

1) Dilute 5 µL fresh blood in 495 µL phosphate-buffered saline (PBS) or any other flow cytometry measurement (FCM)-compatible buffer.
2) Incubate 500 µL diluted blood with 100 µL anti-GPA (CD235a fluorescein isothiocyanate [FITC] clone KC16; use at 20 µL/100 µL cells) antibody and 10 µL anti-CD59 antibody (clone MEM43; use at 2 µL/100 µL cells) for 15 minutes in the dark.
3) Incubate 110 µL diluted blood with 22 µL anti-GPA antibody for 15 minutes.
4) Spin at 500 g for 3 minutes and carefully discard supernatant.
5) Resuspend in 1 mL CellFix™.
6) Incubate at room temperature for 30 minutes in the dark.
7) Add 2 mL PBS then spin at 500 g for 3 minutes and carefully discard supernatant.
8) Resuspend in 600 µL and 110 µL PBS, respectively.
9) Rack vigorously (or pipette) to dissociate aggregates.
10) Pipette 100 µL single-stained RBCs in an FCM tube and acquire 250,000 events (tube 1).
11) Distribute 90 µL double-stained RBCs in tubes 2-6 and 100 µL in tube 7.
12) Take 10 µL single-stained RBCs and dilute serially in tubes 2-6 by carrying 10 µL each time to the next tube.
13) Acquire 1,000,000 events.

| Tube | CD235a CD59 | CD235a | PNH clone |
|---|---|---|---|
| **1** | 0 | 100 | 100% |
| **2** | 90 | 10 | 10% |
| **3** | 90 | 10 | 1% |
| **4** | 90 | 10 | 0.10% |
| **5** | 90 | 10 | 0.01% |
| **6** | 90 | 10 | 0% |
| **7** | 100 | 0 | 0% |



## Data analysis

The tables below show the results of a spiking experiment in which a PNH blood sample was spiked with normal blood. The same experiment can be used to check the sensitivity of detecting PNH monocytes, although the number of monocytes is typically lower and thus results in lower sensitivity than that achieved with granulocytes.

**4-colour granulocyte assay sensitivity**

| Dilution | PNH granulocytes | Sensitivity, % |
|---|---|---|
| Neat | 51,420 | 91.3 |
| 1:10 | 5799 | 9.4 |
| 1:100 | 573 | 0.94 |
| 1:1000 | 91 | 0.089 |
| 1:10,000 | 9 | 0.01 |

**4-colour monocyte assay sensitivity**

| Dilution | PNH monocytes | Sensitivity, % |
|---|---|---|
| Neat | 12,718 | 89.8 |
| 1:10 | 1227 | 4.1 |
| 1:100 | 112 | 0.4 |
| 1:1000 | 13 | 0.044 |
| 1:10,000 | ND | ND |

ND, not detected

**2-colour RBC assay sensitivity (based on 1,000,000 RBCs collected in data files)**

| Dilution | Type III RBCs | Sensitivity, % |
|---|---|---|
| Neat | 348,626 | 34.86 |
| 1:10 | 17,665 | 1.77 |
| 1:100 | 1822 | 0.18 |
| 1:1000 | 203 | 0.02 |
| 1:10,000 | 18 | 0.0018 |

RBCs, red blood cells

*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.