

REDQUANT PNH

Kit for Paroxysmal Nocturnal Haemoglobinuria diagnosis
on red blood cells by flow cytometry

For In Vitro Diagnostic Use in combination with
CELLQUANT PNH kit (Ref. 7201)



Ref. 7301

Kit for 12 determinations



1 INTRODUCTION

Paroxysmal Nocturnal Haemoglobinuria (PNH) is an acquired rare and clonal disorder characterized by an intravascular haemolytic anemia due to red cell lysis.

PNH results from a defect in the PIG-A gene involved in the synthesis of glycosyl-phosphatidylinositol (or GPI) linkage of proteins to the membrane. CD55 and CD59 are GPI-anchored proteins involved in the cell protection against complement mediated lysis. In PNH disorder, cells are deficient in CD55 and CD59 and are thus sensitive to complement mediated lysis.

2 METHOD

Single color flow cytometric analysis of CD55 and CD59 antigens on red blood cells. The relative amounts of CD55 and CD59 deficient red blood cells are determined using a threshold gating method. The method establishes an analysis region in which CD55 and CD59 deficient red blood cells migrate and can be differentiated from normal red blood cells.

3 REAGENTS

- **Reagent 1 :** 1 x 15 mL vial , diluent, 10-fold concentrated.
- **Reagent 2a :** 1 x 240 µL vial, MAb anti CD55.
- **Reagent 2b :** 1 x 240 µL vial, MAb anti CD59.
- **Reagent 3a :** 1 x 480 µL vial, precalibrated α bead. This α bead is characterized by an α value for CD55 deficiency interpretation.
- **Reagent 3b :** 1 x 480 µL vial, precalibrated β bead. This β bead is characterized by a β value for CD59 deficiency interpretation.
The α and β values are indicated in the assay value insert provided in each kit. The α and β values may vary from lot to lot.
- **Reagent 4 :** 1 x 960 µL vial, staining reagent, polyclonal antibody anti mouse IgG-FITC.

The tray is designed to hold the vials and the corresponding caps to avoid reagent contamination.

4 WARNING

- Follow the conventional laboratory practices.
- All reagents contain sodium azide as a preservative. Reagents containing sodium azide should be discarded with care to prevent the formation of explosive metallic azides. When dumping waste materials into sinks, use copious quantities of water to flush plumbing thoroughly.
- Blood must be considered as potentially infectious.
- Follow the appropriate reglementation for waste disposal.

5 MATERIAL REQUIRED BUT NOT PROVIDED

- Vortex.
- Timer.
- Cytometer.
- Test tubes for cytometer.
- Adjustable pipettes with disposable tips (10 µL to 1 mL).
- Pipettes (1 and 2 mL).
- Distilled water.

6 REAGENT PREPARATION AND STORAGE

Unopened kits and contents remain stable until the expiration date printed on the box label, when stored at 2-8 °C. *

- **Reagent 1 ** :**
Stability after opening : 2 months at 2-8 °C when free of contamination.
Prepare a **1:10 dilution** with distilled water. Prepare the appropriate volume required for the series to be tested.
Stability after dilution : 15 days at 2-8 °C.
- **Reagents 2a, 2b and 4 :**
Ready for use.
Stability after opening : 2 months at 2-8 °C when free of contamination.

- **Reagents 3a and 3b :**

Ready for use after resuspension by **vortexing for 5 seconds**.

Stability after opening : 2 months at 2-8 °C when free of contamination.

Notes : * Do not freeze the kit.

** The presence of crystals does not affect the quality of the reagent. Incubate at 37 °C until the crystals are completely dissolved.

7 SPECIMEN COLLECTION AND TREATMENT

7.1 Specimen collection :

- Use non-wettable (plastic or siliconized glass) blood collection tubes.
- Anticoagulant : EDTA (K3).

7.2 Specimen storage :

- The blood specimen must be treated within **8 hours** after collection to avoid a drop in CD55 and CD59 antigen expression.
- Whole blood must be stored at room temperature before testing (18-25 °C).
- Do not freeze the specimen.

8 PROCEDURE

Note : For good results exercise great care in the pipetting of small reagent volumes by depositing them at the bottom of the test tubes.

All the reagents must be at **room temperature**.

One preparation of α and β beads must be run for each series. One series could contain up to 6 samples.

We recommend to run a normal sample and a known PNH sample in parallel of each series as quality control.

8.1 Sample preparation :

Label a tube T0.

- After mixing the blood sample, pipette **10 µL** of whole blood into tube T0.
- Add **1.5 mL** of **diluted Reagent 1** (diluent).
- Mix the tube using a vortex for **5 seconds**.

8.2 α and β bead preparation :

Label 2 tubes respectively T1 and T2.

After resuspension of reagents 3a and 3b using a vortex :

- Pipette **40 µL** of **Reagent 3a** (α bead) into tube T1.
- Pipette **40 µL** of **Reagent 3b** (β bead) into tube T2.

8.3 Immuno-labelling of the samples :

Label 2 tubes respectively T3 and T4.

In each of the tubes T3 and T4 :

- Pipette **20 µL** of diluted sample from tube T0.

Note : any drop of sample present at the top or along the inner side of the test tube must be clean off in order to avoid any contamination leading to incorrect results.

- Pipette **20 µL** of **Reagent 2a** (MAb anti CD55) into tube T3.
- Pipette **20 µL** of **Reagent 2b** (MAb anti CD59) into tube T4.
- Mix the tubes using a vortex for **2 seconds**.
- Incubate for **8-12 minutes** at room temperature.

8.4 Fluorescent Staining :

In each of the 4 tubes T1, T2, T3 and T4 :

- Add **20 µL** of **Reagent 4** (staining reagent).
- Mix the 4 tubes using a vortex for **2 seconds**.
- Incubate for **8-12 minutes** at room temperature.
- Add **2 mL** of **diluted Reagent 1** to each tube.

The prepared test samples may be stored for **4 hours** at **2-8 °C** before cytometric analysis.

8.5 Cytometric analysis :

Refer to the Operator's Manual of the cytometer for instructions on how to perform cytometric readings. The selected MFI statistics is the geometric mean (Mn(x) or GeoMean depending upon the cytometer).

Vortex each tube before analysis.

The test requires 10,000 red blood cells (or beads) to be analyzed per tube.

• **α and β bead analysis : tubes T1 and T2 (Figs. 1)**

Create a FS LOG vs SS LOG cytogram.

Draw an analysis region "A" around the main single α or β bead population (Fig. 1a).

Create an FL1 LOG histogram gated by the region "A".

Note the MFI of the α and β beads on the whole histogram (Figs. 1b and 1c, linear regions "B" and "E").

For optimum analysis conditions, the peak of the β bead fluorescence intensity (FL1) must be set in the third decade. To achieve this, adjust the FL1 PMT voltage.

Fig. 1a : α or β bead cytogram
FS LOG

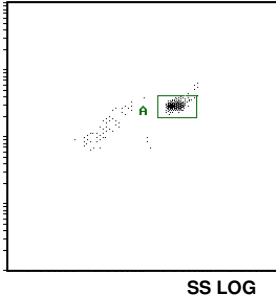


Fig. 1b : β bead histogram

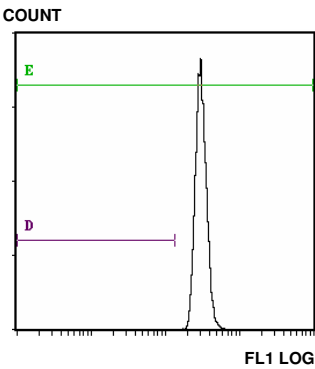
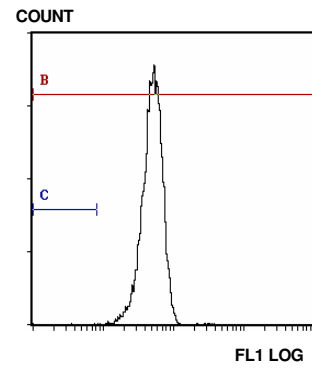


Fig. 1c : α bead histogram



• **Setup of interpretation cursors (Figs. 1b and 1c)**

On each of the two FL1 LOG histograms gated by the "A" region, set up two linear analysis regions ("C" and "D") corresponding to the expected location of deficient cells. To position the cursors (markers), set the Min (left) boundary in the first channel and the Max (Right) boundary at a Fluorescence Intensity (FI) value obtained from the following calculations :

CD55 cursor (marker) ("C") $\alpha FI = \alpha \times \alpha \text{ bead MFI}$
 CD59 cursor (marker) ("D") $\beta FI = \beta \times \beta \text{ bead MFI}$

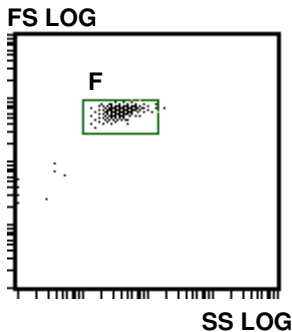
The α and β values are indicated in the assay value insert included in the kit.

• **Sample analysis : tube T3 (CD55) and T4 (CD59) (Figs. 2)**

Do not modify the FL1 PMT voltage.

Draw an analysis gate "F" around the red cell population on the FS LOG vs SS LOG histogram (Fig. 2).

Fig. 2 : Setup of the region "F" around the red cell population



In the FL1 LOG histogram gated by region "F"

Tube T3 : note the percentage of cells included in region "C".

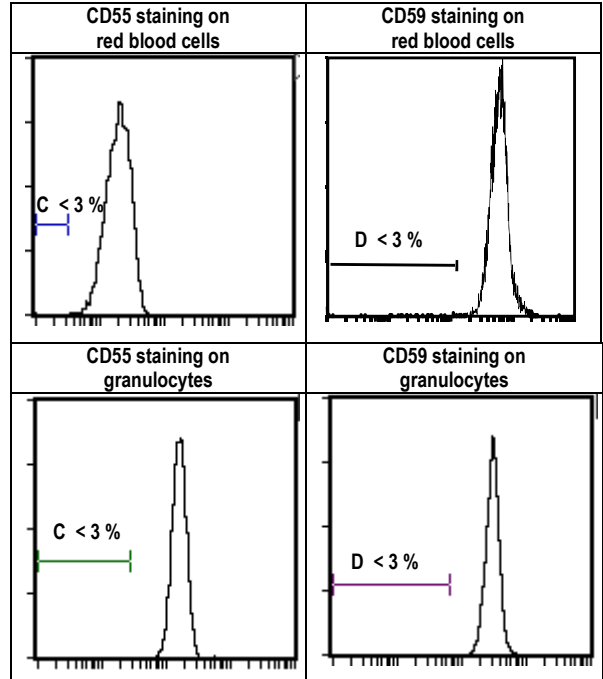
Tube T4 : note the percentage of cells included in region "D".

9 RESULTS AND TEST INTERPRETATION

Note : The test is only applicable when the MFI are expressed as linear values and not as channel number.

Using the suggested procedures for samples without deficient clone the "C" and "D" regions were found to contain not more than 3 % of the cells.

Example of CD55 and CD59 staining on red blood cells and granulocytes (normal sample) :



Test interpretation :

Nb of parameters > 3 %	REDQUANT PNH		CELLQUANT PNH		Conclusion
	CD55	CD59	CD55	CD59	
4	> 3 %	> 3 %	> 3 %	> 3 %	PNH
3	> 3 %	> 3 %	> 3 %	< 3 %	PNH
3	> 3 %	> 3 %	< 3 %	> 3 %	PNH
3	< 3 %	> 3 %	> 3 %	> 3 %	PNH
3	> 3 %	< 3 %	> 3 %	> 3 %	PNH
2	> 3 %	> 3 %	< 3 %	< 3 %	PNH
2	< 3 %	< 3 %	> 3 %	> 3 %	PNH
2	< 3 %	> 3 %	< 3 %	> 3 %	Non PNH
2	< 3 %	> 3 %	> 3 %	< 3 %	Non PNH
2	> 3 %	< 3 %	> 3 %	< 3 %	Non PNH
2	> 3 %	< 3 %	< 3 %	> 3 %	Non PNH
1	> 3 %	< 3 %	< 3 %	< 3 %	Non PNH
1	< 3 %	> 3 %	< 3 %	< 3 %	Non PNH
1	< 3 %	< 3 %	> 3 %	< 3 %	Non PNH
1	< 3 %	< 3 %	< 3 %	> 3 %	Non PNH
0	< 3 %	< 3 %	< 3 %	< 3 %	Normal

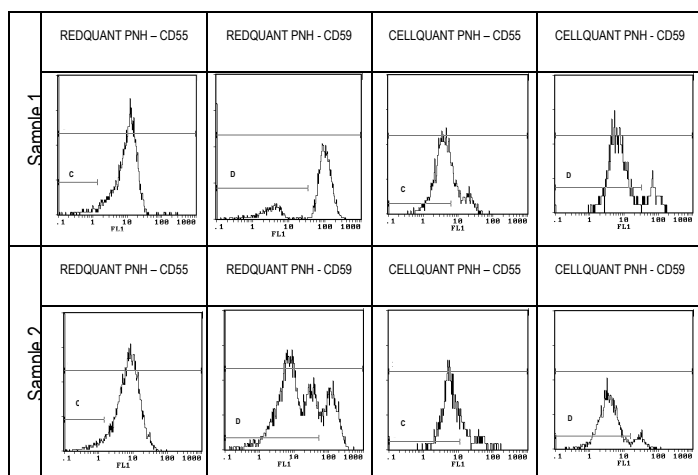
a - If 3 or 4 parameters / 4 are > 3 %, the sample is declared **PNH**.

b - If 2 parameters CD55 and CD59 on the same population (either red blood cells or granulocytes) are > 3 % then the sample is declared **PNH**.

c- If 2 parameters / 4 are > 3 % (any other case than case b mentioned above) or 1 parameter / 4 is > 3 %, then the sample is declared non PNH. It is recommended to perform another test during the disease course in a short time interval.

d- If 0 parameter / 4 is > 3 %, the sample is normal.

Examples of two PNH samples tested on red blood cells and granulocytes :



10 LIMITATIONS

10.1 Microcytosis :

Samples presenting a microcytosis (small size red cells) will artefactually generate percentage of deficient cells > 3 %.

10.2 Transfusion :

Patients that have been recently transfused can not be tested using REDQUANT CD55/CD59 kit. Indeed transfusion can mask potential CD55 and/or CD59 deficiency leading to a false negative result (sample declared falsely normal).

11 PERFORMANCES

The test has been validated on Becton Dickinson / FACScan and Beckman Coulter / XL and XL MCL instruments (software system II).

11.1 Sensitivity (for the combined use of REDQUANT PNH and CELLQUANT PNH kits) : 100%.

23 PNH samples were confirmed PNH with the combined use of REDQUANT PNH and CELLQUANT PNH kits⁽³⁾.

11.2 Detection limit :

0% of CD55- and CD59-deficient cells.

11.3 Measure range :

From 0% to 24.8 % of CD55 deficient cells.

From 0.1 % à 58 % of CD59 deficient cells.

11.4 Repeatability :

Four normal samples treated 5 times with the same kit.

All the tests present percentages of CD55- and CD59-deficient cells < 3 %.

11.5 Intra lot Reproducibility :

One normal sample treated with 6 different kits from the same lot.

All the tests present percentages of CD55- and CD59-deficient cells < 3 %.

12 TROUBLESHOOTINGS

Problems observed	Probable causes	Solutions
On a normal sample a staining equivalent to background is observed on CD55 and CD59 tubes (>3 % of deficient cells)	20 µL of whole blood has been used instead of 20 µL of diluted whole blood. The MABs are thus not at saturating conditions	Perform another test taking care to dilute the blood sample
On a normal sample a staining equivalent to background is observed on CD55 and/or CD59 tubes (> 3 % of deficient cells)	R2a and/or R2b have not been added	Perform another test taking care to pipet all the reagents
On a normal sample a staining equivalent to background is observed on CD55 and/or CD59 tubes (> 3 % of deficient cells)	R4 reagent has not been added	Perform another test taking care to pipet the reagent
Few or none α and/or β beads are detected on FS LOG vs SS LOG histogram.	Bad resuspension of the α and/or β beads	Take care to vortex for 5 seconds prior R3a and/or R3b vial opening and pipeting.
On a normal sample, more than 3 % of CD55 deficient cells are detected and the CD59 peak is far away from cursor "C"	The CD55 and CD59 parameters have not been interpreted with the appropriate cursors ("C" or "D")	Cursor "C" is for CD55 analysis and Cursor "D" is for CD59 analysis

13 LIABILITY

The *in vitro* diagnostic use is only valid within the strict application of the package insert as well as **the use of the REDQUANT PNH kit in combination with the CELLQUANT PNH kit**. Any modification or use of a reagent from other batch can influence the results of the tests. In these cases no contestation or replacement of the product will be accepted.

14 BIBLIOGRAPHY

- 1- KISHIMOTO T. *et al.*, Leucocyte Typing VI, Garland Publishing Inc, White Cell Differentiation Antigens. 1996, 519-520, 521-522.
- 2- SCHLOSSMAN SF. *et al.*, Leucocyte Typing V, Oxford University Press, White Cell Differentiation Antigens. 1995, 1468-1471.
- 3- OELSCHLAEGEL U. *et al.*, Clin Lab Haem. 2001, 23 : 81-90.

15 SYMBOLS

REF	Catalogue number	Use by
IVD	In vitro Diagnostic Medical Device	Contains sufficient for n° tests
Temperature limitation	LOT	Batch code
Manufacturer		

BIOCYTEX
 140 ch. ARMEE D'AFRIQUE
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