

CELLQUANT PNH

Kit for Paroxysmal Nocturnal Haemoglobinuria diagnosis
on granulocytes by flow cytometry

For In Vitro Diagnostic Use in combination with
REDQUANT PNH kit (Ref. 7301)



Kit for 12 determinations

Ref. 7201



Out of Europe, this product is dedicated for research use only.

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 granulocytes. The relative amounts of CD55 and CD59 deficient granulocytes are determined using a threshold gating method. The method establishes an analysis region in which CD55 and CD59 deficient granulocytes migrate and can be differentiated from normal granulocytes.

3 REAGENTS

- **Reagent 1 :** 1 x 15 mL vial, diluent, 10 fold concentrated.
- **Reagent 2a :** 1 x 300 µL vial, MAb anti CD55.
- **Reagent 2b :** 1 x 300 µL vial, MAb anti CD59.
- **Reagent 3a :** 1 x 600 µL vial, precalibrated $\alpha\beta$ bead. This $\alpha\beta$ bead is characterized by α and β values for CD55 and CD59 deficiency interpretation, respectively. 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 3b :** 1 x 300 µL vial, saturation reagent.
- **Reagent 4 :** 1 x 900 µL vial, staining reagent, polyclonal antibody anti mouse IgG-FITC.
- **Reagent 5 :** 3 x 3.2 mL vials, red cell lysing solution.

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.
- Centrifuge.
- Cytometer.
- Test tubes for cytometer.
- 15 mL tubes.
- Adjustable pipettes with disposable tips (20 µL to 1 mL).
- Pipettes (1, 2, 10 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, 3b, 4 and 5 :**
Ready for use.
Stability after opening : 2 months at 2-8 °C when free of contamination.

- **Reagents 3a :**

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 minimize the degranulation of the granulocytes.
- Whole blood must be stored at room temperature before testing (18-25 °C).
- Do not freeze the specimen.

8 PROCEDURE

Note : Exercise great care in the pipetting of small reagent volumes by depositing them at the bottom of the test tubes.

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

One $\alpha\beta$ bead preparation 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 :

- Pipette **800 µL** of **Reagent 5** (lysing solution) into a 15 mL tube marked T0.
- Add **7.2 mL** of distilled water (room temperature).
- Mix the tube using a vortex for **5 seconds**.
- After mixing the blood sample, add **400 µL** of whole blood to 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 by unlyzed red blood cells leading to incorrect results.

- Mix the tube using a vortex for **5 seconds**.
- Incubate for **8-12 minutes** at room temperature.
- Centrifuge for **5 minutes** at **300 g**.
- Discard supernatant.
- Add **2 mL** of **diluted Reagent 1** (diluent).
- Resuspend the cell pellet using a vortex.
- Centrifuge for **5 minutes** at **300 g**.
- Decant tube T0 and aspirate (with pipette or vacuum) the remaining supernatant.
- Visually check the presence of the cell pellet at the bottom of the tube.
- Resuspend the cell pellet with the pipette by adding **150 µL** of **diluted Reagent 1**.
- Mix the tube using a vortex for **2 seconds**.

Note : an incomplete red cell lysis may be observed for some samples.

In this case, it is recommended to bring the distilled water used to dilute the lysing reagent to 37°C (using a waterbath) prior to lysing the specimen.

8.2 $\alpha\beta$ bead preparation :

Label a plastic tube T1.

- After resuspension of Reagent 3a using a vortex, pipette **50 µL** of **Reagent 3a** ($\alpha\beta$ bead) into tube T1 and add **25 µL** of **Reagent 3b** (saturation reagent).
- Mix the tube using a vortex for **2 seconds**.

8.3 Immuno-labeling of the samples :

Label two tubes respectively T2 and T3. Into both tubes :

- Pipette **50 µL** of cell suspension from the tube T0.
- Pipette **25 µL** of **Reagent 2a** (MAb anti CD55) into tube T2.
- Pipette **25 µL** of **Reagent 2b** (MAb anti CD59) into tube T3.
- Mix the tubes using a vortex for **2 seconds**.
- Incubate for **8-12 minutes** at room temperature

8.4 Fluorescent Staining :

In each of tubes T1, T2 and T3 :

- Add **25 µL** of **Reagent 4** (staining reagent).
- Mix the 3 tubes using a vortex for **2 seconds**.
- Incubate for **8-12 minutes** at room temperature.
- Add **3 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 5,000 granulocytes (or beads) to be analyzed per tube.

• $\alpha\beta$ bead analysis : tube T1 (Figs. 1)

Create a FS vs SS cytogram.

Draw an analysis region "A" around the $\alpha\beta$ bead population (Fig. 1a).

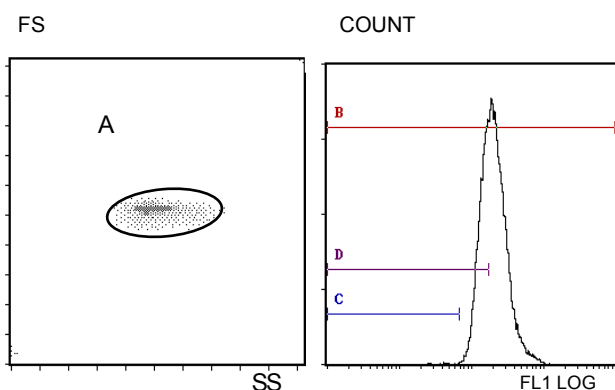
Create a FL1 LOG histogram gated by region "A".

Note the MFI of the $\alpha\beta$ bead histogram gated on the whole population (linear region "B") (Fig. 1b).

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

Fig. 1a : $\alpha\beta$ bead cytogram

Fig. 1b : $\alpha\beta$ bead histogram



• Setup of interpretation cursors (Fig. 1b)

On the FL1 LOG histogram gated by region "A", 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")
CD59 Cursor (marker) ("D")

$$\alpha FI = \alpha \times \alpha\beta \text{ bead MFI}$$

$$\beta FI = \beta \times \alpha\beta \text{ bead MFI}$$

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

• Sample analysis : tubes T2 (CD55) and T3 (CD59) (Figs. 2)

Do not modify the FL1 PMT voltage.

Draw an analysis region "A" around the granulocyte population on the FS vs SS cytogram (Fig. 2a). **Side scatter gain (SS) must be adjusted to optimize placement of the population.**

This region "A" must have an ellipse shape and must be restricted to the dense part of the granulocyte cloud in order to avoid inclusion of non granulocyte cells (monocytes for example).

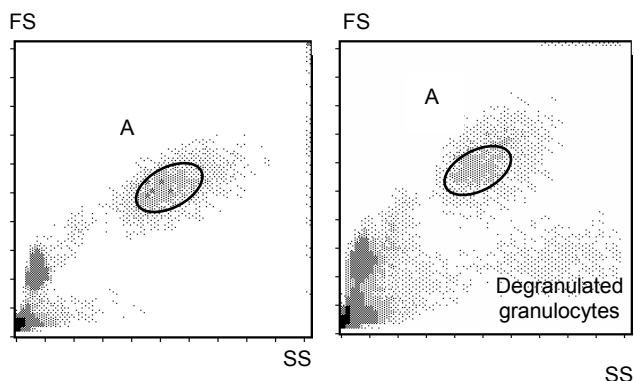
In the FL1 LOG histogram gated by region "A":

Tube T2 : note the percentage of cells included in region "C".

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

Fig. 2a : Setup of region "A" around the granulocyte population

Fig. 2b : Setup of region "A" around non degranulated granulocytes



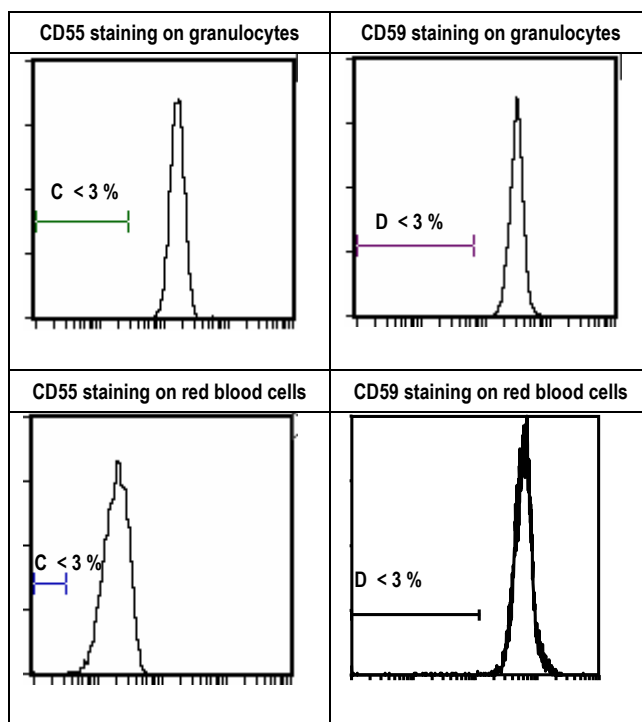
Note : sample ageing leads to the presence of a granulocyte sub-population of lower size (Fig. 2b). It is necessary to set the region "A" around the non degranulated granulocytes.

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 a 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 granulocytes and red blood cells (normal sample) :

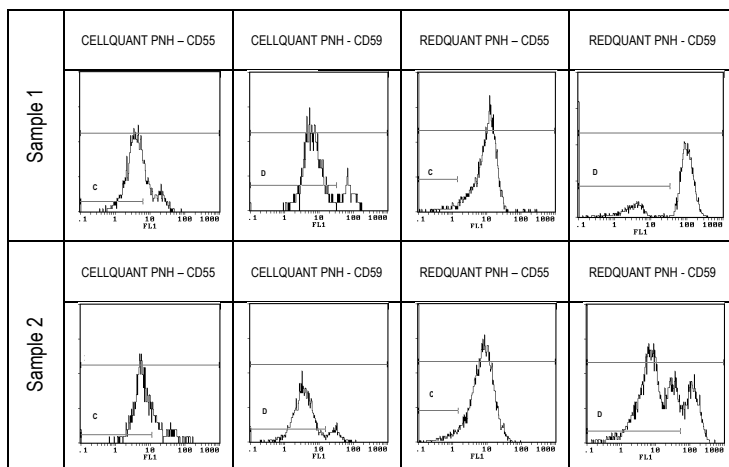


Test interpretation :

Nb of parameters > 3 %	CELLQUANT PNH		REDQUANT 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.

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



10 LIMITATIONS

An incomplete red cell lysis can be observed on some samples. This can generate false positive result on CD59 (> 3 % CD59 deficient cells) due to the fixation of CD59 antibody on residual red cells.

11 PERFORMANCES

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

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

23 PNH samples were confirmed PNH with the combined used 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 98.8 % of CD55 deficient cells.
From 0 % to 99.9 % of CD59 deficient cells.

11.4 Interpretation range :

From 0% up to 100% of CD55- and CD59-deficient cells.

11.5 Repeatability :

4 normal samples treated 5 times with the same kit.
All the tests present percentages of CD55- and CD59-deficient cells < 3 %.

11.6 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 : CD59 is > 3 %	Non optimal red cell lysis	Bring the distilled water used to dilute the lysing reagent to 37°C (using a waterbath) prior to lysing the specimen
On a normal sample a staining equivalent to background signal 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 signal is observed on the beads and on the granulocytes (> 3 % of deficient cells)	R4 reagent has not been added	Perform another test taking care to pipet the reagent
Few or none αβ beads are detected on FS vs SS histogram	Bad resuspension of the αβ beads	Take care to vortex for 5 seconds prior R3a vial opening and pipetting
After bead analysis, granulocytes are not detected on FS vs SS histogram	PMT SS has not been correctly set	Side scatter gain (SS) must be increased to optimize placement of the granulocyte population
Granulocyte population is heterogenous and CD55 and/or CD59 are > 3 % on a normal sample	Sample must have been drawn more than 8 hours ago and granulocytes are degranulated	Set the region "A" around the non degranulated granulocytes. This region "A" must have an ellipse shape and must be restricted to the dense part of the granulocyte cloud in order to avoid inclusion of non granulocyte cells
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 CELLQUANT PNH kit in combination with REDQUANT 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.

14 SYMBOLS

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

BIOCYTEX
140 ch. ARMEE D'AFRIQUE
13010 MARSEILLE
FRANCE
TEL : +33 (0) 4 96 12 20 40
FAX : +33 (0) 4 91 47 24 71
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