



## Screening Kit for Paroxysmal Nocturnal Hemoglobinuria (PNH)

Ref: CYT-HPN-1

*For Research Use Only*

### INTENDED USE

Screening Kit for Paroxysmal Nocturnal Hemoglobinuria (PNH) is a four-color direct immunofluorescence reagent for use in FC to evaluate the possible loss of expression of the CD16 molecule on neutrophils and loss of expression of the CD14 molecule on monocytes.

Although a rare disease, the PNH assay is frequently requested since the screening of PNH should be performed in patients with hemoglobinuria, patients with Coombs-negative intravascular hemolysis, specially patients with concurrent iron deficiency, patients with venous thrombosis involving unusual sites, patients with aplastic anemia, patients with refractory anemia-MDS and patients with episodic dysphagia or abdominal pain with evidence of intravascular hemolysis <sup>(1)</sup>.

### SUMMARY AND EXPLANATION

FC (FC) is a powerful tool for the analytical and quantitative characterization of cells which provides rapid, quantitative and multiparametric analysis of heterogeneous cell populations on a cell-by-cell basis. FC is performed on cells in liquid suspension that have been incubated with fluorescently-labeled antibodies directed against specific cellular proteins. The relative fluorescence intensity of the positive cells indicates the amount of antibody bound to specific binding sites on the cells, and therefore provides a relative measure of antigen expression.

PNH is an acquired clonal hematopoietic stem-cell disorder related to the occurrence of a somatic mutation in the PIG-A gene, located in the human X chromosome. This genetic alteration results in a partial or total deficiency of all proteins normally linked to the cell membrane by a glycosyl-phosphatidyl-inositol (GPI) anchor (GPI-anchored proteins) <sup>(2-3)</sup>. Typical clinical features of PNH are bone marrow failure of variable severity, thrombosis in unusual sites, chronic intravascular hemolytic anemia that leads to hemoglobinuria, iron deficiency anemia, and increased incidence of acute myeloid leukemia.

FC-based immunophenotypic techniques for the analysis of different markers expression on the major cell populations present in blood are the preferred method for the diagnostic screening of PNH <sup>(4)</sup>. Recent studies have analyzed the expression of a high number of GPI-APs on different subsets of peripheral blood cells. As conclusion of these studies, the best combination of markers for the screening of PNH would include evaluation of CD14 on monocytes and of CD16 on neutrophils, because these two markers allowed unequivocal identification of PNH cells in all patients analyzed and the largest sizes of the PNH clone were systematically found among these two cell subsets<sup>(5)</sup>. Screening Kit for PNH includes the following monoclonal antibodies combination: CD16-FITC/CD64-PE/CD45PerCP-Cyanine5.5/CD14-APC. The antibodies anti-CD45 and anti-CD64 allow the identification of the populations to study: neutrophils and monocytes. The further evaluation of a possible loss of expression of the CD16 molecule on neutrophils and loss of expression of the CD14 molecule on monocytes allows the identification of the PNH clone in a fast and simple way.

### PRINCIPLES OF THE PROCEDURE

FC (FC) is an innovative technology by means of which different cell characteristics are simultaneously analyzed on a single cell basis. This is achieved by means of hydrodynamic focusing of cells that pass aligned one by one in front of a set of light detectors; at the same time they are illuminated by a laser beam. The interaction of the cells with the laser beam generates signals of two different kinds: those generated by dispersed light (FSC/SSC), which mainly reflects the size of the cell and its internal complexity, and those related to the emission of light by the fluorochromes present in the cell. These signals become electric impulses which are amplified and registered as digital signals to be processed by a computer.

When the reagent is added to the sample, the fluorochrome-labeled MAb present in the reagent bind specifically to the antigens they are directed against, allowing the detection by FC of the cell populations carried by the antigen.

The erythrocyte population, which could hinder the detection of the target population, is eliminated by the use of a red blood cell lysing solution previous to acquire the sample on the cytometer. The use of QuickLysis™ (CYT-QL-1) erythrocyte lysing solution is recommended, since it requires no further washing step and contains no fixative, therefore minimizing the handling of the sample and avoiding the cell loss associated to the centrifuge process.<sup>(6, 7)</sup>

### REAGENT COMPOSITION

The Screening Kit for PNH is provided in phosphate buffered saline with < 0.1% sodium azide. It contains the following mixture of MAb:

- Fluorescein isothiocyanate (FITC) anti-human CD16, clone: 3G8; isotype IgG1.
- Phycoerythrin (PE) anti-human CD64, clone: 22, isotype IgG1.
- Peridinin chlorophyll protein-Cyanine 5.5 tandem (PerCP-Cyanine5.5) anti-human CD45 (LCA); clone: HI30, isotype IgG1.
- Allophycocyanin (APC) anti-human CD14, clone 47-3D6; isotype IgG1.

Purification: Affinity chromatography

Amount per 1.250 ml vial: 25 tests (50 µl reagent to 10<sup>6</sup> cells)

Reagents are not considered sterile.

### STORAGE CONDITIONS

The reagent is stable until the expiration date shown on the label, when stored at 2-8° C. The reagent should not be frozen or exposed to direct light during storage or during incubation with cells. Keep the reagent vial dry. Once opened, the vial must be stored in a vertical position to avoid any possible spillage.

### WARNINGS AND RECOMMENDATIONS

1. For research use only.
2. Peripheral blood is the most suitable specimen for immunophenotyping PNH. As the expression of GPI-anchored proteins depends on the maturity stage of the different hematopoietic series, the study in bone marrow samples unnecessarily complicates the analysis <sup>(1)</sup>.
3. This product is supplied ready to use
4. The reagent is stable until the expiration date shown on the label if it is properly stored. Do not use after the expiration date shown on the label. If the reagents are stored in conditions different from those recommended, such conditions must be validated by the user.

- Alteration in the appearance of the reagent, such as the precipitation or discoloration indicates instability or deterioration. In such cases, the reagent should not be used.
- It contains 0,09% (m/v) sodium azide (CAS-No. 26628-22-8) as a preservative, but even so care should be taken to avoid microbial contamination of reagent or incorrect results may occur.

**Indication(s) of danger:**

H302 Harmful if swallowed

**Safety advice:**

P264 Wash thoroughly after handling.

P270 Do not eat, drink or smoke when using this product.

P301+P312 If swallowed, call a poison center or doctor/physician if you feel unwell.

P301+P330 If swallowed, rinse mouth.

P501 Dispose of contents/container in accordance with local/regional/national/international regulation.

- All patient specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection <sup>(8)</sup>, and disposed according to the legal precautions established for this type of product. Also recommended is handling of the product with appropriate protective gloves and clothing, and its use by personnel sufficiently qualified for the procedures described. Avoid contact of samples with skin and mucous membranes. After contact with skin, wash immediately with plenty of water.
- Use of the reagent with incubation times or temperatures different from those recommended may cause erroneous results. Any such changes must be validated by the user.

## PROCEDURE

### Material included

Screening Kit for PNH is sufficient for 25 determinations (50 µl reagent to 10<sup>6</sup> cells).

### Material required but not included

- Flow cytometer equipped with 488 nm ion argon laser and 633 red laser and appropriate computer hardware and software.
- Test tubes suitable for obtaining samples in the flow cytometer used. Usually tubes with a rounded bottom for 6 mL, 12x 75 mm are used.
- Automatic pipette (100µL) and tips.
- Micropipette with tips.
- Chronometer
- Vortex Mixer
- Isotypic control reagent
- Quicklysis™ lysing solution
- Wash buffer as phosphate buffered saline (PBS) containing < 0.1% sodium azide.

### Preparation

Whole blood sample must be taken aseptically by means of a venipuncture <sup>(9, 10)</sup> in a sterilized tube for blood collection containing an appropriate anticoagulant (use of EDTA is recommended). The analysis requires one hundred (100) µl of the whole blood sample per tube, assuming a normal range of approximately 4 to 10 x 10<sup>3</sup> leucocytes per µl. For samples with a high white blood cell count, dilute samples with PBS to obtain a concentration of cells approximately equal to 1 x 10<sup>4</sup> cells/µL. Store the blood samples at 18-22°C until they are to be tested. It is advisable to test blood samples within the 24 hours after their extraction. Hemolyzed samples or samples with suspended cell aggregates should be rejected.

- Mix 100µl of peripheral blood with 50µl of screening Kit for PNH (CD16-FITC/CD64-PE/CD45-PerCP-Cyanine5.5/CD14-APC). To evaluate the non-specific binding of the antibody, an appropriated isotype control tube can be prepared.
- Incubate for 15 minutes at room temperature in the dark.
- Add 2 ml of Quicklysis™ erythrocyte lysing solution and incubate the sample for 10 minutes at room temperature in the dark.
- Acquire directly on the flow cytometer within the first four hours of finishing the sample preparation. If the samples are not acquired immediately after preparation, they should be stored in the dark at 2-8°C. To ensure the accuracy of the assay is recommended to acquire 100,000 events, especially in samples from patients with aplastic anemia or refractory anemia-MDS without clinical evidence of hemolysis.

Calibration of the instrument must be done according to the manufacturer's advice. Before acquiring samples, adjust the threshold or discriminator to minimize cellular debris and ensure populations of interest are included. Before acquiring the sample on the flow cytometer, mix the cells on the vortex at low speed to reduce aggregation.

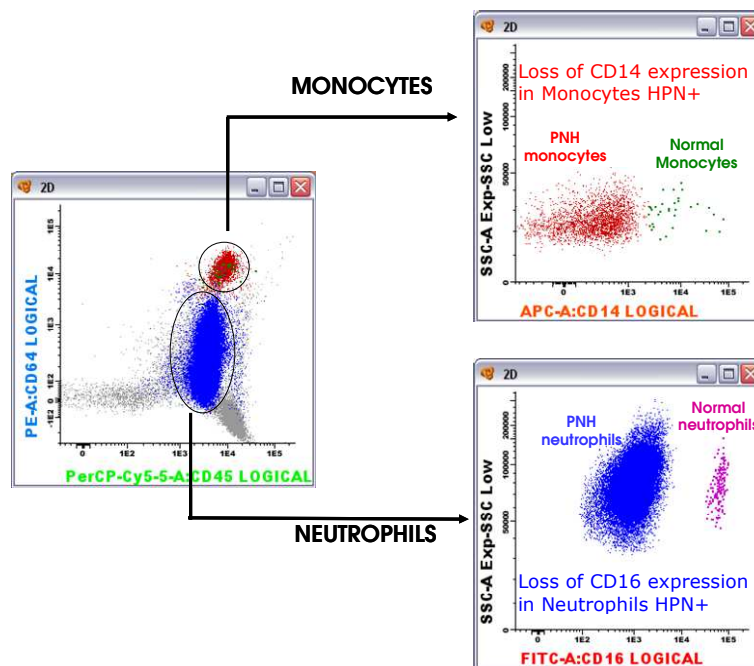
\*Note: The use of other lysing solutions may require the elimination of the lysed red blood cells. Follow the manufacturer's recommended protocol of the lysing solution used.

### FC analysis

Check that the cytometer is correctly aligned and standardized for light dispersion (FSC/SSC on linear scale) and fluorescent intensity (FL1, FL2, FL3 FL4 on logarithmic scale) and that the right color compensation has been set following the instructions of the cytometer manufacturer.

- Use a CD45/CD64 dot plot to define monocytes (CD45+/CD64++) and neutrophils (CD45+/CD64+) regions. It is important to exclude eosinophils from the region of neutrophils (eosinophils are CD16 negative and may lead to error).
- Evaluate the possible loss of expression of the CD16 molecule on neutrophils and loss of expression of the CD14 molecule on monocytes.
  - When there are two groups of expression (bimodal expression) of CD16 on neutrophils and two groups of expression of CD14 on monocytes the sample may be considered PNH positive.
  - When the expression bimodal is observed only in one of the two populations to study (neutrophils or monocytes), or when there is bimodal expression neither neutrophils nor monocytes, the sample is PNH negative.
- Only the samples suspected of being HPN positive would require further studies which provide additional clinically useful information (e.g. for management of the PNH-related anemia)

The following figure shows representative dot plots of CD14 and CD16 expression on peripheral blood monocytes and neutrophils from a PNH patient.



### LIMITATIONS

- Blood samples should be stored at 18-22°C and be tested within the 24 hours after they are obtained.
- It is advisable to acquire stained samples on the cytometer as soon as possible to optimize the results. Non-viable cells may stain nonspecifically. Prolonged exposure of whole blood samples to lytic reagents may cause white cell destruction and loss of cells from the target population.
- Results obtained by FC may be erroneous if the cytometer laser is misaligned or the gates are improperly set
- Certain patients may present special problems due to altered or very low number of certain cellular population.

### QUALITY CONTROL

- To obtain optimum results it is advisable to verify the precision of pipettes and that the cytometer is correctly calibrated.
- The fluorochromes fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), peridin chlorophyll protein-Cyanine 5.5 tandem (PerCP-Cyanine5.5) and allophycocyanin (APC) emit in different wavelengths but show a certain spectral overlapping which must be corrected by means of electronic compensation if combinations of different monoclonal antibodies are used conjugated with these fluorochromes. The optimum levels of compensation can be established by analysis in a dot-plot diagram of cells from healthy individuals stained with mutually exclusive monoclonal antibodies conjugated with the fluorochromes to be used in the test.
- To evaluate the non-specific binding of the antibody, an appropriated isotype control tube can be prepared.









### REFERENCES

1. Hernández-Campos PM et al. Hemoglobinuria paroxística nocturna. Med Clin 2008; 131 (16): 617-630.
2. Richards SJ, Hillmen P. Advances in the laboratory diagnosis of paroxysmal nocturnal hemoglobinuria. Clin Appl Immunol Rev 1 2001; 1:315-330.
3. Takeda J et al. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. Cell 1993; 73:703-711
4. Richards SJ et al. Application of FC to the diagnosis of paroxysmal nocturnal hemoglobinuria. Cytometry 2000;42:223-233.
5. Hernández-Campos PM et al. Detailed immunophenotypic characterization of different major and minor subsets of peripheral blood cells in patients with paroxysmal nocturnal hemoglobinuria. Transfusion 2008; 48:1403-1414.
6. Menéndez P, et al. Comparison between a lyse-and-then-wash method and a lyse-non-wash technique for the enumeration of CD34+ hematopoietic progenitor cells. Cytometry (Comm. Clin. Cytometry) 34: 264-271 (1998)
7. Gratama JW, Menéndez P, Kraan J, Orfao A. Loss of CD34+ hematopoietic progenitor cells due to washing can be reduced by the use of fixative-free erythrocyte lysing reagents. J Immunol. Methods 239: 13-23 (2000)
8. Protection of Laboratory Workers from occupationally acquired infections. Second edition; approved guideline (2001). Villanova PA: National Committee for Clinical Laboratory Standards; Document M29-A2.
9. Procedures for the collection of diagnostic blood specimens by venipuncture- approved standard; Fifth edition (2003). Wayne PA: National Committee for Clinical Laboratory Standards; Document H3-A5.
10. Clinical applications of FC: Quality assurance and immunophenotyping of lymphocytes; approved guideline (1998). Wayne PA: National Committee for Clinical Laboratory Standards; Document H42-A.

### WARRANTY

This product is warranted only to conform to the quantity and contents stated on the label. There are no warranties that extend beyond the description on the label of the product. Cytognos' sole liability is limited to either replacement of the product or refund of the purchase price.

#### EXPLANATION OF SYMBOLS

	Use by (YYYY-MM)
	Storage temperature limitation
	Keep out of sunlight
	Consult instructions for use
	For research use only
	Batch code
	Catalogue number
	Manufacturer

#### PRODUCED BY

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