# PNH Paroxysmal Nocturnal Hemoglobinuria

| REF | Σ |
|---|---|
| PNH-25T | 25 test |
| PNH-50T | 50 test |
| PNHWBC-25T | 25 test |
| PNHRBC-25T | 25 test |
| LyoPNH-25T | 25 test |
| LyoPNHWBC-25 | 25 test |
| LyoPNHRBC-25T | 25 test |

## 1. PRODUCT DESCRIPTION

**Reagent provided:**

- PNH: CD157/CD45/CD64
- LYOPNH: FLAER/CD157/CD45/CD64

**Tested application:** Flow Cytometry
**Storage buffer:** aqueous buffered solution containing protein stabilizer and 0.09% sodium azide (NaN3 ).
**Recommended usage:** Immunostep's PNH, is intended for the identification and enumeration of Paroxysomal Nocturnal Hemoglobinuria (PNH) to evaluate the possible loss of expression of the glycosylphosphatidylinositol (GPI) anchor molecule on neutrophils and monocytes by flow cytometry. This reagent is effective for direct immunofluorescence staining for flow cytometric analysis using 1 test or tube /10 6 cells.
**Presentation:**

- LYOPNH: freeze dried tube
- PNH: liquid

**The vial contains:**

- FLAER iFluor488 (only in LYOPNH reference)
- PE Anti-Human CD157, clone SY11B5, isotype IgG1
- PerCP Anti-Human CD45, clone D3/9, isotype IgG1, 4th International Workshops on Human Leucocyte Differentiation, WS Code 825
- APC Anti-Human CD64, clone 10.1, isotype IgG1, 6th International Workshops on Human Leucocyte Differentiation, WS Code MA36
- CD15 CF-Blue (MCS-1)
- CD235a FITC (HI264)
- CD59 PE (MEM-43)

**Clones and fluorochromes of reagents:**

| REFERENCE | FORMAT | CD45 | CD157 | CD64 | FLAER | CD15 | CD235a | CD59 | TARGET |
|---|---|---|---|---|---|---|---|---|---|
| PNH-25T | Liquid | X | X | X | | | | | Leukocytes |
| PNH-50T | Liquid | X | X | X | | | | | Leukocytes |
| PNHWBC-25T | Liquid | X | X | X | X | X | | | Leukocytes |
| PNHRBC-25T | Liquid | | | | | | X | X | Leukocytes |
| LYOPNH-25T | Liophilized | X | X | X | X | | | | Leukocytes |
| LYOPNHWBC-25T | Liophilized | X | X | X | X | X | | | Leukocytes |
| LYOPNHRBC-25T | Liophilized | | | | | | X | X | Erythrocytes |

## 2. CLINICAL RELEVANCE

Although a rare disease (2) , the PNH assay is frequently requested since the screening of PNH should be performed in patients with hemoglobinuria, patients with coombs-negative intravascular hemolysis, especially 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. (1,3,4).

## 3. ANTIGEN DETAILS

**Large description:**

- **FLAER (Fluorescent Aerolysin):** A probe that binds specifically to the GPI anchor, allowing for the detection of cells lacking this anchor, which is characteristic of PNH. FLAER is conjugated to iFluor488 for enhanced fluorescence detection.
- **CD157:** Expressed by macrophages, neutrophils, and other immune cells, it plays a role in neutrophil migration and adhesion. CD157 is expressed by macrophages, neutrophils, bone marrow stromal cells, endothelial cells, follicular dendritic cells, and T and B cell progenitors prior to the rearrangement of the antigen receptors.
- **CD45:** A human leukocyte antigen present on all leukocytes, weakly expressed on hematopoietic progenitor cells. CD45 recognizes a human leucocyte antigen that is a member of the leucocyte common antigen (LCA) family.
- **CD64:** The high-affinity receptor for IgG, important in immune response. CD64 is also known as the high-affinity receptor for IgG (FcγRI).

## 4. APPROPRIATE STOREAGE AND HANDLING CONDITIONS

**Store in the dark, refrigerated between 2 °C and 8 °C. DO NOT FREEZE. The antibody is stable until the expiry date stated on the vial label if kept at 2°C-8°C.**

Do not use after the date indicated. Once the vial is open, the product is stable for 90 days.

## 5. EVIDENCE OF DETERIORATION

Reagents should not be used if any evidence of deterioration is observed. For more information, please contact our technical service: tech@immunostep.com

The product's normal appearance is a semi-transparent, colourless liquid. It should not be used if liquid medium is cloudy or contains precipitate. It should be odourless.

## 6. RECOMMENDATIONS AND WARNINGS

- The reagents contain sodium azide. In acid conditions, it is transformed into hydrazoic acid, a highly toxic compound. Azide compounds must be diluted in running water before being discarded. These conditions are recommended so as to avoid deposits in plumbing, where explosive conditions could develop. The safety data sheet (SDS) is available online at www.immunostep.com
- Avoid microbial contamination of the reagent.
- Protect from light. Use dim light during handling, incubation with cells and prior to analysis.
- Never mouth pipette.
- In the case of contact with skin, wash in plenty of water.
- The samples should be handled in the same way as those capable of transmitting infection. Appropriate handling procedures should be guaranteed.
- Do not use after the expiry date indicated on the vial.
- Deviations from the recommended procedure could invalidate the analysis results.
- FOR RESEARCH USE ONLY.
- For professional use only.
- Before acquiring the samples, it is necessary to make sure that the flow cytometer is calibrated and compensated.

## 6. FLOW CYTOMETRY-BASED TEST FOR PNH DETECTION

**Overview:**
Flow cytometry is a powerful analytical technique used to measure the physical and chemical characteristics of cells or particles as they flow in a fluid stream through a beam of light, typically a laser. This method allows for the rapid analysis of multiple parameters at the single-cell level, making it ideal for immunophenotyping and the detection of specific cell populations, such as those in Paroxysmal Nocturnal Hemoglobinuria (PNH).

Flow cytometry is utilized to identify and quantify the loss of GPI-anchored proteins on neutrophils and monocytes:

1. **Staining with Antibodies:** Cells are stained with a combination of monoclonal antibodies, including FLAER (Fluorescent Aerolysin), which specifically binds to GPI anchors. CD157 is a GPI-anchored marker utilized in the detection of PNH, while FLAER serves as a tool for identifying the presence or absence of GPI anchors on the surface of cells. Additionally, other antibodies that are not GPI-linked, such as CD45 and CD64, are also used in the staining process.

2. **Analysis of Cell Populations:** The flow cytometer analyzes the stained cells, allowing for the differentiation between normal and PNH-affected cells. A significant reduction in GPI-anchored proteins on the surface of these cells indicates the presence of PNH.

3. **Quantification:** The results are presented as histograms or dot plots, showing the distribution of cell populations based on their fluorescence intensity. This quantitative data aids in the diagnosis and monitoring of PNH.

## 8. PROTOCOL

**Sample Preparation**

1. Collect whole blood aseptically by venipuncture in a sterile tube containing EDTA anticoagulant.
2. Perform a white blood cell (WBC) count on all samples.
3. If WBC count exceeds 10 x $10^3$ cells/$mm^3$, dilute the sample with 1X PBS ($Ca^2$ $Mg^{2+}$ free) containing 0.5% fetal calf serum (FCS) and 0.1% sodium azide.
4. Prepare 100 µL of whole blood sample per tube, assuming a normal range of 4-10 x $10^3$ leucocytes/µL.

**Staining Procedure**

**Liquid Format (PNH Reference)**

1. Antibody Cocktail Preparation
   - Pipette 20 µL of PNH reagent (CD157/CD45/CD64) + 5 µL FLAER iFluor™488 (Ref. FLAER) into the tube. FLAER not included in PNH reference.
2. Sample Addition
   - Add 100 µL of sample directly to the tube containing lyophilized FLAER/CD157/CD45/CD64 to resuspend the reagents.

**Lyophilized Format (LYOPNH )**

1. Rehydration
   - Add 100 µL well-mixed blood sample directly to lyophilized pellet. Contains pre-formulated FLAER/CD157/CD45/CD64. After sample addition, vortex vigorously for 10 seconds, repeating the process three times to ensure complete mixing.

**Shared Protocol Steps**

1. Gently vortex tubes and incubate for 15 minutes at room temperature (20-25°C) in the dark.
2. Add appropriate volume of lysing solution according to the manufacturer's instructions.
3. Gently vortex tubes and incubate for 10 minutes at room temperature in the dark.
4. Centrifuge for 5 minutes at 540 x g.
5. Carefully aspirate the supernatant using a Pasteur pipette or vacuum system, leaving approximately 50 µL of residual volume to avoid disturbing the cell pellet.
6. Add 2 mL of PBS containing 0.5% FCS and 0.1% $NaN_3$.
7. Gently vortex tubes to resuspend the cell pellet.
8. Centrifuge for 5 minutes at 540 x g.
9. Carefully aspirate the supernatant as in ste
10. Resuspend the cell pellet in 200 µL of PBS containing 0.5% FCS and 0.1% $NaN_3$.

**Flow Cytometry Analysis**

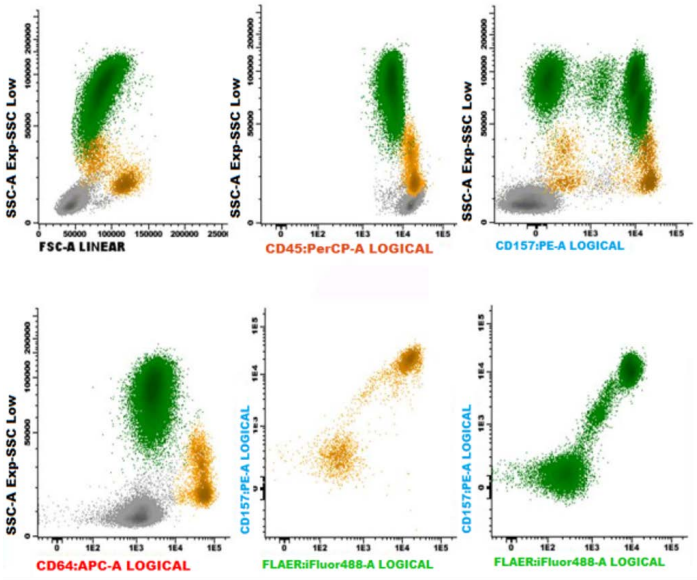
1. Analyze samples within 1 hour of staining or store at 4°C for a maximum of 3 hours before measurement.

2. If samples are not analyzed immediately, vortex thoroughly just before acquisition on the flow cytometer.

**Quality Control Notes**

- Ensure all reagents and samples are at room temperature before use.
- Protect samples from light throughout the staining procedure.
- Optimize centrifugation speed and time if necessary for your specific cell type.
- Always use appropriate personal protective equipment and follow good laboratory practices.
- Include FLAER-positive control (normal neutrophils) and FLAER-negative control (PNH clone) in each run.
- Spectral compensation: Required for iFluor™488/FITC channel overlap.
- Do not use FLAER for erythrocyte/platelet analysis - maintain CD59 testing for these lineage.

This modification follows ISCC guidelines for PNH testing while maintaining compatibility with both reagent formats. The FLAER addition enhances sensitivity for detecting small GPI-deficient clones (<0.01% detection limit).




The histograms are biparametric representations (Side Scatter versus Fluorescence Intensity) of a lysate stabilized whole-blood PNH sample gated on Leukocytes. Human peripheral blood was stained with PNH. Monocytes are represented in yellow and Neutrophils in green.

## 9. CONCLUSION

Flow cytometry provides a rapid, sensitive, and quantitative method for detecting PNH. By analyzing the expression of specific cell surface markers, it enables clinicians to identify patients with this rare hematological disorder efficiently. The ability to perform multiparametric analysis in a single test enhances the understanding of the disease's pathophysiology and guides clinical decision-making.

Please, refer to www.immunostep.com for technical information.

## 10. WARRANTY

Warranted only to conform to the quantity and contents stated on the label or in the product labelling at the time of delivery to the customer. Immunostep disclaims hereby other warranties. Immunostep's sole liability is limited to either the replacement of the products or refund of the purchase price.

## 11. ADDITIONAL INFORMATION

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Not for resale. Immunostep will not be responsible of violations that may occur with the use of this. Unless otherwise indicated by Immunostep by written authorization, this product is intended for research only and is not to be used for any other purpose, including without limitation, for human or animal diagnostic, therapeutic or commercial purposes.

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## 12. REFERENCES


1. Hernández-Campos PM et al. Hemoglobinuria paroxística nocturna. Med Clin 2008; 131 (16): 617-630.
2. Richards ST, Hillmenen P. Advances in the laboratory diagnosis of paroxysmal nocturnal hemoglobinuria. Clin Appl Immunol Rev I 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. 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.


## 13. MANUFACTURED BY


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