Chapter 1

Accurate and High Sensitivity Identification of PNH Clones by Flow Cytometry

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Additional information is available at the end of the chapter

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Abstract

Flow cytometry performs a key role in the diagnosis of paroxysmal nocturnal hemoglobinuria (PNH). Careful selection and validation of antibody conjugates have allowed the development of reagent cocktails suitable for the high sensitivity detection of PNH red blood cells (RBCs) and white blood cells (WBCs) in PNH and related diseases such as aplastic anemia (AA) and some subsets of myelodysplastic syndromes (MDS). A CD235a-FITC/CD59-PE assay was developed capable of detecting Type III PNH RBCs at a limit of quantification (LOQ) of 0.01% or better. While separate 4-color Fluorescent Aerolysin (FLAER), CD24, CD15 and CD45-based neutrophil and FLAER, CD14, CD64 and CD45-based monocyte assays were developed to detect PNH WBC phenotypes, 5-, 6- and 7-color assays have subsequently been developed for more modern cytometers equipped with five or more fluorescence detectors. For instrumentation with five detectors, a single tube 5-color FLAER, CD157, CD15, CD64 and CD45-based assay to simultaneously detect PNH neutrophils and monocytes has been developed. For instruments with six or more detectors and multiple lasers, a variety of 5-, 6- and 7-color assays have been developed using combinations of FLAER, CD24, CD14 and CD157. All WBC assays have a limit of quantification (LOQ) of 0.1% or better. Using these standardized approaches, results have demonstrated good intra- and inter-laboratory performance characteristics even in laboratories with little prior experience performing PNH testing.

Keywords: PNH, flow cytometry, high sensitivity assay, validation, standardization

1. Introduction

Paroxysmal nocturnal hemoglobinuria is a rare, life-threatening acquired hematopoietic stem cell disorder resulting from the somatic mutation of the X-linked phosphatidylinositol glycan

complementation Class A (PIG-A) gene [1]. PIG-A normally encodes an enzyme involved in the first stage of glycosylphosphatidylinositol (GPI) biosynthesis but in PNH, as a result of the mutation(s) in this gene, there is a partial or absolute inability to make GPI-anchored proteins, including complement defense structures such as CD55 and CD59 on red blood cells (RBCs) and white blood cells (WBCs) [2, 3]. Absence of CD59 in particular [4] and CD55 on RBCs is responsible for intravascular hemolysis associated with clinical PNH. Clonal expansion of the PNH population frequently occurs in patients with aplastic anemia in which normal hematopoiesis has failed, and with modern, high sensitivity assays, up to 70% of AA patients have detectable PNH clones [5]. Small populations of GPI-deficient PNH phenotypes have been reported in patients with early stage myelodysplastic syndrome (MDS) [6, 7]. Patients present with a wide range of clinical features, including intravascular hemolysis (that leads to hemoglobinuria), bone marrow failure and thrombosis, with the latter being a major cause of morbidity and mortality [5, 8]. As PNH is an acquired stem cell disease, it is important to demonstrate the loss of GPI-linked cell surface structures in at least two hematopoietic cell lineages, traditionally RBCs and neutrophils, although as more data have recently accumulated, monocytes should also be assessed as monocytes often exhibit a higher 'clone size' than is present in neutrophils. For true high-sensitivity assay design, it is critical to include carefully validated lineage-specific gating reagents such as CD235a (Glycophorin A) for RBC identification, CD15 for neutrophil identification and CD64 for monocyte identification. Examination of RBCs in the non-transfused PNH patient provides the most accurate assessment of the distribution of Type III PNH RBCs (complete CD59 deficiency), Type II PNH RBCs (partial CD59 deficiency) and normal Type I RBCs (normal CD59 expression). The distributions of these populations show a wide variation from patient to patient and delineation between the various types is not always clear-cut [9]. RBC analysis is important in PNH, as accurate determination of the distribution of Type II and III cells; patients with greater than 20% Type III RBCs almost always show clinical evidence of hemolysis [5]. While the loss of GPI-linked CD55 and CD59 was traditionally used to detect PNH RBCs [10, 11], 'routine' CD55 and/or CD59-based approaches are neither accurate nor sensitive below the 1–2% clone size, rendering them inadequate to detect small PNH clones typically found in PNH+ AA and MDS cases [5] or even in some heavily transfused PNH cases.

2. Pre-analytical phase

2.1. Red blood cells

2.1.1. Sample and reagent requirements

Freshly drawn EDTA (preferred) or heparin anti-coagulated whole peripheral blood is used for analysis. If samples are shipped or need to be stored prior to analysis, the blood sample should be kept at 4°C and should generally be used within 48 hours of sample draw. For high-sensitivity RBC analysis, the International Clinical Cytometry Society (ICCS) Guidelines recommended the use of CD235a (for RBC gating) and CD59 (to detect GPI-deficient cells) [12]. In the follow-up 'Practical Guidelines' [13], a large number of clones/conjugates to CD235a-FITC and CD59-PE were tested but only a few were found to have acceptable performance

Target	Antibody Conjugates	Purpose	Clone and Vendor
RBC	CD235a-FITC	Gating on RBC	10F7MN (eBio) YTH 89.1 (Cedarlane) KC16 (BC) JC159 (DAKO)
	CD59-PE	GPI-linked for RBC	OV9A2 (eBio) MEM-43 (Invitrogen) MEM-43 (EXBIO/Cedarlane)

Table 1. Recommended CD235a-FITC and CD59-PE conjugates for high-sensitivity PNH RBC assay.

characteristics and further validated for a variety of instrument platforms (**Table 1**). Of note, even selected conjugates required extensive titration on an individual basis to minimize aggregation prior to premixing or 'cocktailing' for this assay. Only by performing extensive titrations, we were able to identify conjugates with the best performance characteristics for this specific assay. Premixing of reagents once adequately titrated is critically important in PNH assays as both RBC and WBC assays are designed to detect GPI-deficient phenotypes, i.e., cells unstained by the GPI-specific reagents. Given the very small volumes of reagents used for the RBC assay in particular, it is usually necessary to make a dilution of the RBC cocktail such that accurately pipettable volumes of reagent can be employed.

2.1.2. Staining procedure

Blood samples are diluted 1:100 with fresh clean PBS and 100 μL is carefully pipetted using reverse pipetting techniques directly into the bottom of the staining tube taking care to avoid aerosols and blood trails on the inside of the tube. The appropriate volume of diluted CD235aFITC/CD59PE is then pipetted directly into the bottom of the tube and admixed with the diluted sample by gently up-and-down pipetting. After careful removal of the tip, the sample can be gently 'swirled' on a vortex set at a very low speed to avoid aerosol generation. After 20 minutes, the sample must be washed twice with clean phosphate buffered saline (PBS), resuspended in 1 ml of PBS and then 'racked' immediately before data acquisition to disrupt any RBC aggregates generated during the staining process [13].

2.2. White blood cells

2.2.1. Sample and reagent requirements

For high-sensitivity WBC analysis using a single tube approach, CD45 is employed for pattern recognition and to exclude unlysed RBCs, other debris not excluded by light scatter thresholding. Thereafter, carefully selected/validated conjugates of CD15 and CD64 are used to accurately delineate/'gate' neutrophils and monocytes, respectively. To detect GPI-deficient CD15-gated neutrophils, FLAER is used in combination with either carefully selected/validated CD24 or CD157 conjugates, and to detect GPI-deficient CD64-gated monocytes, FLAER is used in combination with either carefully selected/validated CD14 or CD157 conjugates (**Table 2** and **3**) [13–16].

Target	Antibody Conjugates	Purpose	Clone (Vendor)
WBC	FLAER-Alexa488	GPI-linked (Neuts + Monos)	NA (Cedarlane)
	CD24-PE CD24-APC	GPI-linked (Neuts)	SN3 (eBio), ALB9 (BC) SN3 (eBio, EXBIO)
	CD14-PE CD14-APC700	GPI-linked (Monos)	61D3 (eBio), RMO52 (BC) Tuk4 (Invitrogen) RMO52 (BC)
	CD157-PE	GPI-linked (Neuts + Monos)	SY11B5 (eBio, EXBIO, BD, BC, Sysmex)
	CD64-PC5 CD64-ECD CD64-PC7	Gating on Monocytes	22 (BC) 22 (BC) 22 (BC), 10.1 (EXBIO)
	CD15-PC5 CD15-PerCP-eF710 CD15-PerCPCy5.5	Gating on Neutrophils	80H5 (BC) MMA (eBio) MEM158 (EXBIO)
	CD45-PC7 CD45-KO CD45-eF450	Debris/unlysed RBC exclusion + pattern recognition	J33 (BC) J33 (BC) 2D1 (eBio)

Table 2. Recommended clones/conjugates to determine high-sensitivity detection of PNH WBC on Beckman Coulter Cytometers.

Target	Antibody Conjugates	Purpose	Clone (Vendor)
WBC	FLAER-Alexa488	GPI-linked (Neuts + Monos)	NA (Cedarlane)
	CD24-PE CD24-APC	GPI-linked (Neuts)	SN3 (eBio), ML5 (BD) SN3 (eBio, EXBIO)
	CD14-PE CD14-APC	GPI-linked (Monos)	61D3 (eBio), Tuk4 (Invitrogen) MoP9 (BD)
	CD157-PE	GPI-linked (Neuts + Monos)	SY11B5 (eBio, EXBIO, BD, BC, Sysmex)
	CD64-APC CD64-PECy7	Gating on Monocytes	10.1 (BD, eBio) 10.1 (EXBIO), 22 (BC)
	CD15-APC CD15-PerCP-eF710 CD15-PerCPCy5.5	Gating on Neutrophils	HI98 (BD) MMA (eBio) MEM 158 (EXBIO)
	CD45-eF450 CD45-PerCP CD45-APC-H7	Debris/unlysed RBC exclusion + pattern recognition	2D1 (eBio) 2D1 (BD) 2D1 (BD)

Table 3. Recommended clones/conjugates for high-sensitivity detection of PNH WBC on Becton Dickinson Cytometers.

2.2.2. Staining procedure

Undiluted anti-coagulated whole blood is the preferred sample source for the analysis of PNH phenotypes in WBCs. Reverse pipetting is used to dispense 100 μL of sample into the

staining tube, taking all the precautions noted above for the RBC assay to avoid aerosols and ensure that the sample is not left on the wall of the tube. The reagent set in use should be cocktailed, once optimal volumes of each reagent have been determined by titrations. The appropriate volume of cocktail is added directly into the blood sample at the bottom of the tube and gently admixed by up-and-down pipetting. After gentle swirling to avoid aerosols, the sample is incubated in the dark for 20–30 minutes at room temperature before RBC lysis. There are a variety of commercial lysing agents available such as Versalyse, FACSLyse and Immunoprep and most do a very good job. After lysis following manufacturers' recommendations, the sample is centrifuged and washed with PBS supplemented with 1% serum albumin. The sample is resuspended in 0.5–1 ml of PBS and acquired.

3. Analytical phase and data reporting

3.1. Instrument setup and standardization

Optimal instrument setup and standardization is a prerequisite for reproducible results over time and among laboratories. For the analysis of RBCs, the forward scatter (FS) and side scatter (SS) voltages are set in logarithmic mode and voltages adjusted to bring all unstained RBCs into the middle of the plot and above any FS threshold/discriminator. For WBCs, light scatter voltages are set in linear mode at such values that all unstained leukocyte subsets including lymphocytes scatter above the FS threshold and are clearly clustered on scale. Photomultiplier tube (PMT) voltage optimization, standardization and computer-assisted spectral overlap compensation are mandatory steps for instrument standardization of multiparameter assays and can be performed using an instrument platform-based approach (BD Biosciences, Beckman Coulter) or interplatform-based approach [17–20].

3.2. Data acquisition and analysis

3.2.1. Red blood cells

For high-sensitivity RBC analysis, the ICCS Guidelines recommended the use of CD235a (for RBC gating) and CD59 (to detect GPI-deficient cells) [9]. Based on subsequent publications that included rigorous testing and validation of various CD235a and CD59 clones and conjugates, optimal reagent combinations of CD235a-FITC and CD59-PE were identified [13]. Once extensively titrated, these reagents in combination did not cause major aggregation of RBCs while still maintaining a good signal-to-noise ratio and the ability to adequately separate Type II and Type III PNH RBCs from normal (Type I) RBCs [13–15]. Red blood cells are analyzed by a series of gating dot plots beginning with TIME versus SS, FS versus SS with detectors set in logarithmic mode, and CD235a-FITC versus FS to gate singlet RBCs and to quantify and exclude any remaining RBC aggregates (**Figure 1**). TIME is collected as a parameter and monitored during acquisition so that if fluidics problems are encountered, the sample can be reacquired if possible, or if not, data acquired prior to the fluidics hiatus can be 'gated' and only that portion of the data file subsequently analyzed. It is important to adjust the threshold (discriminator) for the FS so that no RBCs are excluded from acquisition. The diagnostic plots include a bivariate CD59 versus CD235a dot plot, a bivariate CD59 versus CD235a density

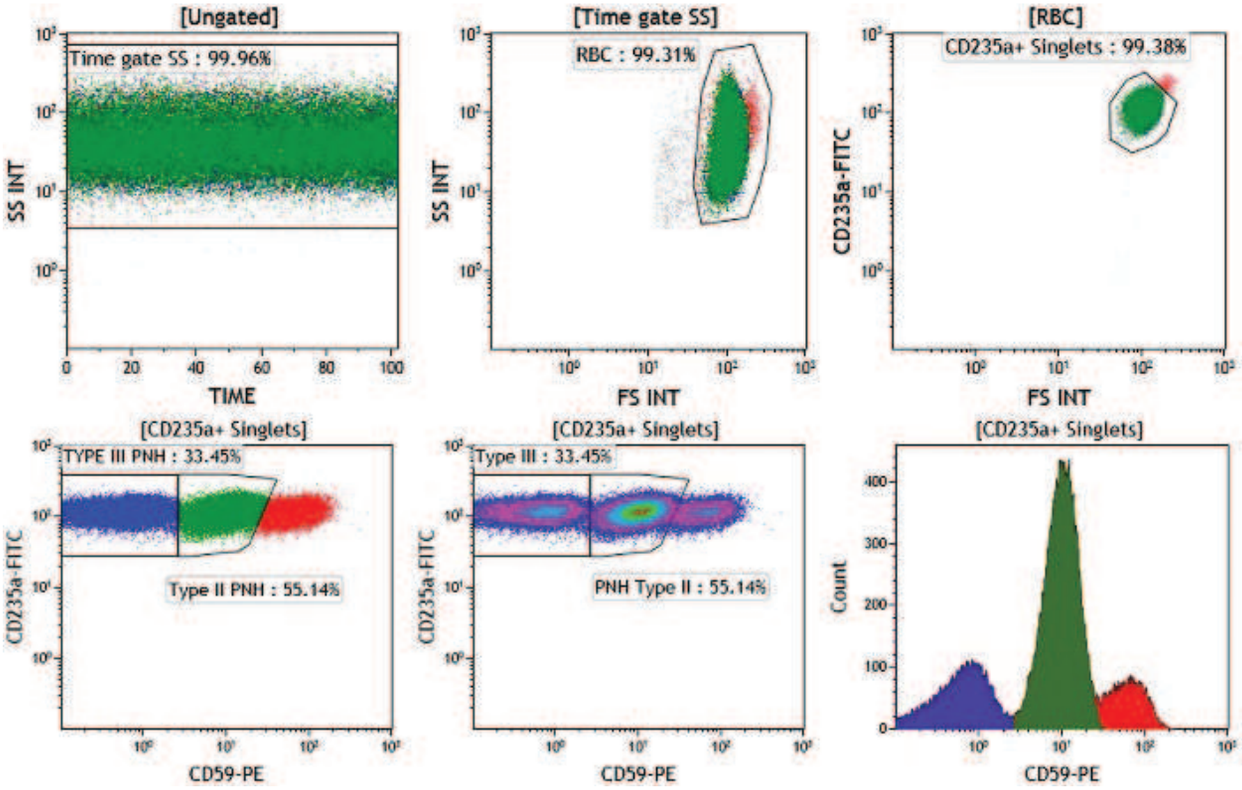

Figure 1. Sequence of bivariate gating and diagnostic dot-plots for analysis of PNH RBCs.

plot and a single parameter histogram of CD59 staining (**Figure 1**). Bivariate dot plots and/or density plots are recommended over single-parameter histograms, especially for samples containing small numbers of PNH phenotypes, for identifying poorly stained samples that need to be re-stained and for detecting media contamination and troubleshooting instrumentation issues [13]. However, while data regarding clone sizes come predominantly from the two-dimensional plots, in which the gating regions are linked across the dot plot and density plots, the single parameter histogram can also be useful in some situations. All three plots work in concert for optimal adjustment of the regions for Type III PNH cells and Type II PNH cells. An additional utility of the single parameter histogram is in comparing old versus new plots of CD235a-FITC/CD59-PE cocktails when tested on non-PNH samples.

3.2.2. White blood cells

High-sensitivity methodologies to detect PNH phenotypes in neutrophils and monocytes have been published previously. These methods were initially based on two separate 4-color neutrophil (FLAER, CD24, CD15 and CD45) and monocyte (FLAER, CD14, CD64 and CD45) tubes. In this earlier setting, samples were stained first with the RBC and neutrophil cocktails and if PNH phenotypes were detected, the 'reflex' monocyte tube was thereafter set up. The current document uses the same gating strategy used in earlier assays but discuss the more modern single tube assays on newer flow cytometers with 5, 6, or more PMTs that allow the simultaneous detection and quantification of both neutrophils and monocytes.

3.2.2.1. FLAER/CD24/CD14-based assay

For laboratories equipped with modern cytometers with 6-, 8- or 10 PMTs (Canto, Canto II and Navios), it is possible to configure 6-color cocktails based on FLAER, CD24 and CD14 (**Tables 2** and **3**). **Figure 2** shows the sequence of bivariate gating and diagnostic dot-plots from a Navios-specific reagent set comprising FLAER-Alexa488, CD24-PE (clone ALB9), CD15-PC5 (clone 80H5), CD64-PC7 (clone 22), CD14-APCA700 (clone RMO52) and CD45-KO (clone J33). The FS versus SS plot is gated from TIME versus SS plot (not shown) and light scatter voltages are set so that all WBC subsets are clearly visible and optimally separated; the threshold/discriminator is set to ensure that no lymphocytes are excluded. A debris exclusion gate (or WBC inclusion gate) can then be established to exclude any debris above the threshold/discriminator but below the

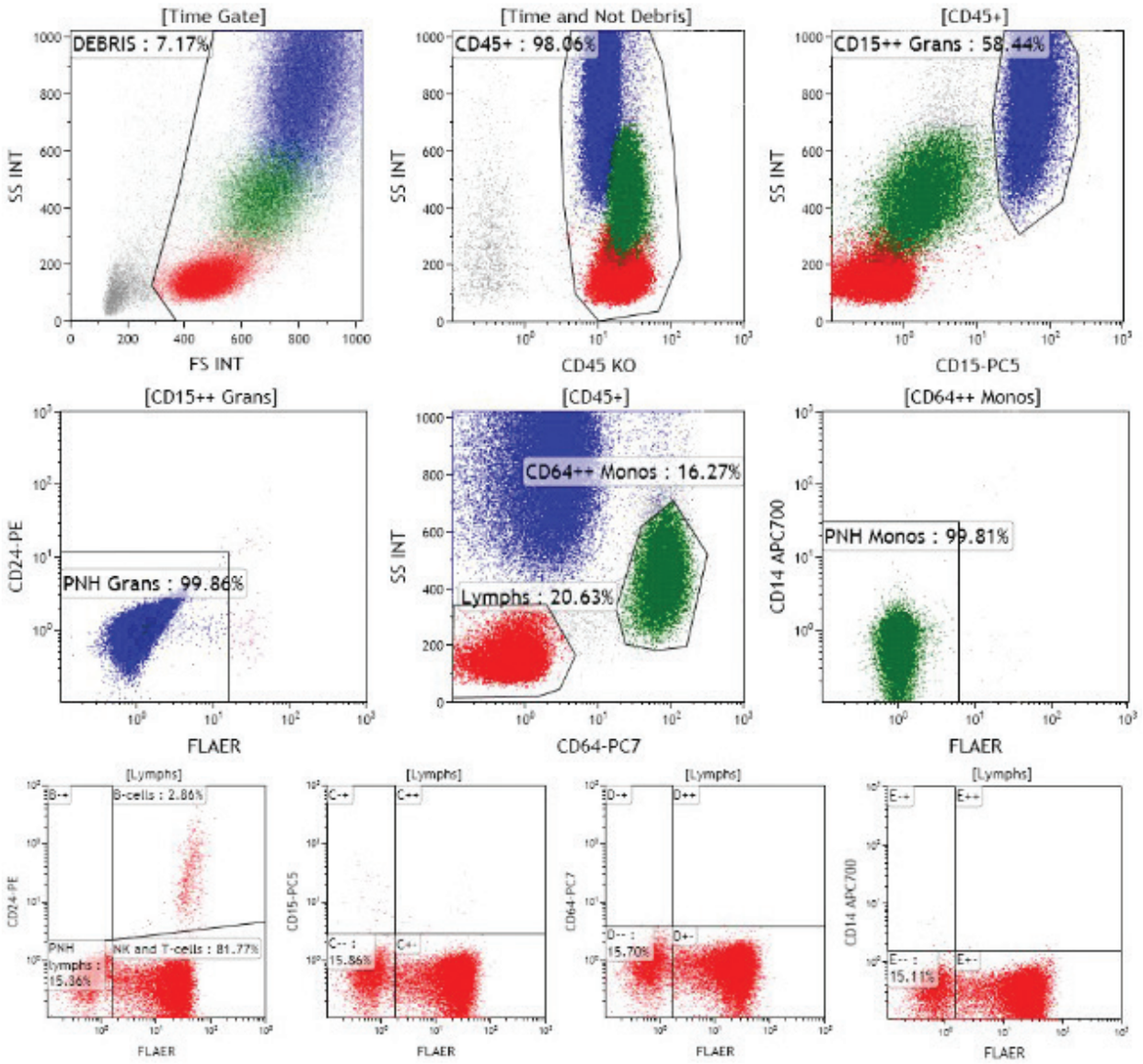

Figure 2. Sequence of bivariate gating and diagnostic dot-plots for FLAER/CD24/CD14- based analysis of PNH neutrophils and monocytes.

smallest lymphocytes. The CD45 versus SS plot is then gated through Boolean gating on Time and "not debris" with a gate drawn around the CD45+ cells. The CD45 versus SS plot is useful not only for pattern recognition but also for excluding any unlysed RBCs and other debris not removed by the debris exclusion gate. The CD15 versus SS plot is gated on the CD45+ populations and includes a gate drawn around the CD15++ neutrophils excluding as well as possible the CD15 dim + eosinophils visible to the left of the neutrophil/granulocyte population. The diagnostic FLAER/CD24 plot is gated on the CD15++ neutrophils and a region is drawn to encompass the FLAER-negative/CD24-negative cells, which represent the PNH neutrophils. The CD64 versus SS plot is also gated on the CD45+ cells and a region is drawn around the CD64++ monocytes. The FLAER versus CD14 dot plot is gated on the CD64++ monocytes and a region is drawn to delineate the FLAER-negative/CD14-negative cells, which represent the PNH monocytes. The lymphocytes gated on the CD64-negative/low SS plot are not a suitable target population for the PNH clone quantification due to their long lifespan. However, they serve as internal controls for verification of antigen expression and compensation settings. Plotting FLAER versus CD24, CD14, CD15 and CD64 verifies the instrument voltage and compensation settings as visible and clustered populations in the "correct" location. Plot FLAER versus CD24 shows B-cells (FLAER+/CD24+) verifying that both reagents were added, FLAER+/CD24-negative NK and T-cells and no dual-negative cells as this is a PNH-negative sample.

3.2.2.2. FLAER/CD157-based assay

ADP-ribosyl cyclase 2 (CD157) is a GPI-anchored cell surface enzyme encoded by the bone marrow stromal cell antigen-1 gene, which plays a role in pre-B cell growth [21]. Within the hematopoietic system, CD157 is highly expressed on both mature neutrophils and monocytes [22] leading to the possibility that CD157 could replace both CD24 and CD14, allowing the development of a single tube, high sensitivity 5C assay to identify and quantify both PNH neutrophils and PNH monocytes on cytometers with five or more PMTs [14–16]. The ability to perform simultaneous evaluation of both PNH neutrophils and PNH monocytes is particularly attractive to laboratories equipped with 5-C instruments such as the FC500 due to the major cost and time savings involved over running two separate 4-color assays for neutrophils and monocytes [14–16]. The gating and analysis strategies are similar to the ones used for the above described single-tube 6-color assay, except for the diagnostic FLAER/CD157 dot plots gated on CD15++ neutrophils and CD64++ monocytes (**Figure 3**). Three control lymphocyte plots (FLAER/CD15, FLAER/CD64 and FLAER/CD157) are also shown to monitor instrument setup and compensation.

It is important to note that several CD157-negative, non-PNH cases have been observed in the authors' laboratories (unpublished data). For these rare cases, the inclusion of the second GPI reagent (FLAER) as part of the built-in robustness of the assay prevents the misinterpretation of the data as a PNH clone-containing sample. Furthermore, in keeping with current state-of-the-art guidelines [12, 13], the RBC lineage should also be analyzed on every sample tested for the presence of PNH WBCs. As these rare CD157-negative non-PNH samples only contain normal (Type I) RBCs, there is even less chance of misinterpretation. An example of a CD157-negative case is shown in **Figure 4**. The sample was stained with a 7-color combination

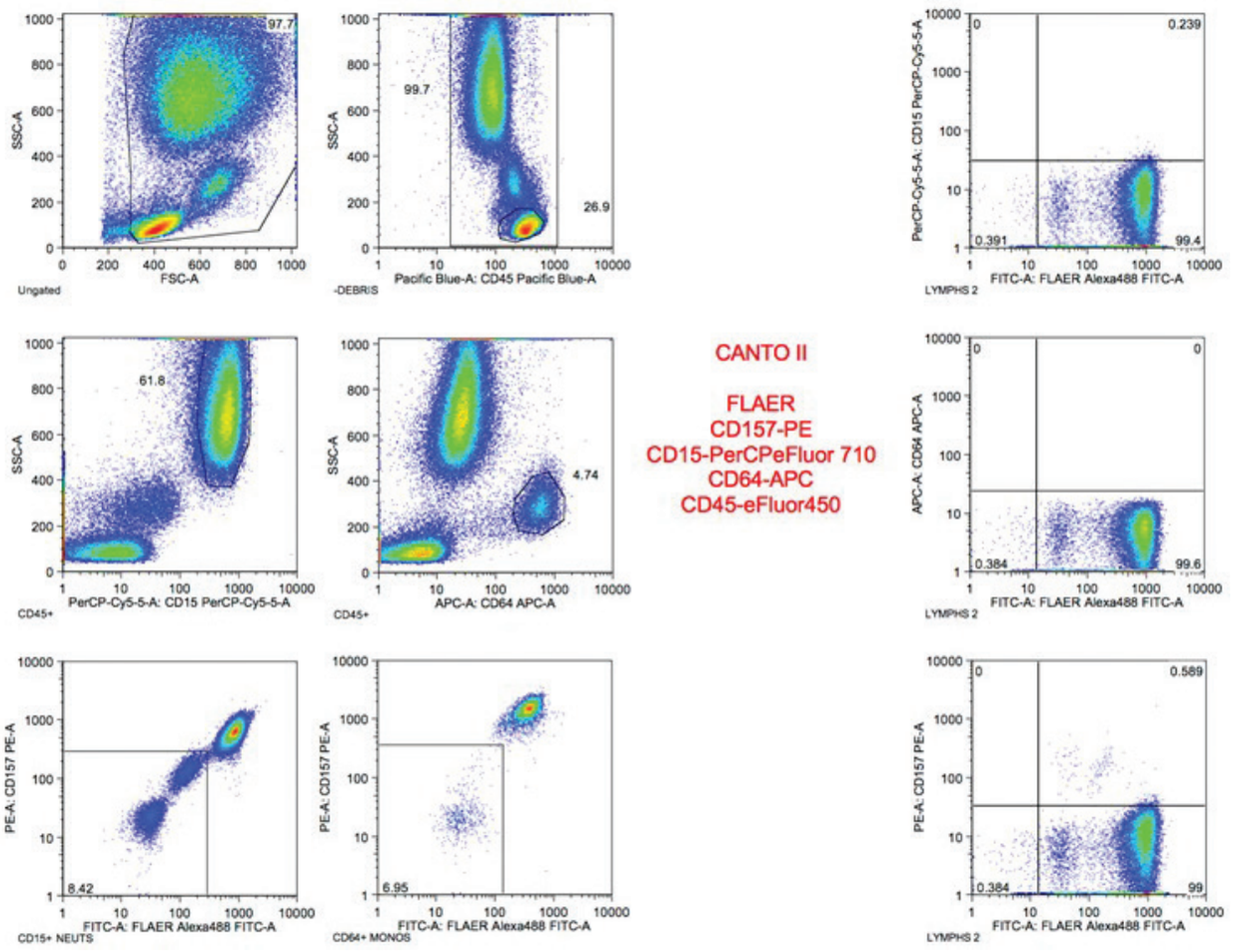

Figure 3. Sequence of bivariate gating and diagnostic dot-plots for FLAER/CD157- based analysis of PNH neutrophils and monocytes.

of FLAER, CD157, CD24, CD14, CD15, CD64 and CD45. While the CD157 failed to stain normal neutrophils in this sample, FLAER and CD24 stained the neutrophils in the expected manner. Similarly, while CD157 failed to stain normal monocytes in this sample, FLAER and CD14 stained monocytes in the expected manner.

3.3. Reporting

The following components are recommended for a PNH report:

1. The presence or absence of PNH clones. It is important to be clear and to avoid potentially misleading ambiguous terminology. A report stating that a CD59 test is negative may imply to some providers that the target population is negative for the GPI marker CD59 (thus indicating a PNH clone) or that absence of CD59 is not seen (thus indicating the absence of a PNH clone).

2. PNH clone size in the RBCs (total PNH clone size as well as the percentages for Type II and Type III PNH populations). There is a clinical significance associated for Type II and

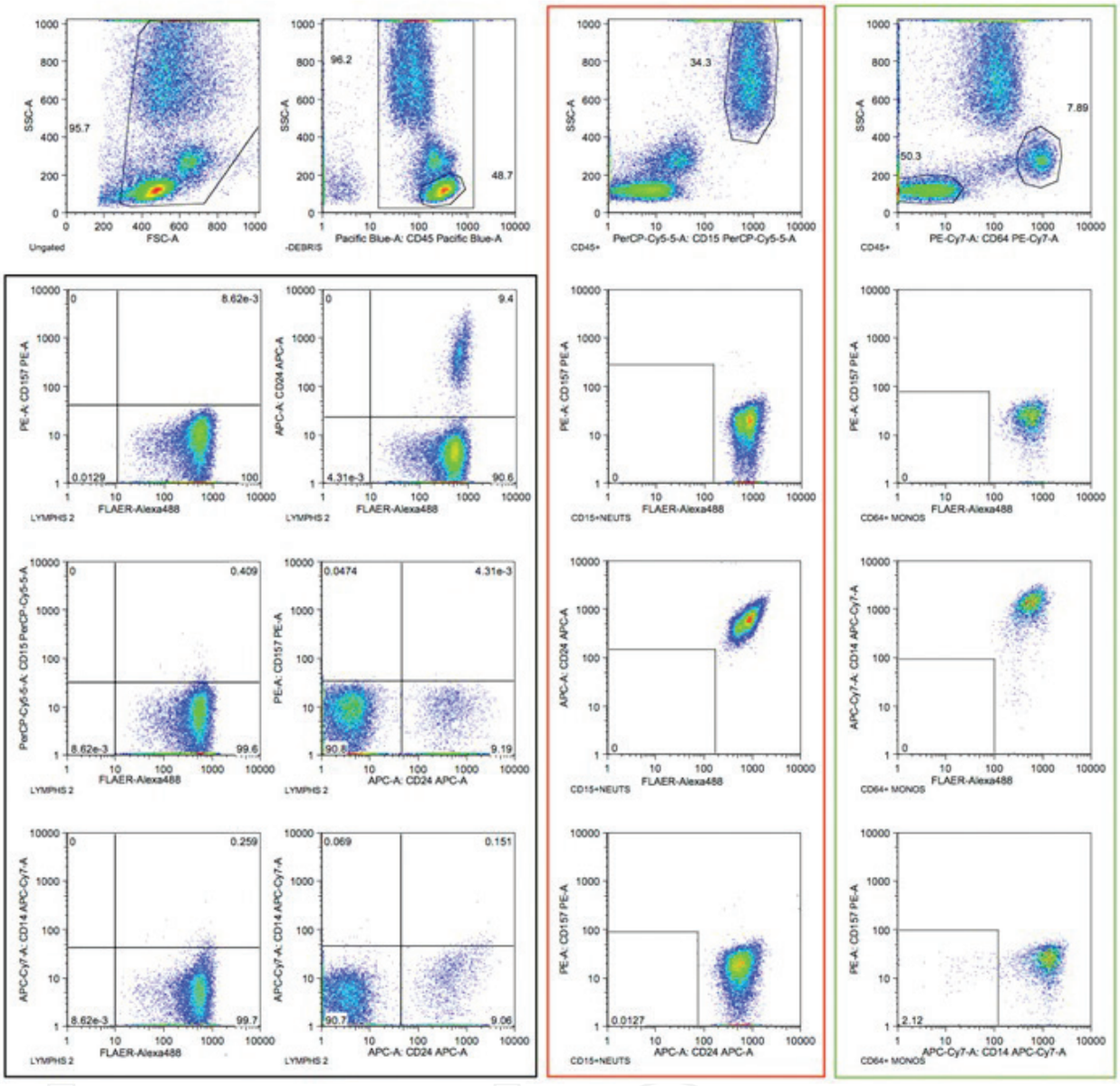

Figure 4. Example of CD157-negative, non-PNH case, 7-color FLAER/CD24/CD14/CD157-based protocol.

Type III RBCs. Type I RBCs are normal red blood cells with bright CD59 expression and a lifespan of approximately 120 days. Type III PNH RBCs have complete CD59 deficiency, which results in no protection from complement-mediated lysis and a shortened lifespan of 10–15 days. Type II PNH RBCs have partial CD59 deficiency resulting in partial protection from complement-mediated lysis. Just as the expression of CD59 on Type II RBCs varies considerably from patient to patient, the lifespan of Type II cells reflects this being intermediate between Type I normal RBCs and Type III PNH RBCs. Since the clinical significance of Type II PNH RBCs and Type III PNH RBCs is well established, it is recommended to report them separately and combined as the total PNH RBC clone.

3. PNH clone size in both lineages for the WBCs (neutrophils and monocytes). The PNH monocyte clone is often larger than the neutrophil PNH clone and reporting only the PNH neutrophil/granulocyte clone may underestimate the PNH clone size in the WBCs. Neutrophils

and monocytes may also show the presence of Type II populations but the clinical and biological significance of these populations has not been established at this time. It is therefore recommended to report only the total PNH clone size in the neutrophils and monocytes.

4. Interpretive terminology of reporting PNH clones based on CSLI H52-A2 [23]:
   a. PNH population > 1%: "PNH clone."
   b. PNH population 0.1–1%: "minor population of PNH cells" or "minor PNH clone."
   c. PNH population < 0.1%: "rare cells with GPI deficiency" or "rare cells with PNH phenotype.
5. List of all gating and diagnostic markers used for the PNH assay.
6. Levels of the limit of quantification (LOQ) for the neutrophil assay and the RBC assay, stating the recommended LOQ of 0.05% or better for RBCs (100,000 gated cells) and 0.1% or better for neutrophils (50,000 gated cells). It is important to include this information to the provider as an LOQ of 1% means that the possibility of a minor clone (less than 1%) cannot be excluded based on this LOQ.
7. Histograms or dot plots if possible because the dot plots may provide powerful visual supportive evidence of the PNH clone and also provide evidence of the quality of the assay.

4. Post-analytical phase and assay validation

The results of PNH testing by flow cytometry are usually reported as percentage of type II and III PNH cells from the total gated neutrophils, monocytes and red blood cells. Assays reporting numeric data are considered as semi-quantitative, therefore the post-analytical validation process should comprise confirmation of accuracy, specificity, sensitivity, repeatability, reproducibility and stability [24, 25].

4.1. Accuracy of PNH assays

The accuracy of a measurement is described by its trueness, which refers to the closeness of agreement between the average value of a large number of test results and the true or accepted reference value [25]. For PNH assays, we do not have cellular reference standard, therefore accuracy cannot be determined directly. Alternatively, interlaboratory comparison and/or external quality assessment represent the only available option for assay validation and mandatory step for ISO accreditation [26].

4.2. Specificity of PNH assays

4.2.1. Analytical specificity

The analytical specificity of PNH testing assays reflects the choice and validation of all antibodies/reagents and corresponding fluorochromes (**Tables 1–3**).

4.2.2. Clinical specificity

The clinical specificity or the ability to exclude abnormal specimen defined by true negatives/true negatives + false positives should be determined by assay of a series of samples and scoring for abnormality in comparison to a suitable reference method, such as clinical diagnosis [27]. The clinical specificity of well-established PNH assays is usually >99%.

4.3. Sensitivity of PNH assays

4.3.1. Analytical sensitivity

The analytical sensitivity of PNH assays is determined by the limit of blank (LoB) defined by the highest apparent signal detected in replicates of a sample containing no measurand and the limit of detection (LoD) defined by the lowest level of measurand that can be reliably distinguished from the LoB [28]. LoB for PNH assays could be determined by measuring a few replicates of a few negative specimens run over a few separate days and calculating the mean and standard deviation (SD) according to: LoB = mean of blank +1.645 SD of blank, assuming that 95% of negative values will be below this limit. Typically, the LoB for well-established PNH assays is <0.001% (<10 PNH phenotypes out of 1,000,000 acquired events). LoD for PNH assays is closely related but usually greater than LoB and could be determined by measuring a few replicates of a few negative specimens run over a few separate days and calculating the mean and SD according to: LoD = mean of blank + 2SD (3SD) of blank or by measuring a few replicates of a few low positive specimens run over a few separate days and calculating the SD according to: LoD = LoB + 1.645 SD of low positive. Alternatively, target LoD could be estimated by measuring a few replicates of a few low positive specimens run over a few separate days and calculating the reproducibility (inter-assay imprecision) expressed as coefficient of variation (CV%) or by confirming that no more than 5% of the values for a target LoD fall beyond the LoB. The generally accepted smallest number of events required to reproducibly detect a PNH population and determine LoD is 20 PNH events, lower levels should be validated in each laboratory.

4.3.2. Functional sensitivity

The functional sensitivity of PNH assays is determined by the limit of quantification (LoQ), which is the lowest level of measurand that can be reliably detected at predefined levels of bias and imprecision [28]. LoQ is usually greater than LOD and for PNH assays could be determined by measuring a few replicates of a few positive (near the expected LoQ) specimens run over a few separate days and calculating the reproducibility (inter-assay imprecision) expressed as CV%, which should be acceptable at levels below 10%. The generally accepted smallest number of events required to reproducibly quantify a PNH population and determine LoQ is 50 PNH events, lower levels should be validated in each laboratory.

4.3.3. Clinical sensitivity

The clinical sensitivity or the ability to detect an abnormal specimen and distinguish from normal specimens defined by true positive/true positive + false negative should be determined

by assay of a series of abnormal samples and scoring for abnormality in comparison to a suitable reference method, such as clinical findings [27]. The values for clinical sensitivity of well-established PNH assays is usually >99%.

4.4. Repeatability and reproducibility

The validation of assay performance characteristics comprises the determination of repeatability (intra-assay imprecision) and reproducibility (inter-assay imprecision). It is generally recommended to assay a few replicates from at least five samples within a single analytical run for repeatability and a few replicates from at least five samples in separate analytical runs for reproducibility [29]. For confirmation of good performance characteristics, CV% below 10% should be obtained for samples with more than 1% target PNH cells and below 20% for samples with minor clones (<1%).

4.5. Stability

The validation of specimen, processed specimen and reagent stability has been reviewed in Section 2 [13, 16, 23].

5. Accreditation

Flow cytometry is a highly versatile and complex technology, which is routinely applied in clinical diagnostic laboratories. The vast majority of assays are laboratory developed tests based on publications, without any gold standard reference [30] mostly with poor validation. For the purpose the ICSH/ICCS workgroup published in 2013 the practice guidelines for validation of cell-based fluorescence assays [31], subsequently several relevant publications addressed various aspects of the validation of PNH testing by flow cytometry [12, 13, 32]. Each laboratory applying for ISO 15189 accreditation should confirm optimal validation of instrument setup, assay performance characteristics, laboratory information system and result reporting [33].

6. Conclusion

Highly sensitive and specific PNH testing of all three lineages (RBC, neutrophils and monocytes) has become the standard of care for patients with suspected PNH. This is a rare disease and therefore often overlooked as a diagnostic possibility. It is important for the ordering physician to test the high-risk patients for PNH [12] and also to receive informational reports as an important part for best patient management. The laboratories are challenged with the validation of multiple steps, including instrument optimization, selection of best antibody clones/conjugates, panel design and targeted acquisition and interpretation of data. Developing competency in PNH testing and reporting is critical for laboratories and is directly related to awareness of best practices, following guidelines which are developed by experts based on extensive evaluation of

various approaches. Standardized reporting based on currently available guidelines is important to communicate to the physician the size of the PNH clone, which aids him/her in the decision-making for optimal treatment of the patient.

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