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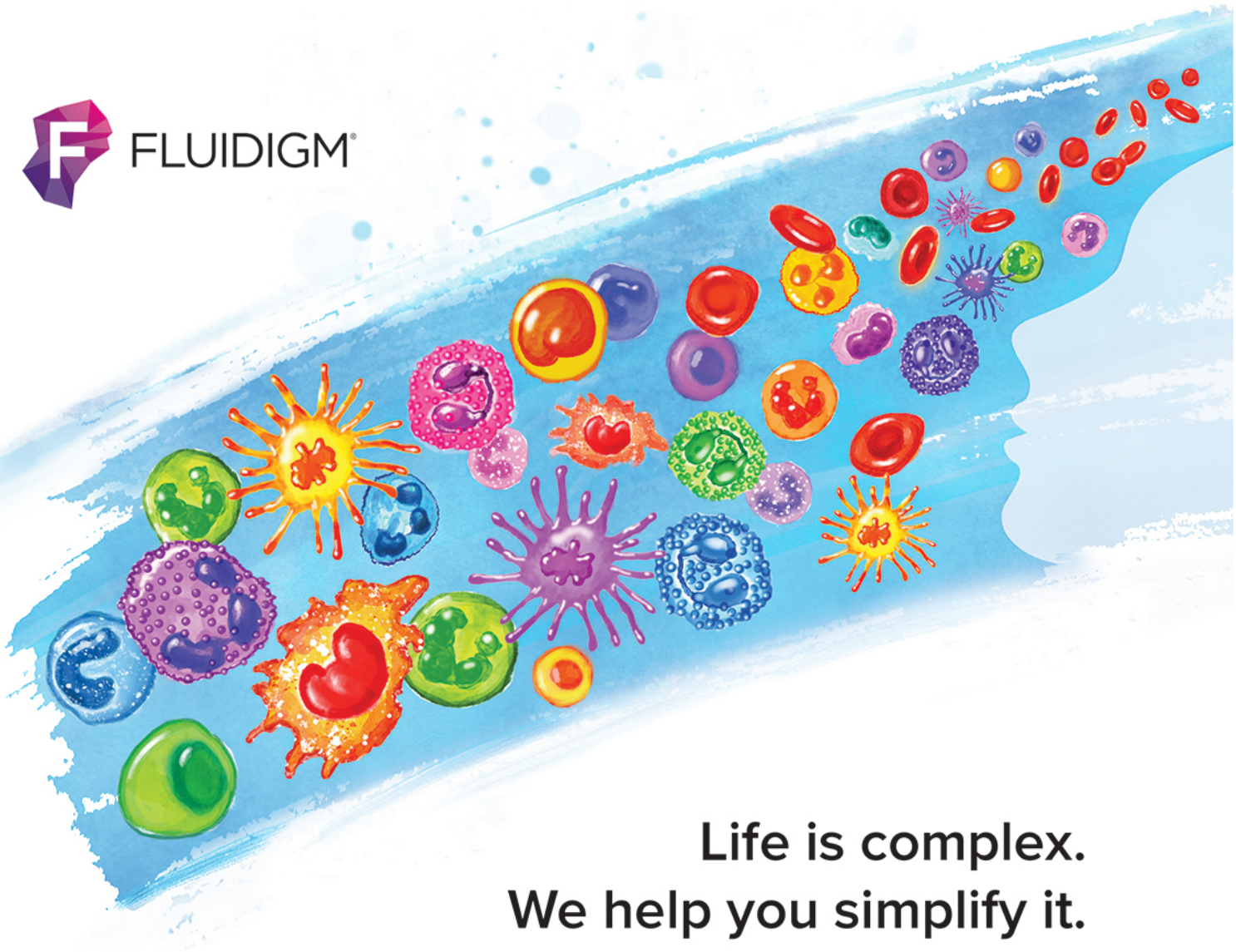

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Original Article

Performance Characteristics of a Non-Fluorescent Aerolysin-Based Paroxysmal Nocturnal Hemoglobinuria (PNH) Assay for Simultaneous Evaluation of PNH Neutrophils and PNH Monocytes by Flow Cytometry, Following Published PNH Guidelines

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Background: CD157 has been recently reported as a useful glycosylphosphatidylinositol (GPI)-linked marker for the detection of paroxysmal nocturnal hemoglobinuria (PNH) clones in patients with suspected paroxysmal nocturnal hemoglobinuria by flow cytometry as it targets both neutrophils and monocytes. The aim of this study is to test the feasibility of a non-fluorescent aerolysin (FLAER) approach and propose an alternative for laboratories, where FLAER is not available.

Methods: We validated a non-FLAER-based single-tube, 6-color assay targeting the GPI-linked structures CD157, CD24, and CD14. We determined its performance characteristics on 20 PNH patient samples containing a variety of clone sizes and compared results with a previously validated FLAER-based approach.

Results: Coefficient of variation (CV) for intra-/interassay precision analyses ranged from 0.1%/0.2% to 3.02%/7.58% for neutrophils and from 0.10%/0.3% to 5.39%/6.36% for monocytes. Coefficient of determination (r^2) for linear regression analysis of PNH clones from 20 patients ranging from 0.06% to 99.7% was 0.99 in all cases, Wilcoxon ranks test showed no statistically significant differences ($P > 0.05$), Bland–Altman analysis demonstrated performance agreement with mean bias ranging from 0.06 to 0.2.

Conclusion: Our results confirm very good performance characteristics for both intra- and interassay precision analyses, favorable correlation, and agreement between the FLAER and non-FLAER-based approaches, using the CD157 GPI marker. Our experience suggests that a rapid and cost-effective simultaneous evaluation of PNH neutrophils and monocytes by flow cytometry without using FLAER is possible in areas where FLAER may not be widely available.

Key terms: PNH; flow cytometry; CD157; non-FLAER-based approach

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Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired hematopoietic stem cell disorder resulting from the somatic mutation of the X-linked phosphatidylinositol glycan complementation Class A (*PIG-A*) gene (1–3). This mutation leads to a partial or total inability to biosynthesize GPI-anchored proteins including complement-defense structures such as CD55 and CD59 on red blood cells (RBCs) and white blood cells (WBCs) (4–6). PNH is characterized by intravascular hemolysis (hemolytic form), immunologically determined bone marrow aplasia (hypoplastic form), and elevated risk for thrombosis at unusual sites (7–10). Thirty-five percent of PNH patients die within 5 years of diagnosis due to related complications, even with supportive care (7). Early PNH detection in patients with bone marrow failure may impact treatment decisions and outcomes, and the presence of small PNH clones usually predicts favorable response to immunosuppressive therapy (8–11).

Since 1996, flow cytometry has become the method of choice for identifying GPI deficient cells (12,13). In 2010, the International Clinical Cytometry Society (ICCS) published the first Consensus Guidelines (14) for the diagnosis and monitoring of PNH by flow cytometry, although specific reagents and combinations of reagents for high sensitivity testing were not provided. Sutherland et al. published the follow-up 2012 Practical Guidelines (15), addressing the importance of instrument optimization, standardized procedures utilizing specific reagent cocktails, and describing detailed protocols and analytic strategies for high sensitivity detection of PNH RBCs and PNH WBCs (both neutrophils and monocytes). Both WBC assays employed a fluorescent aerolysin (FLAER)-based approach comprising FLAER-Alexa488, CD24-PE, CD15-PECy5, and CD45-PECy7 (for neutrophils) and FLAER-Alexa488, CD14-PE, CD64-PECy5, and CD45-PECy7 (for monocytes). FLAER is an Alexa-488-conjugated derivative of the bacterial lysin—proaerolysin, which binds directly to the GPI moiety of all GPI-linked structures on WBCs, and has been shown to be a very reliable reagent for detecting GPI-deficient WBCs in PNH and related disorders (14–19). In a subsequent multicenter study (20), our group confirmed very good performance characteristics for both intra- and interassay precision analyses, excellent correlation, and agreement for different target PNH clone sizes based on the Practical Guidelines WBC reagent sets. These results confirmed that the 2-tube/4-color FLAER-based approach to detect PNH neutrophils and monocytes represents an excellent basis for validation and standardization of PNH testing by flow cytometry (20–22).

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 (23,24). Within the hematopoietic system, CD157 is highly expressed on normal neutrophils and monocytes (25–27). Two subsequent publications of Sutherland et al. (28) and Marinov et al. (29) reported the potential clinical relevance and performance characteristics of an FLAER-based single-tube/5-color WBC assay for rapid and cost-effective simultaneous detection of PNH neutrophils and monocytes by flow cytometry using CD157 in combination with FLAER, CD15, CD64, and CD45.

Table 1a
Specific Gating or GPI-Linked Reagents and Fluorochromes Used in the Non-FLAER-Based Panel Used in the Study

Reagent	Clone	Fluorochrome	Source
CD14	MφP9	APC-H7	BD Biosciences
CD15	MEM-158	FITC	Exbio
CD24	SN3	APC	eBioscience
CD64	10.1	PE-Cy7	BD Biosciences
CD45	2D1	PerCP-Cy5.5	BD Biosciences
CD157	SY11B5	PE	eBioscience

Table 1b
Specific Gating or GPI-Linked Reagents and Fluorochromes Used in the Predicate FLAER-Based Panel Used in the Study

Reagent	Clone	Fluorochrome	Source
FLAER	-	Alexa 488	Cedarlane
CD15	HI98	APC	BD Biosciences
CD45	2D1	PerCP-Cy5.5	BD Biosciences
CD64	10.1	PE-Cy7	BD Biosciences
CD157	SY11B5	PE	eBioscience

Since FLAER is not universally available and/or may be too costly to obtain in certain parts of the World, we report in this study, the results of the validation and the performance characteristics of a non-FLAER-based PNH assay using CD157, along with CD24, CD14, CD15, CD64, and CD45. The assay described herein thus follows the ICCS (14), Practical Guidelines (15), CLSI H52-A2 (30), and Current Protocols in Cytometry (31) recommendations to use at least 2 GPI reagents for each specifically gated lineage of neutrophils and monocytes.

MATERIALS AND METHODS

PNH Patient Sample

Following informed consent, peripheral blood (PB) from a PNH patient was appropriately diluted with compatible blood of a healthy donor to obtain three target PNH clone sizes for neutrophils and monocytes: large (> 20%), small to intermediate (1–20%), and minor (<1%) PNH clone, respectively. Twenty additional patients with confirmed PNH diagnosis were also analyzed for the correlation and comparison studies.

Monoclonal antibodies

The MoAbs used in the study are listed in Table 1a and Table 1b.

Flow Cytometry

Acquisition and analysis were performed on a BD FACSCanto™ cytometer equipped with 3 lasers and BD FACSDiva™ 6.1.3. software. For initial setup and photomultiplier tube (PMT) optimization, we used unstained leukocytes. Target mean fluorescent intensity (MFI) values for each fluorochrome used in the assay were set

Table 2
Main Characteristics of the Gating Strategy for the Non-FLAER-Based Panel Used in the Study

Method	Target population	Gating strategy	GPI-linked reagents
1-tube/6 color	Neutrophils	FSC/SSC/CD45/CD15	CD157/CD24
	Monocytes	FSC/SSC/CD45/CD64	CD157/CD14

Table 3
Results From Intra-/Interassay Precision Analysis with Non-FLAER-Based Panel

Clone size	PNH clone	*N*	Min.	Max.	Mean	SD	CV (%)
Large	Ne	3/10	97.7/97.9	97.9/98.4	97.8/98.2	0.08/0.18	0.08/0.18
	Mo	3/10	88.6/96.9	88.9/97.9	88.9/97.3	0.24/0.26	0.27/0.27
Intermediate	Ne	3/10	30.1/14.1	30.7/14.8	30.4/14.5	0.23/0.19	0.75/1.29
	Mo	3/10	34.5/15.7	34.6/16.7	34.5/15.9	0.03/0.44	0.08/2.75
Minor	Ne	3/10	0.4/0.5	0.4/0.5	0.4/0.5	0.01/0.04	2.95/7.58
	Mo	3/10	0.5/0.7	0.6/0.8	0.6/0.8	0.03/0.05	5.39/6.36

$\geq$2.5 robust standard deviation (rSD) of the electronic noise as defined from baseline setting. Computer-assisted compensation matrix was generated using single stained compensation tubes acquired without changing PMT. Daily performance check for instrument performance and PMT standardization was performed using the CS&T module and beads.

1-tube/6-color non-FLAER-based analysis of PNH neutrophils and monocytes

The reagents used in this approach are listed in Table 2. Briefly, 100 μL of well-mixed peripheral blood (EDTA) were incubated with the appropriate amount of pretitrated MoAbs for 30 min in the dark at room temperature. RBCs were lysed with 2 mL of fresh ammonium chloride solution diluted to 1× concentration (BD Pharm Lyse™) for 10 min in the dark at room temperature, then WBCs were washed twice in phosphate-buffered saline containing 1% bovine serum albumin (PBS/PBA) and resuspended in 0.5–1.0 mL of PBS/PBA prior to acquisition. WBCs were analyzed using a Boolean gating strategy using forward scatter channel (FSC)/side scatter (SSC), CD45/SSC and CD15/SSC for neutrophils, and FSC/SSC, CD45/SSC, and CD64/SSC for monocytes. 50,000 CD15+ neutrophils were then acquired and PNH clone assessment was determined by CD157/CD24 deficiency for neutrophils and CD157/CD14 deficiency for CD64+ monocytes.

Statistical Analysis

Intra- and interassay precision analysis (performance characteristics) were performed by replicate analysis, results were reported as mean, standard deviation (SD), and CV (%). Correlation of results obtained from intra- and interassay was performed by linear regression analysis and Pearson's correlation coefficient (r) significant at level 0.01. Comparison between methods was determined by the Wilcoxon-signed rank test for paired samples significant at level 0.05. Performance agreement was evaluated by Bland and Altman analysis of the relationship between the differences and the mean of differences reported as mean bias (mean of the differences ± 2 SD). Mean bias equal to zero shows absolute performance agreement.

RESULTS

For intra-assay precision analysis, we performed 3 replicate determinations in a single analytical run of the large (>20%), small to intermediate (1–20%), and minor (<1%) PNH clones within the neutrophil and monocytic populations. For the large PNH clone, CV/SD was 0.08%/0.08% for the "total" PNH neutrophils and 0.27%/0.24% for the "total" PNH monocytes, respectively. Similarly, for the small-to-intermediate PNH clone, CV/SD was 0.75%/0.23% for total PNH neutrophils and 0.08%/0.03% for total PNH monocytes, respectively. Finally, for the minor PNH clone, CV/SD was 2.95%/0.01% for total PNH neutrophils and 5.39%/0.03% for the total PNH monocytes (Table 3 and Fig. 1).

For the interassay precision analysis, we performed 10 consecutive determinations in separate analytical runs (instrument power-down and recalibration) for all target PNH clone sizes within 24 h. For the large PNH clone, CV/SD was 0.18%/0.18% for total PNH neutrophils and 0.27%/0.26% for total PNH monocytes, respectively. Similarly, for the small-to-intermediate PNH clone, CV/SD was 1.29%/0.19% for total PNH neutrophils and 2.75%/0.44% for the total PNH monocytes. Finally, for the minor PNH clone, CV/SD was 7.58%/0.04% for total PNH neutrophils and 6.36%/0.05% for total PNH monocytes, respectively (Table 3 and Fig. 1).

For the correlation and comparison studies, we analyzed 20 patients with confirmed PNH clones, using the non-FLAER-based panel containing CD157 in parallel to a previously validated 1-tube 5-color FLAER-based protocol (28,29). Linear regression analysis of PNH neutrophils (type II + III) showed good correlation: $r^2 = 0.99$ ($r = 1.0$), slope 0.99, intercept 0.06. The Wilcoxon-signed rank test did not prove statistically significant differences between approaches: $P = 0.18$ (>0.05). Bland–Altman analysis of compatibility confirmed agreement between methods with mean bias 0.2. Similarly, linear regression analysis for PNH monocytes (type II + III) showed good correlation: $r^2 = 0.99$ ($r = 1.0$), slope 1.0, and intercept −0.09. The Wilcoxon-signed rank test did not prove statistically significant differences between

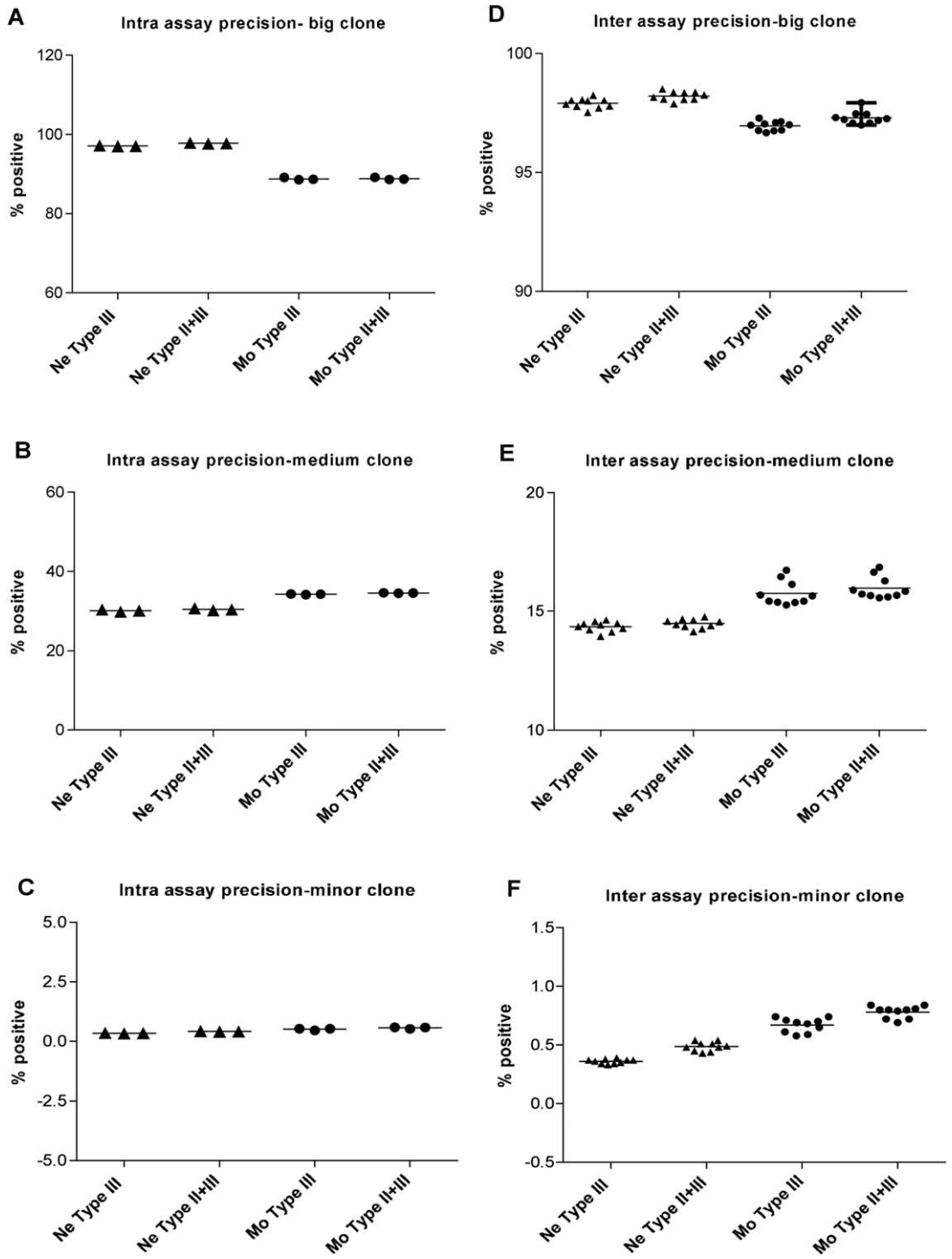

FIG. 1. Performance characteristics: results from (A,B,C) intra- and (D,E,F) interassay precision analysis for neutrophils and monocytes.

protocols: $P = 0.99$, Bland–Altman analysis confirmed agreement between methods with mean bias 0.06. Figure 2 and Table 4 show data from correlation and Bland–Altman compatibility analyses of the non-FLAER-based versus FLAER-based protocols using CD157 for evaluation of both PNH neutrophils and monocytes.

DISCUSSION

The ICCS consensus guidelines (14), Practical Guidelines (15), H52-A2 Guideline (30), and most recent Current Protocols in Cytometry document (31) emphasized the need to include two GPI-specific reagents along with specific gating reagents for each WBC lineage assessed.

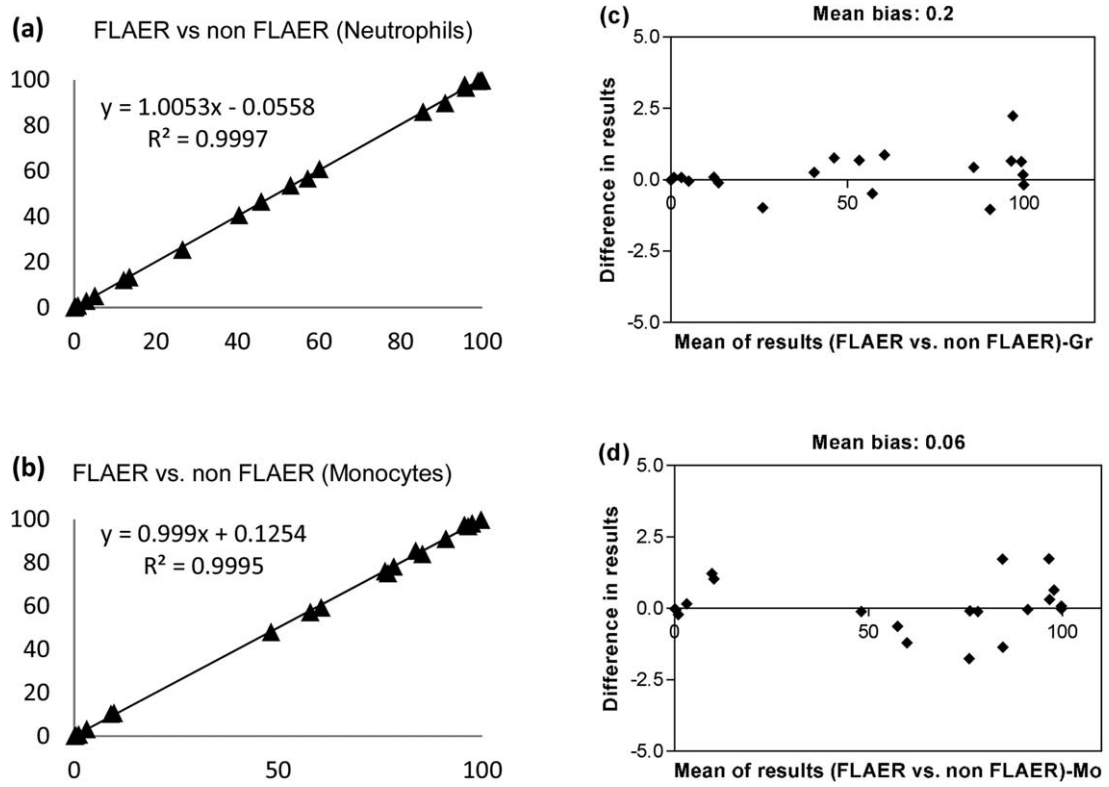

FIG. 2. Linear regression and Bland–Altman agreement analysis of FLAER vs non-FLAER-based evaluation of (a,c) PNH neutrophils and (b,d) monocytes.

The use of two GPI-specific reagents per lineage is a fundamental aspect of robust assay design for the accurate detection and quantitation of PNH phenotypes even bone marrow failure syndromes, in which PNH phenotypes below 0.1% are routinely detectable. The Practical Guidelines (15) validated FLAER-based approaches for high-sensitivity detection of neutrophils (using CD24PE and FLAER) and monocytes (using CD14PE and FLAER). For gating neutrophils and monocytes, specific conjugates of CD15 and CD64, respectively, were identified and validated (15). Subsequently, it was shown that CD157, which is expressed on both neutrophils and monocytes (24–26) could be used (with FLAER) in place of CD24 and CD14, thus keeping to the two-GPI-linked structures per WBC lineage recommendations (28,29).

Together, data generated from the Practical Guidelines (15) and CD157-based approaches (28,29) suggested the possibilities to use a variety of validated reagent sets in 4-color, 5-color, and 6-color combinations with detailed analytic strategies optimized for a variety of instrument platforms equipped with 4, 5, and 6 (or more) PMTs (31). The ability to deploy CD157-based high-sensitivity assays for simultaneous detection of PNH neutrophils and 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 (28–31). However, for laboratories equipped with instruments with 6 or more PMTs, the deployment of CD157-based WBC assays is less compelling. Other than the small extra cost incurred in deploying a single-tube 6-color (FLAER, CD24, CD14, CD15, CD64, and CD45) versus single-tube 5-color (FLAER, CD157, CD15, CD64, and CD45) assay, it is important to note that several CD157-negative, non-PNH cases have been observed in the authors' laboratories (Sutherland, unpublished data) and beyond. For these rare cases, the inclusion of the second GPI reagent is part of the built-in robustness of the assay, and prevents the misinterpretation of the data as a PNH clone-containing sample. Furthermore, in keeping with current state-of-the-art guidelines (30,31), the RBC lineage should also be analyzed on every sample tested for the presence of PNH RBCs. All rare CD157-negative non-PNH samples detected to date contained only normal (Type I) RBCs confirming the finding of an isolated single (CD157) GPI deficiency.

In all the above approaches, FLAER has been a key reagent in the development of robust high-sensitivity assays to detect GPI-deficient neutrophils and monocytes and in recent years, it has become much more widely used (14–22,28–31). However, as a relatively recently developed non-monoclonal antibody reagent, it is not universally available and the cost of the reagent may be beyond the resources available to some laboratories around the world. Thus, the aim of this study was to explore whether a non-FLAER approach based on the

Table 4
Results from Analysis of PNH Patients with FLAER/Non-FLAER-Based Panel

PNH clone	Protocol	*N*	Min.	Max.	Median	SD	95% CI of mean	
							Lower	Upper
Neutrophils	FLAER	20	0.06	99.98	49.3	39.52	30.74	67.73
	Non-FLAER	20	0.05	99.80	50.15	39.74	30.85	68.04
Monocytes	FLAER	20	0.09	99.77	76.55	39.28	40.14	76.91
	Non-FLAER	20	0.07	99.78	76.52	39.25	40.22	76.96

use of CD24 and CD157 (for neutrophils) and CD14 and CD157 (for monocytes) could generate equivalent data compared to the predicate 5-color FLAER/CD157-based approach (29). This study showed the high level of correlation between the FLAER-based and non-FLAER-based approaches for delineating and quantitating PNH neutrophils and PNH monocytes and thus the non-FLAER-based approach could serve as suitable alternative for laboratories that do not have access to FLAER. The CVs for intra- and interassay precision analyses of "total" PNH neutrophils and monocytes in our hands were $\leq$5% and $\leq$10%, respectively, even for the minor PNH clones, where values below 20% are still acceptable. Higher values for CVs and more heterogeneous results were obtained for separate analyses of "total" PNH monocytes, which is mainly due to significantly lower numbers of acquired events. More importantly, the results from analysis of PNH patients obtained from the non-FLAER-based and a previously reported FLAER-based approach (29) demonstrated perfect correlation (linear regression analysis and Pearson $r = 0.99$), very good agreement (Bland–Altmann's bias ranging from 0.06 to 0.2) and no statistically significant difference ($p > 0.05$) as assessed by the Wilcoxon's rank test.

An important practical issue for PNH WBC testing is the use of internal control populations of leukocytes to assess optimal cytometer settings (PMTs and spectral compensation) as well as the confirmation of appropriate antibody specificity. For the FLAER-based approaches, the FLAER vs CD24 dot plot, gated on lymphocytes can be used; the clear distinction of T lymphocytes (FLAER+/CD24−), B lymphocytes (FLAER+/CD24+), and PNH lymphocytes (FLAER−/CD24−) is an excellent benchmark for all of the above parameters. Similarly, for the non-FLAER-based cocktail described herein, the CD24 vs CD157 dot plot gated on all CD45+ leukocytes provides a means to assess assay quality; the clear distinction of B lymphocytes (CD24+/CD157−), T lymphocytes (CD24−/CD157−), basophils (CD24−/CD157dim+), monocytes (CD24−/CD157+), neutrophils (CD24+/CD157+), and eosinophils (CD24+/CD157 dim+) can be delineated (Fig. 3).

FLAER-based PNH assays have been tested extensively and confirm FLAER as the GPI-reagent of choice due to its high sensitivity, broad specificity, and robustness over

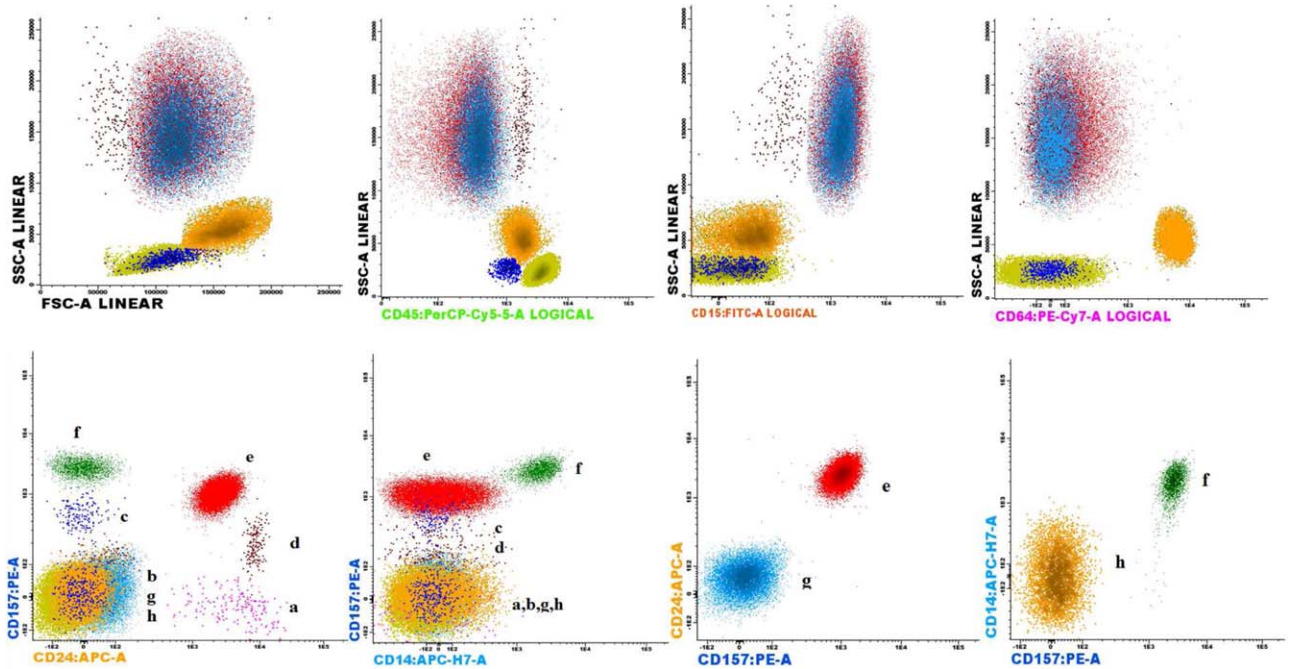

FIG. 3. Representative dot plots from a PNH patient using the non-FLAER-based protocol: (a) B-Ly, (b) T-ly, (c) Ba, (d) Eo, (e) non-PNH Ne, (f) non-PNH Mo, (g) PNH Ne, and (h) PNH Mo. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

a wide range of clinical samples and the authors would continue to strongly promote its use in places where it is readily available at an economic price. The non-FLAER based assay described here will need to be tested further to confirm its performance over a more challenging range of sample types.

CONCLUSION

We have demonstrated very good performance characteristics of a non-FLAER-based approach for high-sensitivity PNH WBC (neutrophils and monocytes) testing which could represent an alternative for laboratories, where FLAER is not available. Future studies thus combining GPI-specific reagents FLAER, CD24, CD14, and CD157 in a single tube will address whether all combinations of FLAER, CD24, and CD157 generate identical data for CD15-gated PNH neutrophils and whether all combinations of FLAER, CD14, and CD157 similarly generate identical data for CD64-gated PNH monocytes on a per sample basis.

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