*Original Article*

# Automated Analysis of GPI-Deficient Leukocyte Flow Cytometric Data Using GemStone™

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**Background: Flow Cytometry is the standard for the detection of glycosylphosphatidylinositol (GPI)-deficient clones in paroxysmal nocturnal hemoglobinuria (PNH) and related disorders. Although the International Clinical Cytometry Society (ICCS) and the International PNH Interest Group (IPIG) have published guidelines for PNH assays, data analysis has not been standardized. Current analyses use manual gating to enumerate PNH cells. We evaluate an automated approach to identify GPI-deficient leukocytes using a GemStone™ (Verity Software House) probability state model (PSM).**

**Methods: Five hundred and thirty patient samples were assayed on BD Canto II flow cytometers using a stain-lyse-wash technique. Populations were defined using CD15, CD45, CD64 and side scatter. GPI-deficient myeloid cells were recognized as FLAER-, CD24-, and dim or absent CD16. GPI-deficient monocytic cells were identified as FLAER- and CD14-. The data were not censored in any way. A PSM was designed to enumerate monocytic and myeloid cells by adjusting the peaks and line spreads of the data, and recording the normal and GPI-deficient counts. No operator adjustments were made to the automated analysis.**

**Results: By human analysis, 53 of 530 samples showed GPI-deficient clones. Automated analysis identified the same 53 clones; there were 0 false positives and 0 false negatives. Sensitivity was 100% and specificity 100%. Gating and automated results (percent GPI-deficient cells) were highly correlated: $r^2$ = 0.997 for monocytic cells, and $r^2$ = 0.999 for myeloids. Mean absolute differences were 0.94% for monocytes and 0.78% for myeloid cells.**

**Conclusions: Automated analysis of GPI-deficient leukocytes produces results that agree strongly with gate-based results. The probability-based approach provides higher speed, objectivity, and reproducibility.** © 2012 International Clinical Cytometry Society

**Key terms: flow cytometry; multivariate classification; automated analysis; PNH; GemStone; probability state model; FLAER; GPI-deficient; leukocytes**

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The problems associated with analysis of flow cytometry listmode files by gating on plots of univariate or bivariate data are well-documented (1–4). Gating approaches are subjective, difficult to reproduce, and challenging to automate. Attempts have been made to solve these shortcomings using multivariate classification techniques, including clustering algorithms, support vector machines, and other approaches. All of these efforts have been driven by the idea that automated analysis could improve the objectivity, standardization, and speed of flow cytometry data analysis.

Additional Supporting Information may be found in the online version of this article.

Disclosure: Authors Benjamin Hunsberger and Bruce Bagwell have a financial interest in and serve as officers for Verity Software House [(VSH; VSH owns and markets GemStone software and owns the patent rights for Probability State Modeling (US Patent Number 7,653,509)].

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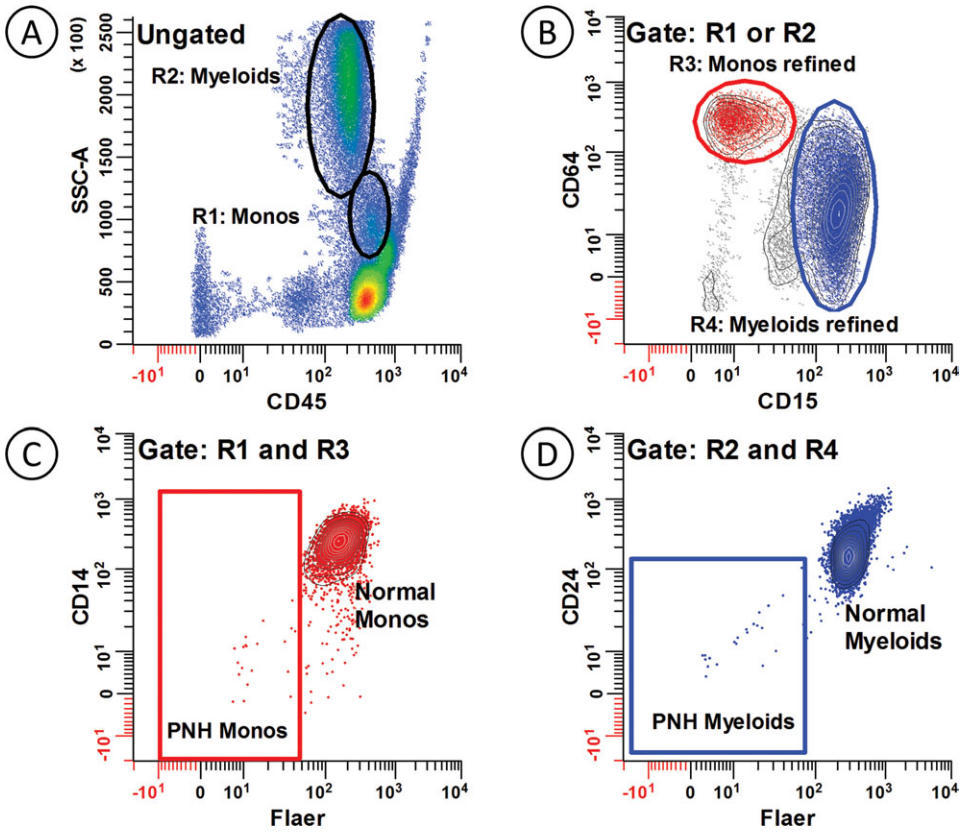


FIG. 1. Traditional gating method for GPI-deficient leukocyte analysis. Regions defining the monocytes and myeloid cells are drawn on a plot of CD45 by linear side scatter (**A**). Cells in these two lineages are further refined using a plot of CD15 by CD64 (**B**). The GPI markers are gated using the lineage regions and displayed in plots (**C**) and (**D**), and regions on these plots quantify the number of GPI-deficient leukocytes.

One flow cytometry application that would benefit from automation is the detection of glycosylphosphatidylinositol (GPI)-deficient clones in paroxysmal nocturnal hemoglobinuria (PNH) and other bone marrow failure disorders. Although the International Clinical Cytometry Society (ICCS) and the International PNH Interest Group (IPIG) have published guidelines (5,6) for the performance of PNH assays, data analysis has not been standardized. Current analysis requires trained analysts using subjective gating to enumerate normal and GPI-deficient cells (7). Guidelines require the analysis of two cell lines, red cells and leukocytes, to confirm a diagnosis of PNH. In this study, we propose an automated multivariate classification approach for GPI-deficient leukocyte analysis, based on GemStone™ (Verity Software House). We compare the results of automated analysis with a traditional gating analysis of GPI-deficient leukocytes performed by a trained expert.

## METHODS

Five hundred and twenty-seven peripheral blood samples and three bone marrow samples submitted for PNH analysis were assayed in a clinical laboratory using four Becton-Dickinson Canto II flow cytometers. While the laboratory performs the recommended PNH screening of both red cells and leukocytes, this study focuses on the analysis of leukocytes for GPI-deficiency.

Machine-to-machine consistency was verified using a patient sample with a large clone size. The within-run, machine-to-machine coefficients of variation were 0.25% for myeloid cells and 0.28% for monocytes. The white blood cells were prepared using a stain-lyse-wash technique with the markers FLAER-AF488™ (Pinewood/Cedarlane, Ontario, Canada), CD24-PE (Beckman Coulter, Miami, FL), CD16-PerCP-Cy5.5 (Becton-Dickinson, San Jose, CA), CD14-PE-Cy7 (Pharmingen, San Diego, CA), CD64-AF647 (BioLegend, San Diego, CA), CD45-APC-H7 (Becton-Dickinson), and CD15-PacBlue (IgG1) (Becton-Dickinson). Typically, 90,000 ungated events were acquired. Monocytic and myeloid cells were defined using CD15, CD45, CD64 and linear side scatter. GPI-deficient myeloid cells were recognized as FLAER-, CD24-, and dim or absent CD16. GPI-deficient monocytic cells were identified as FLAER- and not CD14+. Forward scatter was not utilized in the analysis. At least 10 GPI-deficient cells in each lineage were required to report an abnormality.

The data were not censored for data quality, the presence of a hematologic malignancy, or other factors. Samples were 1–5 days old; the data set was not selected for age-of-sample. All patient identifiers were removed from listmode files before analysis. This study was considered exempt under 45 CFR 46.101(b)(4) as defined by the

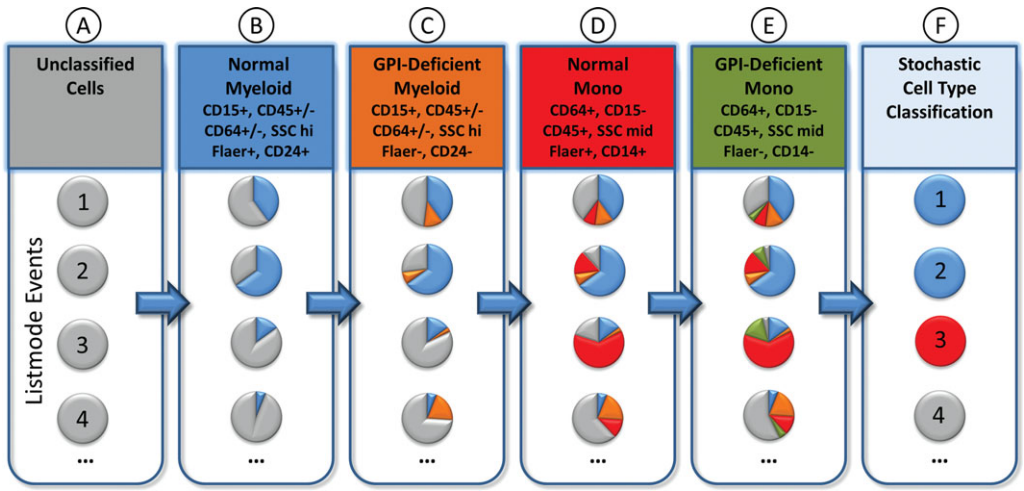


FIG. 2. Probability state modeling process for GPI-deficient leukocytes. For each event, the Probability State Model (PSM) determines the probability that the event belongs to each of the four cell types: Normal Myeloid, GPI-deficient Myeloid, Normal Monocyte, and GPI-deficient Monocyte. The probabilities are used to assign events to cell types. In the figure, listmode events in the leftmost column (A) are unclassified. The PSM starts by evaluating the likelihood that the event is a normal myeloid event (B) based on CD15, CD45, CD64, SSC, Flaer, and CD24. It proceeds to evaluate probabilities for remaining cell type based on marker expression levels (C, D, E). Finally, events are stochastically assigned to one cell type (F) based on probabilities of the prior steps. Events with low probabilities for all defined cell types are left unclassified.

Office of Human Research Protections (OHRP), U.S. Department of Health and Human Services (HHS). WinList™ (Verity Software House) was used for the traditional gating method by a trained expert. The gating results were considered to be the predicate method. The gating method used is shown in Figure 1.

A Probability State Model (PSM) was designed using GemStone to enumerate normal and GPI-deficient monocytic and myeloid leukocytes. Four cell types are defined in the model: Normal myeloids, normal monocytes, GPI-deficient myeloids, and GPI-deficient monocytes. Each cell type defines a set of marker expression characteristics for one subset of cells. As with the gating approach, the model uses CD15, CD45, CD64 and linear side scatter to identify monocytes and granulocytes. The model uses FLAER and CD24 to separate normal and GPI-deficient granulocytes. FLAER and CD14 are used in the model to distinguish normal and GPI-deficient monocytes. When the model is applied to a listmode data file, each event (cell) is assigned to one cell type by means of the PSM's competitive probability algorithm (8,9), Figure 2. The automated analysis performed on each data file adjusted the PSM to the peaks and line spreads of the data, and recorded the normal and GPI-deficient counts for monocytic and myeloid cells. No operator adjustments were made to the PSM automated analysis, and computers of modest performance (dual-core processor) were used. The PSM automated analysis process is illustrated in a video, available online as Supporting Information.

To assess the sensitivity of the PSM, gating and PSM results were compared with predicted values in a serial dilution experiment. A patient sample with a white cell count of 4,200/μl and a clone size of 93% of the myeloid cells was diluted 1:100 with a normal sample with a white cell count of 8,400/μl and a clone size of 0%. This dilution then underwent serial twofold dilutions to a level of 1:3,200. The samples for the two greatest dilutions (1:1,600 and 1:3,200) were acquired at 200,000 events instead of 90,000. Only myeloid cells were considered. The smallest predicted clone size is 0.014%.

## RESULTS

Typical PSM output for a GPI deficient clone is shown in Figure 3. By human analysis, 53 of 530 samples were reported using the traditional gating method as showing the presence of a GPI-deficient clone. The clone sizes ranged from 0.10 to 99.92% for the myeloid cells. Automated analysis by PSM identified the same 53 samples as having a GPI-deficient clone. There were 0 false positives and 0 false negatives; the sensitivity of the PSM analysis is 100% and the specificity is also 100%.

Gating and PSM percentages of GPI-deficient cells were highly correlated: $r^2 = 0.997$ for monocytic cells, and $r^2 = 0.999$ for myeloids, Figure 4. Linear regression analysis also showed favorable results: monocytes $y = 0.9841x + 0.4778$, and myeloid cells $y = 1.0112x - 0.1866$. The mean absolute differences were 0.94% points for monocytes and 0.78% points for myeloid cells. The maximum absolute difference was 5.2% points. PSM results produced by two operators were identical ($r^2 = 1.00$, data not shown).

Another measure of the performance of the PSM model was obtained by comparing the PSM clone size result to the predicted value in a serial dilution experiment (Fig. 5). As a point of reference, traditional gating of the serial dilution data produced results that were highly correlated with the expected concentrations ($r^2 = 0.9944$), slightly underestimating the predicted values ($y = 0.935x + 0.004$). The PSM results were also highly correlated with expected values ($r^2 = 0.9914$), with

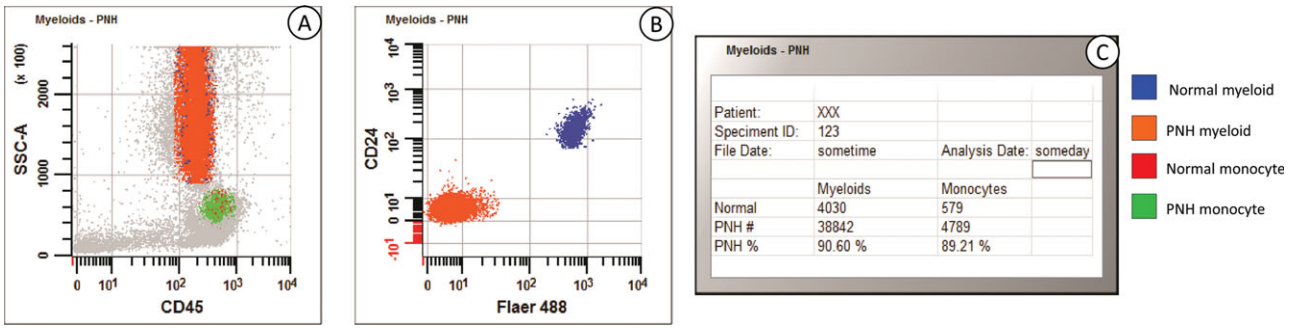


FIG. 3. Typical PSM Output (Myeloid Cells with a Large Clone). The results of the PSM modeling approach can be viewed with conventional dot plots. The myeloid and monocytic lineage determinations made by the model are shown in the CD45 vs SSC-A plot (**A**). The classification of normal (blue) and GPI-deficient (orange) myeloids is shown in the FLAER vs CD24 plot (**B**). It is important to note that no gating is used in these determinations; the PSM uses probability-based decisions to assign events to each subset. Typical statistics are also output from the model (**C**).

results slightly overestimating the predicted values ($y = 1.1022x + 0.093$).

Taking the dataset as a whole, four of the samples showed sizeable clones of myeloid blasts. PSM correctly demonstrated that the blasts had weak expression of FLAER and were negative for the GPI anchored markers tested (data not shown). Both PSM and gating methods appropriately displayed the immature cells in the three bone marrow samples (data also not shown).

## DISCUSSION

Standardized criteria for the evaluation of the performance of automated, multivariate classification programs in the analysis of flow cytometry listmode data are not available. Comparison with a predicate, manual method as such was done in this study would seem to be a minimum requirement. By this criterion, the PSM method performed accurately against the gating method in this mixture problem setting.

The assessment of performance with less-than-perfect data is a multifaceted problem that must be dealt with by any multivariate classification system. In this study we have only considered the issue of cellular viability and integrity. The PSM model performed well in its ability to handle data that showed extensive loss of viability and integrity. In fact, one of the great strengths of the PSM model is its ability to adjust to intensity shifts in the data. There are, however, other reasons for spuriously variable data. Unintended changes in reagent or cytometer performance can have a profound effect on measurement values. The ability of the PSM model to detect large, artifactual shifts has not been explored.

Human analyst-to-analyst or even analyst-to-self precision in the manual analysis of flow cytometry listmode data is never perfect (personal observation). Thus manual analysis can never be completely objective nor can the analysis be entirely standardized. By contrast, the automated results were invariant regardless of the operator using the PSM model. The goals of precision, objectivity, and standardization were achieved with the automated PSM approach.

The PSM analysis is intrinsically faster than the manual analysis: 35 seconds compared with 300 seconds per data file on average. This improved analysis time is in

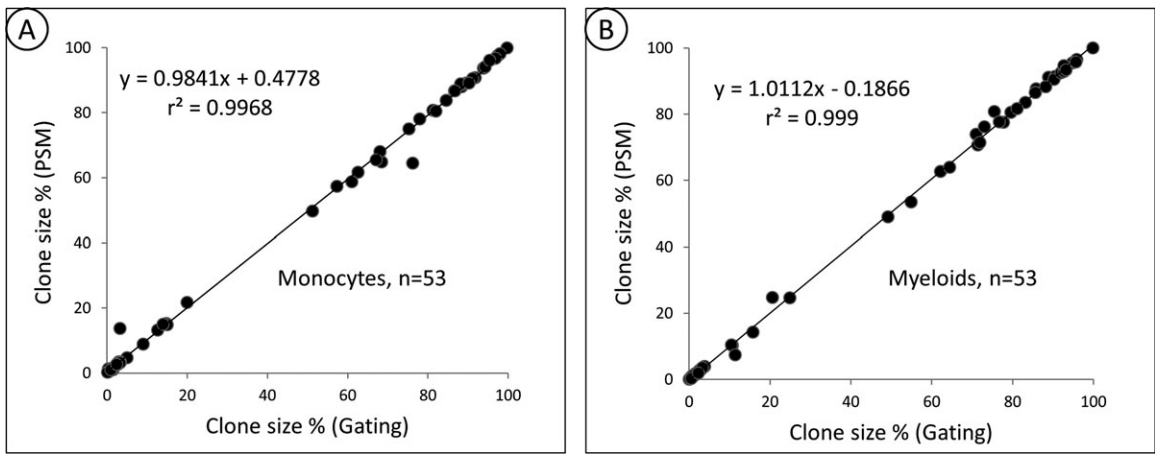


FIG. 4. Comparison of Gating and PSM Results for GPI Deficient Clones. Clone size percentage is compared between traditional gating by an expert and automated PSM methods for 53 GPI-deficient cases. Results are highly correlated for both monocyte (A) ($r^2 = 0.9968$) and myeloid (B) ($r^2 = 0.9990$) subsets.

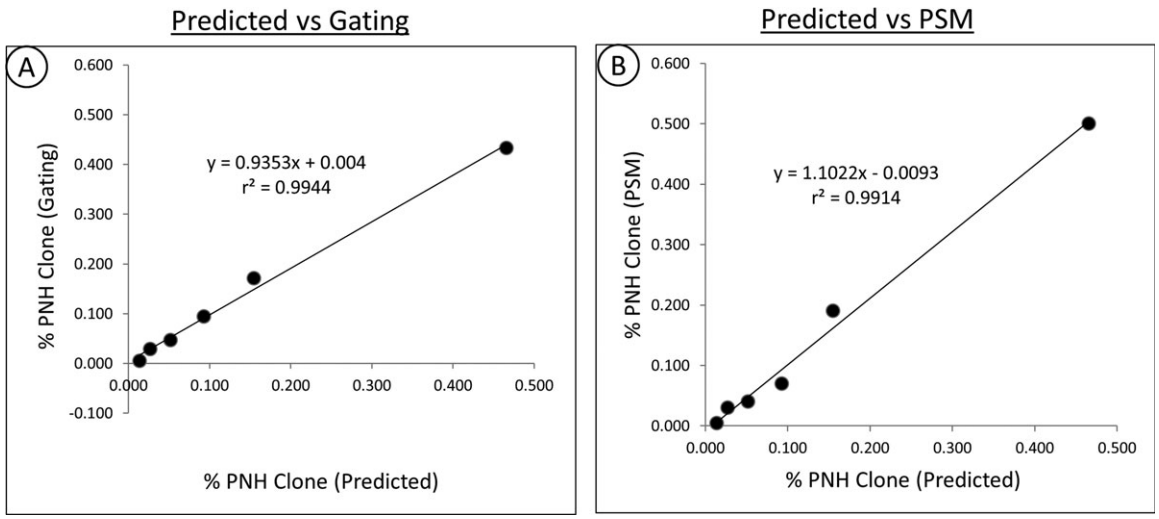


FIG. 5. Comparison of automated and traditional results to predicted results in a serial dilution experiment. A sample from a patient with a large clone size was serially diluted with normal peripheral blood down to a predicted clone size of 0.014%. This is a cellular dilution, rather than a volume dilution. Traditional gating (**A**) and automated analysis with PSM (**B**) performed equally well in comparison with the predicted results, with $r^2$ = 0.9944 and 0.9914, respectively.

part due to the automated production and routing of reporting figures and data. The operator's time was only required to select files for batch analysis by GemStone and to trigger the process. No operator time or intervention was required during the automated analysis.

The PSM analysis offered additional insights into the panel of markers that were used for the detection of GPI-deficient granulocytes. For the samples in this study, the laboratory used three markers to identify GPI-deficient granulocytes: FLAER, CD16, and CD24. The original PSM in our study included components to model all three of these markers, and that model achieved reasonable correlations with the expert results (data not shown). Since modeling reveals the relative contribution of each marker in the panel, we created additional PSMs to explore whether we could achieve similar results with fewer markers. Surprisingly, we found that we achieved better correlations with the expert results by using two markers: FLAER and CD16, or FLAER and CD24. The use of CD16 and CD24 without FLAER produced lower correlations (data not shown). The model used in our final analysis made use of FLAER and CD24, which produced the highest correlations with the expert analysis. These data provide support for the published ICCS recommendations for using two reagents to identify GPI-deficient granulocytes. On the basis of these findings, the laboratory subsequently removed CD16 from PNH-screening panel. This allowed for the addition of 7AAD into the tube and the elimination of a separate viability tube in the panel. This change enables a considerable savings in both time and money, and only became apparent as a result of using modeling.

In this study, the use of linear Side Scatter follows the recommendations in current guidelines and practice (5,6). For myeloids (granulocytes), the linear side scatter distribution is very broad and presents challenges for automated peak-finding routines in GemStone. The model used in this study makes limited use of side scatter in the myeloid cell types for this reason. The use of a logarithmic transformation for side scatter may be a better choice when modeling is used, presenting a more compact myeloid distribution with a distinct peak. This should be the subject of further study.

## CONCLUSIONS

GemStone is a commercially available computer program that uses PSMs for the analysis of cytometry list-mode data. PSMs have been used in other unpublished studies to understand cell lineage maturation sequences and for discovery. In this study, we find GS is equally well suited to handle mixture problems. Automated analysis of GPI-deficient leukocytes by the PSM model produces results that correlate strongly with expert analyst. The probability-based approach provides higher objectivity, speed, and reproducibility than the gating analysis. Automation capabilities of the PSM model required no case-by-case operator decisions. PSM decisions are made by the software and are based on probability distributions in the data and defined in the model. The PSM model showed a robust capability to accommodate data variability due to compromised cellular integrity and viability. Although GemStone has not been approved for clinical use at the time of publication, it is the intention of the manufacturer to seek approval for routine use of this application in the clinical laboratory.

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