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Automated quantitation of fetomaternal hemorrhage by flow cytometry for HbF-containing fetal red blood cells using probability state modeling

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SUMMARY

Background: Flow cytometric methods (FCMs) are the contemporary standard for fetal red blood cell (RBC) quantitation and fetomaternal hemorrhage (FMH) detection. FCM provides greater sensitivity and repeatability relative to manual microscopic Kleihauer–Betke methods. FCM assays are not totally objective, employing subjective manual gating of fetal RBCs with measureable interobserver imprecision. We investigated Probability State Modeling to automate analysis of fetal RBCs using an assay for hemoglobin F (HbF)–containing RBCs.

Methods: Two hundred human bloods were processed using the FMH QuikQuant™ assay (Trillium Diagnostics, Brewer, ME, USA). A Probability State Model (PSM) was designed to enumerate fetal RBCs by selecting the three RBCs subpopulation based on differences in intensity levels of several parameters. The GemStone™ program uses a PSM that requires no operator intervention. Routine manual analysis by experienced users was performed, along with replicate analyses for both methods.

Results: The PSM by GemStone™ correlates strongly with the expert manual analysis, $r^2 = 0.9986$. The mean absolute difference of the FMH results between GemStone™ and manual 'expert' analysis was 0.04% with no intermethod bias detected. Manual gating demonstrated coefficient of variations (CVs) of 10.6% for intra-analyst replicates and 22.6% for interanalyst imprecision. The interanalyst agreement in GemStone™ is a perfect correlation, $r^2 = 1.00$, and no imprecision with a 0.00% CV.

Conclusion: Automated PSM analysis of fetal RBCs strongly correlates with expert traditional manual analysis. PSM enumerates fetal RBCs accurately with significantly greater objectivity and lower imprecision than the traditional manual gating method. Thus, PSM provides a means to markedly improve interlaboratory variance with FMH assays based upon subjective gating strategies.

INTRODUCTION

Fetomaternal hemorrhage (FMH) occurs normally in minute amounts throughout pregnancy and increases during parturition [1, 2]. If there is a significant difference in the red blood cell (RBC) antigenicity between the fetus and mother, this can result in allosensitization of the maternal immune system, leading to morbidity and mortality of that pregnancy and future pregnancies. Flow cytometric method (FCM) for the detection and enumeration of fetal RBCs in pregnant women with antihemoglobin F (HbF) and anti-RhD can provide rapid and accurate results for clinical management [3–9]. Such FCM is now considered the preferred method of FMH detection where accuracy is considered clinically important.

Flow cytometric FMH detection offers improved sensitivity, reproducibility, and precision over the widely used Kleihauer–Betke (KB) method of visual microscopic counting [4–9]. Despite its technical improvement over the KB method, flow cytometric quantitation of fetal RBCs still suffers from moderate interlaboratory variation and the lack of analytical rules for list mode data analysis. The reproducibility and precision of flow cytometric FMH assays are limited by the subjectivity of traditional gating. For example, sources of data analysis variance include using user-generated gating region definitions of red cells (are small aggregates included or excluded?), varying approaches of different efficacy for leukocyte exclusion, which are a source of false-positive fetal red cell events [8] and most importantly the method for distinguishing adult F cells and true fetal RBCs. FMH QuikQuant™ (Trillium Diagnostics, Brewer, ME, USA) is an accurate, precise, and streamlined FCM addressing all the aforementioned concerns requiring about 30 min to complete, with <10 min of hands-on technologist time [10, 11]. However, this assay, like all other FCM assays for FMH detection, still has the problems associated with FCM list mode file analysis by visual gating on plots of univariate or bivariate data [12–17].

Probability State Modeling is a patented approach to flow cytometric data analysis, which has the potential advantage for *in vitro* diagnostic (IVD) assays in that it allows fully automated list mode data analysis [12–14]. A Probability State Model (PSM) has several advantages over manual subjective gating approaches, including proposed multivariate data classification techniques of clustering, attractors, and support vector algorithms [15–18]. Thus, potentially, a probabilistic algorithm for the analysis of the data list mode files replaces the subjectivity of manual gating of FCM data. Furthermore, it allows for the integration of software flags or dialog boxes with the user to build into an analysis template or model features of quality control for the particular assay.

With a PSM approach to data analysis, each cell is evaluated for the probability that it belongs to each cell type in the model. It is then stochastically assigned to a cell type. For example, suppose that the model determines a cell to have a 90% probability of being an adult RBC, in a stochastic assignment, nine of ten times that cell would indeed be assigned to the adult RBC type and one of ten times the cell would be assigned to another cell type or left unassigned. This probability-based approach accounts for variability in measurements on the cytometer resulting in natural overlaps in adjacent populations. With manual gating, such population overlaps will consistently cause inter- and intraobserver variance, but with a PSM, the results are identical for every software user.

In this study, we propose and validate an automated PSM approach for quantitating HbF-containing fetal RBCs, based on GemStone™ (Verity Software House, Topsham, ME, USA) and FMH QuikQuant™ (Trillium Diagnostics), to improve the precision and reproducibility of the FMH detection assay. We compare the results of automated analysis with traditional manual gating of fetal RBCs by experienced data analysts.

METHODS

Control and patient samples

Blood samples were collected from patients into vials containing K_3EDTA following institutional approved ethics guidelines. Blood specimens included in this study are those collected for other medically indicated testing that has been completed and are considered 'discarded specimens'. No patient demographic data were collected, and all patient identifiers were removed from list mode files to ensure patient confidentiality. This study was considered exempted from

an IRB registration in accordance with US regulation 45 CFR 46.101(b) [4]. Patient selection was directed to those where FMH is a possibility or appropriate controls where pregnancy can be excluded as a co-incident physical condition. All clinical samples were stored refrigerated, if analysis could not be completed within 6 h. From sample collection to completion of testing, samples did not exceed a storage time of more than 72 h. Other specimens for testing included various mixtures of FETALtrol™ (Trillium Diagnostics), a US FDA-cleared IVD stabilized human red blood controls containing various levels of adult and fetal RBCs. The number of specimens totaled 200 and was comprised of 68 FETALtrol™ control samples and 132 human blood samples.

Sample preparation and data acquisition

Fetomaternal hemorrhage (FMH) QuikQuant™ (Trillium Diagnostics) was the FCM used for FMH detection and fetal RBC quantitation. The FMH QuikQuant™ kit contains cell permeabilizing and phosphate buffer concentrates and an antibody reagent composed of a fluorescein-labeled monoclonal antibody to hemoglobin F and propidium iodide (PI) as a specific marker of nucleated cells [10, 11]. Clinical samples were processed as detailed in the instructions for use in the FMH QuikQuant assay, including the following steps: sample dilution, cell fixation and permeabilization, and incubation with the antibody reagent. The samples were analyzed on standard configuration BD FACSCanto II cytometer (BD Biosciences, San Jose, CA, USA).

Manual gating strategy

WinList™ (Verity Software House) was used for the traditional, manual gating method of list mode data analysis, as previously described [6, 9]. These results were considered to be the predicate or standard method for comparison. A gate was set around the RBCs on a FSC *vs.* SSC plot to exclude cell aggregates. Nucleated cell populations were then excluded from the RBCs using a propidium iodide exclusion gate. The RBCs were populated on an antihemoglobin F single-parameter histogram to define the three subpopulations of RBCs: adult RBCs, adult F cells, and fetal RBCs. Gating strategy is shown in Figure 1. Fetal RBCs were by defined by their high expression of hemoglobin F (HbF), adult F cells by their intermediate expression of HbF, and adult RBCs by their absence of HbF. The 200 samples were divided into data sets, each with their associated high FETALtrol™ control. The fetal RBC region based upon anti-HbF

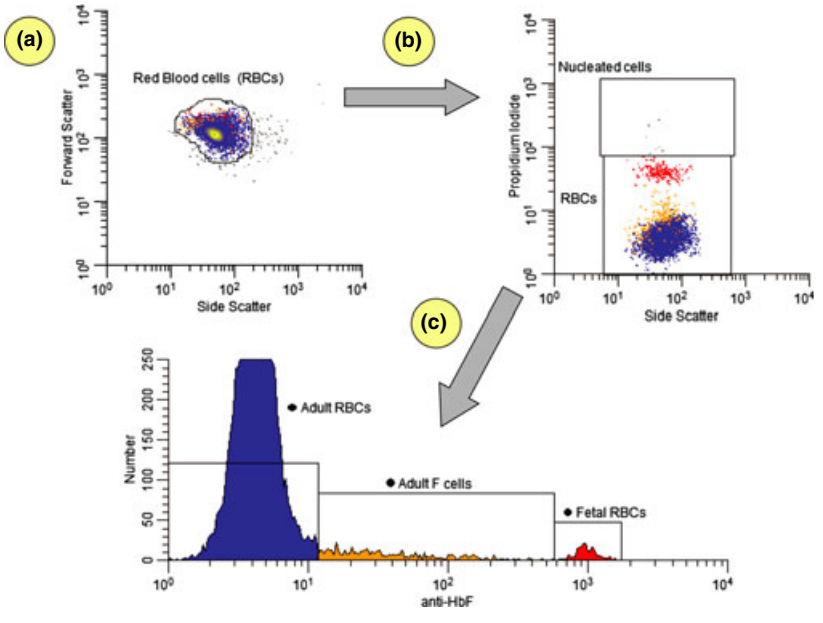

Figure 1. Traditional gating strategy for fetal red blood cells (RBCs) quantitation. RBCs were defined on a FSC and SSC plot to exclude aggregates (**a**). A gate was placed on the PI-negative population to exclude nucleated leukocytes and RBCs (**b**). The RBCs were replotted on an anti-HbF single-parameter histogram (**c**). Fetal RBCs defined by their high expression of hemoglobin F (HbF), adult F cells by their intermediate expression of HbF, and adult RBCs by their absence of HbF.

expression was set using a high FETALtrol™ control sample (approximately 1.5% fetal RBCs) to define the region for fetal RBC enumeration before analyzing the associated clinical samples. Results are expressed as percent of fetal RBCs comprised in the gated RBC population with the limit of detection determined to be approximately 0.04% fetal cells [10, 11].

Triplicate manual WinList analysis was performed by an experienced analyst to determine intra-analyst imprecision and compared to the GemStone™ analysis. These results were compared with those results from two other experienced data analysts to determine interanalyst imprecision by determining the coefficient of variation.

Probability state modeling

A Probability State Model was designed using GemStone™ to quantitate the percentage of cells of interest [12–14]. Four cell types were defined in the model: adult RBC, fetal RBC, F cells, and junk (cell debris, etc.). Each cell type uses a set of expression profiles for side scatter signal (SSC-A), PI, and anti-HbF to identify the appropriate subset of cells (Figure 2).

Adult RBCs were characterized as having moderate SSC-A, dim PI, and the absence of anti-HbF expression. Fetal RBCs have identical SSC-A, slightly higher PI, and approximately two decades higher anti-HbF, by comparison. Adult F cells have similar SSC-A and PI as adult RBCs, but the intermediate anti-HbF expression for adult F cells was defined as being not the fetal RBCs and not the adult RBCs. The junk cell type was used to capture cells that would otherwise interfere with the cells of interest. In particular, the PI for the junk cell type was defined as not the PI for the other three cell types, but PI positive for junk cell type. This nicely removed nucleated cells from the other cell types.

In use, the modeling process is designed to take advantage of the most prominent peaks in the markers to position the expression profiles. A high positive or level III FETALtrol™ control file was opened and the GemStone™ automatically adjusted the model's expression profiles to the intensities of the nearest peaks. This adjusted model was saved to disk. With each associated test file, the adjusted model was reopened and events in the test file were classified based on the adjusted model's peak locations. This allows the model to account for the known assay to assay differences in fetal RBC staining using intra-assay control samples, as is optimally performed for manual data analysis as well.

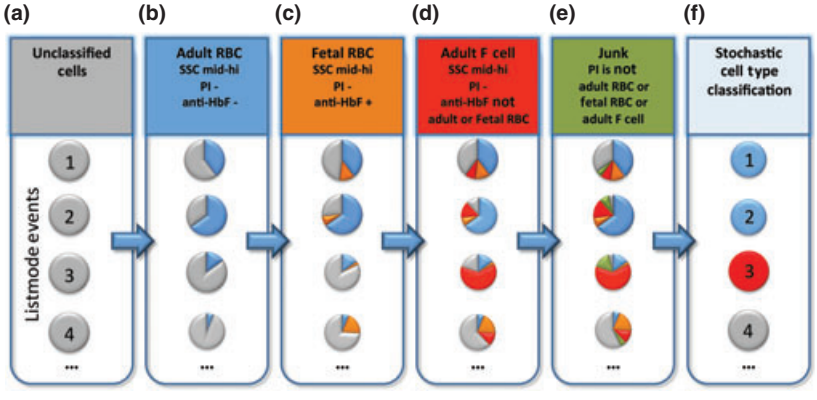

Figure 2. Probability State Modeling Process for fetomaternal hemorrhage (FMH) analysis. For each event, the PSM determines the probability that the event belongs to each of the four cell types: adult red blood cell (RBC), fetal RBC, F cells, and junk (includes nucleated cells). The probabilities are used to assign events to cell types. In the figure, list mode events in the leftmost column (a) are unclassified. The PSM starts by evaluating the likelihood that the event is an adult RBC event (b) based on SSC, PI, and anti-HbF. 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.

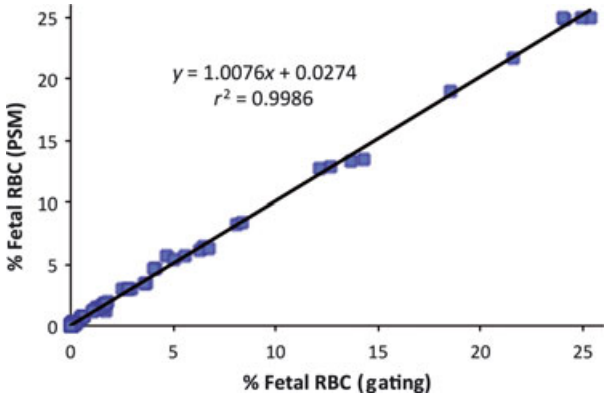

Figure 3. A comparison between GemStone™ automated analysis [%fetal red blood cell (RBC) Probability State Modeling, *y* axis] and Manual Expert Analysis using WinList™ [%fetal RBC (gating), *x* axis] of 200 blood samples stained with fetomaternal hemorrhage (FMH) QuikQuant™. Results from both methods of analysis are highly correlated.

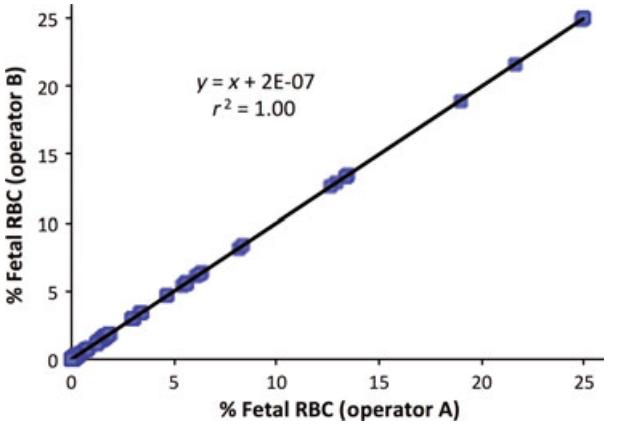

Figure 4. Reproducibility of GemStone™. The operator-to-operator agreement with the Probability State Modeling using GemStone™ is a perfect correlation, $r^2 = 1.00$.

No operator adjustments were made during the PSM analysis. Analysis of the 200 cases was performed by two different operators to evaluate the reproducibility of GemStone™.

Statistical analysis

Correlation of the two data analysis methods was analyzed using linear regression analysis and a Bland–Altman plot to examine the bias between the derived percent of fetal RBCs reported by the various methods of data analysis. Precision was expressed as a CV. This statistical analysis was performed using MedCalc™, version 12.2.10 (MedCalc Software, Ostend, Belgium).

RESULTS

Expert manual analysis reveals fetal RBCs percentages ranging from 0.00% to 24.9% for the data set. The results generated by the GemStone™ PSM correlate strongly against the predicate, manual method of analysis: $r^2 = 0.9986$ (see Figure 3). Linear regression showed favorable comparison of the GemStone™ and manual gating methods ($y = 1.0076x + 0.0274$, see Figure 4). The mean absolute differences were 0.04% for the fetal RBCs between the automated and manual methods. The maximum absolute difference was 0.48% points. A Bland–Altman plot reveals that there is no inherent bias between the two methods (see Figure 5).

The average imprecision between the expert triplicate manual analyses (analyst–self) was 10.6%. The imprecision between the expert and two other expert-level individuals (analyst–analyst) using the manual gating approach was 22.6%. GemStone™ results produced by two operators are identical, =1.00 (see Figure 4). GemStone™ analyst–self comparisons were also completely reproducible with a 0.00% CV (Table 1).

Average required time for processing the 200 samples with WinList™ and manual gating analysis was 150 min. Typical processing time required for 200 samples with the automated PSM analysis with GemStone™ was 16 min, which included generation of a four-page PDF report for each sample. This is nearly a 90% reduction in technical time required for data analysis.

DISCUSSION

Standardized criteria for the evaluation of the performance of automated programs in the analysis of flow cytometry list mode data are not available, as no consensus has been reached. Comparison of the GemStone PSM with the predicate method of traditional gating used by the most clinical laboratories was the

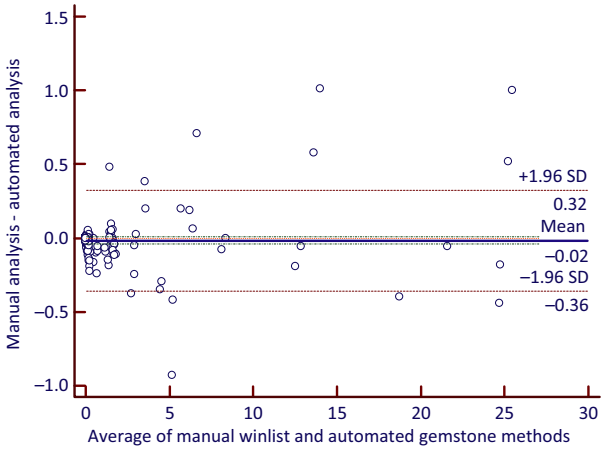

Figure 5. Bland–Altman bias plot comparing manual WinList™ gating to the automated GemStone™ analysis on 200 specimens processed with fetomaternal hemorrhage (FMH) QuikQuant™ shows no significant bias between the two methods of data analysis.

Table 1. GemStone™ Probability State Modeling and manual gating reproducibility. Imprecision between two expert-level operators using manual gating was 22.6%. Even intra-analyst imprecision of manual analysis is not perfect, as evident by the 10.6% variation between the same expert's triplicate analyses. GemStone achieved perfect reproducibility with a 0.00% coefficient of variation (CV) in both intra-analyst and interanalyst comparisons even on samples with low or absent fetal red blood cells (RBCs)

Replicate analyses	Average % CV
Manual Analysis–intra-analyst	10.6
Manual Analysis–interanalyst	22.6
GemStone PSM–intra-analyst	0.00
GemStone PSM–interanalyst	0.00

basis of our evaluation. By this criterion, the automated analysis using GemStone yielded results that correlate strongly with the manual 'expert' analysis with no bias between the two methods detected.

The average CV for the WinList™ replicate analysis was higher than expected. These CVs are elevated because there are many low fetal RBC percentages (<0.03%) among the samples tested, which would expectedly increase the CV. The CV of samples with fetal RBCs between 0.00 and 0.01 was 141.42%. These 'low' values artificially inflate the imprecision between the WinList replicate analyses. If these values are excluded, then the CVs would be approximately 4% and 7% for the manual intra-analyst and interanalyst analyses, respectively, which is in keeping with previous reports [6, 9–11]. If such variation is observed in expert analyses, we anticipate that it would even higher for nonexperts, where CV is typically reported in the range of 10-30% from regional proficiency testing programs for FMH testing by flow cytometry, such as those run by the College of American Pathologist or UK NEQAS programs. Our replicate analyses demonstrate that human analyst to analyst analysis and even analyst–self precision in manual analysis of list mode data never correlate perfectly, suggesting that subjective manual data analysis alone contributes approximately 5% to the imprecision of flow cytometric FMH assays and that interindividual subjectivity adds another approximately 5% to the imprecision. The GemStone™ analysis demonstrated no variance with repetitive analysis of the same list mode file, as one would expect for a fully automated analytic approach.

Inconsistency between laboratories can be even greater. Proficiency testing by the College of American Pathologist and UK NEQAS consistently documents interlaboratory variation to be 10–30% [9]. The poor reproducibility of manual analysis originates from the various subjective decisions that are required for data analysis. The tradition
al method of gating requires operators to define gating regions, consistently exclude interfering cell populations (e.g., nucleated cells) and set regions for adjacent and overlapping RBC subpopulations (adult RBCs, adult F cells, fetal RBCs) on a case-by-case basis. GemStone™ PSM decisions are made entirely by the software based on probability distributions in the data and as defined in the model. This eliminates the need for case-by-case operator decisions, removing intra-analyst and interanalyst variability in the traditional method of data analysis, and significantly reduced by nearly 90% the amount of time required for analysis. The operator's time was only required to select files for batch analysis by GemStone. GemStone's improved analysis time is also in part due to the automated production of figures and data.

In our GemStone™ analysis, we assume that the FETALtrol™ positive control allows us to determine expected intensity for the fetal RBCs and that the

patient samples will have populations with the same intensity. However, improper sample preparations can lead to minor intensity shifts, which might be further improved using fully automated sample preparation. Our current model does not provide criteria for detecting sample preparation problems that may end up causing intensity shifts, but does integrate the use of batch external control samples and thereby including quality control into data analysis of patient samples. Model detection of potential sample preparation problems can provide valuable information to clinical laboratories for the troubleshooting of problems. Our study determined that PSM automated data analysis is a robust alternative to the traditional method of gating; however, more investigation is required to evaluate the adaptability of the PSM used for this study to flow cytometric data generated by a variety of FCM instrument models.

Our study provides support for the routine use of PSMs in clinical laboratories for the analysis of flow cytometric data for FMH testing. Our findings add to the recently reported applications of PSMs, such as improved enumeration of CD34+ stem cells for bone marrow or blood harvests prior to bone marrow transplantation [19] and the enumeration and identification of abnormal phosphatidylinositol-linked protein expression in paroxysmal nocturnal hemoglobinuria detection [20]. Automated PSM analysis of clinical samples using GemStone™ performed accurately against manual expert analysis and provides a more robust alternative to the current practice of subjective gating. GemStone™'s greater objectivity and reproducibility are due to the elimination of operator gating decisions, which also greatly reduce the time required for analysis. We anticipate that the use of PSMs or other improved software algorithms will significantly improve flow cytometry IVD assays and remove a major source of imprecision from clinical FCM testing.

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