Improved algorithms for the analysis and classification of HbF-containing Red Blood Cells

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1. Abstract

Fetomaternal hemorrhage (FMH) occurs normally throughout pregnancy with an increment occurring during the birthing process. In cases with a significant difference in red blood cell antigenicity between the fetus and mother, allosensitization of the maternal immune system can occur, which causes significant morbidity and mortality in that or subsequent pregnancies. Flow cytometric methods for detection and enumeration of fetal red blood cells (RBCs) in pregnant women using anti-hemoglobin F (HbF) methods can provide rapid and accurate results for clinical management. However, current methods suffer from large inter-laboratory variability and the lack of analytical rules for listmode data file analysis.

The current approach to data analysis requires subjective gating decisions and region placement, usually based upon a one parameter approach evaluating the anti-HbF parameter in log space. It is possible to double-count some cells and miss other cells altogether. Our study examines the use of a Probability State Model (PSM) for classification of human blood samples into the three recognized sub-populations of fetal RBCs (high HbF expression), adult F cells (intermediate HbF expression), and adult RBCs (lacking HbF expression), as typically used in clinical FMH detection. A PSM uses probabilities to assign cells to populations based on a model definition and is not dependent on subjective gating decisions. Samples used for comparisons where composed of fresh human blood samples or stabilized FMH assay controls (FETALtrol, Trillium Diagnostics, Bangor, Maine, www.trilliumdx.com) and were assayed using the FMH QuikQuant assay kit (Trillium Diagnostics) for anti-HbF staining. PSM analysis was performed using GemStone ™ software (Verity Software House, Topsham, Maine, www.vsh.com).

Results of the PSM approach are compared with expert analysis using the standard one parameter identification of fetal RBCs to assess the utility of PSM for FMH detection. Each approach was done in triplicate to assess reproducibility on 115 sample files with fetal cells ranging between 0– 25% fetal cells. The results show an improvement in reproducibility using the PSM approach and indicate the PSM approach to data analysis for rare event determinations, such as FMH determinations, is a more robust approach to result reporting and should significantly reduce inter-laboratory variation in results reported for proficiency testing.

2. Study Aims

1. Develop a Probability State Model (PSM) for analysis of flow cytometric listmode data files using Anti-HbF methods for the detection of fetal red blood cells (RBCs) in maternal blood samples as used in diagnostic assessment of fetomaternal hemorrhage.

2. Compare PSM model in GemStone ™ software (Verity Software House) to manual data analysis with WinList ™ software (Verity Software House) as performed by blinded analysis by an expert (developer of the Anti-HbF method) in >100 data files from analysis of diagnostic clinical blood samples, artificial mixtures of fetal (cord) blood and adult blood, and stabilized control product for FMH assays (FETALtrol, Trillium Diagnostics).

3. Examine data from above comparative analysis to determine the correlation between the methods and determine if any bias is identified between PSM and expert manual analysis.

4. Determine potential for PSM data analysis to provide automated analysis of flow cytometric data files for clinical fetomaternal hemorrhage testing and provide a more robust alternative to the current practice of subjective gating and subpopulation region definition that contribute to the documented imprecision or variation between various diagnostic laboratories performing flow cytometric fetomaternal hemorrhage testing for pregnant women.

3. Methods

1. **Materials:**
   1. Clinical blood samples submitted for analysis for fetomaternal hemorrhage as EDTA anti-coagulated blood
   2. Artificial mixtures of ABO and Rh blood type matched adult blood and cord (fetal) blood samples
   3. FETALtrol, stabilized three level control material for fetomaternal hemorrhage assays (Trillium Diagnostics, Bangor, Maine, USA)
   4. Phosphate buffered saline (PBS) with 0.5% bovine albumin, pH 7.4
   5. FMH QuikQuant assay for flow cytometric detection of fetomaternal hemorrhage (Trillium Diagnostics, Bangor, Maine, USA)
   6. Flow cytometer: BD Biosciences (San Jose, CA) BD FACSCanto II instrument with acquisition set-up protocol as described by FMH QuikQuant instructions for use
   7. WinList software (Verity Software House, Topsham, Maine, USA)
   8. GemStone software (Verity Software House, Topsham, Maine, USA)

2. **Sample Processing and Preparation:**
   All samples were processed as detailed in the instructions for use for the FMH QuikQuant assay including:
   1. 10 microliters of 1:20 diluted blood or control samples added to 0.75 mL 0.05% glutaraldehyde for 10 minutes
   2. Add 1.5 mL Trillium IntraCell permeabilizing reagent (component of FMH QuikQuant) for 10 minutes
   3. Centrifuge sample and decant supernatant
   4. Add 10 microliters of FMH QuikQuant reagent A (anti-HbF-FITC antibody and propidium iodide) and 40 microliters PBS/BSA, incubate 15 minutes
   5. Analyzed on flow cytometer, collecting at least 50,000 events for parameters of FALS, SS (log), FL1 (anti-HbF-FITC), FL2 ("auto-fluorescence"), and FL3 (propidium iodide)

3. **Manual "Expert" Data Analysis with WinList software:**
   1. Data files analyzed independently by one of the authors (BHD) using the approach outlined previously described (Davis BH, Davis KT: Enumeration of Fetal Red Blood Cells, F Cells, and F-Reticulocytes in Human Blood. In *Current Protocols in Cytometry*, Unit 6.17, Editors Robinson JP, Orfao AJ, John Wiley and Sons, Inc. 2004)
   2. Specific gating and analysis approach shown in Figure 1.

4. **Probability State Model with GemStone software:**
   1. Model developed by author (BH) based upon principles used by expert analysis
   2. Data files analyzed independently of "expert" with minimal adjustments to sub-population regions

4. Current FMH Assay Approach: Gating Strategy and Problems

1. Proficiency testing by College of American Pathologist and UK NEQAS programs consistently documents inter-laboratory variation between 10 – 30%. While vastly superior to non flow cytometric methods for FMH detection, this variation is higher than the intra-laboratory imprecision with a typical CV of ~5%.
2. Sources of inter-laboratory variation include the subjectivity of defining gating regions, the gating approach, consistency of excluding interfering cell populations (nucleated cells), and setting regions for the defined subpopulations of Adult RBCs, F cells, and Fetal RBCs. Automated data analysis could remove these variables and reduce the imprecision.
3. Education on data analysis is required and often this too can be variable between products for FMH detection and between technical "experts".
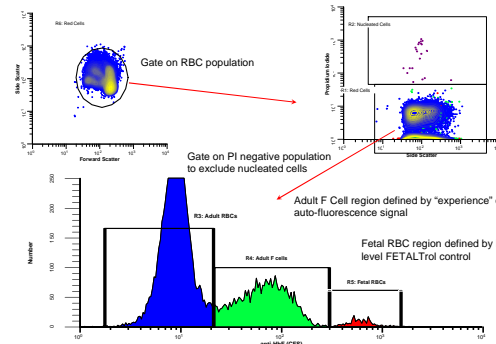

Figure 1. Manual data analysis of listmode file with WinList. The principals of analysis are to gate on red cells, excluding aggregates, and to also exclude any nucleated cells (leukocytes and nucleated red cells, identified by PI staining), then to identify fetal RBCs by their high Hemoglobin F (HbF) content, Adult RBCs by the absence of HbF, and adult F cells by the intermediate level of HbF.

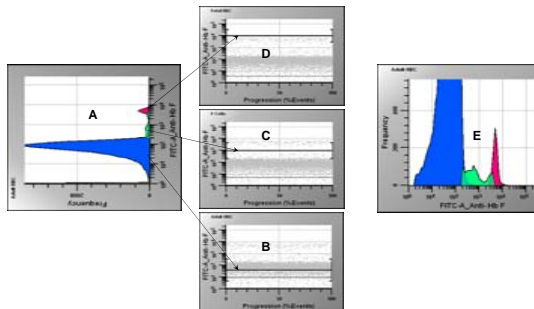## 5. Benefits of Probability State Modeling

Figure 2. A Probability State Model (PSM) uses a set of expression profiles for one or more listmode parameters to assign events to states and cell types probabilistically. A PSM can be designed to minimize subjective decisions in FMH analysis. The operator visually examines intensity and line spread of Anti-HbF for a FETALtrol high control file (A, ~1.5% fetal cells) and makes a simple intensity estimate for the Adult RBC population (B, HbF negative cells, blue population). The model automatically adjusts for the HbF intensities of the F Cells (C, green population) and Fetal RBCs (D, red population).

A PSM can account for population overlaps (E) as well as continuums that can occur in FMH analysis. The expression profiles in the model used in this study are designed to overlap, allowing the modeling process to assign events to the most probable cell type. This provides for a more reproducible analysis from operator to operator, and sets the stage for future total automation of the data analysis.

Unlike most PSMs, the model used here does not attempt to define a progression of cells in the sample. It is designed simply to resolve the mixture of the three cell types: Adult RBC, F-Cells, and Fetal RBC. This allows the use of "constant" parameter profiles set at different intensity levels to select the cells of interest.
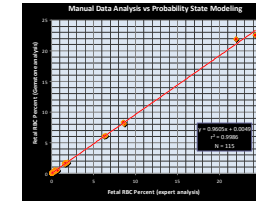
6. Results

Figure 3. Correlation between manual "expert" analysis and GemStone probability state modeling (Verity Software House) of 115 listmode files from FMH QuikQuant (Trillium Diagnostics, LLC) stained and flow cytometric analysis human blood, artificial mixtures of adult and cord blood (values with > 55 fetal RBCs), and stabilized controls samples (FETALtrol, Trillium Diagnostics) in the range of 0 – 25% fetal RBCs in adult blood. The two methods of data analysis give essentially equivalent results and a high degree of correlation even at levels higher than seen in clinical practice.
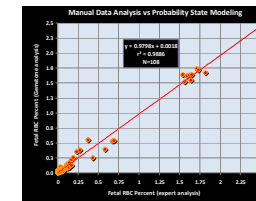

Figure 4. Correlation between manual "expert" analysis and GemStone probability state modeling (Verity Software House) of 108 listmode files from FMH QuikQuant (Trillium Diagnostics, LLC) stained and flow cytometric analysis human blood and stabilized controls samples (FETALtrol, Trillium Diagnostics) in the range of detection typically seen with clinical assessment of fetomaternal hemorrhage. The two methods of data analysis give essentially equivalent results.
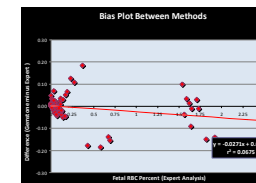

Figure 5. Bias plot between the GemStone probability state modeling and manual "expert" analysis shows no significant bias between the two methods for fetal RBC detection as a function of the incidence of fetal cells in the adult blood sample.

7. Conclusions

1. Probability State Modeling provides equivalent results to manual data analysis with standard listmode analysis software by highly experienced individuals for Fetal RBC analysis, a form of rare event analysis.
2. The GemStone PSM protocol requires no or minimal adjustment of gate or regions, providing a high likelihood that FMH analysis could be done in an automated format, thereby removing an important source of inter-individual variability in the current methods of data analysis.
3. PSM analysis by GemStone and the improved, no-wash method of FMH QuikQuant together promise to further improve the accuracy, precision, and technical simplicity of FMH detection in clinical flow cytometry.
4. Further study is warranted to further improve the PSM protocol for data analysis of clinical samples and document the degree of improved performance over current manual methods of flow cytometric data analysis.