Peripheral blood contamination in cerebrospinal fluid - Automated quantitative analysis.

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INTRODUCTION

The implementation of the HeMoStep kit, designed for the quantitative measurement of blood contamination in cerebrospinal fluid (CSF), presents significant challenges in clinical practice. This assay, based on microspheres for flow cytometry and using hemoglobin (Hb) as a biomarker, involves several analytical steps beyond sample preparation, such as the construction of a calibration curve, interpolation of fluorescence readings from the cytometer, result calculation, and ultimately, interpretation within a clinical context. Initially, this analysis would require the use of various software tools, complicating its application. Additionally, reproducibility is a particular concern in flow cytometry, as traditional analyses rely on expert users to manually define "gates"—thresholds, ellipses, or polygons used to identify specific cell populations—which hinders the consistency of results. To address these challenges and streamline the process, an automated software application, FlowStep, has been developed.
This application is designed for non-expert users and, when used in conjunction with the HeMoStep kit, provides a comprehensive solution for quantifying contamination in CSF.

MATERIAL AND METHODS

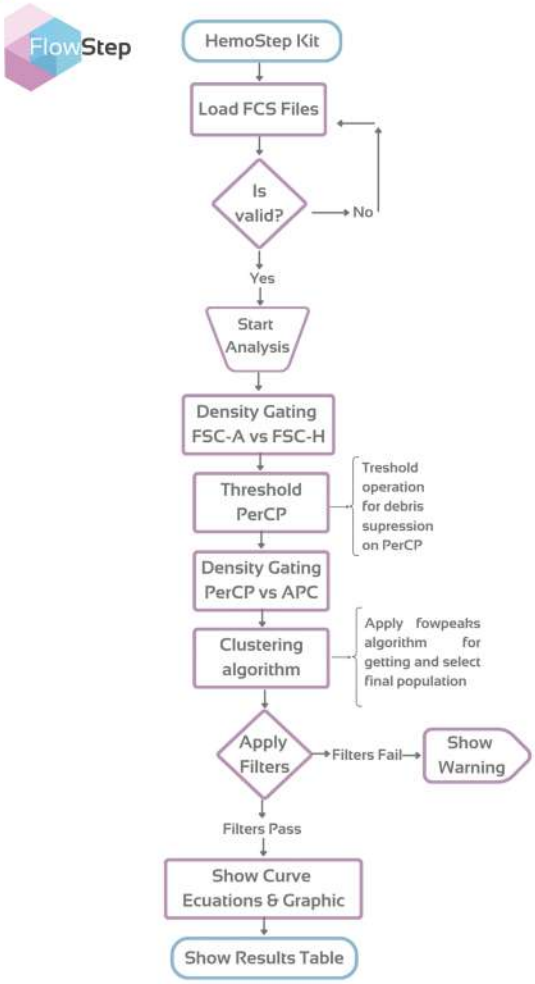

Figure 1: Flowchart illustrating the sequential steps employed by the software algorithm for identifying the microsphere population, along with the parameters utilized in this process.

Classification approach for the analysis of flow cytometry datasets

The data set used in this paper included flow cytometry data obtained by measuring 5 different parameters corresponding to samples and standard curves processed according to the HeMoSep kit instructions. Two of these attributes are light scattering data (FSC-A and FSC-H) and three are emission measurements of different wavelengths associated with bead autofluorescence (PerCP, APC) and others (PE) for analyte detection. Therefore, for the automated analysis of these parameters, the software sequentially performs gating and compensation operations, leveraging Cytoflow, a Python library specifically designed for the analysis of flow cytometry data. For manual (operator independent) flow cytometry analysis, the INFINICYT software program was used.

Automatic search for microsphere population

For the correct identification of the microsphere population, the first step of the algorithm implanted (Fig. I) is to discriminate microsphere doublets through gating. It should be noted that the microsphere population typically forms a well-defined cluster of events in the two light scatter parameters (FSC-A and FSC-H). Once classified and quantified, the singlet population, a threshold was applied on attributes in PerCP for then selected by gating and clustering based on the fluorescent data in PerCP and APC, allowing only those events corresponding to the microsphere population whose PE fluorescence emission is to be measured. Cytoflow facilitates the processing of FCS files, compatible with all standards, allowing for density-based gating to identify cell subpopulations. Additionally, it enables the application of the FlowPeaks algorithm, a fast, unsupervised clustering method for flow cytometry data that uses K-means and density peak finding to group events based on shared characteristics.

Generation and Analysis of Data-Files

The Matplotlib library, known for its ability to create static, animated and interactive visualizations in Python, was used to generate custom graphs such as scatter plots and scatter diagrams to facilitate interpretation of the results. (Fig. 2) In parallel, SciPy, a Python library offering a wide range of advanced mathematical tools and algorithms, was used to perform complex mathematical operations, including curve fitting and data interpolation, to calculate the concentration of the analyte Hb (ng/ml) and to estimate the number of RBCs and WBCs present as contaminants in the CSF sample. For manual (operator independent) data analysis, the GraphPad Prism 8 software program was used.

Figure 2: Software screenshot example of the automated algorithm implemented for the construction of the calibration curve. In each step, the groups of events/populations listed to apply the statistical analysis are defined together (A) fcs files corresponding to each of the tubes acquired by the cytometer, corresponding to each of the concentrations of the curve (B) automatic tree-based classification of the curve files (C) identification of the microsphere population of interest using the FlowStep algorithm, which performs FSC-A FSC-H gating, followed by PerCP thresholding and finally gating and clustering using PerCP and APC microsphere autofluorescence as parameters, (D) the representation of the constructed curve with its parameters, derived from the set of FCS files, (E) data table is associated with the calibration curve and the inverse standard calculation, which is used to evaluate the quality of the curve fit. In addition to the R², other metrics are considered.

Finally, for report generation, the software provides a set of HTML+ templates that are converted to PDF using the pdfkit library.

RESULTS

Correlation between automated software and manual method for MFI data for construction of a Standard Curve

The new automated software for constructing the calibration curve demonstrated its ability to perform batch analysis of all FCS files corresponding to the calibration curve, eliminating the need for sorted loading and parameter analysis. The result showed a high correlation (r^2 = 0.999) between the MFIs obtained through manual analysis by an independent expert operator and the automatic method.

Manual operator						Automated Software (HeMoStep)					
Sample	MFI Mean	[ng/ml]	[g/dl]	RBC's/ul	N° WBCs	MFI Mean	[ng/ml]	[g/dl]	RBC's/ul	N° WBCs	CV (%)
1	28376179	227,08	2,27E-05	7,50	31,03	28461,25	234,42	2,3442E-05	7,83	32,40	3,055%
2	467790,60	514,18	5,14E-05	16,98	70,27	468279,81	537,03	5,3703E-05	17,74	73,10	2,792%

Sample ID	MFI (median)	Concentration(ng/ml)(log)	RBC's / (µL)	N° WBC's	CSF Blasts N°	Result
15523121	1896.99	-	-	-	-	Negative
15523122	1566632.28	-	-	-	-	Dilution Required
sample1	1523184.56	-	-	-	-	Dilution Required
sample2	284611.25	2.37	7.83e+00	3.24e+01	-	Positive
tube4	468279.81	2.73	1.77e+01	7.31e+01	-	Positive

Degree of agreement between automated software and manual method for the analysis of blood contamination in cerebrospinal fluid (CSF)

Similarly, the results of peripheral blood contamination in CSF showed a high degree of agreement between the automated software and the manual analysis (Table I). While the software exhibited superior reproducibility in accordance with expectations. The FlowStep software is preset to report the degree of contamination in ng/ml of Hb. However, if the user provides the hemoglobin concentration (g/dL), as well as the number of red blood cells (RBCs) and white blood cells (WBCs) present in the patient's blood count, the software calculates and reports the contamination in RBCs and WBCs in the cerebrospinal fluid (CSF) sample using the following algorithm.

The FlowStep software is preset to report the degree of contamination in ng/ml of Hb. However, if the user provides the hemoglobin concentration (g/dL), as well as the number of red blood cells (RBCs) and white blood cells (WBCs) present in

Finally, FlowStep software has no minimum system requirements and runs in a Windows environment. The processing speed of FCS files depends on the CPU performance of the computer on which it is used. For a computer with conventional hardware, this speed is less than 1 second per file.

CONCLUSIONS

The FlowStep software, developed for use with the HeMoStep kit, proved to be an automated, efficient and accurate solution for the quantification of blood contamination in cerebrospinal fluid (CSF). It showed a high correlation (r^2 = 0.999) with the manual method and better reproducibility, reducing dependence on expert operators. Its integration of advanced algorithms and automatic report generation facilitates rapid and standardized analysis, improving efficiency and reproducibility in clinical laboratories.

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