FULL-LENGTH ARTICLE

Manufacturing

Validation of a flow cytometry-based method to quantify viable lymphocyte subtypes in fresh and cryopreserved hematopoietic cellular products

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ABSTRACT

Background aims: Adoptive cellular therapy with immune effector cells (IECs) has shown promising efficacy against some neoplastic diseases as well as potential in immune regulation. Both inherent variability in starting material and variations in cell composition produced by the manufacturing process must be thoroughly evaluated with a validated method established to quantify viable lymphocyte subtypes. Currently, commercialized immunophenotyping methods determine cell viability with significant errors in thawed products since they do not include any viability staining. We hereby report on the validation of a flow cytometry-based method for quantifying viable lymphocyte immunophenotypes in fresh and cryopreserved hematopoietic cellular products. **Methods:** Using fresh or frozen cellular products and stabilized blood, we report on the validation parameters accuracy, uncertainty, precision, sensitivity, robustness and contamination between samples for quantification of viable CD3+, CD4+ T cells, CD8+ T cells, CD3–CD56+CD16+/- NK cells, CD19+ B cells and CD14+ monocytes of relevance to fresh and cryopreserved hematopoietic cellular products using the Cytomics FC500 cytometer (Beckman Coulter).

Results: The acceptance criteria set in the validation plan were all met. The method is able to accommodate the variability in absolute numbers of cells in starting materials collected or cryopreserved from patients or healthy donors (uncertainty of $\leq 20\%$ at three different concentrations), stability over time (compliance over 3 years during regular inter-laboratory comparisons) and confidence in meaningful changes during cell processing and manufacturing (intra-assay and intermediate precision of 10% coefficient of variation). Furthermore, the method can accurately report on the efficacy of cell depletion since the lower limit of quantification was established (CD3+, CD4+ and CD8+ cells at 9, 8 and 8 cells/ μL , respectively). The method complies with Foundation for the Accreditation of Cellular Therapy (FACT) standards for IEC, FACT-Joint Accreditation Committee of ISCT-EBMT (JACIE) hematopoietic cell therapy standards, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use Q2(R1) and International Organization for Standardization 15189 standards. Furthermore, it complies with Ligand Binding Assay Bioanalytical Focus Group/American Association of Pharmaceutical Scientists, International Council for Standardization of Hematology/International Clinical Cytometry Society and European Bioanalysis Forum recommendations for validating such methods.

Conclusions: The implications of this effort include standardization of viable cell immunophenotyping of starting material for cell manufacturing, cell selection and in-process quality controls or dosing of IECs. This method also complies with all relevant standards, particularly FACT-JACIE standards, in terms of enumerating and reporting on the viability of the “clinically relevant cell populations.”

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Introduction

Adoptive cellular therapy with immune effector cells (IECs) continues to face several challenges: manufacturing processes, logistic and coordination aspects and toxicity profiles. Accordingly, the consensus from academic cellular therapists, transplant programs,

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commercial cell manufacturers and regulatory agencies was toward the creation of adapted guidelines that meet many of these above-mentioned challenges. Therefore, the Immune Effector Cell Task Force was created, and it formulated standards and a corresponding accreditation program for IEC [1]. The Foundation for the Accreditation of Cellular Therapy (FACT) Standards for Immune Effector Cells, First Edition, Version 1.1, March 2018, has the major objective to promote quality practices when administering IECs destined to therapeutically modulate, elicit or mitigate an immune response [2].

Going into the specifics of the guideline, a relevant and validated assay should be employed to evaluate cellular therapy products undergoing manipulation that alters the nature/function of the target cell population. IECs currently comprise cells that confer broad cytotoxicity against tumors—for example, *ex vivo* activated/expanded, engineered or selected natural killer (NK) cells [3] or targeted cytotoxicity against tumor-associated antigens, such as chimeric antigen receptor (CAR)-transduced T cells [4], T-cell receptor-engineered T cells [5] and tumor peptide-directed cytotoxic T cells [6]. Cells that induce tolerance by suppressing inflammatory responses—for example, regulatory T cells [7], tolerogenic dendritic cells [8], regulatory macrophages [9] or regulatory B cells [10]—or enhancing immune recognition, such as peptide-loaded or genetically engineered dendritic cells [11], are equally included. Whether or not these cellular therapy products undergo manipulation, their identity, enumeration and viability are critical information that is needed on both sides of the process: initiating manipulation and releasing the products for infusion.

Therefore, a relevant and standardized assay for quantifying the initial and final cell population needs to be established and validated. To that end, multi-color flow cytometry is the technology of choice in cell manufacturing facilities for cell surface marker detection, viability and enumeration [12]. Measurement of viable absolute counts of cells is additionally performed using single-platform panels recommended by International Society for Hematotherapy and Graft Engineering and Joint Accreditation Committee of ISCT-EBMT (JACIE) standards. This includes a cell viability dye and counting beads with a lyse/no-wash preparation using commercial kits for CD34+ cells and lymphocyte subpopulations, yet without a viability dye for the latter [13–15]. The challenging aspect is validating laboratory-developed flow cytometry methods. Hence, recommendations/guidelines ensued in three waves, the first formulated in 2011 by the flow cytometry subcommittee of the Ligand Binding Assay Bioanalytical Focus Group of the American Association of Pharmaceutical Scientists [16,17], the second in 2013 by the International Council for Standardization of Hematology and the International Clinical Cytometry Society [18–21] and the last in 2017 by the European Bioanalysis Forum [22].

In this work, we focus on fresh and cryopreserved mononuclear cell and hematopoietic progenitor cell products and the cell manufacturing of IEC products by reporting on the validation of an analytical method: quantification of viable absolute counts of lymphocyte immunophenotypes using the Cytomics FC500 (Beckman Coulter) in compliance with (i) FACT Standards for Immune Effector Cells; (ii) FACT-JACIE hematopoietic cell therapy standards; (iii) International Council for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use Validation of Analytical Procedures: Text and Methodology, Q2 (R1); and (iv) International Organization for Standardization (ISO) 15189 standards (see supplementary Table 1). The intended use of the data generated by this assay is decision-making and not exploratory.

Methods

Samples

Fresh cellular products (leukapheresis, bone marrow, cord blood) from healthy donors previously collected at the apheresis unit and processed/cryopreserved at the cell processing facility operated by Institut Paoli-Calmettes were used. All studies were performed

according to institutional and Helsinki guidelines regarding human ethics. All informed consent forms are available on record. Leukapheresis products were collected in anticoagulant citrate dextrose solution A (Terumo BCT Europe N.V, Arcueil, France), bone marrow products were collected in heparin solution, and cord blood was collected in citrate phosphate dextrose. For cryopreservation, leukapheresis and cord blood products were centrifuged to reduce the volume before distribution into two identical bags, followed by addition of cryopreservation solution (6% hydroxyethyl starch plus 20% dimethyl sulfoxide) to a final dimethyl sulfoxide concentration of 10% and placement in a controlled-rate freezer (Minidigitcool, Cryobiosystem), followed by storage in gas phase liquid nitrogen containers. Bone marrow products were reduced in volume or red blood cell-depleted using the Spectra Optia apheresis system. Thawed products were washed using the Sepax system and 6% hydroxyethyl starch solution. The other sample used for this validation was the StatusFlow flow cytometry control tube, which is a stable preparation of human peripheral blood, with expected values used to monitor the immunophenotyping panels (R&D Systems Inc, MN, USA, EuroCell Diagnostics, Noyal Chatillon/Seiche, France) daily. Samples were diluted in phosphate-buffered saline/0.5% human immunoglobulin/ethylenediaminetetraacetic acid to reach a target concentration of 20×10^6 cells/mL.

Antibodies, reagents and buffers

Antibodies were supplied by Immunotech SAS (Beckman Coulter, Marseille, France). Tube 1: IOTest conjugated antibody anti-CD45-FITC (clone J33), anti-CD3-PE (clone UCHT1), anti-CD4-ECD (clone SFC12T4D11), 7-Aminoactinomycin D (7-AAD) viability dye, anti-CD8-PC7 (SFC121Thy2D3). Tube 2: anti-CD45-FITC (clone J33), anti-CD3-PE (clone UCHT1), anti-CD16-ECD (clone 3G8), 7-AAD viability dye, anti-CD56-PC7 (clone N901, NKH-1). Tube 3: anti-CD45-FITC (clone J33), anti-CD19-PE (clone J3-119), anti-CD14-ECD (clone RMO52), 7-AAD viability dye. All antibodies contained 0.1% sodium azide. All lot numbers are retained on record. The choice of monoclonal antibodies was based on the Tetra panel (Beckman Coulter), in addition to integrating 7-AAD as viability dye.

For sample erythrolysis, IOTest3 lysing solution (PN IM3514, Immunotech SAS, Beckman Coulter, Marseille, France) diluted at 1X was used. For determination of absolute counts of cells, Flow-Count fluorospheres (PN 7507992-MD, Beckman Coulter, CA, USA) were added to each tube and their concentration logged into the CXP acquisition software of the Cytomics FC500 (Beckman Coulter) cytometer (instrument settings and compensation matrix are detailed in Supplementary Table 2). The fluorochrome-monoclonal antibody pairing, lysis using ammonium chloride solution and gating strategy (Figure 1A–C) followed the same logic as that behind the International Society for Hematotherapy and Graft Engineering recommendations for stem cell enumeration and the seminal work by Koehl *et al.* [13]. Gating strategy for CD4+ and CD8+ T cells allows for elimination of CD3–CD8+ or CD3–CD4+ cells, yet does not eliminate double positive CD4+CD8+ T cells (of minor prevalence in cellular products).

Lyse/no-wash single-platform viable cell enumeration

A 100- μ L aliquot sample (concentration adjusted to 20×10^6 cells/mL) was added per tube, each containing 80 μ L of the corresponding antibody mix, and incubated for 20 min at room temperature in the dark. Afterward, 2 mL of 1X working lysis solution was added to fresh products, followed by a 10-min incubation period at room temperature in the dark. For thawed products, 2 mL of phosphate-buffered saline replaced the 1X working lysis solution, bypassing the incubation period. Finally, 100 μ L of Flow-Count fluorospheres was added to each tube, and acquisition on the flow cytometer followed immediately. The sample was acquired using the Cytomics FC500 (compliant with the European Union *in vitro*

Figure 1. Gating strategy allows for specificity in reporting viable lymphocyte counts. If leukocyte concentration was high, sample was diluted to 20×10^6 cells/mL and stained. Fresh samples were lysed without washing and beads added and acquired using Cytomics FC500 (Beckman Coulter). (A) Mix 1: for CD3+, CD4+ and CD8+ absolute T-cell counts, gating starts with an ungated dot plot to quantify beads, followed by “no beads” dot plots on viable cells, then CD45+ cells (to remove debris), and then CD3+ cells gated, clustered (to isolate a clear signal) and counted (absolute counts); same applies for CD3+CD4+ cells and CD3+CD8+ cells. (B) Mix 2: the same strategy is followed for CD3–CD56+CD16+/- NK cells but with gating on lymphocytes instead of CD45+ cells. (C) Mix 3: the same strategy is followed for CD14+ monocytes and CD19+ B cells, with gating on CD45+ cells. Limit of acquisition is set at 60 000–70 000 events on viable CD45+ cells, excluding beads. (Color version of figure is available online).

Table 1

Validation plan with relevance to cell therapy products.

Parameter	Description	Relevance to cell therapy product	Experiment	Acceptance criteria
Accuracy <ul style="list-style-type: none"> • Comparison with another method • External quality assessment/proficiency testing 	Closeness of the measured result to the true value for the sample	<ul style="list-style-type: none"> • Accurate quantification of viable absolute numbers of cells in the starting material from patients as well as healthy controls • Stability of reported results over time necessary in longitudinal studies and multicenter trials 	<ul style="list-style-type: none"> • Staining using stabilized blood samples (n = 33) or cellular products (n = 11 leukapheresis, bone marrow or cord blood from healthy donors) and acquisition using FC500 (Beckman Coulter) or FACSCanto II (BD Biosciences) (TBNK kit) the same day • Three annual campaigns of staining and analysis of stabilized blood at three concentrations, distributed to 38 QC labs 	Bias (difference between methods) $\leq 10\%$ $-3.0 \leq SDI \leq +3.0$
Measured uncertainty	Quantification of the doubt around a measured result, an essential indicator of result quality and important for a meaningful interpretation	Correct interpretation of results when knowing uncertainty that is derived from analytical and biological variability	Calculation involving bias, imprecision and biological variability over 3 years; monthly campaigns of staining and analysis of stabilized blood, distributed to 43 QC labs	$\leq 20\%$
Precision <ul style="list-style-type: none"> • Intra-assay • Inter-assay 	Closeness of individual measures of an analyte when the test is applied repeatedly (testing for variations introduced by operator, reagent, instrument)	<ul style="list-style-type: none"> • Confidence in meaningful changes during product processing (e.g., in-process monitoring) 	<ul style="list-style-type: none"> • Staining using stabilized blood samples (n = 30) or DLI (n = 30), followed by acquisition and analysis in a single batch • Staining using stabilized blood samples (normal or low concentration, n = 30) or DLI (n = 30), followed by acquisition and analysis in batches (five replicates per run on six occasions) 	CV $\leq 10\%$ CV $\leq 20\%$
Sensitivity Limits of quantification	Determination of the linearity of dilution and lowest and highest counts measured by acceptable accuracy and precision	Ability to report on efficacy of cell depletion and selection	Staining using serially diluted stabilized blood samples (n = 8), followed by acquisition and analysis	$R^2 \geq 0.99$ between predicted and measured viable absolute cell count; exploratory
Robustness	Measurement of susceptibility of the test to changes	Ability to get reproducible results despite fluctuations in room temperature	Staining using stabilized blood samples (n = 10) at 4°C or 37°C, followed by acquisition and analysis	CV $\leq 20\%$
Carryover	Of relevance in Cytomics FC500 (Beckman Coulter) with carousel design	Measure of inter-specimen contamination	Staining and analyzing sequentially; replicates of fluorospheres, then water	$\leq 1\%$

Acceptance criteria extrapolated from O'Hara *et al.* [17], Wood *et al.* [21] and Chandran *et al.* [25].

CV, coefficient of variation; DLI, donor lymphocyte infusion; QC, quality control; SDI, standard deviation index.

diagnostic medical device directive 98/79/EC), operated using the CXP acquisition software, version 2.2 (Beckman Coulter, CA, USA). Representative cytometer settings and compensation matrix are provided in supplementary Table 2. The resulting data were saved as list-mode files. CXP software was linked to a database of patient details, enabling automated reporting of absolute counts of cells.

Assay validation procedure

Accuracy, measured uncertainty, precision (intra- and inter-assay), sensitivity, limits of quantification, robustness, stability and carryover were performed for quantification of viable CD3+, CD4+ T cells, CD8+ T cells, CD3–CD56+CD16+/- NK cells, CD19+ B cells and CD14+ monocytes. Table 1 provides an overview of the evaluation of these parameters, the predefined acceptance criteria and the relevance to apheresis products (as starting material) and IEC products.

Statistics

Coefficient of variation (CV) is reported as percentage of standard deviation to mean. Bland-Altman graphs were used to compare

absolute counts of lymphocyte immunophenotypes between the Cytomics FC500 (Beckman Coulter) and FACSCanto II (BD Biosciences). For inter-laboratory comparisons, standard deviation index is reported as (mean value at our center – mean value of group of centers)/standard deviation of the group, and precision index is reported as the ratio of our center's %CV to the group's %CV. All analyses were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

Results

Accuracy

Accuracy of the quantification of viable lymphocyte immunophenotypes is defined by closeness of the measured result to the true value for the sample [21]. To assess accuracy of our method, we followed the two strategies defined in applicable guidelines: comparison with another method and acceptable performance as defined in College of American Pathologists proficiency testing [17]. In comparison to the TBNK panel performed using the FACSCanto II (BD Biosciences) cytometer, when assaying fresh samples, the bias for all

Figure 2. Accuracy of viable lymphocyte immunophenotype quantification method was established. Stabilized blood (StatusFlow; Eurocell) ($n = 33$) and cellular products from healthy donors ($n = 11$ fresh products, six bone marrow and five cord blood; $n = 14$ thawed products, five cord blood and nine leukapheresis products) were stained with each of the three panels and acquired using the Cytomics FC500 (Beckman Coulter) or stained using the BD Multitest 6-color TBNK kit (BD Biosciences) and FACSCanto II (BD Biosciences) cytometer. Bland-Altman plots represent the comparison data from fresh (A) and frozen (B) products using both techniques. Line represents the bias (% value measured using the Cytomics FC500 [Beckman Coulter] value measured using the FACSCanto II [BD Biosciences]/mean value of both methods), and dotted lines represent the 95% limits of agreement. (C) EQA/proficiency testing results reported with no discrimination between platforms of cell quantification from 38 French labs using different cytometers and reagents over 3 years (2017–2019). SDI (equal to Z score) is shown on the graph. Results from the last 3 years (several campaigns/year at three concentrations/campaign) are reported along with the mean $\pm$ SD of SDI. Dashed lines represent the limit between acceptable values (between -3 and $+3$) and unacceptable values ($> +3$ or < -3). EQA, external quality assessment; SD, standard deviation; SDI, standard deviation index.

Figure 2 Continued.

subpopulations was close to zero between both methods (Figure 2A). The bias values (differences between methods) were consistent across the range of mean concentrations (low, medium and high values), at $\leq 10\%$. The acceptance criteria for our facility, set at bias $< 10\%$ between methods, were met for all subpopulations. Data for CD14+ cells were not reported since only the Beckman Coulter panel includes reagents to stain for these cells. When cryopreserved samples were assessed, the bias was high, ranging from 9.4% (CD8+ cells) to 45.6% (CD19+ cells) (Figure 2B), pointing toward major differences between the two methods in quantifying absolute counts of viable subpopulations. This was mostly observed at low mean concentrations. The discrepancy between fresh and thawed samples can be explained by the fact that the TBNK panel measures absolute values of viable CD45+ cells, which are then extrapolated to subpopulations, and is thus an indirect measurement. By contrast, the method developed for the Cytomics FC500 (Beckman Coulter) directly measures the viability of each individual subpopulation and is thus a direct

measurement. This argues in favor of adopting the second method, particularly when working with cryopreserved products.

To assess our lab's results compared with other labs, we participate every year in one of the triannual French campaigns of external quality assessment (EQA)/proficiency testing held by the Centre Toulousain pour le Contrôle de qualité en Biologie clinique (CTCB). Figure 2C shows results of this comparison, all reagents and cytometers taken together. All results fall within the acceptable values identified by the CTCB ($-3 \leq \text{standard deviation index} \leq +3$). Therefore, both approaches are suitable for checking the accuracy of the quantification of viable lymphocyte immunophenotypes using the Beckman Coulter platform at all levels of cell absolute counts.

Measured uncertainty

Measured uncertainty provides information about compliance with analytical performance characteristics and provides objective

Table 2

Measured uncertainty for each viable immunophenotype accounts for analytical and biological variability and complies with acceptance criteria.

Variable	Expected range (cells/ μL)	Measured mean value (cells/ μL)	Measured uncertainty (%)		
			2017	2018	2019
CD3	980–1306	1040	9.9	14.1	9.0
CD4	652–860	673	10.8	14.2	7.9
CD8	304–480	315	10.1	18.2	9.4
CD16/56	127–218	207	18.9	ND	9.6
CD19	195–315	163	12.6	ND	10.8

ND, not detected.

Table 3

Precision assessment meets acceptance criteria.

Material	Precision intra-assay experiments, measured values (n = 30)									
	CD3 (cells/ μL)		CD4 (cells/ μL)		CD8 (cells/ μL)		CD19 (cells/ μL)		CD16/56 (cells/ μL)	
	Mean $\pm$ SD	CV %	Mean $\pm$ SD	CV %	Mean $\pm$ SD	CV %	Mean $\pm$ SD	CV %	Mean $\pm$ SD	CV %
StatusFlow	1198 $\pm$ 50	4.1	772.5 $\pm$ 36	4.8	378 $\pm$ 18	4.7	236 $\pm$ 7	3.1	180 $\pm$ 11	6.1
Leukapheresis	10 459 $\pm$ 939	9	6607 $\pm$ 546	8.4	3482 $\pm$ 334	9.7	ND	ND	ND	ND
Precision inter-assay experiments, measured values (n = 30)										
StatusFlow	1171 $\pm$ 48	4.1	766 $\pm$ 32	4.2	350 $\pm$ 15	4.2	279 $\pm$ 15	3.1	154 $\pm$ 12	8.1
StatusFlowLO	896 $\pm$ 37	4.1	138 $\pm$ 5	3.6	645 $\pm$ 23	3.5	417 $\pm$ 22	5.3	393 $\pm$ 23	5.9

Precision was assessed by staining and acquiring samples using a Cytomics FC500 (Beckman Coulter) for all three mixes in a single batch or on several occasions using stabilized blood (normal or low values) or leukapheresis products. Intra-assay is reported as mean $\pm$ SD of viable absolute counts of cells performed in a single batch by the same operator. The CV percentage is equally reported. Inter-assay was performed over several batches by different operators. N = 30 per assessment.

CV, coefficient of variation; ND, not detected; SD, standard deviation.

Figure 3. Linearity of the viable lymphocyte immunophenotype quantification method and lower limit of sensitivity were established. Linearity and lower limit of quantification of the method were established using serially diluted samples acquired 10 times using two lots of stabilized whole blood on several days. (A) Predicted absolute counts are plotted against measured absolute counts, and goodness of fit (linear regression) is elucidated. (B) Lower limit of quantification with an acceptable uncertainty is identified for CD3+, CD4+ and CD8+ cells. Dashed line represents the acceptable uncertainty.

information that helps in interpretation of measurement results [23]. Deriving from analytical and biological variability (ISO 15189), uncertainty needs to be known when reporting and interpreting viable immunophenotype counts on cell products. Measured uncertainty for the last 3 years is represented in Table 2, showing all values $\leq 20\%$ set as acceptance criteria.

Precision

Precision describes the closeness of individual measures of an analyte when the assay is repeatedly applied, in a single batch by the same operator (intra-assay) or in batches on several occasions by different operators (inter-assay) [24,25]. The CV percentages of both

Table 4
The quantification method is robust at different staining temperatures.

Staining temperature	CD3 (cells/ μ L)		CD4 (cells/ μ L)		CD8 (cells/ μ L)		CD19 (cells/ μ L)		CD16/56 (cells/ μ L)	
	Mean $\pm$ SD	CV %	Mean $\pm$ SD	CV %	Mean $\pm$ SD	CV %	Mean $\pm$ SD	CV %	Mean $\pm$ SD	CV %
37°C	1041 $\pm$ 43	4.2	668 $\pm$ 22	3.2	328 $\pm$ 12	3.5	149 $\pm$ 10	6.7	255 $\pm$ 10	4
4°C	1158 $\pm$ 116	10	767 $\pm$ 81	10.6	338 $\pm$ 31	9.2	198 $\pm$ 25	12.8	267 $\pm$ 26	9.7

Stabilized blood was stained at two temperatures (4°C or 37°C) and acquired using a Cytomics FC500 (Beckman Coulter). N = 10 per assessment. CD14 (cells/ μ L) is not reported on the product insert.

starting materials (at two concentrations) fall within the predefined acceptance criteria of <10% or <20%, respectively, for all subpopulations (Table 3). All in all, these data show an acceptable assay precision, giving confidence in reporting meaningful changes in viable immunophenotypes of starting material for IEC manufacturing and account for acceptable inter-operator variations.

Sensitivity and lower limit of quantitation

Several cell therapy products will undergo cell selection or depletion; hence, of peculiar relevance in these situations is setting the lower limit of quantification, whose total error meets the predefined acceptance criteria for accuracy [21]. We explored the linearity of the method using serially diluted samples. A high goodness of fit for a linear regression for predicted versus measured absolute counts ($R^2 \geq 0.99$) was revealed (Figure 3A). Using the same data set, the lower limit of quantification, with an acceptable uncertainty (variation from theoretical values), was identified for CD3+, CD4+ and CD8+ cells: 9, 8 and 8 cells/ μ L, respectively (Figure 3B).

Robustness

A method needs to withstand changes in assay conditions to accommodate the reality in cytometry core labs. From our experience, temperatures fluctuate in our room, which could have an influence on the results. Considering that our procedure required staining at 18–25°C, according to the manufacturer's recommendations, testing the robustness of our method at two extremes of staining temperature (4°C and 37°C) was required by the accreditation authority. Samples of stabilized whole blood were stained at both temperatures and acquired in four batches on different days ($n = 10$ runs per temperature). The method proved to be robust at both temperatures, with a CV percentage ranging from 3% to 13%, falling within our predefined acceptance criteria (<20%) (Table 4). This parameter provides further assurance as to the resistance of the assay to temperature changes.

Carryover

The design of the Cytomics FC500 (Beckman Coulter) cytometer, being a 32-tube circular rack, risks carryover between samples. Carryover needs to be evaluated according to the dedicated guidelines (Clinical and Laboratory Standards Institute CLSI-H52-A, 2001, ISO15189 standards) when an automatic sample loader is used routinely. Carryover was assessed by acquiring in triplicate Flow-Check pro fluorospheres (high concentration: H1, H2, H3, 30-second acquisition time for each), followed immediately by triplicate water samples (blank: B1, B2, B3); the process was repeated in five cycles. Accordingly, carryover was assessed by acquiring to the formula $([\text{mean B1} - \text{mean B3}]/[\text{mean H} - \text{mean B3}]) \times 100$. Set at less than 1% contamination, the method proved that no carryover took place (0.0056%), fostering further confidence in proper assessment of individual samples.

Discussion

We report on the validation of a method to enumerate viable lymphocyte immunophenotypes on the Cytomics FC500 (Beckman Coulter) cytometer. The predefined acceptance criteria for accuracy, measured uncertainty, precision, linearity of dilution, sensitivity, robustness and carryover were all met. The method complies with the FACT standards for IEC (first edition) requirement for an “appropriate and validated assay,” FACT-JACIE hematopoietic cell therapy standards, ICH Q2(R1) and ISO15189 standards. Furthermore, it complies with validation recommendations/guidelines (Ligand Binding Assay Bioanalytical Focus Group/American Association of Pharmaceutical Scientists,

International Council for Standardization of Hematology/International Clinical Cytometry Society and European Bioanalysis Forum) applicable to such a method. The method can help manufacturers draw meaningful comparisons during the process of scaling up a laboratory-based manufacturing protocol. It is equally useful in reporting on variability in cell content of collected leukapheresis products, a challenge faced by manufacturers that need to adapt their manufacturing protocols.

Since, for the time being, cellular therapy products that have obtained marketing authorization are being manufactured and released by pharmaceutical stakeholders, starting with leukapheresis products collected in hospitals, the applicability of the method is limited to quality control of the starting material, with variability inherent to such collections [26]. For example, target cell number of CD3+ T cells is one of the criteria for collecting starting material for CAR T-cell manufacturing. Nonetheless, the manufacturing of IECs in a point-of-care process would benefit from such a validated quantification method [3,27–29]. Another application involves performing quality control of allogeneic products (hematopoietic progenitor cell or donor lymphocyte infusions) where dosing of CD3+ T cells is necessary [30].

The gating strategy adopted for NK cell absolute counts differs from that adopted by Koehl *et al.* [31] in their inclusion of CD14+ monocytes in the mix and initially gating on live CD45+ cells. We argue that we excluded monocytes (via size and granularity) by gating on live lymphocytes instead of CD45+ cells. Furthermore, we agree with Koehl *et al.* [31] regarding the absence of CD14+ monocytes in expanded NK cell products, despite their detection upon selection/prior to expansion (Institut Paoli-Calmettes, clinicaltrials.gov NCT01853358).

An accurate quantification of viable absolute numbers of cells is a prerequisite for several activities: standardization of input cellular material for CAR-transduced T or NK cell manufacturing [32,33], cell selection [3,14,34,35] and dosing of donor lymphocyte infusions [36]. As reported by others, participation in EQA indeed helped improve the accuracy of the method [37,38]. Our data on high accuracy and the measured uncertainty over 3 years across a range of values of lymphocyte immunophenotypes give reassurance of the method's capacity to quantitate them in starting material from lymphopenic patients as well as from healthy donors [39,40]. Our participation in the quarterly campaigns of EQA held by the CTCB shows that despite the different instruments, reagents, counting systems and biological materials used, our method compares with the different immunophenotyping methods used by other labs. Additionally, we participated in an inter-laboratory campaign using stabilized blood, which allowed for the establishment of “real-life” accepted variation in results of absolute counts of lymphocyte immunophenotypes [40]. Although this study reports a snapshot of this variation in a given month, our data from the past 3 years show a mean standard deviation index between –1 and +1 in reference to the participating groups, further validating our method beyond one lab and one instrument.

In cell therapy, the precision aspect of a viable lymphocyte immunophenotype quantification assay gives confidence in meaningful changes in data collected throughout the processing of a product [41], particularly during cryopreservation [42] or over longitudinal studies with IECs [43]. All in all, this successful validation renders the method readily available for adaptation by quality control labs processing, cryopreserving or manipulating leukapheresis products for IEC manufacturing. Such validated flow-based methods for immunophenotyping reinforce the efforts of more detailed monitoring of immune cells in patients using established panels and standardized processes (sample collection, staining, acquisition and analysis) [44,45].

A limitation to this method is its inability to respond to quality controls required for extensively manipulated products like TCR α /phbeta/CD19-depleted progenitor cell grafts [46]. To that end, a more advanced 10-color single-platform method has been validated for graft composition and is currently being used in an ongoing

multi-center phase 1/2 trial (Miltenyi Biotec, Eudract No. 2011-005562-38) [14].

Possible causes for interference in flow-based immunophenotyping are erythrocytes, fat, Orthoclone OKT3 and fluorescein, as listed in Beckman Coulter's antibody product sheets. The use of lysis solution and CD45 gating eliminates the effects erythrocytes and/or lipids could have on fluorescence signals. The validation runs were performed on leukapheresis products or lymphocytes of healthy donors. Hence, Orthoclone OKT3 and fluorescein were not tested—another limitation of this validation work.

Since many labs worldwide use the Cytomics FC500 (Beckman Coulter) for CD34 enumeration via CXP software, adapting to this validated single-platform method may prove less challenging to implement if processing IECs—or even collecting and shipping leukapheresis products to pharmaceutical manufacturers—is foreseen in their short-term plans. Furthermore, with the cytometer settings provided, this method can be used with other flow cytometers that can capture fluorescence signals from the five colors we used, forward scatter and side scatter. We used StemCount beads (StemKit, Beckman Coulter) for absolute counts, so there is no restriction on using this method, unlike volumetric measurement or TruCount, with other systems. In fact, Veluchamy *et al.* [47] have shown that this is feasible in the setting of harmonizing NK cell immune phenotyping across flow cytometers.

Acceptance criteria were set based on seminal work from dedicated consortia [17,21] and are an essential part of the validation plan. Likewise, relevance of the validation criteria to cell therapy products is reported in the absence of consensus in the field. This assay has been established and validated owing to the accreditation procedures required by the French authority Cofrac. In our efforts to continuously optimize the assay, we participated in harmonization panels and inter-laboratory comparisons. Throughout this process of validation and accreditation, the experience gained enabled us to better set up validation plans for other assays and better set the acceptance criteria.

In summary, we have reported on the validation of a viable flow cytometry-based immunophenotype enumeration assay. This validation procedure is of relevance to fresh and cryopreserved/thawed starting material destined for use in the manufacturing of advanced therapy medicinal products, T-cell dosing in donor lymphocyte infusion and the evaluation of the efficacy of depletion/selection of cells, among other applications.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: CL, CC and BM. Acquisition of data: JG, JC, BM and CL. Analysis and interpretation of data: JG, JC, BM and CL. Drafting or revising the manuscript: BM, JG, JC, BC, CC and CL. All authors have approved the final article.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2020.06.005.

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