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REVIEW ARTICLE

CLINICAL CYTOMETRY WILEY

European flow cytometry quality assurance guidelines for the diagnosis of primary immune deficiencies and assessment of immune reconstitution following B cell depletion therapies and transplantation

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Abstract

Over the last 15 years activity of diagnostic flow cytometry services have evolved from monitoring of CD4 T cell subsets in HIV-1 infection to screening for primary and secondary immune deficiencies syndromes and assessment of immune constitution following B cell depleting therapy and transplantation. Changes in laboratory activity in high income countries have been driven by initiation of anti-retroviral therapy (ART) in HIV-1 regardless of CD4 T cell counts, increasing recognition of primary immune deficiency syndromes and the wider application of B cell depleting therapy and transplantation in clinical practice. Laboratories should use their experience in

standardization and quality assurance of CD4 T cell counting in HIV-1 infection to provide immune monitoring services to patients with primary and secondary immune deficiencies. Assessment of immune reconstitution post B cell depleting agents and transplantation can also draw on the expertise acquired by flow cytometry laboratories for detection of CD34 stem cell and assessment of MRD in hematological malignancies. This guideline provides recommendations for clinical laboratories on providing flow cytometry services in screening for immune deficiencies and its emerging role immune reconstitution after B cell targeting therapies and transplantation.

KEYWORDS
B cell depleting therapy, flow cytometry, hematopoietic cell transplantation, immune reconstitution, primary immune deficiencies, solid organ transplantation

1 | INTRODUCTION

A major change in flow cytometric laboratory practice in high income countries has been the decline in requests for CD4 T cell counts to monitor disease progression and immune reconstitution in patients with HIV-1 infection. World Health Organization guidelines (WHO, 2016) no longer recommend that CD4+ T cell counts should be used to start ART. Results from multicentre clinical trials demonstrated that starting ART in patients with CD4 T-cell counts over 500 cells/μL improved clinical outcomes (Danel et al., 2015; Lundgren et al., 2015). Measurement of CD4+ T counts is still recommended at initial diagnosis to guide antimicrobial prophylaxis in patients with advanced HIV-1 infection (Kestens & Mandy, 2017). Introduction of up to 10 color single-tube monoclonal antibody panels with improved flow cytometry (FC) software for analysis have contributed to better outcomes in hematology oncology. It should now be extended to screen for primary immune deficiencies and to assess immune reconstitution following B cell-depleting therapy and transplantation.

1.1 | Application of FC in the diagnosis and classification of primary immune deficiencies (PID)

FC is now well established in the diagnosis and classification of primary immune deficiency disorders (PID). Absolute T cell counts, total B cells and class switched memory B cell percentages are components of the diagnostic criteria for severe combined immune deficiency (SCID) (Dvorak et al., 2023; Shearer et al., 2014), x-linked agammaglobulinaemia (XLA) (Conley et al., 1999), and common variable immune deficiency (CVID) (Seidel et al., 2019). The use of FC to determine specific protein defects or to analyze immune function in PID is beyond the scope of this guideline however further information is available in several excellent review articles (Delmonte & Fleisher, 2019; Knight, 2019; Richardson et al., 2018).

FC protocols for the diagnosis and screening of PID vary enormously in high income countries, with significant differences in sample processing, composition of monoclonal antibody panels, antibody staining protocol, instrumentation, sample measurement, and data analysis (van Dongen et al., 2019). Although screening for PID using single tubes to enumerate T, B, and NK can detect major immune deficiencies including SCID and XLA, several disorders including CVID, the most common major antibody deficiency syndrome in adults, may have normal B cell and T cell counts despite the presence of significant defects in composition of memory B- (Wehr et al., 2008) and naïve CD4 T- (Malphettes et al., 2009) cells.

The EuroFlow consortium have established standardized flow cytometric protocols to screen for PID (Blanco et al., 2018; Glier et al., 2019; Pedreira et al., 2019) culminating in the release of a CE approved 8-color Primary Immune Deficiency Orientation Tube (PIDOT) screen with follow up panels to evaluate immune defects affecting T and B cell compartments (van der Burg et al., 2019; van Dongen et al., 2019). The PIDOT can be analyzed in EuroFlow Infinicyt software, which has automatic gating algorithms and a database of healthy controls providing age-matched reference ranges (Linskens et al., 2020). In a diagnostic tertiary care laboratory, the sensitivity and specificity of the PIDOT tube to identify various PID compared with disease controls was 87% and 62% respectively (Neirinck et al., 2022).

There has been long-standing interest in using flow cytometric platforms to sub-classify CVID, the most common symptomatic primary antibody deficiency syndrome, into distinct subgroups (Piqueras et al., 2003;Warnatz et al., 2002; Wehr et al., 2008). The EUROclass protocol (Wehr et al., 2008) has been widely adopted in European and Australian laboratories, however inconsistent results have been reported when relating composition of memory B cell subsets (Al Kindi et al., 2012; Driessen et al., 2011; Piątosa et al., 2013; Piqueras et al., 2003; Warnatz et al., 2002) or transitional B cell frequency (Warnatz et al., 2002; Wehr et al., 2008) to clinical outcomes in CVID. The application of different flow cytometric protocols, use of different monoclonal antibodies to identify B cell subsets, reporting of B cell in terms of absolute counts or percentages may account, in part, for discrepant results (Al Kindi et al., 2012; Driessen et al., 2011;

Piątosa et al., 2013; Piqueras et al., 2003; Rösel et al., 2015; Warnatz et al., 2002; Wehr et al., 2008; Yazdani et al., 2017). Notably, except for a few studies (Blanco et al., 2018; Blanco et al., 2019; Glier et al., 2019), FC classification protocols have not incorporated age-specific reference ranges covering narrow enough age groups (younger children, older children, adults, and those over the age of sixty as a minimum). The use of standardized protocols, expansion of quality assurance schemes and more multi-centre studies with appropriately aged-matched controls are needed to demonstrate the clinical utility and feasibility of introducing more recent PID panels into routine practice. Additionally, lymphocyte immunophenotyping may also be useful in the management of patients with secondary antibody deficiency syndrome, particularly for identifying patients who may need long term IgG replacement and those who will recover antibody production (Chapel & Patel, 2019).

1.2 | FC to assess B cell depletion and repopulation after B cell targeting therapies

The expanding use of drugs, monoclonal antibodies, and cellular therapies targeting B cells in the management of hematological malignancies and autoimmune diseases has led to the identification of post-treatment residual immune dysfunction. This is characterized by antibody deficiency, which in some patients, results in severe and recurrent infections (Dhalla & Misbah, 2015). The risks for antibody deficiency are best characterized for rituximab and include reduced baseline immunoglobulins, exposure to prior immunosuppressive therapies and maintenance (multiple) cycles of rituximab (Patel et al., 2019). Rituximab is currently licensed by the European Medicines Agency (EMA) for some B cell cancers (non-Hodgkin's lymphoma and chronic lymphocytic leukemia), rheumatoid arthritis (RA), severe pemphigus vulgaris and the ANCA (anti-neutrophil cytoplasmic antibody) associated vasculitides (AAV); granulomatous with polyangiitis (GPA), and microscopic polyangiitis (MPA), however successful off-label use of rituximab in nearly all autoimmune diseases is increasingly common (Brando & Gatti, 2023).

Laboratory monitoring for infection risk in these patients include serum immunoglobulins, antibody responses to vaccines and assessment of B cells. (Otani et al., 2022; Patel et al., 2019). Baseline B cell counts can help to distinguish PID syndromes from rituximab-induced antibody deficiency (Kaplan et al., 2014). Patients with AAV have a greater risk of secondary antibody deficiency and infection than RA and systemic lupus erythematous (SLE) patients (Thiel et al., 2017; Wade & Kyttaris, 2021). Baseline B cell counts can also identify AAV patients at increased risk of infection (Odler et al., 2023) but are not associated with infection in RA and SLE patients (Md Yusof et al., 2019).

Successful use of anti-CD20 agents in maintaining remission in autoimmune diseases (RA, SLE, AAV, multiple sclerosis, and neuromyelitis optica spectrum disorders) has stimulated interest in the most effective strategy to achieve an appropriate balance between preventing disease and the risks of drug toxicity (severe infection) (Arnold et al., 2021; Baker et al., 2017; Dass et al., 2008; Kim et al., 2019; Lebrun et al., 2018; Md Yusof et al., 2015; Roll et al., 2008; Vital et al., 2011). Failure to achieve complete B cell depletion or repopulation with predominantly memory B cells has been associated with early disease relapse, whereas more sustained remission of disease is linked to absent B cells or immune constitution with naïve B cells. The use of highly sensitive FC protocols similar to those developed for paroxysmal nocturnal hemoglobinuria (PNH) (Sutherland et al., 2012), leukemia minimal residual disease (MRD) analysis (Flores-Montero et al., 2017; Riva et al., 2021), and CD34 stem cell enumeration (Lanza et al., 2013) is essential to measure accurately extremely low B cell subsets counts and proportions following anti-CD20 therapy (Gatti et al., 2021). Multi-centre prospective studies using highly sensitive FC protocols to assess B cell reconstitution paired with clinical outcomes in autoimmune disease patients are needed before there can be widespread adoption into clinical practice.

1.3 | Use of FC to assess immune reconstitution after HCT and SOT

Restoration of CD4 T cell immunity is the most critical factor for successful outcomes following allogeneic hematopoietic transplantation (Bertaina et al., 2022). Delays in immune reconstitution are associated with increased risks of infection, graft versus host disease (GvHD), disease relapse, and reduced overall survival (Ogonek et al., 2016). CD4 T cell reconstitution is complex as several variables such as recipient's age, disease, timing of conditioning regimens, source and composition of graft, HLA compatibility, onset of GvHD, and/or infection can affect the kinetics of immune reconstitution (Bosch et al., 2012; Velardi et al., 2021). A CD4+ T cell count over 50 cells/μL within 100 days of transplantation is associated with improved overall survival, reduced risk of GvHD and reduced infection in transplant recipients for malignant and nonmalignant disease (Admiraal et al., 2015; Admiraal et al., 2017; Lakkaraja et al., 2022; van Roessel et al., 2020). Those patients who develop GvHD also have improved survival if they attain CD4+ T cell count over 50 cells/μL by 3 months post-transplant (de Koning et al., 2021). Prophylaxis against opportunistic infections (OI) following allogeneic transplantation for hematological cancers is usually given for 6–12 months, irrespective of CD4+ T cell counts or percentage (Holtick et al., 2015). A significant number of OI occur within 3 months of stopping antimicrobial prophylaxis and it is not known whether immune monitoring to guide cessation of antibiotic therapy will reduce this risk. This is likely an important issue following treatment with chemotherapeutic drug combinations such as purine analogues, which can induce marked and prolonged CD4+ T cell depletion when used in the treatment of lymphoproliferative (Gauthier et al., 2019; Seymour et al., 1994) and autoimmune diseases (Comi et al., 2019; Rolfes et al., 2022).

Immune monitoring to modify anti-thymocyte globulin (ATG) dose reduced the risk of infection without increasing graft rejection in two small trials of heart and kidney solid organ recipients (Abouna

et al., 1995; Koch et al., 2005), demonstrating that personalized immunosuppressive dosing and immune monitoring can improve the management of hematopoietic (Admiraal et al., 2015) and solid organ transplantation (SOT) (Mohty et al., 2014). Studies of immune reconstitution in SOT are limited by the poor correlation between absolute lymphocyte counts and function and short term (within one year) follow up. There is no data to support the use of lymphocyte subsets to guide antimicrobial prophylaxis or to monitor for emergence of post-transplant associated pathogens (Imlay et al., 2023). Future diagnostic application of high-resolution FC is likely to include analysis of γδ T cell reconstitution post haplo-identical transplantation (Aversa et al., 2019; Dadi et al., 2023; Takahashi & Prockop, 2022) and assessment of lymphocyte depletion before and after CAR-T cell therapy (Ghilardi et al., 2022; Wudhikarn & Perales, 2022).

In conclusion, there is accumulating evidence to support the use of immune based biomarkers as primary endpoints in clinical trials to stratify the continuing use of antimicrobial agents to prevent OI, adjust dose of conditioning/rejection medication, commence vaccination and manage GvHD in transplant populations (Admiraal et al., 2015). Translation to clinical practice will depend on standardization of sample handling and processing, agreement on composition of flow panels, data analysis and reporting (Boelens et al., 2020).

2 | LABORATORY SAFETY

All healthcare scientists should be familiar with local safety guidelines and the laboratory quality management system must have policies in place for sample handling, processing, and disposal, which conform to national or international guidelines. Sample preparation for analysis of lymphocyte subsets for patients with primary and secondary immune deficiencies should take place in a designated laboratory area. A lyse-no-wash procedure reduces sample handling and avoids the need to fix specimens provided the sample loader is sealed when in use. All samples should be inactivated prior to disposal. The flow cytometer must be decontaminated after use in accordance with the manufacturer's instructions. Local rules and national guidelines should be followed for sample disposal.

3 | SPECIMEN COLLECTION AND TRANSPORT

Specimens should be collected by venepuncture into either ethylenediamine tetra-acetate (EDTA) or sodium heparin (Davis et al., 2013). The use of acid citrate dextrose (ACD) is not recommended for absolute counting technology as its addition to blood collection tubes means it is difficult to calculate absolute counts (Mandy et al., 2003).

All samples must be labeled with unique patient identifiers complying with ISO 15189:2022 laboratory standards. Depending on local guidelines, Soundex or hospital reference numbers can be used to ensure confidentially. Laboratories should regard all samples as potentially high risk and take standard precautions (Health and Safety Executive, 2003).

Samples should be transported and maintained at 15–25°C prior to analysis. Packaging and posting of samples to laboratories sited in external locations to phlebotomy facilities should also follow local and national guidelines. Samples should be processed following venesection within the timeframe recommended by the flow cytometer supplier. The FC laboratory should validate stability of samples if extended services are offered to clinical users beyond this timeframe. Protocols to screen for primary immune deficiency emphasize sample processing within 36 h (Neirinck et al., 2022). There should be policies in place for the use of extended stabilization preparations if required. Blood samples should be inspected for clots and hemolysis prior to analysis. The laboratory should have protocols for urgent requests.

4 | SPECIMEN PROCESSING

4.1 | Analysis of T cell (CD4 and CD8), B and NK lymphocyte counts

Single platform flow cytometers where absolute lymphocyte counts are generated without the need for hematology analyzers are strongly recommended (Barnett et al., 1999). Single platform flow cytometry platforms using internal calibrator counting microbeads have clearly superior intra- and inter-assay coefficient of variation compared with dual platform methods (Barnett et al., 1999; Reimann et al., 2000). Semiautomated single platform bead FC platforms continue to be widely used in diagnostic practice, however fully automated single platform volumetric flow cytometers, where absolute lymphocyte counts are generated without the need for internal reference microbeads to enumerate lymphocyte subsets are also in use (Gossez et al., 2017; Grossi et al., 2018). Automation reduces the potential for user error inherent in existing systems that require numerous manual steps to set up and run samples as well as minimizing exposure to potentially biohazardous material. Automated software is available to select lymphocyte gates, but processes must be in place to verify the accuracy of this gating (Lemoine et al., 2022).

Laboratories which employ semi- or fully automated specimen preparation, processing, staining, and fixation protocols should follow the manufacturer's recommendations. If using manual steps laboratories should ensure that blood samples should first be placed on gentle blood rockers for 5 min to ensure that the contents of specimen tube are evenly distributed. Use of electronic adjustable pipettes are recommended particularly for large workloads to avoid repetitive strain injury. Reverse pipette techniques must be used for addition of blood, monoclonal antibodies, and beads for single platform technology to reduce error (Whitby et al., 2015). Laboratories should provide evidence that pipettes are regularly calibrated to the level outlined in ISO15198:2022. Once aliquoted, samples tubes should be vortexed to mix blood and reagents and disperse cellular aggregates. Cells should be fixed with 2% paraformaldehyde or proprietary fixative for 30 min after monoclonal staining prior to acquisition on flow cytometer. If red cell lysis is performed, it should be undertaken without washing. This is essential for enumeration of T cell subsets, B, and NK

cell counts as wash steps could result in cell loss or the removal of beads (depending on the bead type used). On addition of beads, the sample must be analyzed within 2 h. Following red cell lysis and fixation, the sample can be stored for up to 24 h at 4°C before flow beads are added. Samples should be vortexed before analysis.

4.2 | Analysis of naïve and memory T and B cell subsets

The EuroFlow PIDOT uses an 8-color flow panel to screen for antibody and cell mediated immune deficiencies syndromes, which is now supported by several commercial companies (Glier et al., 2019; Neirinck et al., 2022; van der Burg et al., 2019; van Dongen et al., 2019). Standardized operating procedures for sample processing rely on bulk lysis and staining of between of 300 µL and 2 mL of whole blood. The EuroFlow consortium recommends that laboratories closely follow standard operating procedures for sample processing and washing steps. Laboratories should adhere to recommendations regarding placement of specimen tubes on gentle blood rockers, use of electronic adjustable pipettes, reverse pipetting, and calibration of pipettes as described in Section 4.1. Cells are either acquired immediately or stored at 4°C for 1 h until flow cytometric analysis. Provision of lyophilized/dried tubes saves time and reduces the potential for operational error. It also saves a considerable amount of the time in managing monoclonal antibody supplies and validation of new monoclonal antibody batches. It is recommended that at least 10^6 PBMC (ideally up to 5×10^6) be acquired so that sufficient events are obtained to identify all B and T cell subsets. A dual platform approach (multiplying the percentage of each lymphocyte subset by absolute lymphocyte count derived from a separate full blood counts and differential instrument) is needed for B and T cell counts as subsequent washing steps following red cell lysis leads to loss of lymphocytes.

Automated software to define gates and compare results with age-matched reference and disease controls are available, however it is important that laboratories have alert systems in place to ensure that automated gating does not lead to the release of erroneous reports (Lemoine et al., 2022).

4.3 | Analysis of B cell and T immune reconstitution after anti-CD20 treatments and transplantation

Laboratories must have agreement with clinical users about the lower limits of quantitation for B and T cell subsets for immune reconstitution analysis after B cell depletion therapies and transplantation. Freshly drawn (within 4 hours of venesection) blood should be used to minimize white cell aggregation (Gatti et al., 2021). Bulk lysis, monitoring of the time parameter, and doublet discrimination is recommended to acquire sufficient high quality CD45+ lymphocyte events to assess B cell subset proportions and count (Gatti et al., 2021). As for PID screening, a dual platform approach (using lymphocyte count obtained from full blood count differential) is needed to enumerate absolute B cell count, and at least 1×10^6 CD45+ white cells (ideally up to 5×10^6) should be acquired. Analysis of B cell subsets is possible and clinically relevant when at least 200–300 clean B cell events are acquired at counts greater than 0.5 cells/µL. The lowest level of quantitation for high sensitivity FC lies in the range of 0.002% or 0.2–0.3 cells/µL: complete B cell depletion is defined at counts below 0.1 cells/µL. Measurement of T cell subsets in the same tube is necessary to monitor CD4 T cells counts, which can be reduced by anti-CD20 therapies (Capasso et al., 2021; Lavielle et al., 2016; Lee, 2022) and other immunosuppressive drugs (Spiezia et al., 2022). Initial reports on the use of rituximab maintenance regimens emphasized the importance of *Pneumocystis jirovecii* pneumonia (PJP) prophylaxis in patients with CD4 T cell counts below 250 cells/µL (Guillevin et al., 2014), and the EMA "A Summary of Rituximab Product Characteristics" states that rituximab is contra-indicated in severely immunocompromised patients (European Medicine Agency, 2021). The EMA do not give a definition for severe immune compromise however some European national guidelines highlight increase of infection with rituxiamb in patients with inflammatory rheumatic diseases who have a total lymphocyte count less 500 cells/uL or CD4+ T cell count less than 200 cells/µL (Schulze-Koops et al., 2020). The wide variation of individual B cell memory subsets in healthy anti-CD20 treated patients means that it is difficult to establish reliable thresholds or reference values. This can be circumvented in part by longitudinal monitoring of individual patients where each patient acts as their own internal control. A limitation of current protocols includes the need for fresh blood as the expression of surface immunoglobulins is labile, an important consideration if blood samples are referred to distant laboratories. It should also be noted that analysis of peripheral blood may not always capture what is happening in tissues. There is a need for external quality assurance providers to establish schemes to assess laboratory performance for analysis of immune constitution after anti-CD20 treatments and transplantation, which should improve assay standardization, and performance.

Finally, laboratories should be able to provide documentation consistent with ISO 15189:2022 laboratory standards for the steps associated with specimen processing including temperature logs of fridges and freezers, maintenance records for Class I/II safety hoods, pipettes, and, automated sample processing instruments. Evidence documenting the criteria used to validate existing flow cytometric protocols and the introduction of new tests should also be available (Davis et al., 2013; Devitt et al., 2023; Scott et al., 2017).

5 | IDENTIFICATION OF LYMPHOCYTE POPULATIONS

5.1 | Gating strategy

The CD45/Side Scatter (SSC) gate is recommended for the identification of the lymphocyte population using single platform technology (Devitt et al., 2023). The CD45/SSC gate minimizes the acquisition of

cellular debris, reduces monocyte contamination, discriminates basophils (CD45low) from lymphocytes (CD45bright), and avoids the need for T cell isotype controls. However, there may be loss of lymphocytes with weak CD45 expression such as large granular lymphocytes (LGL) from the CD45/SSC gate. In addition, it may still be difficult to discriminate lymphocytes from small monocytes.

5.2 | Monoclonal antibody panels

Six-color panels using lineage markers CD45/CD3/CD4/CD8/CD16-56/CD19, covering the major lymphocyte subsets in blood have superseded three- or four-color panels used to monitor primary and secondary immune deficiencies. In addition, the single-tube 6-color tube can also detect CD4 + CD8dim, and CD3 + CD8 + CD56+ T cell populations, which are associated with HIV infection, and can also provide internal quality control checks to verify results (Ashman et al., 2007; Devitt et al., 2023). Either the manufacturers' (e.g. FACS DIVA) or commercially available software programmes (e.g. Flow Jo) can be used to analyze lymphocyte subsets.

Biomarker panels to screen for immune deficiencies affecting the composition of naïve and memory T and B cell subsets (such as CD3, CD45, CD4, CD8, CD16/CD56, CD19, CD27, CD45RA, smIgD, smIgM, CD19, and TCRγδ, utilized by EuroFlow) can be deployed in diagnostic laboratories and software programmes to support more complex data analysis are commercially available (Neirinck et al., 2022; van der Burg et al., 2019; van Dongen et al., 2019). A similar panel (CD3, CD4, CD8, CD19, CD20, CD27, CD38, CD45, sIgM, and sIgG) has been recommended for analysis of B cell reconstitution after B cell depleting therapies (Gatti et al., 2021).

6 | INSTRUMENT QUALITY CONTROL

It is important to monitor instrument performance over time since data generated by flow cytometers are not derived from international reference standards (Cossarizza et al., 2019). The performance of the flow cytometer can vary during acquisition of data, hence there is a need retrieve information about the actual status of the instrument and compare it with baseline and/or ideal standards. Signals generated by a flow cytometer are influenced by the composition and rate of flow of sheath fluid, the power and alignment of lasers, alignment and performance of optics (mirrors and filters), photomultiplier tubes (PMT), and ambient environmental conditions (Maecker & Trotter, 2006; Wood, 2009; Wood & Hoffman, 1998). Predetermined standards for instrument performance (optical alignment, PMT target values, linearity, and sensitivity) are set up on installation of instruments and adjusted following major service or replacement of lasers, filters, mirrors, and PMT.

Daily checking of the flow cytometer is recommended to ensure optimal performance (Tangri et al., 2013). Commercially available fluorochrome tracking beads should be used to check quality of fluidics, laser alignment and predefined PMT voltage for target MFI (mean fluorescence intensity) and linearity. Automated software checks confirm that PMT voltages and fluorochrome scatter for each filter meet manufacturers' instructions. Compensation beads should then be run daily after fluorescent tracking beads as the PMT voltages set the template for appropriate adjustment of spectral spill over (Tangri et al., 2013). A compensation matrix to adjust for spectral overlap among different detectors is then usually automatically applied by flow cytometer software. FC performance should be recorded and monitored. In some fully automated FC systems instrument performance and internal quality controls checks are automatically tracked (Gossez et al., 2017; Grossi et al., 2018). Laboratories must verify that they can reproduce characteristics of assay performance outlined in the manufactures' standard operating procedures. Validation steps should include assessment of accuracy, precision, (intra-assay and inter-assay), analytical sensitivity, analytical interference, and stability (Devitt et al., 2023).

7 | QUALITY CONTROL

All laboratories should use internal quality control samples—commercially obtained preparations to monitor reagent performance, staining, lysis, and analysis. Internal quality control samples should be run every day or at least once a week and should include at least 2 target values; one with normal counts and one at clinically relevant decision thresholds. The results of internal quality control samples should be plotted on a Levy Jennings plot to provide a visual indication of drift or bias over time. Westgard rules should be used to establish criteria to monitor instrument performance. If more than one instrument is used to provide a diagnostic service, the laboratory should ensure that comparable results are obtained from each cytometer. Internal quality control samples must also be run if there has been a change in reagent/laboratory personnel or after an instrument has been serviced or recalibrated and if there are concerns about the accuracy and precision of immune monitoring panels. Laboratories using single platform tubes for immune monitoring should run either daily duplicate or triplicate CD3+ T cell values at normal/low normal CD4+ T cell counts to check for pipetting errors (Mandy et al., 2003). An alternative strategy based on data that the flow rate through a flow cytometer is constant and stable is to monitor bead rate count (Bergeron et al., 2003; Scott & Glencross, 2005). The use of fresh normal blood specimens must not be used as a process control due to the inherent variability between individuals. Laboratories should ensure that they comply with relevant ISO 15189:2022 laboratory standards for selection and acquisition, installation and initial calibration, routine maintenance, service and repair records, and disposal of FC instruments used in diagnostic services.

Laboratories undertaking immune monitoring for immune deficiencies and reconstitution should consider adopting quality control targets already established in HIV-1 infection. A multi-centre French study showed that maximum CV mean (+ 3SD) was 4.0% for CD4 T cell percentage and 7.4% for CD4 T cell counts of 608 cells/μL in HIV-1 infection and was not influenced by choice of QC FC

instrumentation, absolute count system, or choice of manufacturers' IQC material (Ticchioni et al., 2019). Unlike most chemical analytes, the large number of events acquired using FC means that the laboratory need only perform up to 5 replicates to determine sample variance (Davis, McLaren, et al., 2013). Estimation of measurement of uncertainty is an important ISO 15189:2022 laboratory standard. The long-term uncertainty in measurement (LTUM) uses external quality assessment (EQA) reference values only and has the advantage that any variation resulting from cell concentration is taken into account. In addition, it can be applied to lymphocyte thresholds important for clinical decision making as EQA samples offer a larger range of values than internal quality controls and are more representative of what is routinely encountered in the laboratory. In one study the LTUM for CD4 and CD8 T cell percentage were 19% and 16%, respectively for single platform technology (Dannus et al., 2022). It has been proposed that laboratory performance can be assessed using data boundaries defined by measurement of uncertainty and data obtained be used to identify centers of excellence (Kunkl et al., 2002). Quality control targets including LTUM will need to be defined for flow cytometric diagnosis of immune deficiency and the assessment of immune reconstitution after anti-CD20 therapies and transplantation.

8 | SAMPLE ANALYSIS

The analysis of samples is performed by utilizing FC "gating" on the populations of interest. Gates can be selected using automatic computer software or manually, however laboratories must have standard operating procedures in place to determine when automatic gate setting need to be manually adjusted for stressed specimens (Diallo et al., 2017). One large study showed that 25% of results still need manual correction by appropriate skilled laboratory staff despite using an automated walk away platform for lymphocyte phenotyping (Lemoine et al., 2022). Laboratories should determine criteria to accept or reject single tube samples with unacceptable internal variation or ensure that the computer software has a built-in programme to define the limits of acceptable sample variation. If in doubt the laboratory should repeat the staining or request another specimen. Laboratories should define how long primary tube samples are retained post-sample analysis. Primary samples should be stored at room temperature.

9 | DATA ANALYSIS AND REPORTING

Using a CD45/SSC gating strategy lymphocyte counts and percentage should be reported, with lymphocyte counts reported in cells/μL. Automated transfer of data from the flow cytometer analytic software to the laboratory IT system is recommended to prevent transcription errors resulting from manual entry of data. The laboratory report should include all parameters tested in clinically relevant units (cells/μL) and should include reference ranges for age and gender matched populations. Laboratories should demonstrate how reference ranges were derived (manufacturer's guidance, publication, in house) and be aware of the influence of other factors such as ethnic background and different instrumentation on lymphocyte cell counts and the associated reference intervals. There should be policies to cover what results are phoned or emailed, how amended/revised results are communicated, and testing systems should facilitate full audit trails to review any errors or miscommunications (Barnett et al., 2013).

10 | DATA STORAGE

All FC data should be stored as flow cytometry standard (FCS) (Barnett et al., 2013) on a separate secure server or on permanent data media. The integrity of back-up files should be confirmed before original data is deleted from the flow cytometer. The duration of storage for electronic data varies according to local/national guidelines: UK Royal College of Pathologist (RCPath) suggest that all hard copies and/or electronic analytical reports of primary patient files should be retained for 8 years (Wilkins, 2015). Laboratories should ensure that all automated stored records and data transfer procedures comply with local hospital IT security and confidentiality policies.

11 | QUALITY ASSURANCE

All laboratories should meet ISO15198 2022 standards concerning the quality management system (Table 1). Participation and satisfactory performance in national or international proficiency testing (PT)/EQA programmes, shown to be associated with improvements in analytical performance, is essential (Bainbridge et al., 2018; Sack et al., 2013; Whitby et al., 2013). Several organizations provide external quality support to FC laboratories including UK NEQAS, College of American Pathologists (CAP) and the Royal College of Pathologists of Australasia (RCAPA) for analysis of T cell subsets, B

TABLE 1 Checklist of ISO 15189:2022 standards to monitor performance of flow cytometry diagnostic service for immune monitoring.

1. Overview of methods for specimen collection, handling, transport, identification, processing, and storage.
2. Description of content provided on electronic request templates or request forms for CD4 T cell request and reports.
3. Policy for monitoring of regents, equipment, flow cytometer instrument performance, and quality control procedures.
4. Presentation of QC data to meet ISO 15189:2022 accreditation standards (intra-assay variation, precision, bias, linearity, quantitation and detection limits, reagent and sample contamination, agreement between individual instruments, and assessment of measurement of uncertainty for clinical decision thresholds).
5. Standard operating procedures for technical and clinical validation and reporting of test results.
6. Outline of staff training and education, including documentation of practical training, assessment of competency and continuing staff education and development.

TABLE 2 Summary of processes required for optimal application of flow cytometry in immune monitoring.

1. Laboratories should engage with clinical users as to the frequency of testing for immune reconstitution following B cell depletion therapies and after transplantation.
2. Indications for screening for primary and secondary immune deficiencies by flow cytometry should be agreed between clinical services and the diagnostic immunology laboratory.
3. Peripheral blood specimens should be collected in EDTA samples and analyzed within 48 h of venesection for T cell subsets, 36 h if using PIDOT to screen for primary immune deficiencies and within 4 h for bulk lysis protocols following anti-CD20 therapies. Laboratories should validate time scales or the use of specimen stabilizers if samples are to be analyzed after recommended intervals following venesection.
4. Immune monitoring for HIV should be undertaken with single platform flow cytometer technology where absolute lymphocyte counts are generated independently of hematology-derived parameters.
5. The use of electronic adjustable pipettes and automated sample preparation stations are recommended. Laboratories which employ semi or fully automated specimen preparation stations should follow the flow cytometer manufacturer's recommendation for their use.
6. Reverse pipetting is essential for the addition of blood, monoclonal antibodies, and beads for single platform technology.
7. Laboratories should provide evidence that pipettes are calibrated on a regular basis.
8. Laboratories should ensure that they comply with ISO 15189:2022 laboratory standards for selection and acquisition, installation and initial calibration, routine maintenance, service and repair records, and disposal of flow cytometer instruments used in diagnostic services.
9. Laboratories are strongly encouraged to adhere to standardized protocols for screening and diagnosis of primary immune deficiencies and for detection of B cell depletion and recovery following anti-CD20 therapies.
10. A minimum of 1×10^6 (ideally 5×10^6) CD45 positive white cell events should be acquired for the diagnosis of primary immune deficiencies and analysis of immune constitution following anti-CD20 treatments and transplantation.
11. Laboratories should define, in agreement with local guidelines, their lower limits of quantitation for T and B cell counts for monitoring immune reconstitution after transplantation and B cell depleting therapies.
12. Immune monitoring panels incorporating B cell memory markers are advisable to correlate clinical outcomes with B cell reconstitution following anti-CD20 therapies.
13. Internal quality controls to monitor reagent staining and lysis procedures should be run preferably at clinical decision points for all applications of flow cytometric technology.
14. Flow cytometer performance should be monitored daily using unstained and fluorescent latex beads.
15. T and B cell values should be reported as percentage and absolute counts using clinically relevant units (cells/μL) with appropriate age reference ranges.
16. All flow cytometry data should be stored as flow cytometry standard (FCS). All hard copies and/or electronic analytical reports of primary files should also be stored for at least 8 years (UK RCPath) or according to alternative local and/or national guidelines.
17. Laboratories providing diagnostic and/or immune monitoring services should be compliant with ISO 15198:2022 standards and be accredited by an appropriate regulatory agency.

and NK cells. The EuroFlow consortium offers an external quality assessment program for standard lymphocyte subsets and for the PIDOT, which incorporates both a practical laboratory component and data interpretation generated from FCS files using blood donated by patients with immune deficiency. In contrast, CAP only offers EQA for naïve and memory T cell subsets while the RCAPA supports analysis of memory B cells using markers derived from the EuroClass study. The laboratory should be able to show satisfactory performance if requested by current or potential service users (Table 2).

12 | CONCLUSION

Flow cytometric methods for diagnosing PID and assessing immune reconstitution after B-cell depletion are improving. Individual laboratories will need to assess their operational capacity to deliver better services. Studies to verify the utility of primary immune deficiency flow panels to screen for secondary immune deficiencies are needed. There is an increasing need for standardization of laboratory protocols to monitor immune reconstitution after transplantation and B cell depletion therapies. Multi-centre prospective studies are required to evaluate the impact of analysis of B cell and CD4+ T cell immune reconstitution on clinical outcomes after transplantation and B cell depletion therapies.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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