

# Recommendations for quality assurance in multiparametric flow cytometry: first consensus of the Brazilian Group of Flow Cytometry (GBCFLUX)

## *Recomendações para garantia da qualidade em citometria de fluxo multiparamétrica: primeiro consenso do Grupo Brasileiro de Citometria de Fluxo (GBCFLUX)*

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### ABSTRACT

The Brazilian Group of Flow Cytometry (Grupo Brasileiro de Citometria de Fluxo [GBCFLUX]), founded on April 24, 2010, is composed of experts in flow cytometry (FC) area who have the common objective of contributing to technical and scientific advances in Brazilian clinical and research laboratories. Among GBCFLUX working groups, the Quality Control (QC) subcommittee is responsible for discussing data in the literature and contributes to the quality assurance of the pre-analytical, analytical and post-analytical process in FC. The QC subcommittee's actions began through meetings and lectures, in which data from the literature were reviewed and discussed with all participating members of the GBCFLUX. In a second step, it was decided to draw up a text of technical and scientific consensus recommendations, informative and educative, for dissemination to all FC working groups in Brazil. To this effect, a questionnaire with objective responses was designed and sent to 35 recognized Brazilian institutions, in order to evaluate the QC profile of these institutions. Thus, the QC technical-scientific recommendations, which will be described in this updating article, are intended to ensure the process quality, technical standardization, and reproducibility of results in FC.

**Key words:** flow cytometry; quality control.

### INTRODUCTION

Since its founding on April 24, 2010, the Brazilian Group of Flow Cytometry (Grupo Brasileiro de Citometria de Fluxo [GBCFLUX]) brings together experts in order to discuss and promote technical and scientific advances in Brazilian clinical and research laboratories. The GBCFLUX is composed of working committees and, among them, the flow cytometry (FC) quality control (QC) subcommittee, which is responsible for reviewing data of the literature, discussing and proposing recommendations that ensure the reliability of results and minimize potential technical failures, inherent to the method.

The subcommittee determined that a consensus of technical and scientific recommendations on QC should be drawn up and disclosed to all FC working groups in Brazil. To this purpose, a questionnaire with 67 objective questions was sent to 35 recognized Brazilian institutions. From the 35 groups, 27 answered the questionnaire (73%), and the responses were compiled and presented at the 15<sup>th</sup> GBCFLUX meeting, in which participants and members decided what would be mandatory, recommended and optional for all QC topics discussed.

Therefore, this article aims to inform the role of the Brazilian group of FC, as well as the literature recommendations, which could act as a guide for participants, establishing the

minimum necessary to guarantee the process quality, technical standardization, and reproducibility of results in FC.

## QUALITY CONTROL OF THE PRE-ANALYTICAL PHASE

In the laboratory environment, the pre-analytical phase is from the moment of the examination order until the sample arrival at the laboratory. According to the literature, 70% of errors in the clinical laboratory are directly related to the pre-analytical phase<sup>(1)</sup>.

### Anticoagulants for peripheral blood (PB) and bone marrow (BM)

The characteristics of the anticoagulant used for PB and BM collection are described in **Table 1**. For immunophenotypic study, we recommend using ethylenediaminetetraacetic acid (EDTA) or heparin.

### Collection medium for cerebrospinal fluid (CSF), cavity fluids, fine-needle aspiration, and tissue fragments

CSF may be collected in a dry tube without anticoagulant, however, due to the high rate of cell degeneration, it is recommended to collect directly on special medium such as Transfix<sup>®</sup> (Cytomark, UK), or Roswell Park Memorial Institute (RPMI) culture medium, with 5% fetal bovine serum (FBS)<sup>(3)</sup> (**Table 2**).

**TABLE 1** – Characteristics of the anticoagulants used in PB and BM collection for immunophenotyping by FC<sup>(2)</sup>

Anticoagulant	Considerations	Sample stability time (hours)
EDTA	<ul style="list-style-type: none"> <li>• ideal for collecting PB when CBC and FC are ordered</li> <li>• acceptable for FC and molecular biology, but unacceptable for cytogenetic</li> </ul>	12-48
Heparin sodium	<ul style="list-style-type: none"> <li>• ideal for collecting BM for cytogenetic analysis</li> <li>• limitation: affect cell staining in BM smears</li> </ul>	48-72

PB: peripheral blood; BM: bone marrow; FC: flow cytometry; EDTA: ethylenediaminetetraacetic acid.

**TABLE 2** – Conditions for CSF collection and storage for analysis by FC<sup>(3)</sup>

Collection medium for CSF	CSF ratio: collection medium	Storage temperature	Stability time
Transfix <sup>®</sup>	1:4	4°C (2°C to 8°C)	48-72 hours
RPMI 5% FBS	1:1	4°C (2°C to 8°C)	18 hours

CSF: cerebrospinal fluid; FC: flow cytometry; RPMI: Roswell Park Memorial Institute; FBS: fetal bovine serum.

For cavity liquids, collection in a dry tube is recommended; for fine-needle aspirates, lymph node tissue fragments is indicated the use of isotonic transport medium, RPMI for example. Lymph node in sterile saline should be quickly transported to the FC technical sector.

### Sample identification and exam ordering form

It is mandatory to carry out sample identification before or during the material collection, and it must contain at least two identifiers that individualize the patient. The material should always be sent to the laboratory accompanied by the medical order and a duly complete form with the following information: a) service name, assistant doctor, telephone number; b) type of material, date and time of collection; c) the examination order with medical history, diagnosis hypothesis, and previous treatment; d) clinical data related to the investigation moment (diagnosis, relapse, control or survey of minimal residual disease); and e) other laboratory data relevant to the diagnosis.

### Volume of samples

**Table 3** summarizes the recommended volume of samples commonly analyzed by FC.

### Temperature and transport conditions

The samples must be sent to the laboratory immediately after collection and, in the case of PB and BM, they must be properly conditioned at a temperature of 18°C to 25°C<sup>(2)</sup>. CSF, lymph nodes, and tissue fragments must be kept refrigerated at 2°C to 8°C during transportation<sup>(3)</sup>. It is important that the temperature is controlled, monitored and recorded during transport, as well as during material receipt by the laboratory.

**TABLE 3** – Recommended volume of biological samples for analysis by FC

Material	Volume (mL)	Observation
PB	Minimum 5	-
BM	Maximum 2	Avoid dilution with PB
CSF and cavity fluids	Minimum 5*	Except for pediatric patients

\* because it is a material that usually has low cellularity, the higher the volume collected, the greater the assurance of complete immunophenotypic study.

FC: flow cytometry; PB: peripheral blood; BM: bone marrow; CSF: cerebrospinal fluid.

## Receipt of materials and rejection and restrictions criteria

In the laboratory, during the receipt of the material, it is essential to check medical form data, and to evaluate for adverse conditions related to the collection, conditioning and transport<sup>(4)</sup>.

Cell viability below 75% (evaluated by trypan blue, propidium iodide, 4',6-Diamidino-2-phenylindole Dihydrochloride [DAPI], DRAQ-5<sup>®</sup> or 7 amino-actinomycin D [7-AAD]), intense hemolysis, and presence of clots significantly compromise the quality of the immunophenotypic study, and should be considered as rejection criteria for the material. However, it is important not to reject noble samples (BM, CSF, cavity fluids, and tissue fragments), but rather to process and analyze them rigorously to release complete or partial results.

It is required to describe any restriction information in the final report, for example, describing the presence of clot – “suboptimal sample due to the presence of clot”.

## QUALITY CONTROL OF THE ANALYTICAL PHASE

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### Quality control of the equipment (FC)

The equipment functions must be checked daily, corrective and preventive actions (maintenance) must be defined and documented by the laboratory together with the company responsible for technical assistance.

The equipment should be placed on a physical structure without vibration and in a room with a temperature set between 19°C and 25°C. Ideally, the temperature registration and documentation occur during the periods of the day when the equipment is connected.

In the daily boot process, or when the equipment is in use, it is mandatory to check the fluidic systems, pressure, vacuum and laser power<sup>(5)</sup>. Although this assessment is automatically carried out by the equipment, monitoring, recording and documentation are required.

The same is applied to the evaluation of light scattering and fluorescence detection system, which laser alignment and photomultipliers (PMTs) performance are evaluated with specific products for each equipment and supplier<sup>(5,6)</sup>.

It is important that the values obtained are easily accessible and filed in a historical sequence related to each equipment. It

is recommended to create spreadsheets and average/median and standard deviation (SD) graphs analysis, the same procedure for the registration and the traceability of historical occurrences and maintenance is applied.

To validate new equipment, checking and documentation of linearity, reproducibility, resolution and sensitivity of the equipment are optional when the company responsible for technical support of the equipment certifies these items<sup>(7)</sup>.

The equivalence of analytical systems must be evaluated every six months, in the laboratories with different equipment performing the same tests<sup>(5)</sup>. For quantitative assays, such as CD4 and CD8 lymphocytes quantitative measure, and CD34<sup>+</sup> progenitor cells quantitative measure, the use of statistical programs for comparison and/or historical coefficient of assay variation is recommended, and an acceptable limit established for each service.

For qualitative tests, such as immunophenotypic characterization of leukemias and lymphomas, the use of the categorical agreement for the final diagnosis as a comparison criteria is recommended; different equipment must generate the same final diagnosis composed by negative and positive expressions of the markers used.

### Periodicity of color compensation

For services that use combinations of standardized and validated fluorescence, the daily verification of color compensation of the flow cytometer is recommended before starting the routine. Daily compensation of the fluorescence is not recommended, but rather to verify if the predefined compensation is in accordance with the acceptability limits established and documented for each service.

It was decided that the periodicity for a new compensation matrix should be related to laser alignment performance, the variation of fluorescence channels ( $\Delta$ PMT) and the performance of a tube labeled with ideal fluorochromes combinations ideal for checking the compensation. If the evaluated parameters are within the acceptability limit, there is no need to apply a new compensation matrix. However, it is important to apply a new matrix if the values are outside the acceptable limits after preventive and corrective maintenance of the equipment.

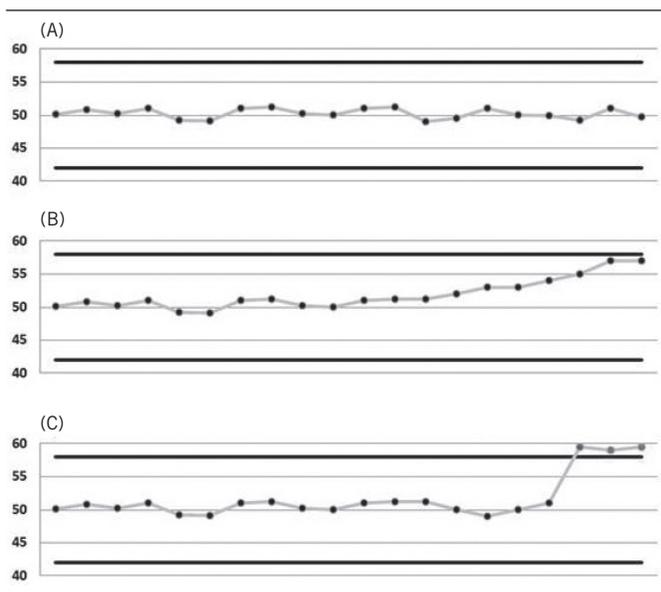
For services using tandem fluorochromes, it is necessary that the color compensation be specific for each fluorochrome-conjugated antibody and for each product lot<sup>(5,8)</sup>. For example, the compensation matrix used for the marker CD19 PE-Cy7 Lot 50 should be different from the one used for CD19 PE-Cy7 Lot 51,

as well as for CD117 PE-Cy7 marker. It is important to mention that when introducing a new tandem marker into the routine, it is necessary to apply a new compensation matrix adding the new product concerned. The previous electronic compensation matrix cannot be used for the calculation, since there may be variations in the fluorescence intensity during the period between compensations.

### Internal quality control

This involves ensuring that the obtained results are accurate, precise, consistent and reproducible. This performance is checked, usually by commercial reagents with reference values that assess the technical process as a whole.

It is important that this verification is performed before the starting or releasing routine, and that the laboratory monitors the performance, assesses trends, and applies corrective action when necessary. It is recommended that monitoring is carried out by scatter plots showing the relationship to the average, such as the Levey-Jennings chart, which allows using Westgard Rules (**Figure 1**).



**FIGURE 1** – Levey-Jennings charts representing the daily assessment of an analyte with mean value and ranges defined by the product manufacturer (mean  $50\% \pm 8\%$ )

*A) chart with acceptable performance and values ranging around average; B) chart shows the trend in which the values progressively deviate from the mean. Corrective actions are required because more than eight consecutive values differ from the average; C) chart with values out of the acceptable upper limit. Corrective actions are required when three consecutive determinations present unacceptable performance, when there is a consecutive violation of two SD, when two SD are violated on the same day by two levels of control, and when a violation of three SD occurs.*

SD: standard deviation.

Care should be taken when using the instruction values provided by the manufacturer, because it is important to ensure that the acceptability limits are two SD. As an alternative to the instruction values, the laboratories may use the determinations history to establish the average and variations of two SD. The use of at least twenty consecutive determinations of the same product lot is recommended for this application.

Despite the problems involving the use of commercial reagents in FC, as low availability, high cost and short shelf-life, it is mandatory that two levels of internal control (clinical decision level) are performed daily, or when performing quantitative tests of CD4/CD8 lymphocytes and quantitative measure of CD34<sup>+</sup> progenitor cells<sup>(9)</sup>. In the absence of commercial reagents, the use of the samples analyzed the previous day with known values and variations established and documented by the laboratory is suggested as an alternative. The acceptability limits can be defined through analysis of at least twenty samples on consecutive days.

For leukemia and lymphoma immunophenotyping panels, monitoring and monthly recording of the performance of the monoclonal antibodies used is recommended, assessing antigen expression in normal and abnormal populations of the sample concerned. This strategy is important to evaluate the performance of reagents during the use, mainly due to decrease or loss of fluorescence, which, if not detected, may induce the interpretation of false negative results.

### Reagents

Regarding the reagents and the working solutions, for example, hemolysis reagents, phosphate-buffered saline (PBS), and solutions for labeling monoclonal antibodies, it is mandatory to validate them before releasing for use. If such reagents are prepared daily, then daily validation is also required.

Regarding the commercial kits, each service must have a written procedure consistent with the manufacturers' recommendations, as well as values within the acceptable limit established by kits that have reference values. Any change in the procedure must be documented and validated by each service<sup>(10)</sup>.

### Monoclonal antibodies conjugated with fluorochromes

The validation of monoclonal antibodies (MAb) must occur before their release for use in routine. Validation needs to be performed according to the lot and the receiving date of

products, according to the following considerations: a) if several MAb bottles from the same lot are received on the same day, the validation of only one bottle is enough to indirectly validate the other bottles; b) if the antibodies are from different lots, each lot must be validated regardless of the receiving date. The same applies to the products of the same lot received on different dates.

This strategy is required since the packaging conditions during transportation may influence MAb quality, even if they are from the same lot.

Among MAb validation criteria, titration is the most important factor and it is intended to define the optimal concentration of antigen-antibody saturation. Although MAb manufacturer recommends an ideal volume for labeling, titration is important to minimize the background fluorescence and the non-specific binding, while optimizing the signal-to-noise ratio relationship<sup>(7)</sup>.

For titration, it is recommended:

- following the same standardized technical procedure for sample preparation, as the concentration of labeled cells, reagents, hemolysis buffers and fixing;

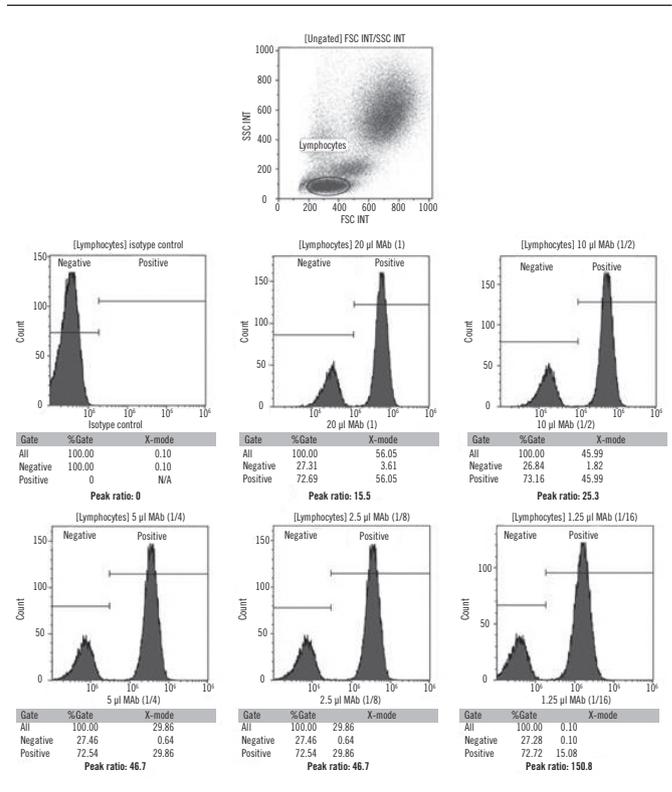
- the positive population should be as bright as possible (high fluorescence intensity), since the negative population behave as the unlabeled sample. When applicable, it is recommended to use the so-called Peak Ratio (PR) calculation, which is to divide the positive population fluorescence by the negative population fluorescence, and the largest PR value is represented by the better distinction between populations (resolution), and therefore by the optimal concentration for use<sup>(11)</sup> (**Figure 2**);

- once titrated, the monoclonal antibody released for use may be used as a reference to validate other antibodies through the comparison between products. If the performance is similar and meet the criteria described above, established by each service, the “new” MAb may be released for use, respecting the same volume used in the comparative test; otherwise, the new Mab should be titrated and an ideal volume for labeling is established.

### Samples processing

It is suggested that the samples are processed immediately upon receipt, or as soon as possible after collection, respecting the maximum limit of 24 to 48 hours post-collection, depending on the anticoagulant used.

For samples with high proliferative and/or apoptosis level, for example, samples suspected of Burkitt’s lymphoma, diffuse large B-cell lymphoma, plasmocyte dyscrasia, BM collected after recent



**FIGURE 2** – Example of MAb titration:  $1 \times 10^6$  PB total cells labeled with IgG1 (mouse) – PE and different volumes/concentrations of CD3-PE, from the volume recommended by the manufacturer

The analysis of lymphocytes region (SSC  $\times$  FSC) shows that the optimal volume ranges from 5.0  $\mu$ l (1/4) to 1.25 (1/16), because in these concentrations there were enough fluorescence intensity to identify the CD3 positive lymphocyte population from the other CD3 negative lymphocytes, which remained restricted to the region of unstained cells. The Peak Ratio values, 46.7 and 150.8 respectively, support the use of these volumes.

MAb: monoclonal antibodies; IgG1: immunoglobulin G1 class; PE: phycoerythrin; SSC INT: side scatter integral; FSC INT: forward scatter integral; PB: peripheral blood.

chemotherapy and CSF, the immediate processing after collection and/or material receiving is mandatory<sup>(2)</sup>.

### Cell concentration for labeling

The recommended cell concentration for labeling samples with monoclonal antibodies conjugated with fluorochromes is  $5 \times 10^5$  to  $1 \times 10^6$  cells/ $\mu$ l. For standard applications by working groups, such as the Euroflow Consortium protocols, the volume 50  $\mu$ l is recommended regardless of the cellularity<sup>(12)</sup>. The Clinical and Laboratory Standards Institute (CLSI) describes important details about the specific cell concentration for different clinical applications<sup>(13, 14)</sup>.

For rare events research and acquisition of million events in which the cell concentration exceeds  $1 \times 10^6$ , concentration of the

sample before labeling is recommended, as well as to titrate again the specific markers for such applications<sup>(15)</sup>. It is important to use one control tube for each patient, in order to evaluate the flow cytometer settings and the negativity and/or the autofluorescence level of the sample. Therefore, it is indicated to use a tube with sample and without monoclonal antibody (unlabelled tube) and/or a tube labeled with fluorescence isotype controls. Additionally, the cell populations of the sample/tube itself labeled as fluorescence internal control may be used<sup>(5, 13)</sup>.

### Data acquisition and analysis

For services that do not use post-labeling fixers, samples should be conditioned at temperature of 18°C to 25°C, protected from light, and acquired in the flow cytometer, no later than two hours after labeling. If the service uses fixer, the samples should be conditioned at a temperature of 2°C to 8°C, and the acquisition carried out within 24 hours post-labeling. It is important to be alert to possible changes in the antigenic expression of the labels in fixed samples. It is recommended that the standardization of acquisition and data analyzing processes (settings, gates strategy, number of acquired events, and post-acquisition analysis on specific software) is implemented, monitored and documented in the services. This action is important to minimize subjectivity and maximize the reproducibility of results<sup>(16)</sup>.

It is worth noting that results interfacing technology, besides providing security to the process, avoids transcription errors of results. Thus, the services should invest in this tool for considerable gain in the quality process.

## QUALITY CONTROL OF THE POST-ANALYTICAL PHASE

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### Consistency analysis and release of the results

The consistency analysis of the results obtained by FC should be carried out prior to release of the final result, and it consists of a critical review of immunophenotypic findings correlated with clinical data and other laboratory data, and double checking for verifying the analyzed and transcribed data<sup>(17)</sup>.

In immunophenotypic evaluation of hematologic malignancies, it is recommended that the diagnostic conclusion is conducted together with the morphology and medical history, and, if necessary, to establish an active communication channel to the assistant doctor. When possible, it is advisable to associate the immunophenotypic results with the histopathological, cytogenetics

and molecular biology findings. For other applications, for example, lymphocyte immunophenotyping, quantitation of CD34<sup>+</sup> progenitor cells and paroxysmal nocturnal hemoglobinuria, the application of consistency analysis established in the literature is recommended<sup>(14, 18)</sup>.

### Conditioning and retention period of biological samples

After the report release, it is recommended to condition the biological samples at temperature of 2°C to 8°C for a period up to three days<sup>(19)</sup>. If performing new procedures and new labelings with the material are required, it is mandatory to carry out a critical analysis of the results obtained due to possible changes in cell viability and antigenic expressions. When these changes are identified, it is appropriate to describe them to described them in the report.

### Data archiving and retention period

Archiving and backing up the data generated by the FC service (raw acquisition file, analyzed files, application forms, and working maps) must be filed in printed and/or electronic form for at least five years<sup>(19, 20)</sup>. It is recommended that the backup data is performed daily.

### External quality control

The external QC aims to assess the technical control of the process, through proficiency external programs and external commercial controls; the external QC samples are processed in the same way as the patient samples<sup>(19, 21)</sup>.

It is essential that all tests performed in the service participate in external proficiency programs; ensuring test accuracy if the commercial products are unavailable, sending samples, in parallel, to another reference laboratory is recommended<sup>(1)</sup>.

In situations where the test is not performed at another service (peculiar methodologies internally validated), the performance of the test/double blind evaluation is recommended. This consists of performing tests by two different analysts, and the result must necessarily be the same. Acceptability limits should be statistically established, monitored and documented by each service.

When an external control delivers results outside the range established by the program, the services should identify the cause of the error and, if necessary, apply corrective, preventive and

educational activities. These actions should be monitored in future tests, especially to investigate and ensure that the results of the patients analyzed in dates close to the external control have not been compromised due to detected error.

## FINAL CONSIDERATIONS

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This document was reviewed and approved by the Quality Control Subcommittee in Flow Cytometry (Subcomitê de Controle de Qualidade em Citometria de Fluxo) together with the GBCFLUX Scientific Subcommittee. New versions and/or updates will be reported.

It is important to report that learning this complex area must be continuous, and training and evaluation of technical skills are essential. The continuous improvement and monitoring processes of knowledge development must be monitored by the responsible doctor and technician.

## ACKNOWLEDGEMENTS

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The authors thank the GBCFLX members, who answered the QC questionnaires. We also thank all the participants and members of the GBCFLUX for their contribution in preparing this first QC document.

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## RESUMO

*O Grupo Brasileiro de Citometria de Fluxo (GBCFLUX), fundado em 24 de abril de 2010, é composto por especialistas da área de citometria de fluxo (CF) que possuem o objetivo comum de contribuir para avanços técnico-científicos em laboratórios clínicos e de pesquisa brasileiros. Entre os grupos de trabalho do GBCFLUX, o subcomitê de Controle de Qualidade (CQ) é responsável por discutir dados da literatura e contribuir para a garantia da qualidade do processo pré-analítico, analítico e pós-analítico em CF. As ações do subcomitê de CQ iniciaram-se por meio de reuniões e palestras, nas quais dados da literatura foram revisados e discutidos com todos os membros participantes do GBCFLUX. Em uma segunda etapa, definiu-se elaborar um texto de recomendações consensuais técnico-científicas, informativas e educativas para divulgação a todos os grupos que trabalham com CF no Brasil. Para tanto, foi elaborado um questionário com respostas objetivas, sendo enviado para 35 instituições brasileiras cadastradas, com a finalidade de avaliar o perfil de CQ dessas instituições. Dessa forma, as recomendações técnico-científicas de CQ que serão descritas neste artigo de atualização têm o objetivo de contribuir para a garantia da qualidade do processo, a padronização técnica e a reprodutibilidade dos resultados em CF.*

*Unitermos: citometria de fluxo; controle de qualidade.*

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