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Quality of advanced therapy products: requirements of extensively manipulated cell used in cell therapy and bioengineering

Qualidade dos produtos de terapias avançadas: requisitos de células extensamente manipuladas usadas em terapias celulares e em bioengenharia

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ABSTRACT

Introduction: Preparations of products for advanced therapies include an extensive manipulation stage of the cell primary culture, which can lead to changes in the final cellular population. Standardization of the technique for obtaining the cellular cultures, in complying with Good Manufacturing Practices (GMP), and carrying out quality controls of process and final product, are essential to ensure patient safety and comparability of clinical outcomes. **Objective:** The purpose of this study was to identify the most prevalent trials cited in the scientific literature or in health standards applied to the evaluation of the quality of human primary cells, which could be inserted in the routine of the Cell Processing Centers. **Method:** A survey was carried out of scientific articles and health standards related to cell therapy and associated quality assays. **Results:** This study showed that the advanced cellular therapy products regulation do not specify the tests that should be used for quality control or, when specified, do not define acceptance or rejection ranges for the products, which makes urgent the discussion of such matter. **Conclusions:** In this article, we highlight the need to prepare the product in the form of a single batch that should be quality-controlled from samples representative of the whole. In addition, we mention some of the trials that define the quality of the advanced cellular therapy product, detailing the points at which they are to be performed in the flowchart.

KEYWORDS: Cell Processing Center; Biotechnology; Primary Culture; Cell Culture; Quality Control

RESUMO

Introdução: O preparo de produtos para terapias avançadas inclui, frequentemente, a manipulação extensa de células primárias *in vitro*, o que pode acarretar em alterações da população celular final. A padronização dos métodos de obtenção das culturas, seguindo as regras das Boas Práticas de Fabricação, com a realização dos controles de qualidade do processo e do produto final, é essencial para garantir a segurança do paciente e a comparabilidade dos resultados clínicos obtidos. **Objetivo:** Este estudo buscou identificar os ensaios, prevalentemente citados na literatura científica ou em normas sanitárias, aplicados na avaliação da qualidade de células primárias humanas, passíveis de serem inseridos na rotina dos Centros de Processamento Celular. **Método:** Foi realizado o levantamento de artigos científicos e de normas sanitárias que tratassem de terapia celular e dos ensaios de qualidade associados. **Resultados:** Foi evidenciado que as normas regulamentares direcionadas a produtos com base em células cultivadas *in vitro* não detalham os ensaios de qualidade, o que torna urgente a discussão dessa matéria. **Conclusões:** Evidenciamos a necessidade de preparo do produto na forma de um lote de células, que deve ser controlado para a qualidade, a partir de amostras representativas do todo e propomos a realização de uma bateria de ensaios, que definem a qualidade do produto de terapia avançada, a base de células cultivadas *in vitro*, com detalhamento dos pontos em que estes devem ser realizados, organizados como fluxogramas de processamento.

PALAVRAS-CHAVE: Centro de Processamento Celular; Biotecnologia; Cultivo Primário; Cultura de Células; Controle de Qualidade

INTRODUCTION

Cells obtained from human tissues and cultured *in vitro* may be used in clinical trials or therapies and may even be commercially available, depending upon the health regulations of the country of origin.

In order to evaluate proposals for the therapeutic use of cells, regulatory agencies require cells to be prepared in compliance with Good Manufacturing Practices (GMPs), with proven safe, reproducible and efficient results[1,2]. They also provide different regulatory treatments according to the laboratory processes that the cells undergo. These processes can be classified as minimal or extensive cell manipulations. The basic difference for these two types of processing is the possibility of significant changes in physiological, functional, or structural cell characteristics relevant for the intended use, such as differentiation state, proliferation potential or metabolic activity in extensive manipulation, while such changes do not occur under minimal manipulation conditions. All the types of cell cultures are characterized as extensive manipulation, while activities like cutting, separating and centrifuging cells characterize minimal manipulation. The second aspect that gives differentiated treatment to the cell-based products is their use. When the cells are used in therapies to perform functions different from the original ones, they are always classified as extensively manipulated *in vitro*, and are classified as advanced therapy products.

In Brazil, the clinical use of cells cultured *in vitro* is currently only permitted in research, provided it is approved by the National Commission for Research Ethics (Conep), or in therapies, when authorized by the Federal Medical Council (CFM) or Federal Council of Dentistry (CFO). They have been handled in laboratories authorized by the Brazilian Health Regulatory Agency (Anvisa), the Cell Processing Centers, as determined by the Collegial Board Resolution (RDC) n. 214, of February 7, 2018, or by resolutions that replace it[3]. Under exceptional conditions, the clinical use of cultured cells is also permitted when it represents the only opportunity for improvement of patients at risk of death. These cases are classified as "Compassionate Use" by the European Medicines Agency or "Expanded Access" by the U.S.A. Food and Drug Administration (FDA), who may authorize the proposed use for a short period based on data published in the scientific literature and on the experience of the involved clinical group.

In the U.S.A. and Canada, advanced therapy products are classified as "" Regenerative Medicine Advanced Therapy Advanced (RMAT) Products, regulated by Section n. 351 of the US Public Health Service Act. They may be available after approval by the Food and Drugs Administration (FDA), when their safety and efficacy had been confirmed. The first advanced therapy product approved in the United States was *Carticell* (autologous chondrocytes), in 1997. It was followed by others, such as *Laviv* (autologous fibroblasts) and *Gentuit* (allogenic keratinocytes and fibroblasts). The first product based on cellular and gene therapy, *CAR-T,* was initially approved by the International Society of Cell Therapy (ISCT) in August 2017 and is under review by the FDA.

In the European Union (EU), therapies using cultured cells *in vitro* are classified as Advanced Therapy Medical Products (ATMP) and regulated by EU directive n. 1394/2007. This applies to all member-countries of the European Community and contains the rules for authorization, inspection and technical requirements related to product characteristics and information, prepared in the industry or in academic institutions. The Committee for Advanced Therapies (CAT) of the European Medicines Agency (EMA) centralizes marketing authorizations for ATMP. In 2014, the EMA approved the first therapy based on adult stem cells taken from the cornea, cultured *in vitro* and applied to the donor patient's own cornea regeneration: *Holoclar*.

Similar to the understanding of advanced therapy products based on cells cultured *in vitro*, which is variable for each community according to its regulatory framework, the quality requirements of the final products also vary. Regulatory agencies state that such products must have granted quality, safety and efficacy, but do not define which quality assays should be applied to each situation. The scarcity of details about quality assays that must be performed, including their acceptance thresholds, makes this a pressing matter. It is necessary to describe the requirements for cell use authorization, which will be fundamental for the comparison of the clinical results obtained both in clinical research and in therapy.

Our proposal is to define here the tests that can be applied directly to cells prepared for clinical use, and that these become tools able to define and harmonize the final products, establishing the equivalence between the batches produced. The assays should be applied to all batches of prepared cells, especially those that are cryopreserved, thawed and returned to *in vitro* culture, up to the time they are requested for clinical use.

The present study describes a series of assays that define the quality of the advanced therapy product whenever one of the components contains cells cultured *in vitro*. It also details the points at which they should be conducted. A flowchart of processes for each of the major cell handling situations is proposed and should guide both the application the of extensively manipulated cells *in vitro* in clinical trials and their use in therapeutic procedures.

METHOD

This study was the survey of scientific papers, with the objective to identify the main tests carried out for the quality control of human primary cells cultured *in vitro*. The literature search was carried out on the PubMed database (using keywords like Quality control, Cell Processing Center, Biotechnology, Primary Culture, Cell Culture, Cell Therapy). The survey included the period from 1990 to 2018. The investigation of the requirements for conducting product and process quality control tests, defined by Anvisa, was made at http://portal.anvisa.gov.br/legislacao.

RESULTS AND DISCUSSION

Primary cell culture - basic material for cell therapies

Mesenchymal stem cells (MSC) are the best example of primary cell cultures used for cell therapies, and they are currently the most frequent cell type cultured *in vitro* for the purpose of clinical application. The first researcher to isolate and characterize these cells from a bone marrow sample was Friedenstein[4]. Ever since then, other tissues have been used as sources, such as adipose tissue, umbilical cord and dental pulp[5,6,7]. Their therapeutic potential was applied to ischemic, degenerative, or inflammatory diseases, such as stroke[8], lower limb ischemia[9], chronic ulcers[10], degeneration of musculoskeletal tissues[11,12], and immunomodulation of graft versus host disease, a serious disease that affects patients after allogeneic transplantation of hematopoietic stem cells[13]. The clinical benefits of the systemic use of MSC are the result of a combination of the different mechanisms of action that are possible. These stem cells show differentiation potential in various cells of the injured tissue: osteoblasts, chondrocytes, adipocytes, blood vessel, skeletal muscle or tendon cells. They produce cytokines that promote angiogenesis and/or attract new tissue stem cells or even act in the modulation and reduction of local inflammatory activity[14]. These processes depend on the type of the original lesion, the quality of the tissue bed and its evolution, before and after the treatment, so that the understanding and the proof of the efficacy of the therapy achieved with the use of MSC is a challenge for both researchers and regulatory bodies.

Many other cell types cultured *in vitro* also have clinical application, such as keratinocytes and human skin melanocytes, combined or not with fibroblasts and mesenchymal cells, used primarily in therapies for skin burn, vitiligo, chronic ulcerations[10,15]. Cultured chondrocytes are also used in cartilage lesions[16].

The common point in these clinical protocols for cell therapies and bioengineering is their requirement of a high number of cells. Therefore, the possibility of increasing the number of cells through *in vitro* culture is a great advantage when compared to the use of cells obtained from fresh tissues followed by immediate transplantation, since It grants the autologous or allogenic use of cells expanded from a small initial volume of donor tissue. However, the *in vitro* culture may have complex and occasionally disastrous consequences. Most primary cultures are heterogeneous, and the constituent cell types have proliferative abilities peculiar to tissue characteristics, donor profile and/or environmental conditions. Cells that have the highest rate of proliferation tend to dominate the culture. Culture conditions at low cell densities may result in clonal selection. One of the major concerns is the possible drift of cultured cells into potentially neoplastic cells, causing cancerous cell growth after their therapeutic implantation into the recipient organism. Furthermore, cultures may exhibit microbiological or chemical contamination, alteration of the normal rate of cell proliferation and differentiation, and they can undergo senescence, a natural procedure of depletion of their replication capacity, which can also reflect to sub-optimal conditions of the system. The cell culture system is complex, dynamic and difficult to standardize, which makes cultured-cell-based advanced therapy products different from therapeutic products with defined chemical properties.

When the desired number of cells for therapy is reached, they are usually cryopreserved at ultra-low temperatures (less than negative 135/166°C). Thus, they maintain their vital capacities until they are required for clinical use.

Advanced therapy products based on cells cultured *in vitro* should be prepared in the form of a homogeneous batch, thereby enabling the use of a small part as a representative sample of the whole in quality control assays.

Cell quality assays

Cell culture should be standardized and provided with relevant quality controls, from obtaining the primary culture to the batch of cells to be released for use. Faced with so many variables and the possible adversities of the system, the controls should ensure the cell quality, provide the harmonization of the advanced therapy product, and provide also the definition of the clinical modifications resulting from its application. For the use of each advanced therapy product, information on the properties listed below is required.

Purity

Purity includes negative results in the assays for detection of fungi, bacteria, including mycoplasmas, and endotoxins. The assays recommended by the national pharmacopoeia for the release of sterile products are used.

Regarding the virus contamination, Brazilian and EU health legislations have a pre-established list of diagnostic assays to be performed for the collection of tissues for therapeutic purposes. However, there are no requirements for diagnostic assays of viral pathogens, potentially present in the products obtained from cultured cells.

RDC-Anvisa n. 214/2018, which regulates the Cell Technology Centers in Brazil, presents in its article 60 the quality and safety assays of autologous and allogeneic cells cultured *in vitro*, namely:

> IV - for cells (other than HPC-BM, HPC-PB or HPC-UCPB for conventional transplantation purposes) and Advanced Therapy Products, in a Final Product sample: a) total cell count; b) appropriate identity or phenotyping test for the product and quantification of the cell populations; c) cell viability; d) purity test: includes, where applicable, the verification of substances or cells that may be harmful to the Recipient and, in the case of Extensive Manipulation, the presence of endotoxins must be verified; e) microbiological tests: the provisions of art. 49 of this Resolution and, when applicable, repeat the respective tests in the Final Product, and, in case of Extensive Manipulation, also include the test for the detection of mycoplasma contamination; f) nucleic acid detection of the following viruses: CMV, HIV-1 and HIV-2, HTLV-I and

HTLVII, EBV, HBV, HCV and B19, and, if applicable, other viral agents of clinical relevance in humans, only in case of Extensive Manipulation for allogeneic use; g) cytogenetic, only in case of Extensive Manipulation; h) potency test, when applicable: the relevant biological activity of cells, if known, or products synthesized by the cell should be defined and quantified.

The FDA-USA requires that the final product containing cells intended for use as a therapeutic product has been tested for the pathogens of Cytomegalovirus (CMV), Human Immunodeficiency Virus types I and 2 (HIV-1 and 2), Cell Lymphotropic Virus (HTLV-1 and 2), Epstein-Barr Virus (EBV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Erythrovirus B19 (B19). The FDA recommends that the general conditions of the process be assessed to identify the need for the search for other pathogens[17].

The reason for indicating the virus search at the end of the culturing is due to the fact that many of them can stay latent in the infected organism. The family of herpes viruses (Herpes simplex virus - HSV), which includes oral herpes (HSV type I), Varicella-Zoster and CMV, may be cited as an example. After primary infection, which can occur with or without symptoms, herpes viruses can remain latent within cells for a long time and reactivate their replication causing disease due to the immunologic deficiency of the individual[18,19]. Cell culture conditions may be conducive to reactivation of the proliferative activity of the virus, present in the tissue of primary culture origin. The infections that cells as advanced therapeutic products can cause are potentially severe and even lethal, according to the general condition of the recipient patient.

Morphological appearance

The evaluation of the quality of primary cultures begins with routine observation, using inverted optical microscope and phase contrast, to determine their morphological characteristics. This practice allows the handler to get accustomed to the normal aspects of the cultures and to perceive, at an early stage, the occurrence of undesired events, such as the presence of morphologically distinct cells or with an appearance that is indicative of suffering. The presence of chemical and/or microbiological contamination and the use of low quality or purity materials or reagents are examples of undesired culture-related events.

Primary cultures should therefore be routinely monitored for morphological appearance, in order to observe the following aspects:

I. Verification of the presence of cells with conventional morphology, expected for their tissue origin;

II. Verification of the confluence percentage of the monolayer;

III. Presence of debris and/or loose and/or dead cells in the supernatant, which is indicative of application of unsatisfactory culture system or presence of chemical or microbiological contamination.

IV. Verification of the presence of cells with modified morphology to increase cell cytoplasm with irregular, serrated and ill-defined borders, increase in organelles or cytoplasmic inclusions, increase in cytoplasmic vacuoles that are signs of cellular senescence, or presence of micronuclei that are signs of apoptosis;

V. Verification of the presence of microorganisms, such as fungi and bacteria (including mycoplasmas); some contaminants can be observed under an optical microscope, but are easily mistaken for other debris. The ideal is to monitor the culture with specific tests to detect microbiological contamination.

Characterization of the cell population

The characterization of a primary culture is done by one or more characteristics of the population, such as gene expression, presence of plasma membrane proteins, cytoskeleton or secreted proteins, metabolic activity, functional capacity, among others. These procedures may include complex laboratory manipulation and, whenever possible, the method chosen should be the least expensive and the least complex, as long as it results in satisfactory information.

The population characterization is necessary, since the tissues are heterogeneous and contain several cellular types, with the possible exception of cartilage. The *in vitro* culturing can modify the original characteristics of the primary cell populations. In human bone marrow, for example, mesenchymal stem cells (MSC) account for 0.001%[20], while in adipose tissue they may reach 2%[21]. However, after the *in vitro* culturing and the expansion period, MSC may reach a percentage over 95%[22] regardless of the original tissue.

Many cell types have characteristics that can be recognized directly through colorimetric or fluorescence assays. These cells include lymphocytes and macrophages. These characteristics are determined with the use of antibodies that are specific for the proteins, lipids or membrane glycoconjugates generally defined with the cluster definition (CD) antigens, cytoskeletal proteins or nuclear components, by the presence of metabolic activities, or by the production of the extracellular matrix *in vitro*.

Other cell types require the combination of exclusion and inclusion of markers and/or methods for their characterization. Examples are hematopoietic stem cells and MSC cells. For MSC, a consensus was established among the researchers for the minimum requirements that the cell culture should present. These requirements were adopted by the ISCT and appear as follows: MSC must be plastic-adherent when maintained under standard culture conditions, more than 95% of the cells must express the CD73, CD90, CD105, but lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR markers, and the must have the ability to differentiate into cells of the osteogenic, chondrogenic and adipogenic lineages when exposed in vitro to the conditions[23].

Cell quantification and determination of the percentage of viable cells

Flow cytometry is currently a widely used technique for cell quantification associated with phenotyping because of its accuracy, reproducibility and traceability. It is used in clinical research and in diagnostic assays in the quantification of specific cell populations, which is granted by the commercial availability of kits for this purpose. The association of appropriate markers enables us to count and subdivide the cellular populations into living, dead, senescent and apoptotic cells, simultaneously. Annexin V, for example, is a protein that has high affinity for the cell membrane phospholipid phosphatidylserine, which is exposed in the early stages of apoptosis. Because of this characteristic, the conjugation of Annexin V to a fluorochrome enables its use as a marker for cells that are at the beginning of the apoptosis process. In addition, its use associated with nucleic acid dyes, such as propidium iodide or 7-aminoactinomycin (7-AAD), enables the identification of cells in the advanced phase of apoptosis or dead cells. Cells with intact membranes exclude these dyes and the damaged membranes are permeable.

Cells collected from bone marrow, peripheral blood or umbilical cord blood and placental blood expressing CD34, used for the transplantation of hematopoietic stem cells, are quantified and have their viability percentage determined by means of the flow cytometry technique of the 7-AAD cell viability marker, according to the SICT recommendation.

However, manual cell counting using the Neubauer chamber (or hemocytometer), is the method routinely used by cell culture laboratories because it is a fast, low cost, simple and direct method. The use of the Neubauer chamber, associated with Trypan blue exclusion dye, allows us to obtain the percentage of dead cells. Therefore, unless the determination of cells in apoptotic activity is necessary, the use of the Neubauer chamber and the Trypan blue dye enables us to obtain reliable results, the total number of cells and their percentage of viability. The accuracy of this technique is directly related to how careful the technician is in the preparation of the cell suspension and dilution of the sample.

Proliferative capacity: doubling time and cell cycle progression

The progressive increase in the generation time of cultured (or diploid) cells *in vitro* is expected, in accordance with the Hayflick limit[25]. Cells increase their generation time after an active replication period, regardless of whether other causes, like nutrient restriction or the presence of contaminants, contribute to this. However, this increase in generation time also occurs when the cells are in unsatisfactory growing conditions. Therefore, for advanced cell-based therapy products that undergo extensive handling, determining the potential for cell proliferation is critical. The generation time (G) can be calculated using the formula $G = 3.322 (\log Y - \log I)$, where (Y) is the total value of cells obtained with the subculture and (I) is the total value of cells used in the beginning of the culture.

Another way of accompanying the proliferative status of the cell culture is through the analysis of cell cycle progression, using the Vindelov method. This is a simple, quantitative, traceable and relevant method for comparing culture conditions by quantifying cells at various stages of the cell cycle. It uses the DNA content analysis of cells, identifying the G0/G1 phase stops, comparing them with cells in the active phase of DNA duplication (S phase), and with those that entered the G2 phase, already with the DNA fully duplicated. Quantification of the DNA/cell is done by flow cytometry[26,27,28].

Assays to determine the malignant potential of cultured cells

At present, there is as no scientific or medical consensus on the development of tumors in humans as a consequence of the use of normal human somatic cells that have undergone prolonged primary culture *in vitro*. Therapies may be categorized as safe, but cells that undergo extensive handling should be monitored for potentially malignant changes. Traditionally, the karyotype assay is performed to identify some type of genetic instability[29]. This assay should be considered a low sensitivity assay, since it analyzes a small number of cells and its relevance is therefore limited[30].

In a complementary approach, the soft-agar cell colony formation assay is used for the detection of clonal cell growth in agar gel, which does not support cell adhesion onto a solid substrate. With exception of lymphoid and myeloid cell lines, all normal human cells depend upon the anchoring on a solid substrate for their proliferation, and they cannot form colonies in soft-agar. The conversion of normal human cells into cancer cells results in acquisition of the growth capacity in a non-adherent gel[31]. Accordingly, soft agar culturing under clonogenic conditions can be applied to a relatively large sample of cultured cells, and the presence of a single colony may be indicative of the presence of a potentially neoplastic cell.

The positive result of the assay should be verified by the collection of the suspected clone and its potential identification as belonging to the hematopoietic lineages, which represents a false positive result. Staining with the traditional dyes for these cells, such as May-Grünwald-Giemsa or Peppenheim, is sufficient. It is understood that in the original harvest of the tissue to be cultured, the donor's blood may have been introduced into the culture, and some normal blood cell lineages could be maintained throughout the culture, producing a false positive result. On the other hand, endogenous occult cancer cells may also circulate in the blood of the donor as individual cells or as micro-metastases[32]. They will also be potentially identified by the soft-agar test. Their identification may be important for a new assessment and review of the cell donor's health in the absence of prior information about the presence of a neoplastic process. They will also be relevant when advanced therapy is directed, in an autologous context, to an application in repair of iatrogenic lesions associated with prior cancer treatment. That is the case of restoration of

extensive tissue ablations, such as a mastectomy, or treatment of difficult-to-heal lesions, such as tissue lesions following chemo or radiotherapy. The detection of circulating neoplastic cells in these cases may also be relevant for the review of health and therapeutic interventions in the patient.

Functional capacity

In addition to their proliferative ability that should ensure the number of cells for therapeutic use, the potential function of cells cultured *in vitro* to be used in advanced therapies depends on the properties relative to their origin and on the maintenance of their natural ability to organize, repair and regenerate the tissue throughout the culture. The need to determine the functional capacity of advanced therapy products containing cells cultured *in vitro* is unanimous for all regulatory agencies. However, the assays were not determined, neither were the acceptance thresholds for the expected results. It is recommended to apply the appropriate tests according to the intended use of the product. Examples of functional assays are those that assess the ability of stem cells to differentiate into osteogenic, chondrogenic, adipogenic, neurogenic or hematopoietic cells and those that assess the absorption of antigens by dendritic cells, among others. The interpretation must, however, be weighted according to the origin of the tissue and the characteristics of its expected actions in the patient. One of the examples is the aforementioned ISCT recommendation regarding the assays that are necessary for the evaluation of the populations of mesenchymal cells, proposed for use in therapies. When exposed to specific conditions, mesenchymal cells must differentiate into adipogenic, chondrogenic and osteogenic cells. However, for acceptance of their specific use, they should be able to differentiate into at least two of these three lineages[23].

The quality assays of cells that are handled *in vitro*, described in the previous sections, should be conducted under the responsibility of the Cell Processing Center, which should essentially guarantee the absence of potentially adverse effects on the patient in all therapies. On the other hand, the definitions of the functional properties of cells essentially address the issue of maintaining or modifying the expected therapeutic effects throughout the cell culture. Accordingly, the documentation accompanying the formal registration of an advanced therapy product should contain a detailed and complete description of the product's functional capacity control assays. The Cell Processing Center should receive and integrate these assays to its operational capacity, since they must be carried out in parallel with the cell quality assays, in all batches to be produced and made available for clinical use.

Proposal for the organization and conduction of quality assurance and functional capacity assays of cells for advanced therapies

Advanced therapy products based on cells cultured *in vitro* should be prepared in a standardized, critical, traceable and controlled manner to ensure their final quality. This should be done with two main purposes: (1) to guarantee the quality of the product, with the consequent safety of the treated patient and (2) to ensure the possibility of achieving the desired clinical and therapeutic outcomes. The agreement of researchers and regulatory agencies on this fact will result in a definition of the assays that should be applied specifically for each of the cell culture categories, as well as the acceptance thresholds for their results.

The Brazilian Health Regulation RDC n. 214/2018 provides for the Good Practices in Human Cells for Therapeutic Use and Clinical Research and applies to all Cell Processing Centers that are fit for the extensive handling of various types of human cells and for producing advanced therapy products. This new denomination includes bone marrow and peripheral blood processing laboratories, cord blood and placental blood banks and cellular technology centers. It defines the particularities for the supply of cell-based products and advanced therapy products, with the objective of ensuring the quality and safety of the products supplied for therapeutic use and clinical research and to minimize the risks to patients. These products comprise: (1) advanced cell therapy products; (2) tissue engineering products; and (3) gene therapy products consisting of or based on cells. These three categories will be released for use only after the individual analysis of each of them, associating the characteristics of the final product with the procedures used to obtain them. At that time, specific quality controls for each of them will be required, analyzed and applied. As the three product categories encompass cell manipulation, the quality of such manipulation will be required and verified specifically for each case. Throughout the development and release of new products and associated manipulation, these requirements can be used as precedents for establishing the quality parameters to be demanded. Cumulatively, they can become a valuable list of qualities to be required to ensure the reliability of advanced therapy products available to the Brazilian population. Public consultation n. 416/2017, which is being now analyzed by Anvisa, addresses the regulation of clinical assays for safety and efficacy purposes to be carried out with investigated advanced therapy products that need registration in Brazil and require the manipulation of cells and advanced therapy products at Cell Processing Centers authorized by Anvisa[33].

There is currently a consensus that the ability of cells to produce the benefits with advanced therapy products depends on the quantity of available cells[34]. The therapeutic efficacy of the product will depend on the conditions in which the cells are expanded *in vitro* and cryopreserved. Accordingly, the batch of cells to be used in a therapy may be obtained in parallel or in cumulative cultures over time, with their partial or full cryopreservation. This generates different batches, and each batch must be submitted to appropriate controls at the time of its release for use. This requirement will be met in three contexts, as described below.

(I) Reception, extensive manipulation and expansion of cells in primary culture, until the necessary amount for the therapeutic procedure is obtained, and their release for use. This is the procedure of autologous cell therapies with basic cell expansion (Figure 1).

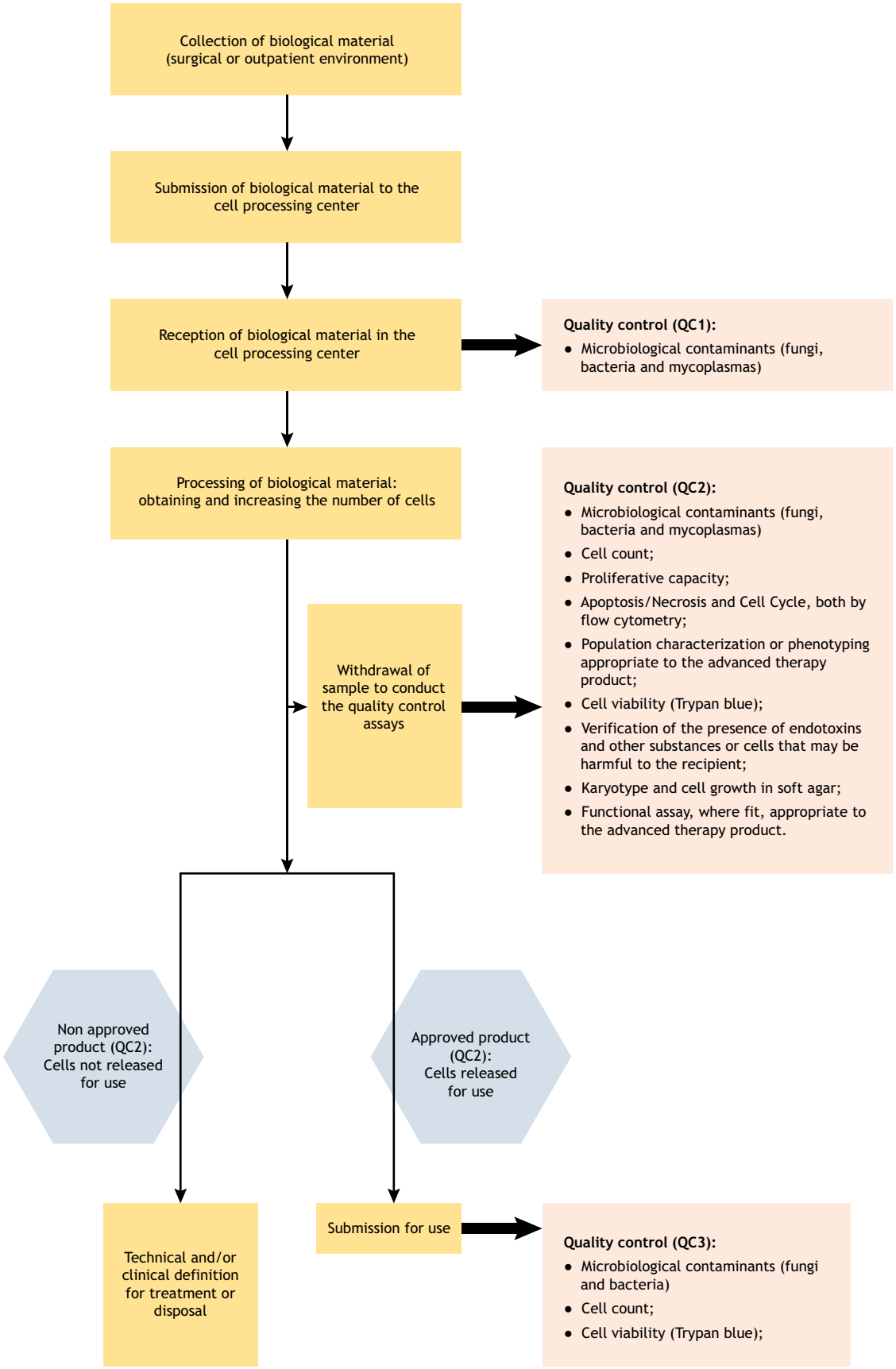

Figure 1. Flowchart of the processing of human biological material that undergoes extensive handling, followed by preparation and delivery of the cells for use (case I); indication of the sample withdrawal points for the conduction of quality control assays. QC: quality control

(II) Reception, extensive manipulation and expansion of cells in primary culture, until the required amount for the therapeutic procedure is obtained, followed by cryopreservation. The preserved batch will be used later at the appropriate time for therapeutic use, and in subsequent times, when repeated treatments may occur, using the same batch of cells. After thawing, cells will be maintained in culture for a limited time, to return to adequate viability and to appropriately prepare them for use with the administration vehicle (Figure 2).

(III) Reception, extensive manipulation and expansion of cells in primary culture, cryopreservation and storage of cells in a Master Bank for future use. Whenever necessary, a batch from the Master Bank will be thawed and then expanded again to be provided for clinical use or for further expansion of the Master Bank. In this case, each new expansion generates a new batch that must pass the required quality controls. It is the most complex procedure, which applies to the use of allogeneic cells producing paracrine mediators or associated with bioengineering products (Figure 3).

In all cases, the Quality Control 1 (QC1) should be performed at the reception of the biological material, in order to verify the presence of microbiological contaminants. For this assay, the vehicle sample used in the packaging of the material may also be used. A representative sample should be stored for future controls, if necessary.

Quality Control 2 (QC2) is the main quality control of cells that are manipulated and expanded *in vitro*. It will be performed along the primary cell expansion, concluding it at the time of its release for use or for cryopreservation. It consists of the following list of tests:

- Cell count;
- Proliferative capacity;
- Apoptosis/Necrosis and Cell Cycle, both by flow cytometry;
- Population characterization or phenotyping appropriate to the advanced therapy product;
- Cell viability (Trypan blue);
- Impurities: this includes verification of the presence of endotoxins and other substances or cells that may be harmful to the recipient;
- Microbiological control (contaminant fungi, bacteria and mycoplasmas);
- Karyotype and Cell Growth in soft agar (*in vitro* assay for detection of possible presence of cancer cells);
- Potency: Functional assay, whenever applicable, appropriate to the final advanced therapy product: the relevant biological activity of cells, if known, or products synthesized by the cell should be determined and quantified. Failure to perform this test should be justified.

In the case I (Figure 1), the cells should be tested for the quality control assays listed above before their release for use. If the results have not been released before their preparation for use, and their use is recommended for clinical reasons, the responsible physician must be aware of that, and take responsibility for the release.

In the case II (Figure 2), cells should be tested for the quality control assays listed above prior to the cryopreservation (prior to the addition of cryoprotectants); representative cell sample(s) of the cryopreserved batch(es) should be tested for the presence of contaminants (fungi, bacteria and mycoplasmas), cell counts and viability (with use of Trypan blue exclusion dye).

In case III (Figure 3), the cells should be tested for the quality control assays listed above, prior to the cryopreservation process (prior to the addition of cryoprotectants); and representative cell sample(s) of the cryopreserved batch(es) should be tested for the presence of contaminants (fungi, bacteria and mycoplasmas), cell counts and viability (with use of Trypan blue exclusion dye). For each expansion step (Master Bank expansion), the cells should again undergo the quality control assays listed above prior to the cryopreservation process (prior to the addition of cryoprotectants); and representative cell sample(s) of the cryopreserved batch(es) should be tested for the presence of contaminants (fungi, bacteria and mycoplasmas), cell counts and viability (with use of Trypan blue exclusion dye).

Figures 1 to 3 show the flowcharts of human biological material processing for obtaining the primary cell culture and preparation of the advanced therapy product. They indicate the sampling points for performing the quality control assays for cases I, II and III, as described above.

In the release of the cells for use, in any of the situations, the prepared cells should be tested for the presence of microbiological contaminants, fungi and bacteria, in addition to being quantified and having their percentage of viability determined.

In conclusion, we recommend following the listed controls in order contribute to the quality of the final product offered for patient's use, and to promote the comparability of the clinical results obtained with advanced therapy products using cells cultured *in vitro*. These products should be prepared in the form of batches, and should be necessarily checked for quality, with the conduction of the following assays: Cell count; Proliferative capacity; Apoptosis/Necrosis and Cell Cycle; Population characterization or phenotyping appropriate to the advanced therapy product; Cell viability (Trypan blue); Presence of endotoxins; Microbiological control (contaminant fungi, bacteria and mycoplasmas); Karyotype and Cell Growth in soft agar; and Functional testing, whenever applicable, appropriate to the advanced therapy product.

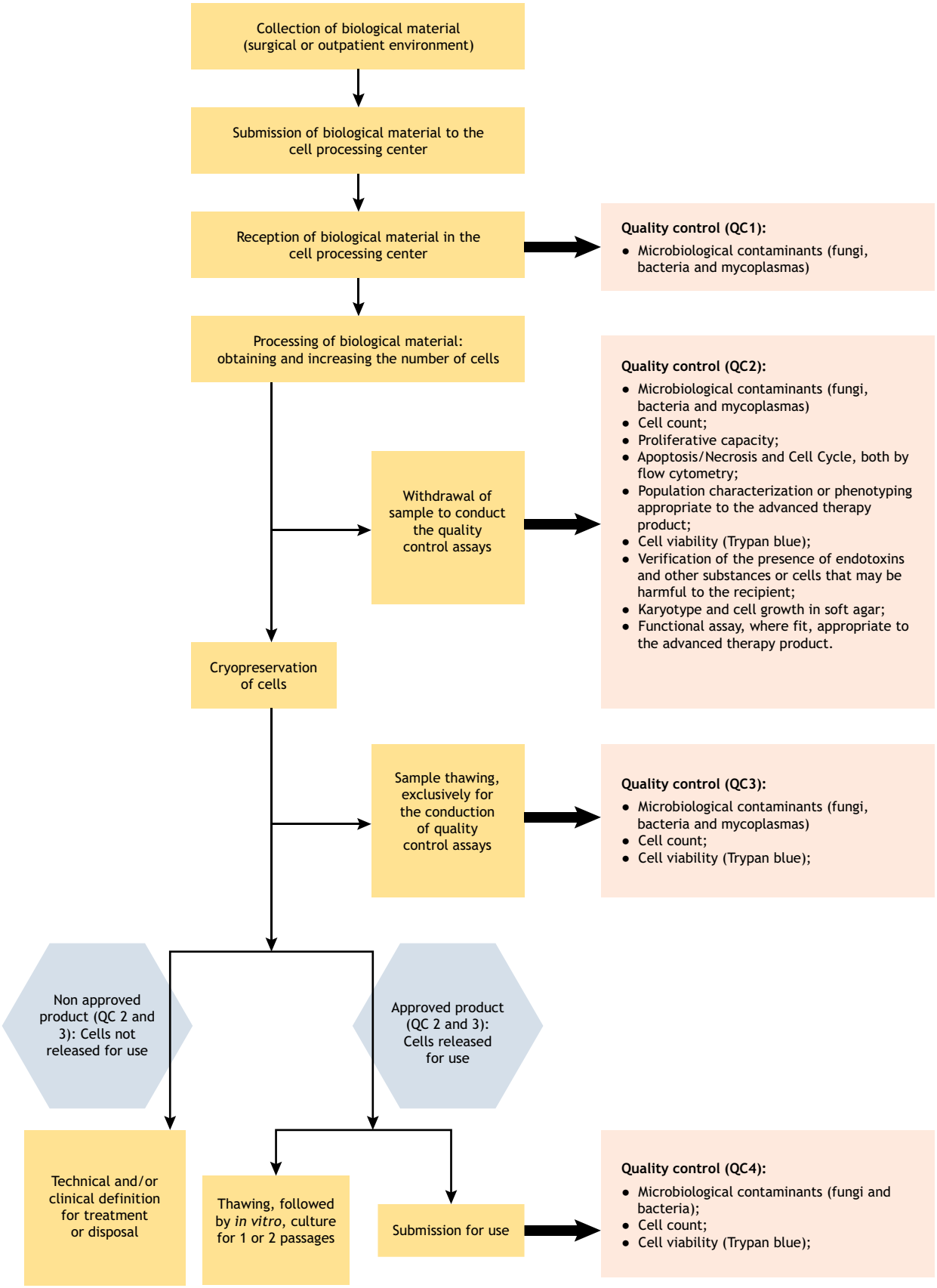

Figure 2. Flowchart of the processing of the human biological material that undergoes extensive handling, followed by cryopreservation of the cells. These cells may be thawed immediately for use or thawed for return to the *in vitro* culture, by one or two passages. They are then prepared and sent for use (case II); indication of the sample withdrawal points for the conduction of quality control assays. QC: quality control.

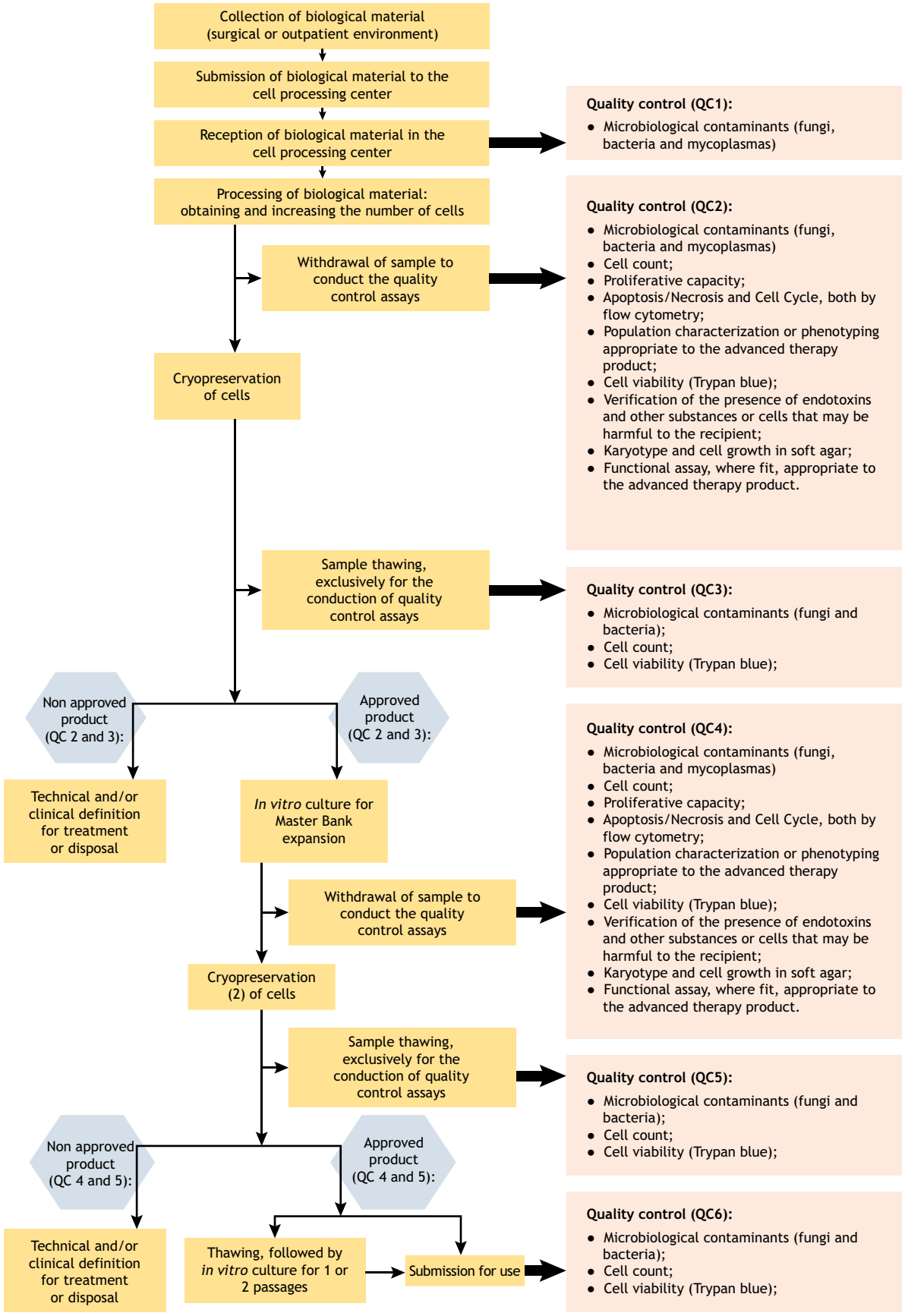

Figure 3. Flowchart of the processing of human biological material that undergoes extensive handling, followed by cryopreservation of the cells, thawing and a second expansion stage (Master Bank expansion). They are then prepared and sent for use (case III); indication of the sample withdrawal points for the conduction of quality control assays. QC: quality control.

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Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.