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Kursad Turksen *Editor*

Stem Cells and Good Manufacturing Practices

Methods, Protocols, and Regulations

Second Edition

Humana Press

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Preface

Great strides have been made in the field of stem cells with respect to their isolation, characterization, maintenance, expansion, and potential for tissue regeneration and repair, but utilizing and delivering these cells for therapeutic purposes in patients in the clinic require very tightly controlled good manufacturing practices (GMP). This need has driven tremendous advances as well. In this second edition of the *Stem Cells and Good Manufacturing Practices* volume, I have brought together a new set of protocols to arm stem cell biologists with protocols that are currently being used in a number of well-established facilities around the world. I am hoping that people in the field will benefit from this compilation, and that the volume will drive continued improvements to GMP and accelerate new stem cell treatments for patients in need.

Once again, the protocols gathered here are faithful to the mission statement of the *Methods in Molecular Biology* series: They are well established and described in an easy-to-follow step-by-step fashion so as to be valuable for not only experts but also novices in the stem cell field. That goal is achieved because of the generosity of the contributors who have carefully described their protocols in this volume, and I am very grateful for their efforts.

My thanks as well go to Dr. John Walker, the Editor-in-Chief of the *Methods in Molecular Biology* series, for giving me the opportunity to create this volume and for supporting me along the way.

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Ottawa, ON, Canada ***Kursad Turksen***

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Swiss Fetal Transplantation Program and Non-enzymatically Isolated Primary Progenitor Cell Types for Regenerative Medicine

Alexis Laurent, Corinne Scaletta, Nathalie Hirt-Burri, Wassim Raffoul, Anthony S. de Buys Roessingh, and Lee Ann Applegate

Abstract

Primary progenitor cell types adequately isolated from fetal tissue samples present considerable therapeutic potential for a wide range of applications within allogeneic musculoskeletal regenerative medicine. Progenitor cells are inherently differentiated and extremely stable in standard bioprocessing conditions and can be culture-expanded to establish extensive and robust cryopreserved cell banks. Stringent processing conditions and exhaustive traceability are prerequisites for establishing a cell source admissible for further cGMP biobanking and clinical-grade production lot manufacture. Transplantation programs are ideal platforms for the establishment of primary progenitor cell sources to be used for manufacture of cell therapies or cell-based products. Well-defined and regulated procurement and processing of fetal biopsies after voluntary pregnancy interruptions ensure traceability and safety of progeny materials and therapeutic products derived therefrom. We describe herein the workflows and specifications devised under the Swiss Fetal Progenitor Cell Transplantation Program in order to traceably isolate primary progenitor cell types in vitro and to constitute Parental Cell Banks fit for subsequent industrial-scale cGMP processing. When properly devised, derived, and maintained, such cell sources established after a single organ donation can furnish sufficient progeny materials for years of development in translational musculoskeletal regenerative medicine.

Keywords Cell therapy, Clinical cell banking, GMP manufacturing, Organ donation, Progenitor cells, Protocols, Transplantation program

Abbreviations

ATMP	Advanced therapy medicinal product
cGMP	Current good manufacturing practices
CMV	Cytomegalovirus
CPMP	European Union Committee for Proprietary Medicinal Products
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
D-PBS	Dulbecco's phosphate-buffered saline
EBV	Epstein-Barr virus
EC	European Commission
ECACC	European Collection of Authenticated Cell Cultures

ECL	Electrochemiluminescence
EDQM	European Directorate for the Quality of Medicines and HealthCare
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELFA	Enzyme-linked fluorescence assay
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	US Food and Drug Administration
FISH	Fluorescence in situ hybridization
GLP	Good laboratory practices
GMP	Good manufacturing practices
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
hCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HE	Hematoxylin and eosin
HHV-6/7/8	Human herpes viruses types 6, 7 and 8
HIV-1/2	Human immunodeficiency viruses types 1 and 2
HTLV-1/2	Human T-cell leukemia-lymphoma viruses types 1 and 2
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPC	In-process control
MCB	Master Cell Bank
PCB	Parental Cell Bank
PCR	Polymerase chain reaction
PS	Penicillin-streptomycin
QFPERT	Quantitative fluorescent product-enhanced reverse transcriptase
QRM	Quality risk management
RNA	Ribonucleic acid
SOP	Standard operating procedure
STD	Sexually transmissible disease
TPHA	*Treponema pallidum* hemagglutination assay
VDRL	Venereal Disease Research Laboratory
WCB	Working Cell Bank

1 Introduction

Numerous considerations and careful planning characterize pragmatic selection of optimal cell sources in view of cell therapy, tissue bioengineering, or cell-based product development. Process robustness and technological simplicity are paramount in obtaining extensive and homogenous quantities of therapeutic cells to be clinically administered or further processed. Traceability and documented quality consistency of regenerative medicine products ensure safety and efficacy for the end patient and must therefore

be continuously maximized [1–3]. Indeed, such parameters necessitate iterative optimization from the inception of a project, starting well before the actual therapeutic cell source is identified, procured, and processed.

Transplantation programs are highly regulated and devised in particular ways that optimally suit the identification and processing workflows of a potential therapeutic cell source. Such platforms are ideal in view of establishing primary progenitor cell sources to be used for manufacture of cell therapies or cell-based products [4–13]. Stringent quality requirements and specifications for raw materials and components, defined processing workflow conditions, and exhaustive traceability are indeed prerequisites for cGMP (current good manufacturing practices) biobanking and clinical-grade production lot manufacture of ATMPs (advanced therapy medicinal products), somatic cell therapies, or combination products, for example [14].

Primary progenitor cell types derived from fetal biopsies during voluntary pregnancy terminations and following specific methods are terminally differentiated and highly stable and enable rapid establishment of extensive and robust cryopreserved cell banks [15]. Multiple organs can be simultaneously harvested and processed for isolation of distinct cell types. Non-enzymatically isolated primary dermal progenitor fibroblasts constitute epitomes of therapeutic cell source optimization and present safe and efficient substrates for cGMP biobanking [16–19]. Trophic paracrine stimulation and extracellular matrix enhancement yielded by therapeutic doses of progenitor fibroblasts have been proven to drastically ameliorate clinical outcomes of patients suffering from wide arrays of acute and chronic cutaneous affections [12, 15, 16, 18]. Advantageous biocompatibility and inherent immune privilege aspects of progenitor cells are complemented by relatively low technical manipulation requirements, high robustness, and industrial scalability [20, 21].

Considering primary dermal progenitor fibroblast sources established as cryopreserved Parental Cell Banks (PCBs), optimal standardized cGMP manufacturing workflows can be devised in order to serially develop extensive consistent Master Cell Banks (MCBs) and multi-tiered Working Cell Banks (WCBs) to be applied in allogenic cell therapy protocols or to furnish progeny materials for medical device and cell-based product development [12, 15]. One single organ donation suffices for non-enzymatic isolation of various progenitor cell types (e.g., dermal fibroblast progenitors, chondroprogenitors, tenocyte progenitors, osteoprogenitors, etc.) and preliminary culture steps, subsequently serving for derivation of progeny cellular materials to be used for many years of translational development [12, 13, 15, 21]. We firstly describe herein the workflows and specifications devised under the Swiss Fetal Progenitor Cell Transplantation Program in order

to traceably isolate primary musculoskeletal progenitor cell types in vitro and to constitute Parental Cell Banks fit for subsequent cGMP processing. Secondly, an optimized selection, isolation, and biobanking method is comprehensively described herein for primary dermal progenitor fibroblasts, allowing for efficient and rapid obtention of high therapeutic value, safe, and consistent cryopreserved banks of progeny cells robustly derived from a single organ donation. Such standardized biological sources are ideally fit for integration in industrial manufacturing campaigns of diverse therapeutic regenerative medicine products, may serve as efficient substrates for vaccine production, or act as potent feeder layers.

2 Materials

All considered materials, consumables, and process or product components should be sterile and GMP cell culture grade. Facilities and equipment are to be qualified and validated appropriately within applicable cGMP guidelines. Waste disposal regulations are to be followed diligently.

2.1 Biological Starting Materials

Whole-fetus donation (without the placenta) and tissue procurement should occur 12 weeks post-amenorrhea (14 weeks of gestation) with the mother donor undergoing a legal, voluntary, and therapeutic pregnancy interruption. Identification of the mother donor and related processing should be defined in a transplantation program, as defined hereafter. Detailed requirements and specifications about the donor and the donation are listed further in Subheadings 3.4–3.6.

2.2 Raw Materials

Raw materials are defined as materials which are present as an appreciable part of the final product when considering specific manufacturing lots. The following raw materials are required for the obtention of PCB cryopreserved vials.

1. DMEM, GMP grade, high glucose, with pyruvate, with phenol red, store at 4 °C until use.
2. FBS, GMP grade, with certificate of animal origin and health, certificate of analysis, irradiation certificate, batch manufacturing record, store at −80 °C until use.
3. DMSO, GMP cell culture grade, store at room temperature until use.

2.3 Ancillary Materials

Ancillary materials are defined as materials which are used and subsequently removed during the manufacturing process, therefore not constituting an appreciable part of the final product. The following GMP grade ancillary materials are required.

1. TrypLE™ Select Enzyme, 1×, sterile, without phenol red, store at room temperature until use.
2. D-PBS, sterile, no calcium, no magnesium, store at room temperature until use.
3. Penicillin-streptomycin, 10^4 U/mL and 10^4 μg/mL, cell culture grade, store at −20 °C until use.

2.4 Contact Process Consumables

Contact process consumables come into direct contact with cell cultures or cell suspensions during the manufacturing process. Store consumables at room temperature until use. The following GMP grade contact process consumables are required.

1. Insulated tissue container with wet ice, sterile, with lock and key
2. T75 sterile cell culture flasks, 75 cm^2, uncoated culture surfaces, with filter screw caps
3. Tissue culture Petri dishes, 10 cm diameter, uncoated culture surfaces
4. Sterile scalpels
5. Sterile forceps
6. Sterile surgical scissors
7. Aspiration pipets, sterile
8. Serological pipets, sterile, 5, 10, and 35 mL, with cotton filters
9. Sterile culture-grade conical centrifuge tubes, 15 mL, 50 mL
10. Sterile culture-grade containers, 500 mL, 1000 mL

2.5 Non-contact Process Consumables

Non-contact process consumables do not come into direct contact with cell cultures or cell suspensions during the manufacturing process. Store consumables at room temperature until use. The following non-contact process consumables are required.

1. Trypan blue solution, 0.4%, filter-sterilized
2. Hemocytometer, Neubauer improved model
3. Micropipette filter tips, 20, 200, and 1000 μL, sterile
4. Microcentrifuge tubes, cell culture grade, sterile
5. Denatured 70% ethanol, in vaporizing bottles
6. Permanent markers, heat, ethanol, and liquid nitrogen resistant

2.6 Product Container System Components

Container system components comprise, in addition to the biological material and cryopreservation solution, the elements constituting a deliverable product unit. The following components are required for the obtention of PCB cryopreserved vials.

1. 1.8 mL cryovials, GMP grade, with internal threading, with star-foot
2. Cryogenic adhesive labels, liquid nitrogen resistant

2.7 Critical Process Facility and Equipment

The critical process facility comprises the production suite and storage unit, along with the installed equipment, instruments, and devices. Wherever applicable, equipment, instruments, and devices should be calibrated and appropriately qualified. The following GMP infrastructure, equipment, instruments, and devices are required.

1. Clean room facility comprising the production suite, B grade, and the storage unit
2. Biological safety cabinet (sufficiently wide for two operators), A grade, vertical laminar flow
3. Complete sterile clean room personal clothing, suit, mask, hood, gloves, boots
4. CO_2 incubators, temperature range 36.5 °C ± 1.5 °C, relative humidity range 80% ± 10%, CO_2 content range 5% ± 1.5%, with conserved distilled water
5. Liquid nitrogen Dewar storage tanks, level alarm fitted
6. Ultra-low temperature freezer, temperature range −80 °C ± 5 °C
7. Freezer, temperature range −20 °C ± 2 °C
8. Refrigerator, temperature range 4 °C ± 2 °C
9. Controlled rate freezing devices, −1 °C/min rate of freezing, CoolCell® LX, CoolCell® FTS30 or Nalgene® Mr. Frosty™, with isopropanol
10. Centrifuge, operational range 230 × *g* ± 10 × *g*, ambient temperature, swing-out buckets with adaptors, 15 mL and 50 mL tubes
11. Optical inverted microscope, 40×, 100×, and 400× optical magnification with phase contrast
12. Binocular magnifying stereo microscope, 10× and 40× optical magnification
13. Water bath, temperature range 37 °C ± 2 °C, with distilled water
14. Aspiration pump, handheld aspiration pipet mount, with tubing
15. Motorized pipet for serological pipet manipulation
16. Micropipettes, 20, 200, and 1000 μL, calibrated
17. Digital timer
18. Cryotube racks, with star-foot adaptors
19. Sterile stainless steel surgical trays

3 Methods

3.1 Generalities About Methods

1. Proper ethical considerations and validations should be obtained before transplantation program initiation. This aspect is covered in Subheading 3.3.
2. Applicable cGMP procedures and recommendations are always to be followed (*see* **Note 1**).
3. Ensure quality risk management (QRM) systems and in-process controls (IPC) are adequately implemented.
4. Legal retention samples (raw and starting materials, in-process control samples, and representative production lot samples), reports, and records shall be constituted and stored appropriately along with full traceability of all manipulations, deviations, and corrective measures.
5. All manipulations requiring the cultures or cell suspensions to be exposed (open dishes, flasks, tubes, or vials) are to be conducted in the class A biological safety cabinet.
6. All materials and equipment entering the production area and biosafety cabinet are to be properly sanitized.
7. Necessary documents and labelling materials are to be prepared before processing operations begin.
8. All media, solutions, or buffers to be warmed at 37 °C are to be placed in a water bath that has been equilibrated at 37 °C for at least 1 h.
9. Procedures are to be carried out at room temperature, unless otherwise specified.

3.2 Transplantation Program Organigram Setup

A defined organigram is necessary for setting up the transplantation program, as varying degrees of compartmentalization need to be ensured (Fig. 1). The following parties must be identified along with their respective responsibilities, and the program must be organized and agreed upon among them.

1. Program manager, with extensive experience in tissue processing and cell banking. Program establishment and coordination, responsible person for the laboratory (*see* **Note 2**)
2. Technical manager, with extensive experience in tissue processing and cell banking. Biopsy processing and primary cell type establishment (*see* **Note 3**)
3. Legal advisor. Support in establishment of the transplantation program within the appropriate legal frameworks and in accordance with Ethics Committee protocols (*see* **Note 4**)
4. Medical doctor, gynecologist with experience in pregnancy interruptions. Donor screening, eligibility establishment, donation procurement, and coding implementation (*see* **Note 5**)

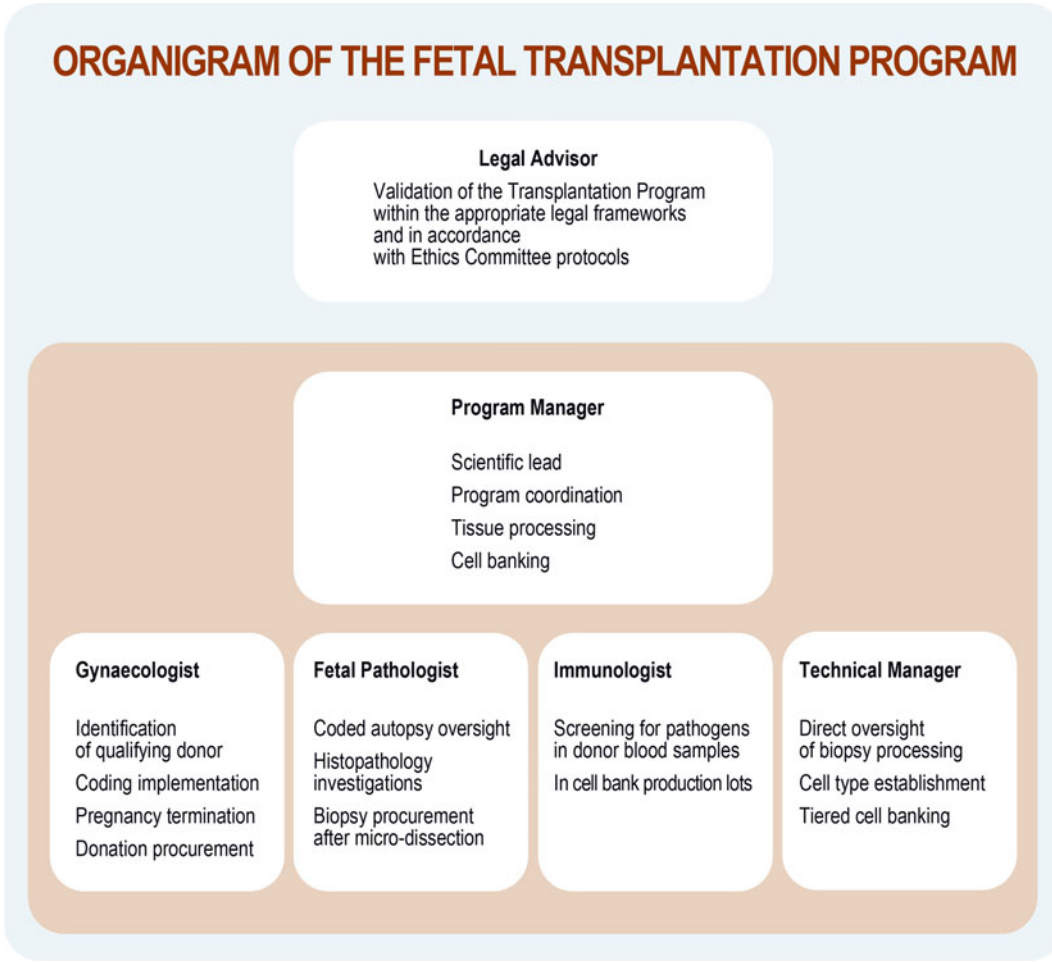

Fig. 1 Organigram of a fetal transplantation program. Close interdisciplinary collaborations and pooling of complementary specialist competences allow for optimal design and implementations of the program. Appropriate compartmentalization between the medical team and the other stakeholders ensures preservation of donor and donation anonymity

5. Fetal pathologist with extensive experience in histopathology. Coded autopsy, histopathology, and biopsy procurement after micro-dissection (*see* **Note 6**)
6. Immunologist. Screening for pathogen detection in donor blood samples and cell bank materials

3.3 Validation of the Transplantation Program

In view of proper and rapid acceptance of the transplantation program by the appropriate authorities, several documents or procedures should be prepared and submitted for validation before initiation of the program. The first document to establish is the Technical Specifications, describing the whole medical and technical parts of the Program, from the donor selection, donation

procurement, and processing to progeny cellular material creation methodologies and workflows. The Technical Specifications should at least cover the following aspects.

1. Definition of the purpose of the transplantation program, the necessary operations, and personnel (*see* **Note 7**)
2. Description of the quality systems organization, specifications, testing, and validations, including the documentation system
3. Description of the specific project, comprising purpose and precise process methods and workflows (*see* **Note 8**)
4. Description of the environmental controls, monitoring, and inspection
5. Description of starting and raw materials, including specifications, testing, and supply chains
6. Description of production systems, including facility, equipment, consumables
7. Description of testing and specifications for the final product (cryopreserved PCB vials) and provisional product usage
8. Index of manufacturing documentation (*see* **Note 9**)

The second important documentation to establish is the Regulations of the Fetal Biobank, in the form of a binding legal framework for the establishment and traceable use of progeny cellular materials derived from the initial donation. The Regulation document should at least cover the following aspects.

1. Scope of application, with reference to the local, state, and national laws and regulations pertaining to organ donations and processing of clinical biological materials
2. Definitions, including the notions of biobank, data, donation, donor, samples, materials, anonymization, and coding
3. Description of the organization of the biobank, comprising the responsible persons, the materials and quality specifications of the materials admitted to the biobank, as well as financing and administration of the latter
4. Description of the admission conditions to the biobank for a donation, progeny materials, and related anonymized data
5. Description of the conservation parameters and requirements of materials and all related data and documentation
6. Description of quality and safety standards to uphold, comprising the quality systems organization, validation, and documentation system
7. Description of the framework for the use of materials in defined projects, comprising the research or clinical applications, the guaranteed irreversible anonymization of materials and data, and the validation of individual projects by the proper authorities

8. Description of the framework for the liberation of materials to other biobanks, including the full traceability of materials, data, and the respective responsibilities of recipients (*see* **Note 10**)
9. Description of mother donor rights and management of mother donor identity

The third important documentation segment to establish is the formal authorizations for storage of the materials, along with import and export licenses, shipping notices, and provisional insurance coverages, if the materials are to be sent abroad. Altogether, the Technical Specifications, Biobank Regulations, and license documents allow for efficient and sound evaluation of the transplantation program by the ethics and regulatory authorities, which need to provide consent to the program as a whole before its initiation.

3.4 Donor Population Screening

Once the appropriate validations have been obtained, initiate the transplantation program with the search for a donor. Screening of potential donors enables the selection of a qualifying donor for the program (Fig. 2). The medical doctor uses information notices, verbal information reviews, and specific consent forms during patient interviews programmed in view of voluntary and therapeutic pregnancy interruptions (*see* **Note 11**). Qualifying donors meet the following criteria.

1. Pregnancy resulting from natural insemination
2. Donor undergoing voluntary and therapeutic pregnancy interruption following local and national legal frameworks
3. Female sex and gender of the donor
4. Donor age between 18 and 25 years
5. Gestational age of 14 weeks (12 weeks post-amenorrhea)
6. Donor of local nationality and residence
7. Good overall health of donor (*see Questionnaire for Health*)
8. Donor not suffering any apparent chronic diseases
9. Donor tests negative for HIV, HBV, and HCV markers
10. Donor not on any anti-inflammatory treatment during the last 6 months
11. Donor capable of giving clear informed consent
12. Donor can be reached 3 months after the donation for additional blood sampling, testing, and consent confirmation
13. Donor consents to genetic and pathological testing to be performed on the donation

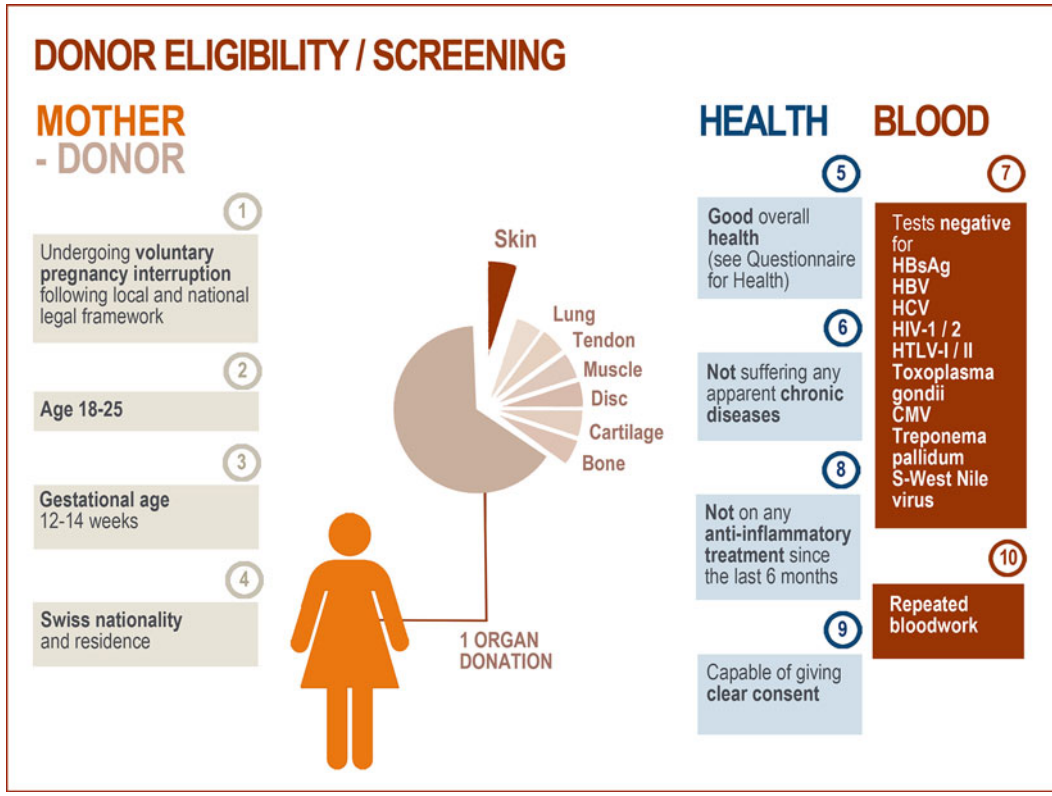

Fig. 2 Donor specifications and screening for inclusion in the transplantation program. The defined parameters and workflows for selection enable the eventual identification of a qualifying donor by the medical doctor. Identified donor screening then allows for evaluation of the quality and safety of the donation, to be confirmed by bloodwork and pathological examination. Methodology for donor inclusion is of paramount importance, as well as the reconsidering periods (renewed bloodwork after 3 months) enabling informed and definitive consent. A single organ donation allows for parallel establishment of various primary progenitor cell types to be used for extensive tiered cell banking and subsequent diversified applications in regenerative medicine or biotechnological manufacturing fields

The anonymous questionnaire for health should at least comprise the following open and closed question elements. The interpretation of the completed questionnaire and eligibility for inclusion in the program are at the discretion of the medical doctor in charge.

1. Is the patient/donor in good overall health?
2. During the last month, did the patient/donor take drugs or medication (specify)?
3. Has the patient/donor ever received immunotherapy (human or animal cells or serum, specify)?
4. Has the patient/donor received vaccines in the last 4 weeks (specify)?

5. Does the patient/donor suffer or has suffered from one or several of the following: circulatory affections, blood pressure problems, cardiac problems, a heart surgery, a stroke, a heart attack, respiratory problems, loss of consciousness, skin disease, allergies, asthma, eczema, hay fever, diabetes, kidney problems, epilepsy, cancer, blood disease, nervous system problems, vascular problems, gonorrhea, or other STDs (specify)?
6. Has the patient/donor been the recipient of human or animal organ transplants (specify)?
7. Was a member of the patients'/donors' family ever been diagnosed with Creutzfeldt-Jakob disease?
8. Has the patient/donor travelled abroad in the previous 6 months (specify)?

3.5 Identified Donor Consent and Screening

Once a potential donor has been identified and eligibility is confirmed by the medical team, the latter shall proceed strictly as follows.

1. Obtain oral and written informed consent for voluntary pregnancy interruption, assorted to a 24-h period of reconsideration.
2. Obtain oral and written informed consent for inclusion of the donor in the transplantation program, for use of fetal organs for research and clinical applications, and for extensive bloodwork and pathological testing, assorted to a 24-h period of reconsideration (*see* **Note 12**).
3. Acquire the following materials from the de-identified donor, along with the coded identity of donor and date and time of material procurement for testing:
   - Four distinct EDTA tubes of venous blood, 5.5 mL tubes
   - Two distinct serum tubes, 10 mL tubes

Transfer the testing material immediately and appropriately for processing, as PCR assays must be performed in the following 6 h. Perform the following serological testing on the mother donors' material at the time of donation and repeat after 3 months to exclude sero-conversion.

1. HBsAg detection by ECL Immunoassay. Specification: negative result
2. HBV core antibody, total, detection by ECL Immunoassay. Specification: negative result
3. HCV antibody, total, detection by ECL Immunoassay. Specification: negative result
4. HCV RNA, detection by PCR. Specification: negative result

5. HIV-1/2 antibodies and HIV Ag (fourth generation), detection by ECL Immunoassay. Specifications: negative results
6. HIV-1 antigen p24, detection by ECL Immunoassay. Specification: negative result
7. HTLV-1/2 antibodies detection by EIA. Specifications: negative results
8. *Toxoplasma gondii* antibody, IgG, detection by ECL Immunoassay. Specification: 1–3 UI/mL
9. *Toxoplasma gondii* antibody, IgM, detection by ECL Immunoassay. Specification: Index 0.8–1.0
10. CMV antibody, IgG, detection by ELFA. Specification: 4–6 EU/mL
11. CMV antibody, IgM, detection by EIA. Specification: Index 0.9–1.1
12. Syphilis, TPHA-VDRL, Ig, detection by immunoassay. Specification: Titer <80 U
13. S-West Nile virus antibody, IgG, detection by ELISA. Specification: negative result
14. S-West Nile virus antibody, IgM, detection by ELISA. Specification: negative result
15. Zika virus antibody, IgG, detection by ELISA. Specification: negative result
16. Zika virus antibody, IgM, detection by ELISA. Specification: negative result

Interpretation of the test results is at the discretion of the immunologist and the medical doctor in charge. In the case of test results out of specifications at the time of donation or after repetition at 3 months, the materials derived from the donation must be destroyed. In the case of a modification of the donors' consent for inclusion in the transplantation program at that time, the materials derived from the donation must also be destroyed.

3.6 Donation Procurement

Once the donor has been identified and has provided appropriate consent, proceed with initiation of the medical and technical part of the program, and procure the donation (*see* **Note 13**).

1. Record date of birth of the coded donor.
2. Initiate the pregnancy interruption protocol (*see* **Note 14**).
3. Record date and time of the coded donation (umbilical cord severing).
4. Rinse the whole donation thoroughly with sterile physiological solution.

5. Cover the whole donation with gauze, and humidify the latter with sterile physiological solution.
6. Place donation in a sterile double-layered plastic bag, wrap the bag in a sterile bed pad, and store on wet ice in a sterile insulated locked transport container.
7. Immediately release the container for transport to the pathology department (<2 h from the time of donation).

3.7 Donation Processing

Once the transport container arrives to the pathology laboratory, immediately proceed as follows.

1. Aseptically unwrap and transfer the donation to a large sterile culture plate in a grade A biological safety cabinet.
2. Sequentially cleanse all exposed surfaces of donation with sterile physiological solution and 70% denatured ethanol.
3. Determine weight and size of donation, confirm macroscopic and microscopic normality, and confirm the gestational age and sex (Fig. 3a).
4. Using sterile surgical tools, perform a full autopsy, with isolation of specific tissues for histology. Tissues, organs, and structures to be specifically investigated during gross pathology examination comprise, i.e., gonads, hypophysis, pancreas, diaphragm, muscles, ribs, vertebrae, thymus, spleen, thyroid, trachea, liver, biliary vesicle, umbilical cord, bladder, kidneys, suprarenal glands, stomach, esophagus, intestines, lungs, heart, tibias, and brain (*see* **Note 15**). Confirm high quality of tissues and no gross abnormalities of donation.
5. Isolate specific tissue sections for conservation at −80 °C (*see* **Note 16**).
6. Isolate 2–4 cm^2 or 1–5 mm^3 of the tissues of interest during microdissection (*see* **Note 17**) (Fig. 3b).
7. Record date and time of tissue isolation.
8. Condition the isolated tissues in properly annotated culture grade centrifuge tubes filled with sterile D-PBS, and store the tubes locked at 4 °C until liberation.
9. Release the biopsied tissues for processing in the cell culture facility (<4 h after reception of the donation), and ensure transport at 4 °C.

3.8 Media Preparation

For Conserved Medium preparation, proceed as follows:

1. Prepare Conserved Medium by diluting penicillin-streptomycin (PS) in sterile D-PBS, so that the final proportion of PS is 1%. Store Conserved Medium at 4 °C until use, and use within 21 days after preparation.

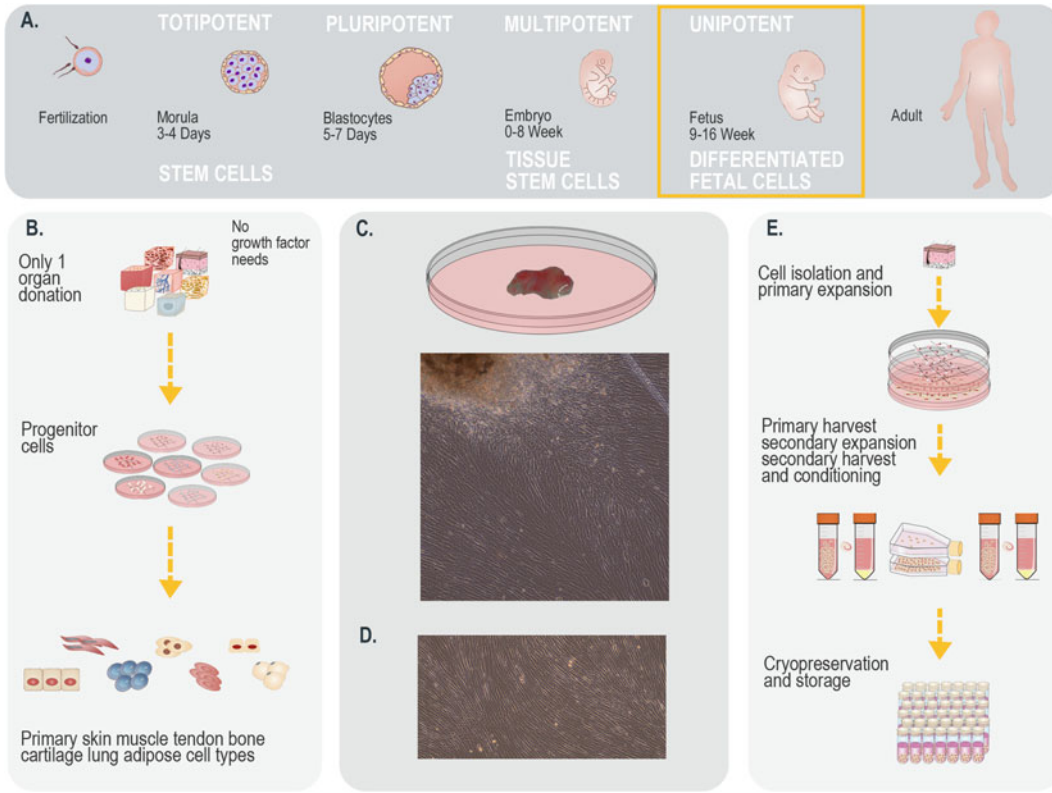

Fig. 3 Overview of fetal donation procurement, primary progenitor cell isolation, and subsequent banking thereof. (**a**) Human developmental continuum with associated relative potency of isolated cell classes. Fetal progenitor cells are isolated within a specific timeframe and are differentiated, unlike stem cells. (**b**) Multiple and parallel tissue-specific culture initiation of progenitor cell types forms one organ donation. Mechanical dissociation is used to produce adequate fragments for cell outgrowth while avoiding deleterious effects of enzymatic tissue treatment. Unlike stem cells, progenitor cells do not require growth factor cocktails to support their growth. (**c**) Photographic imaging of fetal skin tissue after procurement by the pathologist, culture initiation with an initial <2 cm^2 skin tissue biopsy, and primary progenitor dermal fibroblasts emitting in vitro from the biopsy fragments on the treated culture dish. A primary expansion in tissue culture dishes allows for isolation of a primary cell population (Passage 0). (**d**) Confluent progenitor fibroblasts at Passage 1, imaged after the secondary expansion in culture flasks, at the time of harvest for PCB establishment (40$\times$ optical magnification). Progeny cells were characterized and authenticated by ECACC (European Collection of Authenticated Cell Cultures) under the reference code FE002-SK2. (**e**) Overview of primary cell type non-enzymatic isolation and GMP PCB establishment. Harvest of the emitting Passage 0 cells and secondary expansion of Passage 1 cells in culture flasks allow for generation of a large and homogenous cell population

For Complete Medium preparation, proceed as follows:

1. Prepare Complete Medium by diluting FBS in DMEM, so that the final volumetric proportion of FBS is 10% and the final volumetric proportion of DMEM is 90%. Store Complete Medium at 4 °C until use, and use within 21 days after preparation.

For Enriched Complete Medium preparation, proceed as follows:

1. Prepare Enriched Complete Medium by diluting L-glutamine 200 mM 100-fold in Complete Medium. Store Enriched Complete Medium at 4 °C until use, and use within 21 days after preparation.

For Cryopreservation Medium preparation, proceed as follows:

1. Prepare Cryopreservation Medium, by homogenization of 5 parts of Complete Medium, 4 parts of FBS, and 1 part of DMSO. The final volumetric proportions of raw materials in the Cryopreservation Medium are of 45% DMEM, 45% FBS, and 10% DMSO. Prepare Cryopreservation Medium on the day of use, and store at 4 °C until use. Use within the day of preparation.

3.9 Non-enzymatic Primary Cell Isolation

Once the tissue containers arrive to the accredited GMP cell culture laboratory, immediately proceed as follows. The method is described for the isolation of primary dermal progenitor fibroblasts (Fig. 3c).

1. Isolate each tissue fragment in properly annotated sterile tissue culture dishes.
2. Aseptically observe the tissues using the stereo microscope, and discard any adjacent tissues with sterile scalpels and forceps (*see* **Note 18**).
3. Wash cleaned fragments thrice with Conserved Medium for 15 min (*see* **Note 19**).
4. Photographically record appearance of individual tissue fragments.
5. Process individual fragments in new properly annotated sterile culture dishes by dissection into <0.5 mm^3 secondary fragments (*see* **Note 20**) (Fig. 3e).
6. Process sterile culture dishes of 10 cm diameter by deep scoring of the culture surface in a checkboard pattern using a sterile scalpel (*see* **Note 21**).
7. Ascetically transfer secondary tissue fragments to the scored tissue culture dishes, and attach fragments to the scored regions by applying gently mincing motions (*see* **Note 22**).
8. Cover individual tissue fragments with Complete Medium by gentle dispensing (*see* **Note 23**).
9. Transfer the tissue culture dishes to the incubator set at 37 °C, 80% relative humidity, and 5% CO_2.
10. After 24 h of incubation, gently dispense an additional 10 mL of Complete Medium to each dish, and replace cultures in the incubator (*see* **Note 24**).

11. Exchange the Complete Medium every 2 days, by gentle aspiration of the spent medium and gentle dispensing of fresh sterile and warm (37 °C) Complete Medium.
12. Photographically record culture appearances at the time of medium exchanges and before harvest (*see* **Note 25**).
13. Allow cell outgrowth to attain 90% confluency before considering harvest (*see* **Note 26**).

3.10 Parental Cell Bank Establishment

Once optimal banking confluency is attained for the primary isolated cells in the tissue culture dishes, proceed as follows:

1. To initiate harvest, gently remove spent Complete Medium with an aspiration pipet.
2. Gently rinse the cultures twice with warm (37 °C) D-PBS; ensure homogenous rinsing of the cell monolayer by gentle rocking of the vessels.
3. Aseptically and gently dispense 4 mL of warm TrypLE™ (37 °C) to each culture vessel, and ensure homogenous repartition of the solution over the whole culture surface by gentle rocking of the vessels (*see* **Note 27**).
4. Incubate the culture vessels for 5–8 min in the incubator at 37 °C.
5. Microscopically monitor cultures for confirmation of cell detachment. If detachment is incomplete, reincubate the vessels for 2–3-min cycles as necessary until complete detachment.
6. Aseptically add 6 mL of warm D-PBS to the culture vessels for inactivation of the detachment agent.
7. Aseptically transfer and pool cell suspensions in properly labelled centrifuge tubes using sterile serological pipets.
8. Centrifuge the harvested cell suspensions at 230 × *g* for 10 min at ambient temperature.
9. Aseptically remove and discard supernatants using a sterile aspiration pipet and vacuum pump.
10. Resuspend cell pellets in 5 mL of warm (37 °C) Complete Medium, by applying ten successive pipet uptakes and dispensings to ensure homogenous suspension of cells.
11. Pool cell suspensions in an adequate sterile container, and dilute the pool with Complete Medium, so that the final volume equals 10 mL *per* harvested culture vessel. Isolate and separately condition 2 × 0.5 mL of pooled suspension samples in microcentrifuge tubes for total and viable cell count determination.
12. Centrifuge the cell suspension at 230 × *g* for 10 min at ambient temperature.

13. Determine total and viable cell counts by microscopic enumeration using hemocytometers and Trypan blue exclusion dye (1:1 dilution with the cell suspension). Perform two separate counts (2 operators), and use the average results.
14. Aseptically remove and discard supernatants using a sterile aspiration pipet and vacuum pump.
15. Resuspend the cell pellets in warm Enriched Complete Medium, and use the pooled cell suspensions to seed a maximum number of T75 culture vessels at a relative seeding density of 1.5×10^3 viable cells/cm^2, with the final Enriched Complete Medium volume *per* flask being 15 mL (*see* **Note 28**). Ensure homogenous repartition of the suspension over the whole culture surface by gentle rocking of the vessels.
16. Transfer the culture vessels to the incubator set at 37 °C, 80% relative humidity, and 5% CO_2.
17. Exchange the Enriched Complete Medium every 2 days, starting with observation of cultures, both macroscopically and under the microscope, to exclude abnormal growth or contamination. Confirm health of proliferating cells and elongated appearance. In case of contamination, image the cultures, and retain a sample of growth medium for investigation before discarding the vessel. Proceed as follows for medium exchange.
18. Aseptically remove and discard spent medium using a sterile aspiration pipet and vacuum pump.
19. Add 15 mL of fresh warm Enriched Complete Medium to each vessel, ensuring homogenous repartition of the medium over the growth surface and preservation of cell monolayer integrity.
20. Replace the culture vessels in the incubator, and maintain the cultures until 100% confluency is attained (*see* **Note 29**).
21. Photographically record culture appearance (Fig. 3d), and proceed to harvest and viable count determination as described previously in **steps 1–14**, using 2 mL of TrypLE™ *per* T75 flask.
22. Once rinsed and quantified cell pellets are available after harvest, resuspend the cells in chilled (4 °C) Cryopreservation Medium at a final viable cell density of 10^7 cells/mL, and ensure homogenous suspension by applying ten successive pipet uptakes and dispensings to the suspension immediately prior to conditioning in cryovials.
23. Dispense the cell suspension in aliquots of 1.1 mL for conditioning in individual properly labelled cryovials (*see* **Note 30**).
24. Place cryovials in controlled rate freezing devices, and place the devices in an ultra-low temperature freezer set at −80 °C. Maintain the freezer door closed with the devices inside for at least 4 h.

25. Transfer the cryovials constituting the PCB to liquid nitrogen vapor phase storage in a quarantine storage tank (Fig. 3e).
26. Properly test the PCB before the admissibility to the biorepository is assessed. Once the PCB lot is liberated, the vials are to be transferred to the proper storage locations using a dry shipper. For appropriate risk mitigation, split the PCB lot in at least two distinct sublots to be stored in totally independent locations. The final storage conditions are the liquid nitrogen vapor phase of locked and level alarm monitored Dewar storage tanks.

3.11 Parental Cell Bank Testing

In order to verify compliance of the established PCB to cGMP standards and optimal quality thereof, perform the following testing. The testing should be performed on progeny materials serially derived in vitro from the PCB, in order to preserve sufficient amounts of vials of cells in the PCB (*see* Chapter "Non-enzymatically Isolated Dermal Progenitor Fibroblasts for Allogenic Regenerative Medicine").

1. Sterility testing by direct inoculation method, following European, Japanese, and US Pharmacopeias, using a minimum of two vials or at least 1% of the production lot.
2. Qualification of test article materials for sterility by direct inoculation method, following European, Japanese, and US Pharmacopeias, using a minimum of three vials.
3. Testing for the detection of *Mycoplasma* spp., including qualification of test articles, following the European Pharmacopoeia, using a minimum of one vial.
4. Extended in vitro assay (28 days) for the detection of viral contaminants (e.g., picornavirus, orthomyxovirus, paramyxovirus, herpesvirus, adenovirus, reovirus, West Nile virus) in six-well microplates, using three detector cell lines and 10 mL of cell suspension with 10^7 cells/mL.
5. Real-time PCR assay for the detection of human viruses (FDA and CPMP specifications) comprising EBV, HAV, HBV, HCV, hCMV, HIV-1, HIV-2, HTLV-1, HTLV-2, HHV-6, HHV-7, HHV-8, SV40, and B19 parovirus, using 6×10^7 cells.
6. Evaluation of reverse transcriptase activity by ultracentrifugation and QFPERT assay, using 2×2 mL cell free supernatants.
7. Karyotyping of the cell type, using a confluent flask of cells. The number of chromosomes and the gender are to be confirmed. Fluorescence in situ hybridization (FISH) should be used to target chromosome 21 for detection of aneuploidy.
8. Isoenzyme testing to confirm human origin and ethnicity, using one vial (*see* **Note 31**).

4 Notes

1. Good practices are defined for the testing, the acquisition of tissues for cell culture, and the manufacturing environments for cell banking in various official sources, e.g., Directive 2004/23/EC, Commission Directive 2006/17/EC, Commission Directive 2006/86/EC, Directive 2002/98/EC, EDQM's recent "Guide to the quality and safety of tissues and cells for human application," as well as ICH Guidelines and FDA Guidance for Industry documents.
2. The program manager should typically be an experienced biologist or a pharmacist with experience in bioproduction. The program manager provides the leading expertise in establishing the translational aspects of the program, as well as oversight of behavior of the cell type during all steps of processing and manufacturing within cell banking. Choice in the cell source and establishment of the appropriate technical specifications are discussed together with the technical manager. Importantly, clear compartmentalization should exist between the program manager and the medical team performing donor screening and donation procurement.
3. The technical manager should typically be an experienced biologist or a senior laboratory technician with experience in histopathology and cell culture. The technical manager personally participates in the processing of the donation and establishment of the primary cell type of interest.
4. The legal advisor should have experience in local transplantation laws and processing of patient data and tissues for research and development. In Switzerland, federal transplantation laws and ordinances define the frameworks for procurement and use of fetal tissue (9 weeks of gestation to pregnancy term, Fig. 3a) in research and clinical applications. The interpretation of regulatory issues and definition of the therapeutic use of the donation and progeny materials needs to be well thought out and planned, as international use of said materials may be considered. The clinical use of the established cellular material needs to be coordinated with local ethic and regulatory bodies, in order to define proper frameworks.
5. The medical doctor and his team need to be totally independent from the other parties within the program, preferably working in a secondary hospital.
6. Due to the complexity of properly isolating specific tissues from a 14-week-old donation, it is preferable that the fetal pathologist proceeds with the micro-dissection and discards as much adjacent tissue from the biopsies of interest as possible.

7. Usefulness, adequacy, and ethical dimensions of fetal transplantation programs are most easily demonstrable. Indeed, safety and tracing of therapeutic biological materials are essential for ensuring quality of care. Establishment of vast stocks of homogenous progeny cell populations retaining the properties of the initial tissue are of great scientific and medical interest. Pragmatic exploitation of robust cell banks for regenerative medicine applications alleviates the strong and constant demand for standardly defined transplants, which are continuously available in insufficient quantities around the world. Stability of the cryopreserved cellular materials in large quantities allows for long-term projections of the available therapeutic solutions.
8. Within a defined transplantation program, a specific cell type or therapeutic indication may be chosen and defined as an individual project.
9. Great care must be allocated to the constitution and preservation of the appropriate documentation during the bioprocessing of the starting biological material and subsequent cell banking. The established GMP grade materials are indeed worthless without the related, original, and complete documentation to ensure full traceability, following GMP standards.
10. For coherent use of the progeny materials established under the transplantation program, all liberations of cells from the defined Biobank should be the object of material transfer agreements, precisely defining the rights and obligations of the recipient user or biobank, as full traceability of the progeny materials should be available to the direction/responsible person of the original fetal biobank.
11. Standard templates, specific anonymous consent forms, and health questionnaires should be elaborated along with precise SOPs for donation acquisition and processing as parts of the Technical Specifications. This documentation enables clear and unambiguous communication with the medical team while preserving the anonymity of the donor. This methodologic aspect is of major importance, as a guarantee of the ethical quality of the program.
12. The specific methodology for consent obtention is designed to avoid (a) increasing the number of pregnancy terminations, (b) increasing the moral value of pregnancy terminations, and (c) modification of the termination date with influence on the gestational age of the fetus.
13. The donation consists in the whole and intact fetus, without the placenta.

14. The pregnancy interruption should be performed following standard local protocols and preferably by (a) repeated intravaginal misoprostol administrations for monobloc fetus removal and (b) full cavity curettage.
15. A full anonymous autopsy report needs to be generated for the coded donation, establishing evidence of normal physiological development and absence of observable physical or physiological abnormalities. As much as possible, isolate the tissues of interest for cell culture first, to mitigate risks of contamination during the autopsy process. Specific gross abnormality investigations must be carried out, such as particular relative head size and proportions, due to the potential Zika virus effects and limitations regarding validated serological testing thereof.
16. Frozen tissue samples will be kept as retention samples for a defined period of time for supplementary testing if the latter is necessary. Samples are to be conditioned whole in sterile and properly annotated centrifuge tubes withstanding ultra-low temperatures.
17. Depending on the organ and specific cell type of interest, the relative abundance of suitable tissue will vary. For isolation of dermal progenitor fibroblasts, isolate 2–4 cm^2 of dorsal skin.
18. During processing, it is important to keep the tissues hydrated, using minimal amounts of Conserved Medium. When dissecting the biopsies, it is of paramount importance to isolate homogenous tissue, free of any adjacent structures. Make sure to properly identify the structures and tissues of interest before further processing.
19. Immerge the tissue fragments in Conserved Medium for each rinse while making sure to submit them to active rinsing and not only soaking.
20. Keep the fragments hydrated in Conserved Medium during processing.
21. Three to four lines must be drawn in perpendicular setups on the dishes (Fig. 3e). Prepare as many properly annotated dishes as possible for each cell type, depending on the amount of tissue available. Between four and eight dishes should be prepared as a minimum.
22. Ensure homogenous repartition of the tissue fragments on the culture surface, taking care to not place fragments too close to the edge of the dish. Between six and ten tissue fragments should be placed on each culture dish.
23. A minimal amount of medium allows for hydration maintenance of the tissue fragments and favors adherence to the culture surface. Larger volumes of medium would lead to tissue fragment floatation and failure of adherent cell isolation procedures.

24. Great care must be taken when handling the cultures, in order to avoid tissue fragment dissociation from the culture surface and flotation.
25. The first outgrowth of primary cells emitting from tissue fragments can be observed as early as 24 h after culture initiation. At the end of the primary culture period, abundant fibroblasts can be observed migrating outward from the tissue fragments (Fig. 3c).
26. Optimal harvest confluency is typically reached after 6 days of culture. Confluent cells in the tissue culture dishes are defined as Passage 0 cells.
27. TrypLE™ allows for relatively more gentle dissociation of adherent cells than trypsin-EDTA, and inactivation requires simple dilution instead of FBS addition.
28. The ideal quantity of seeded flasks for the secondary expansion is around 80–100 × T75 vessels.
29. After the secondary expansion, the confluent cells are defined as Passage 1 cells. Optimal harvest confluency is generally reached after 7–10 days of culture depending on the cell type.
30. Ideal amounts of PCB vials are 15–50 for a specific cell type (10^6–10^7 viable cells/vial). The final amount depends on the specific cell type.
31. Isotyping enables the ruling out of cross-contamination of the cultures by a secondary cell type.

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GMP Tiered Cell Banking of Non-enzymatically Isolated Dermal Progenitor Fibroblasts for Allogenic Regenerative Medicine

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Abstract

Non-enzymatically isolated primary dermal progenitor fibroblasts derived from fetal organ donations are ideal cell types for allogenic musculoskeletal regenerative therapeutic applications. These cell types are differentiated, highly proliferative in standard in vitro culture conditions and extremely stable throughout their defined lifespans. Technical simplicity, robustness of bioprocessing and relatively small therapeutic dose requirements enable pragmatic and efficient production of clinical progenitor fibroblast lots under cGMP standards. Herein we describe optimized and standardized monolayer culture expansion protocols using dermal progenitor fibroblasts isolated under a Fetal Transplantation Program for the establishment of GMP tiered Master, Working and End of Production cryopreserved Cell Banks. Safety, stability and quality parameters are assessed through stringent testing of progeny biological materials, in view of clinical application to human patients suffering from diverse cutaneous chronic and acute affections. These methods and approaches, coupled to adequate cell source optimization, enable the obtention of a virtually limitless source of highly consistent and safe biological therapeutic material to be used for innovative regenerative medicine applications.

Keywords Cell therapy, Clinical cell banking, Dermal fibroblasts, GMP manufacturing, Optimized protocols, Organ donation, Progenitor cells, Safety testing

Abbreviations

ATMP	Advanced therapy medicinal product
BPyV	Bovine polyomavirus
CD	Cluster of differentiation
cGMP	Current good manufacturing practices
CMV	Cytomegalovirus
CPMP	European Union Committee for Proprietary Medicinal Products
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's phosphate-buffered saline
EBV	Epstein-Barr virus
EOP	End of production

EOPCB	End of Production Cell Bank
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	US Food and Drug Administration
GLP	Good laboratory practices
GMP	Good manufacturing practices
HAV	Hepatitis A virus
HBoV	Human bocavirus
HBV	Hepatitis B virus
hCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HHV-6/7/8	Human herpes viruses types 6, 7 and 8
HIV-1/2	Human immunodeficiency viruses types 1 and 2
HLA	Human leukocyte antigen
HPL	Human platelet lysate
HPV	Human papilloma virus
HSA	Human serum albumin
HTLV-1/2	Human T-cell leukemia-lymphoma viruses types 1 and 2
HuPyV	Human polyomavirus
IPC	In-process control
KIPyV	KI polyomavirus
MCB	Master Cell Bank
PCB	Parental Cell Bank
PCR	Polymerase chain reaction
PDT	Population doubling time
PDV	Population doubling value
PWCB	Pilot Working Cell Bank
QFPERT	Quantitative fluorescent product enhanced reverse transcriptase
QRM	Quality risk management
SOP	Standard operating procedure
TEM	Transmission electron microscopy
WCB	Working Cell Bank
WUPyV	WU polyomavirus

1 Introduction

Innovative cell therapies complement traditional surgeries in combined therapeutic efforts to repair, restore and optimize organ and tissue functions [1–6]. Quality and safety-driven regulations define strict specifications and procedures for manufacturing environments and processes for cell therapies or biological product development [7–11]. Cultured primary human dermal progenitor fibroblasts isolated under defined Fetal Transplantation Programs fulfil requirements for ideal cell types to be traceably processed under modern GMP standards. Non-enzymatically isolated

clinical-grade optimized cell sources of progenitor fibroblasts are of utmost interest for formulation of advanced therapy medicinal products (ATMPs) or medical devices [12–17]. Following controlled fetal organ donations, progeny cellular materials mechanically derived in vitro from defined fetal dermis are differentiated, safe to handle and consistent and can be obtained in large quantities and possess potent therapeutic stimulation potential for repair of cutaneous affections and trauma such as ulcers and deep burns [18–20]. Clinical therapeutic advantages of primary progenitor fibroblasts comprise optimal safety, absence of observable immunogenicity or tumorigenicity, excellent biocompatibility, on-demand availability and demonstrable therapeutic effects exerted through trophic paracrine stimulation of patient cells and tissues [13, 19–22]. Tangible clinical experience with these therapeutic cell types has been gathered for over 30 years in Switzerland, where fetal tissue at the considered timepoint of interest is classified as an organ donation and must be processed under a Federal Transplantation Program [23–26]. Technical advantages of primary progenitor fibroblasts comprise extensive stability of cell populations, consistency of homogenously established cell banks, relative rapidity of in vitro proliferation, no specific nutritional or growth factor requirements, high resistance to oxidative stress and adaptability to industrial transposition processes [24, 25, 27–33]. From a cryopreserved Parental Cell Bank (PCB), standardized tiered Master, Working and End of Production Cell Banks (MCB, WCB, EOPCB) can be manufactured under cGMP standards for allogenic cell therapies or cell-based product development. Ethical advantages of progenitor cell types comprise the need for a single organ donation to potentially treat millions of patients worldwide and drastic reduction of donor-site graft needs. One single qualifying organ donation can potentially yield sufficient quantities of therapeutic material for over 10^{12} individual doses [13, 24, 25, 28, 34, 35]. An optimized tiered biobanking method is described herein for primary dermal progenitor fibroblasts, allowing for standardized, efficient and rapid obtention of high therapeutic value safe banks of progeny cells derived from a robust non-enzymatically isolated cell source. Detailed protocols are provided and annotated for tiered cell bank establishment (MCB, WCB and EOPCB) using non-enzymatically isolated dermal progenitor fibroblasts and for quality and safety testing to be performed on the progeny clinical-grade production lots. Such workflows and specifications may be applied to various similar human primary progenitor cell types in view of GMP manufacture of an array of musculoskeletal regenerative medicine therapeutic products.

2 Materials

All considered materials, consumables and product components should be sterile and GMP cell culture grade. Facilities and equipment are to be qualified and validated appropriately [9–11]. Waste disposal regulations are to be followed diligently.

2.1 Biological Starting Materials

The mother donor included in the Fetal Transplantation Program for progenitor cell isolation and banking should test negative for CMV, HBV, HCV, HIV-1/HIV-2, HTLV-1/HTLV-2, syphilis, toxoplasmosis, West Nile virus and Zika virus serologies (*see* chapter "Swiss Fetal Transplantation Program and Non-enzymatically Isolated Primary Progenitor Cell Types for Regenerative Medicine"). Donation and tissue procurement should occur 12 weeks post-amenorrhea. For optimal tiered GMP cell banking, an equivalent of $\geq 5 \times 10^7$ cryopreserved primary dermal progenitor fibroblasts needs to be processed from the Parental Cell Bank established and tested under strict GMP conditions (conform to Subheadings 3.12, **steps 1–4** and 3.13, **steps 4** and **13**). The dermal fibroblasts from the PCB should be in their frozen state at Passage 1, meaning the cells outgrowing from the mechanically processed biopsies (Passage 0) were culture-expanded twice before harvest and PCB cryopreservation (Passage 1) (*see* chapter "Swiss Fetal Transplantation Program and Non-enzymatically Isolated Primary Progenitor Cell Types for Regenerative Medicine"). Upon initiation of the PCB vials, the cultured cells become Passage 2.

2.2 Raw Materials

Raw materials are defined as materials which are present as an appreciable part of the final product when considering specific manufacturing lots. The following raw materials are required to obtain GMP lots of cryopreserved progeny cell stocks.

1. DMEM, GMP grade, high glucose, with pyruvate, with phenol red, store at 4 °C until use
2. FBS, GMP grade, with certificate of animal origin and health, certificate of analysis, irradiation certificate, batch manufacturing record, store at −80 °C until use
3. DMSO, GMP cell culture grade, store at room temperature until use

2.3 Ancillary Materials

Ancillary materials are defined as materials which are used and subsequently removed during the manufacturing process, therefore not constituting an appreciable part of the final product. The following ancillary materials are required.

1. TrypLE™ Select Enzyme, 1×, sterile, without phenol red, store at room temperature until use
2. D-PBS, sterile, no calcium, no magnesium, store at room temperature until use

2.4 Contact Process Consumables

Contact process consumables are defined as materials which come into direct contact with cell cultures or cell suspensions during the manufacturing process. Store consumables at room temperature until use. The following contact process consumables are required.

1. T175 sterile cell culture flasks, 175 cm^2, uncoated culture surfaces, with filter screw caps
2. Aspiration pipets, sterile
3. Serological pipets, sterile, 5, 10 and 35 mL, with cotton filters
4. Sterile culture-grade conical centrifuge tubes, 15 mL, 50 mL
5. Sterile culture-grade containers, 500 mL, 1000 mL

2.5 Non-contact Process Consumables

Non-contact process consumables are defined as materials which do not come into direct contact with cell cultures or cell suspensions during the manufacturing process. Store consumables at room temperature until use. The following non-contact process consumables are required.

1. Trypan blue solution, 0.4%, filter-sterilized
2. Haemocytometer, Neubauer Improved
3. Micropipette filter tips, 20, 200, 1000 μL, sterile
4. Microcentrifuge tubes, cell culture grade, sterile
5. Denatured 70% ethanol, in vaporizing bottles
6. Permanent markers, heat, ethanol and liquid nitrogen resistant

2.6 Product Container System Components

Container system components comprise, in addition to the biological material and cryopreservation solution, the elements constituting a deliverable product unit. The following components are required to obtain GMP lots of cryopreserved progeny cell stocks.

1. 1.8 mL cryovials, GMP grade, with internal threading, with star-foot
2. Cryogenic adhesive labels, liquid nitrogen resistant

2.7 Critical Process Facility and Equipment

The critical process facility comprises the production suite and storage unit, along with the installed equipment, instruments and devices. Wherever applicable, equipment, instruments and devices should be calibrated and appropriately qualified. The following infrastructure, equipment, instruments and devices are required.

1. Cleanroom facility comprising the production suite, B grade and the storage unit
2. Biological safety cabinet, A grade, vertical laminar flow
3. Complete sterile cleanroom personal clothing, suit, mask, hood, gloves and boots
4. CO_2 incubators, temperature range 36.5 °C ± 1.5 °C, relative humidity range 80% ± 10%, CO_2 content range 5% ± 1.5%
5. Liquid nitrogen Dewar storage tanks, level alarm fitted, with locks
6. Ultra-low temperature freezer, temperature range −80 °C ± 5 °C
7. Freezer, temperature range −20 °C ± 2 °C
8. Refrigerator, temperature range 4 °C ± 2 °C
9. Controlled rate freezing devices, −1 °C/min rate of freezing, CoolCell® LX, CoolCell® FTS30 or Nalgene® Mr. Frosty™, with isopropanol
10. Centrifuge, operating range 230 × *g* ± 10 × *g*, ambient temperature, swing-out buckets with adaptors, 15 mL and 50 mL tubes.
11. Optical inverted microscope, 40×, 100× and 400× optical magnification with phase contrast
12. Waterbath, temperature range 37 °C ± 2 °C, with distilled water
13. Aspiration pump, handheld aspiration pipet mount, with sterile tubing
14. Motorized pipet for serological pipet manipulation
15. Digital timer
16. Micropipettes, 20, 200, 1000 μL, calibrated
17. Cryotube racks, with star-foot adaptors

3 Methods

3.1 Generalities About Methods

1. Applicable cGMP procedures and recommendations are to be followed at all times.
2. Ensure quality risk management (QRM) systems and in-process controls (IPC) are adequately implemented.
3. Proper ethical considerations and validations should be obtained for animal testing studies prior to study initiation.
4. Legal retention samples (raw and starting materials, in-process control samples and representative production lot samples), reports and records shall be constituted and stored

appropriately along with full traceability of all manipulations, deviations and corrective measures.

5. All manipulations requiring the cultures or cell suspensions to be exposed (open flasks, tubes or vials) are to be conducted in the class A biological safety cabinet.
6. All materials and equipment entering the production area and biosafety cabinet are to be properly sanitized.
7. Necessary documents and labelling materials are to be prepared before processing operations begin.
8. All media, solutions or buffers to be warmed at 37 °C are to be placed in a waterbath that has been equilibrated at 37 °C for at least 1 h
9. Procedures are to be carried out at room temperature unless otherwise specified

3.2 Complete Medium Preparation

1. Prepare Complete Medium by diluting FBS in DMEM, so that the final volumetric proportion of FBS is 10% and the final volumetric proportion of DMEM is 90% (*see* **Notes 1** and **2**). Store Complete Medium at 4 °C and use within 21 days after preparation.

3.3 Cryopreservation Medium Preparation

1. Prepare Cryopreservation Medium, by homogenization of 5 parts of Complete Medium, 4 parts of FBS and 1 part of DMSO. The final volumetric proportions of raw materials in the Cryopreservation Medium are of 45% DMEM, 45% FBS and 10% DMSO (*see* **Note 3**). Prepare Cryopreservation Medium on the day of use, and store at 4 °C. Use within the day of preparation.

3.4 Pilot Study and Cell Type Validation

Before considering industrial scale production and tiered GMP banking, the cell type of interest should be sufficiently characterized in terms of identity, safety, purity, potency and stability (Fig. 1). Using a portion of the PCB, perform the following.

1. Initiate two vials of the PCB, perform serial culture expansions (2 passages) and cryopreserve the harvested cells at Passage 4, using the method and materials described in Subheading 3.9, using PCB vials instead of MCB vials, using 80 T175 culture vessels in **step 6** instead of 150 culture vessels, to constitute a Pilot Working Cell Bank (PWCB) (*see* **Note 4**).
2. Perform adequate testing on the PWCB, following Subheadings 3.12, **steps 1–4** and 3.13, **steps 4** and **13** (*see* **Note 5**).
3. Initiate adequate quantities of vials from the PWCB, and perform optimization assays to compare FBS lots and culture vessel manufacturers (*see* **Note 6**).

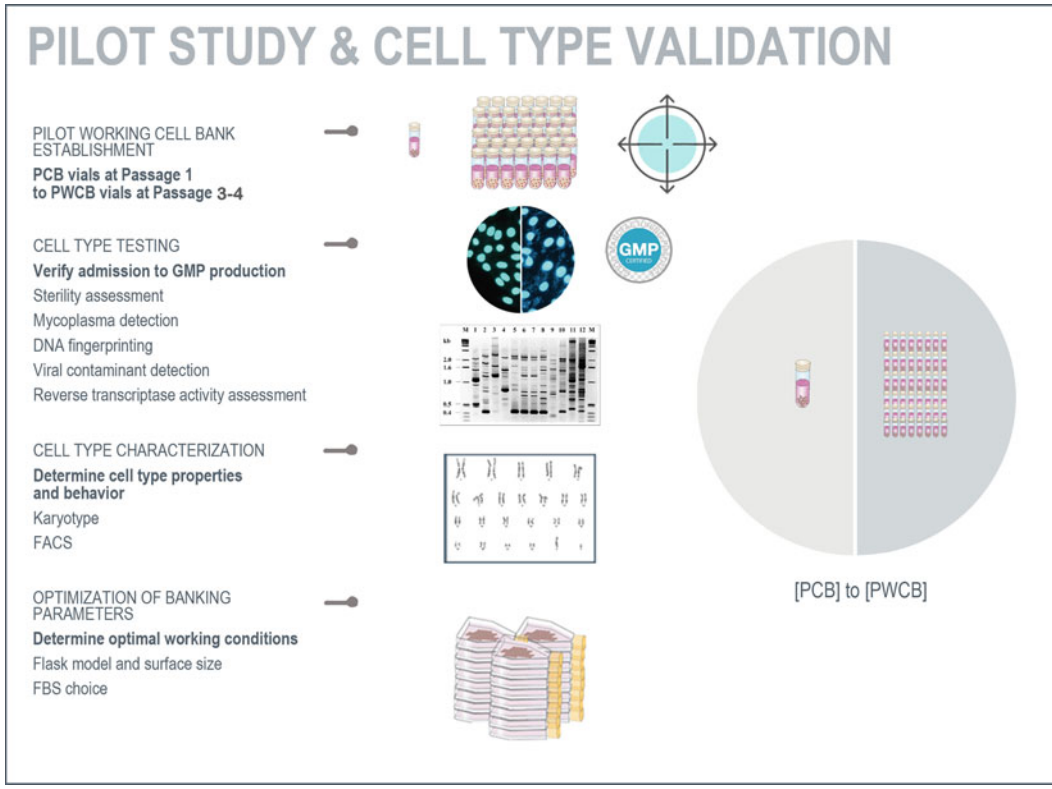

Fig. 1 Pilot study and cell type validation. In order to spare the limited stock of PCB vials, part of the Parental Cell Bank is used to establish a Pilot Working Cell Bank (PWCB) at Passage 3 or 4. The latter is firstly used to determine the admissibility of the considered cell type to GMP production through rigorous testing. Secondly, characterization of the cell type of interest is performed, in order to confirm progenitor dermal fibroblast identity and potency, as well as cell genetic and physiological status. Thirdly, the PWCB serves to optimize the culture expansion conditions by selecting the optimal parameters for the defined cell type

4. Initiate adequate quantities of vials from the PWCB, perform GLP serial culture expansions and cryopreserve the cells at each passage following methods described in Subheadings 3.6–3.8, using PWCB vials instead of PCB vials, using 30 T175 culture vessels *per* passage instead of 150 culture vessels, until cryopreserved vials are available at Passage 15 (*see* **Note 7**).
5. Simultaneously initiate vials at Passages 4, 8, 12 and 15 from the PWCB, and perform culture expansions to furnish cells for karyology stability studies, surface maker profiling using antibodies specific to surface makers CD13, CD14, CD29, CD34, CD45, CD49a, CD54, CD73, CD90, CD106, CD105, CD146, HLA-ABC, HLA-DPQR and multilineage differentiation induction assays (2D adipogenic, osteogenic and chondrogenic induction assays) (*see* **Note 8**).
6. Based on the results of the pilot study and the behaviour of the considered cell type in higher passages, decide to validate or

exclude the cell type for further GMP manufacturing and/or therapeutic applications.

3.5 Master Cell Bank Establishment: Definition

PCB vials of cells at Passage 1 are used for the establishment of the MCB after one in vitro culture expansion. The MCB is therefore constituted of cryopreserved cells at Passage 2.

3.6 MCB: Initiation from Liquid Nitrogen Storage

In order to establish an MCB from PCB vials, proceed as follows (Fig. 2).

1. Transfer 5 PCB cryovials or the equivalent of 5.0×10^7 cryopreserved fibroblasts from liquid nitrogen storage to the culture room on dry ice in a sterile container (*see* **Note 9**).
2. Transfer the cryovials to the waterbath previously equilibrated at 37 °C, and allow the cell suspension to thaw, applying gentle rotations to the vials. Once the last frozen material has just thawed, transfer the cryovials to the laminar flow hood (*see* **Note 10**).

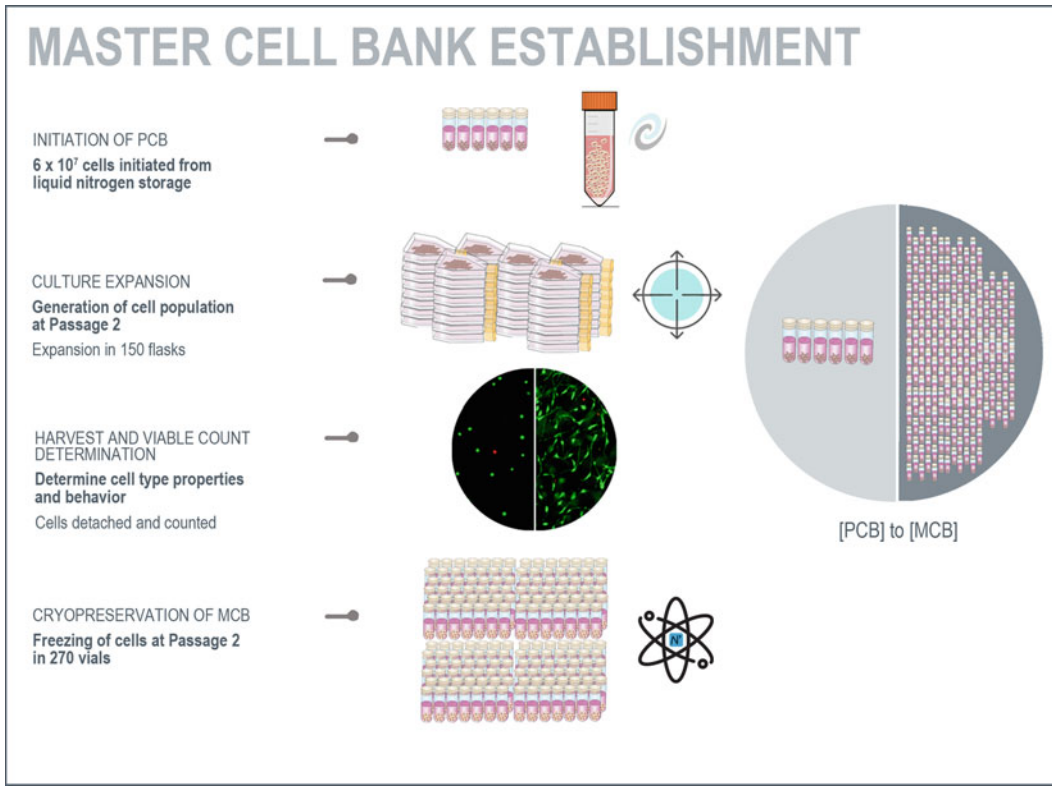

Fig. 2 MCB establishment. Six PCB vials or the equivalent of 6×10^7 cells from the PCB at Passage 1 are initiated, and cells are culture-expanded once before harvest and cryopreservation, yielding approximately 270 vials constituting the MCB at Passage 2

3. Homogenize the cell suspensions by gentle pipetting, ensuring that no air bubbles are formed by expulsion of air in the suspensions.
4. For each cryovial, aseptically transfer the thawed cell suspension to a centrifuge tube containing 14 mL of warm (37 °C) Complete Medium, by slowly dispensing the cell suspension dropwise into the medium. Discard cryovials and collect the original labels, if applicable (*see* **Note 11**).
5. Centrifuge the diluted cell suspensions at 230 × *g* for 10 min at room temperature.
6. Aseptically remove supernatants and condition them for sampling.
7. Resuspend cell pellets homogenously using 10 mL of warm Complete Medium.
8. Pool cell suspensions in an appropriate sterile container and homogenize the pool. Isolate and separately condition 2 × 0.5 mL of pooled suspension samples in microcentrifuge tubes for total and viable cell count determination.
9. Determine total and viable cell counts by microscopic enumeration using haemocytometers and Trypan blue exclusion dye (1:1 dilution with the cell suspension). Perform 2 separate counts (2 operators) and use the average results. Verify that relative cell viability is ≥80% for the initiation process to continue (*see* **Note 12**).
10. Aseptically transfer 4.0×10^7 viable cells to an appropriate sterile container, and dilute the cell suspension homogenously using warm Complete Medium so that the final volume of the suspension becomes 300 mL.
11. Using the diluted cell suspension, seed 150 properly annotated T175 culture flasks with a relative viable cell density of approximately 1.5×10^3 cells/cm^2 by dispensing 2 mL of cell suspension *per* vessel. Further individually dilute the seeding suspensions by dispensing 28 additional mL of warm Complete Medium to each culture vessel. Ensure homogenous repartition of the properly diluted suspensions in the culture vessels (*see* **Note 13**).
12. Transfer the seeded culture vessels to the incubators set at 37 °C, 5% CO_2 and 80% relative humidity (*see* **Note 14**).

3.7 MCB: Maintenance of Cultures and Medium Exchanges

Upon successful initiation, perform medium exchanges every 2 days until a confluency of 100% is attained (*see* **Note 15**). Maintain cultures incubated at 37 °C, 5% CO_2 and 80% relative humidity.

1. Observe cultures macroscopically and under the microscope to exclude abnormal growth or contamination. Confirm

health of proliferating cells and elongated appearance. In case of contamination, image the cultures, and retain a sample of growth medium for investigation before discarding the vessel (*see* **Note 16**).

2. Aseptically remove and discard spent growth medium using a sterile aspiration pipet and vacuum pump (*see* **Note 17**).
3. Add 30 mL of fresh warm Complete Medium to each vessel, ensuring homogenous repartition of the medium over the growth surface and preservation of cell monolayer integrity.
4. Replace the culture vessels in the incubator, and maintain the cultures until 100% confluency is attained (*see* **Note 18**).

3.8 MCB: Harvest and Cryopreservation

Once cultures are confluent, proceed with harvest and cryopreservation procedures as follows.

1. Observe cultures macroscopically and under the microscope to exclude abnormal growth or contamination. Confirm morphological quality of proliferating cells and elongated appearance. Photographically record the appearance of cultures. In case of contamination, image the cultures, and retain a sample of growth medium for investigation before discarding the vessel.
2. Aseptically remove and discard spent growth medium using a sterile aspiration pipet and vacuum pump (*see* **Note 19**).
3. Aseptically add 20 mL of warm (37 °C) D-PBS to each culture vessel, and ensure homogenous rinsing of the cell monolayer by gentle rocking of the vessels.
4. Aseptically remove and discard spent D-PBS using a sterile aspiration pipet and vacuum pump.
5. Aseptically add 5 mL of warm (37 °C) TrypLE™ to each culture vessel, and ensure homogenous repartition of the solution over the whole culture surface by gentle rocking of the vessels.
6. Incubate the culture vessels for 5–8 min in the incubator.
7. Microscopically observe cultures for confirmation of cell detachment. If detachment is incomplete, reincubate vessels for 2–3-min cycles as necessary until complete detachment.
8. Aseptically add 15 mL of warm D-PBS to the culture vessels for inactivation of the detachment agent (*see* **Note 20**).
9. Aseptically transfer and pool cell suspensions in centrifuge tubes using sterile pipets.
10. Centrifuge the harvested cell suspensions at 230 × *g* for 10 min at ambient temperature.
11. Aseptically remove and discard supernatants using a sterile aspiration pipet and vacuum pump.

12. Resuspend cell pellets in 5 mL of warm Complete Medium, by applying 10 successive pipet uptakes and dispensings to ensure homogenous suspension of cells.
13. Pool cell suspensions in an adequate sterile container and dilute the pool with Complete Medium, so that the final volume equals to 5 mL *per* harvested culture vessel. Isolate and separately condition 2 × 0.5 mL of pooled suspension samples in microcentrifuge tubes for total and viable cell count determination.
13. Centrifuge the cell suspension at 230 × *g* for 10 min at ambient temperature.
14. Determine total and viable cell counts by microscopic enumeration using Trypan blue exclusion dye (*see* **Note 21**).
15. Aseptically remove and discard supernatants using a sterile aspiration pipet and vacuum pump.
16. Resuspend the cells in chilled (4 °C) Cryopreservation Medium at a final viable cell density of 10^7 cells/mL, and ensure homogenous suspension of cells by applying 10 successive pipet uptakes and dispensings immediately prior to conditioning in cryovials (*see* **Note 22**).
17. Dispense the cell suspension in aliquots of 1.1 mL for conditioning in individual properly labelled cryovials.
18. Place cryovials in controlled rate freezing devices, and place the devices in an ultra-low temperature freezer set at −80 °C. Maintain the freezer door closed with the devices inside for at least 4 h (*see* **Note 23**).
19. Transfer the cryovials constituting the MCB at Passage 2 to liquid nitrogen vapor phase storage in a quarantine storage tank (*see* **Note 24**).
20. Perform adequate testing on the manufactured MCB lot according to Subheadings 3.11–3.13.

3.9 Working Cell Bank Establishment

MCB vials at Passage 2 are used for the establishment of the WCB after 2 serial culture expansions. The WCB is therefore constituted of cryopreserved cells at Passage 4 (Fig. 3).

1. Following the initiation procedure described in Subheading 3.6, initiate two MCB vials from liquid nitrogen storage, and determine total and viable cell counts in the pooled suspension.
2. Use the obtained cell suspension to seed a maximum number of T175 culture vessels at a relative seeding density of 2.0×10^4 viable cells/cm^2.
3. Transfer the culture vessels to the incubator set at 37 °C, 5% CO_2 and 80% relative humidity.

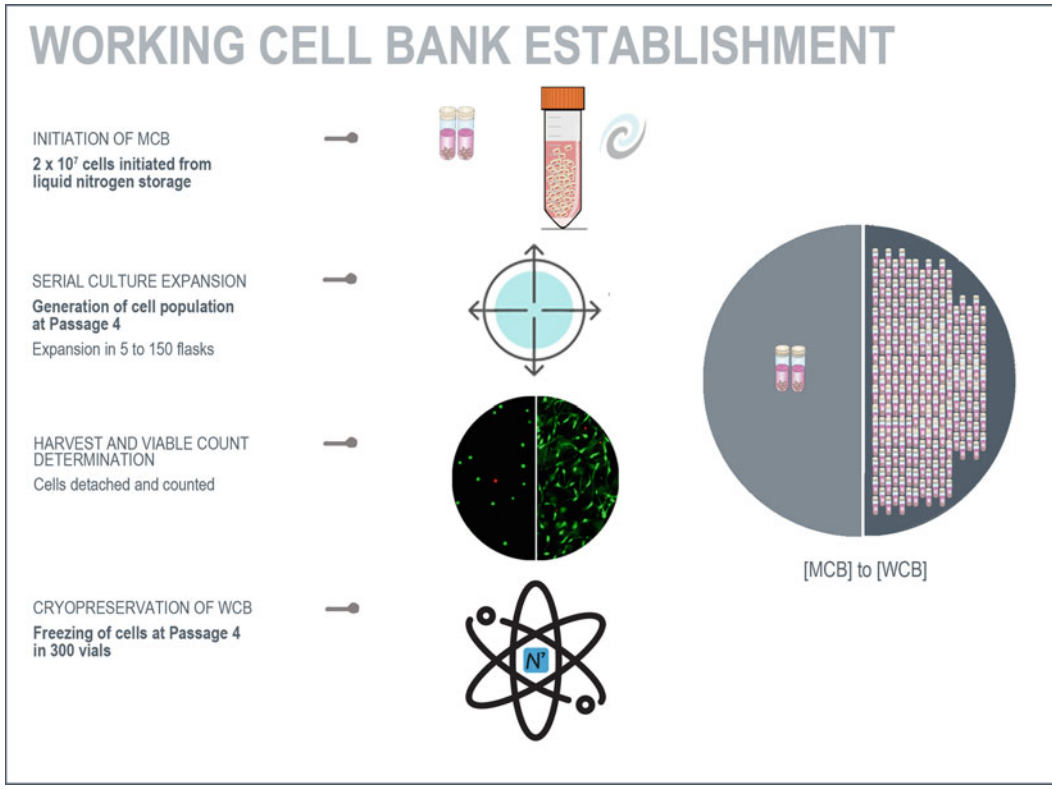

Fig. 3 WCB establishment. Two vials from the MCB at Passage 2 are initiated and cells are culture-expanded in two phases before harvest and cryopreservation, yielding approximately 300 vials constituting the WCB at Passage 4

4. Two days post-initiation, exchange the growth medium following Subheading 3.7.
5. Four days post-initiation and if cells have attained >95% confluency, proceed to harvest and cell count determination following Subheading 3.8.
6. Use the obtained cell suspension to seed 150 properly annotated T175 culture flasks with a relative viable cell density of approximately 1.5×10^3 cells/cm^2.
7. Transfer the culture vessels to the incubators set at 37 °C, 5% CO_2 and 80% relative humidity, and maintain the cultures following Subheading 3.7.
8. Once 100% confluency is reached, proceed to harvest and cryopreserve the cells following Subheading 3.8 to constitute the cryopreserved WCB at Passage 4 (*see* **Note 25**).
9. Perform adequate testing on the manufactured WCB lot according to Subheadings 3.11–3.13.

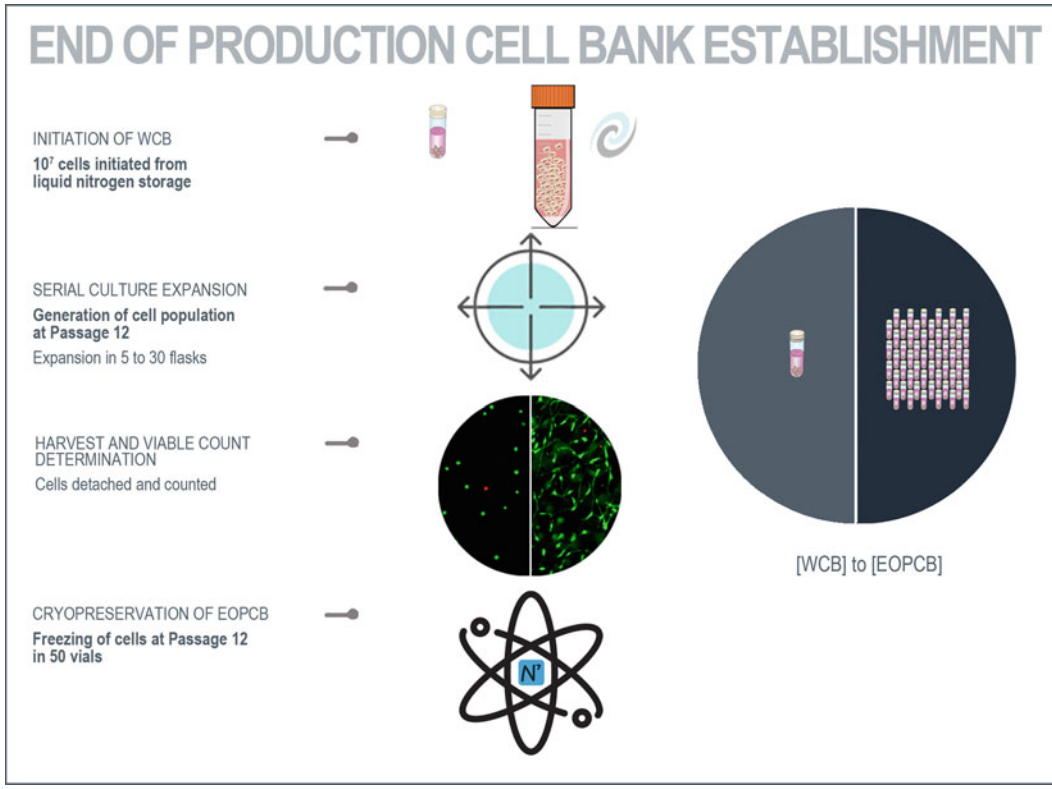

Fig. 4 EOPCB establishment. One vial from the WCB at Passage 4 is initiated, and cells are serially culture-expanded before harvest and cryopreservation, yielding approximately 50 vials constituting the EOPCB at Passage 12

3.10 End of Production Cell Bank Establishment

WCB vials at Passage 4 are used for the establishment of the EOPCB after 8 serial culture expansions. The EOPCB is therefore constituted of cryopreserved cells at Passage 12 (Fig. 4).

1. Following the initiation procedure described in Subheading 3.6, initiate one WCB vial from liquid nitrogen storage, and determine total and viable cell counts.
2. Use the obtained cell suspension to seed a maximum number of T175 culture vessels at a relative seeding density of 2.0×10^4 viable cells/cm^2.
3. Transfer the culture vessels to the incubator set at 37 °C, 5% CO_2 and 80% relative humidity.
4. Two days post-initiation, exchange the growth medium following Subheading 3.7.
5. Four days after initiation and if cells have attained >95% confluency, proceed to harvest and cell count determination following Subheading 3.8.

6. Use the obtained cell suspension to seed five properly annotated T175 culture flasks with a relative viable cell density of approximately 2.0×10^4 cells/cm^2.
7. Transfer the culture vessels to the incubator set at 37 °C, 5% CO_2 and 80% relative humidity, and maintain the cultures following **step 4**.
8. Repeat **steps 5**–7 until serial passages have produced cells at Passage 11 (*see* **Note 26**).
9. Once cells at Passage 11 are harvested and counted, use the obtained cell suspension to seed 30 properly annotated T175 culture vessels at a relative seeding density of 1.5×10^3 viable cells/cm^2.
10. Transfer the culture vessels to the incubator set at 37 °C, 5% CO_2 and 80% relative humidity, and maintain the cultures following Subheading 3.7.
11. Once 100% confluency is attained, proceed to harvest and cryopreserve the cells following Subheading 3.8 to constitute the cryopreserved EOPCB at Passage 12 (*see* **Note 27**).
12. Perform adequate testing on the manufactured EOPCB lot according to Subheadings 3.11–3.13.

3.11 Cell Recovery and Viability Studies

Recovery and viability studies should be performed on MCB, WCB and EOPCB lots before liberation (*see* **Note 28**). The method is described here for the MCB.

1. Wait 48 h following the end of the MCB establishment and cryopreservation, select three vials from the MCB from the start, middle and end portions of the manufactured lot, following the order of conditioning.
2. Initiate the MCB vials and maintain cultures following steps described under Subheadings 3.6 and 3.7, using a maximum number of 15 T175 culture vessels.
3. Monitor cell proliferation until harvest confluency is reached. Photographically record the appearance of several cultures.
4. Harvest and cryopreserve the cells following steps described under Subheading 3.8 to constitute a small GMP PWCB (cells at Passage 3) with vials containing aliquots at 1, 2, 5 and 10×10^6 viable cells/mL (*see* **Note 29**).
5. Compare cell proliferation characteristics (PDT and PDV) from the recovery study with those of MCB establishment in order to release the MCB lot or not. The requirements for validation and liberation of the lot must comprise ≥80% relative cell viability upon initiation and harvest confluency attainment in the same time as the MCB lot.

Fig. 5 Testing of the cellular materials and clinical release. The different testing panels are applied when appropriate in the multi-tiered production process, in order to guarantee the consistency, quality and safety of the lots liberated by the manufacturing facility. The sum of the production and testing reports allows for sound review and final decision on the admission of the cell type and manufactured tiered banks for inclusion in therapeutic protocols or product development (e.g. Progenitor Biological Bandages)

3.12 Product Release Testing

Product release testing should be performed on the MCB, WCB and EOPCB lots before respective liberation (Fig. 5). Using vials from the start, middle and end portions of the production lots/banks, perform the following testing.

1. Sterility testing by direct inoculation methods, following European, Japanese and US Pharmacopoeias, using a minimum of two vials or at least 1% of the MCB.
2. Testing for the detection of *Mycoplasma* spp., including qualification of test articles, following the European Pharmacopoeia, using a minimum of one vial.
3. Qualification of test article materials for sterility by direct inoculation method, following European, Japanese and US Pharmacopoeias, using a minimum of three vials.
4. Endotoxin test, with inhibition/enhancement tests for endotoxins.

3.13 Product Characterization Testing

Product characterization testing should be performed on the MCB, WCB (only **steps 1–4**) and EOPCB lots before liberation (Fig. 5). Using vials from the start, middle and end portions of the production lots/banks or live confluent cultures from recovery studies, perform the following testing.

1. Cell growth and sample preparation, using one vial
2. Isoenzyme testing to confirm human origin and ethnicity, using one vial
3. DNA fingerprinting of the cell type, using a multi-locus probe 33.15, using a cell pellet at 5×10^7 cells
4. Extended in vitro assay (28 days) for the detection of viral contaminants (e.g. picornavirus, orthomyxovirus, paramyxovirus, herpesvirus, adenovirus, reovirus, West Nile virus) in 6-well microplates, using 3 detector cell lines and 10 mL of cell suspension with 10^7 cells/mL
5. Testing for the presence of inapparent viruses using suckling mice, adult mice, guinea pigs and embryonated eggs, using respectively 2×2 mL, 1×4 mL, 1×7 mL, 1×28 mL of cell lysate at 10^7 cell equivalents/mL
6. Real-time PCR for the detection of bovine polyomavirus (BPyV), using a cell pellet at 2×10^7 cells
7. Real-time PCR assay for the detection of human polyomavirus (HuPyV), using a cell pellet with 2×10^7 cells
8. Real-time PCR detection of human papillomavirus (HPV), using a cell pellet with 5×10^7 cells
9. Real-time PCR assay for the detection of human bocavirus (HBoV), using a cell pellet with 2×10^7 cells
10. Real-time PCR assay for the detection of WU polyomavirus (WUPyV), using a cell pellet with 2×10^7 cells
11. Real-time PCR assay for the detection of KI polyomavirus (KIPyV), using a cell pellet with 2×10^7 cells
12. Real-time PCR assays for the detection of human viruses (FDA and CPMP specifications) comprising EBV, HAV, HBV, HCV, hCMV, HIV-1, HIV-2, HTLV-1, HTLV-2, HHV-6, HHV-7, HHV-8, SV40 and B19 parvovirus, using 6×10^7 cells
13. Evaluation of reverse transcriptase activity by ultracentrifugation and QFPERT assay, using 2×2 mL cell free supernatants
14. Quantitative transmission electron microscopy (TEM) of cell sections for the detection of viruses, virus-like particles, mycoplasma, yeasts, fungi and bacteria, observing $\geq$200 cell profiles, using 2 fixed cell pellets at 4×10^6 cells

3.14 Safety Testing

Safety testing is performed on the EOPCB lots and comprises both full product release testing following Subheading 3.12, product characterization testing following Subheading 3.13 and additional testing as listed hereafter (Fig. 5). Using vials from the start, middle and end portions of the EOPCB lots, perform the following testing.

1. In vivo tumorigenicity assays following FDA Points to consider (1993), using athymic mice, using 8 mL of live cell suspension with 7×10^7 cells/mL in FBS free medium
2. Karyology of human cell lines, using a confluent T175 flask of cells treated with colcemid

3.15 Stability Testing of Cryopreserved Cell Banks

In order to assess the stability of the cryopreserved materials, perform recovery studies following Subheading 3.11 on vials from the adequate lots at timepoints of 1, 2, 3, 6, 12 and 18 months after lot manufacture and once yearly after that. Total and viable cell counts, PDVs and PDTs are to be determined, while behaviour of the cells in culture and morphology is to be photographically recorded.

3.16 Banking Campaigns for Clinical Lot Productions

Based on the validation of the EOPCB at Passage 12, which documents and validates the in vitro lifespan of the cell type of interest at least up to Passage 12, clinical applications may be considered for cells at Passages 7 or 8, as they represent two thirds of the documented and validated lifespan. Therefore, using vials from the established WCB at Passage 4, production campaigns may be initiated to obtain cells at optimal passages in view of product manufacturing or clinical application, by performing serial passages in order to establish tier-2 WCBs following methods described in Subheading 3.9. Adequate testing must be performed throughout these supplementary steps as described hereabove, while cell behaviour, PDVs and PDTs must remain consistent and within specifications.

3.17 Production Lot Validation and Clinical Release

Once production lots are ready for validation and clinical release, a final evaluation must be made by the appropriate responsible persons (Fig. 5). Based on a comprehensive assessment of available production and testing documentation, the final decision is made to liberate the respective production lots for subsequent processing or application in the clinic following defined SOPs. Conforming lots may eventually be used for the preparation of bioengineered combinations products such as the Progenitor Biological Bandage, a combined ATMP which has been used for over two decades in the Lausanne University Hospital for treating burns, ulcers and donor-site wounds (*see* chapter "Progenitor Biological Bandages: An Authentic Swiss Tool for Safe Therapeutic Management of Burns, Ulcers and Donor Site Grafts") (Fig. 5) [15, 23, 25].

4 Notes

1. Higher volumetric proportions of FBS do not stimulate primary progenitor cell proliferation in a significative way and do not influence final cell yield nor shorten time to harvest confluency. Lower volumetric proportions of FBS may result in relatively lower final cell yields or longer culture periods.
2. Alternative culture nutrient sources such as human platelet lysate (HPL) supposedly pose less immunologic risks by elimination of xenogeneic raw materials; however this substitution may affect the stability of the cellular materials throughout culture expansions and banking. Furthermore, extensive industrial use of FBS in cell culture processes during the last century has not demonstrated tangible evidence of immunological risks restricting its use as a GMP raw material.
3. As in **Note 2**, the switching out of FBS from cryopreservation media would imply elimination of xenogeneic raw materials, to be substituted by human serum albumin (HSA), cryoprotective sugars, etc.
4. Establishment of a PWCB enables the pragmatic assessment of a considered cell type, sparing precious PCB materials and GMP production time and resources. The rationale for establishment of a GMP PWCB is that testing results within specifications for such materials tacitly imply that cells at lower passages comply as well.
5. If testing results are within defined specifications, proceed. If testing results are not within defined specifications, consider testing the PCB directly. If testing results are within defined specifications, proceed. If testing results are not within defined specifications, reconsider manufacturing processes or discard entire cell type.
6. Often overlooked, these parameters may have a considerable influence on the harvest cell yield or the time to harvest, which translates by relatively elevated costs *per* batch. Compare at least three FBS manufacturers and two lots *per* manufacturer, if materials are available in sufficient quantities. Perform serial dilution subcultures in 10 cm diameter Petri culture dishes, and use Complete Medium prepared with the different test-items. Maintain cultures appropriately and evaluate cell morphology and final cell yield for the different test-items. Choose the FBS lot producing the highest yield of morphologically normal cell populations. Compare at least three manufacturers of culture vessels. Expand the cell type of interest using the selected FBS lot in standard culture conditions in the different test-items. Choose the culture vessel reference and lot producing the highest yield of morphologically normal cell populations.

7. The intended passage to be used in clinical applications is Passage 8. The defined passage for the EOPCB establishment is Passage 12. The ability of the cell type to proliferate up to Passage 15 without drastic reduction of the harvest cell yield or significant extension of the time to harvest needs to be assessed, in order to ascertain and validate the in vitro lifespan of the considered cell type. If the lifespan is shorter than expected, reconsider the passages to be used for clinical application, and determine economic viability of the cell type based on realistic projected material stocks.
8. Investigation of cell surface markers shall help to confirm population purity and the identity of the cell population of interest by comparison with published maker profiles specific to dermal progenitor fibroblasts. Investigation of karyotype evolution along passages yields information about the in vitro stability of the cell type with regard to lifespan, while differentiation induction assays allow to define potency of the cell type of interest and to classify the latter as progenitors and not stem cells.
9. Ensure the vials do not come into direct contact with the dry ice during transport.
10. Following accepted principles of cell cryopreservation, freezing rates should be controlled and slow, while thawing should occur rapidly. Therefore, quickly transfer vials from dry ice to the waterbath to enable rapid transmission of thermal energy and initiate thawing.
11. New generations of cryovials are not manually annotated or labelled individually but may comprise predefined barcodes or identification matrixes. In such cases, photographically record the material before discarding it.
12. If proper cryopreservation and initiation procedures have been followed, expected viability of progenitor fibroblasts upon initiation ranges from 90% to 100% for storage periods reaching up to 10 years.
13. Identify culture vessels with cell type, passage number, date and time of seeding, seeding density and individual vessel identifiers, serving for referencing of deviations, suspected deviations or particularities. Different types of culture vessels comprising higher culture surface densities, e.g. Nunc™ Cell Factories™ or Corning® HYPERFlasks®, could be considered but must first be validated for the cell type of interest. Choice of culture vessels, directed by final cell yield requirements, should be defined in the pilot and validation phase.
14. The total expected process time from the removal from liquid nitrogen storage to flask seeding and incubation is of 2 h.

15. Record culture appearances at days 2 and 6 after initiation and on the day of harvest by photographic imaging.
16. In a normal expansion, the medium should be clear and bright red to red-orange when higher cell densities are present in cultures. No evidence of contamination such as discoloured medium or thick depositions are observed. Flasks are free of cracks and defects.
17. Do not disturb the cell monolayer during medium exchange phases, aspirate, and dispense medium against the surface opposite of the monolayer.
18. Full 100% confluency is expected in 12–14 days of culture. Primary dermal progenitor fibroblasts behave well at high confluency and should not be harvested before reaching optimal final cell yield, which is determined in the pilot and validation phases.
19. Up to five flasks should be processed at a time, in order to not overload the laminar flow hood workspace.
20. Unlike trypsin, TrypLE™ does not require FBS adjunction for discontinuation of the enzymatic effect but requires dilution instead.
21. The expected harvest yield for the MCB lots is around 2.7×10^9 cells, representing around 250–270 vials to be cryopreserved. The expected average population doubling values for such expansions are to be around 6.3 ± 0.5, while the population doubling times are expected to be around 47 ± 7 h. A relative viability <85% at harvest would indicate a problem with the harvest procedure, as relative viability values typically range between 95% and 100%. Harvest confluency is expected to be attained in 12 to 14 days of culture.
22. The specific composition of the cryopreservation solution enables easy homogenous resuspension of the cells, but high cell densities favour rapid deposition to the bottom of the container. Regular resuspensions are paramount in ensuring batch homogeneity and comparable average cell counts *per* vial.
23. Ensure the controlled rate freezing devices are equilibrated at ambient temperature before transferring the cryovials and that system specificities are considered, e.g. adequate levels of isopropanol in Nalgene® Mr. Frosty™ or sufficient space is available in the freezer for non-stacked CoolCell® FTS30. Due to excellent cell viability maintenance and robustness during the cryopreservation and initiation processes, additional measures such as intermediate precooling of freezing devices are not necessary. Maintain the freezer door closed once devices have been transferred, to ensure that an optimal freezing curve and a

rate of −1 °C/min rate of freezing is obtained. Devices can be left in the freezer overnight but not any longer.

24. The MCB lot must be properly tested before the admissibility to the biorepository is assessed. Once the MCB lot is liberated, the vials are to be transferred to the proper storage locations using a dry shipper. For appropriate risk mitigation, split the MCB lot in at least two distinct sublots to be stored in totally independent locations.
25. The expected harvest yield for the WCB lots is around 3.0×10^9 cells, representing around 280–300 vials to be cryopreserved. The expected average population doubling values, population doubling times, and viabilities are to be similar to those of the MCB lots.
26. Surplus cell suspension left over during the passage procedures may be cryopreserved, used in other assays or discarded to waste. Cellular materials should always be revalorized as much as possible before considering discarding to waste.
27. The expected harvest yield for the EOPCB lots is around 0.7×10^9 cells, representing around 60–70 vials to be cryopreserved. The expected average population doubling values, population doubling times, and viabilities are to be similar to those of the WCB lots.
28. These studies can be combined with cell growth and sample preparation assays, for pragmatic use of cellular materials.
29. The GMP PWCB may be used for optimization steps that must be performed under GMP standards, following the GMP establishment of MCBs and WCBs.

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Progenitor Biological Bandages: An Authentic Swiss Tool for Safe Therapeutic Management of Burns, Ulcers, and Donor Site Grafts

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Abstract

Clinical experience gathered over two decades around therapeutic use of primary human dermal progenitor fibroblasts in burn patient populations has been at the forefront of regenerative medicine in Switzerland. Relative technical simplicity, ease of extensive serial multitiered banking, and high stability are major advantages of such cell types, assorted to ease of safety and traceability demonstration. Stringent optimization of cell source selection and standardization of biobanking protocols enables the safe and efficient harnessing of the considerable allogenic therapeutic potential yielded by primary progenitor cells. Swiss legal and regulatory requirements have led to the procurement of fetal tissues within a devised Fetal Progenitor Cell Transplantation Program in the Lausanne University Hospital. Proprietary nonenzymatic isolation of primary musculoskeletal cell types and subsequent establishment of progeny tiered cell banks under cGMP standards have enabled safe and effective management of acute and chronic cutaneous affections in various patient populations. Direct off-the-freezer seeding of viable dermal progenitor fibroblasts on a CE marked equine collagen scaffold is the current standard for delivery of the therapeutic biological materials to patients suffering from extensive and deep burns. Diversification in the clinical indications and delivery methods for these progenitor cells has produced excellent results for treatment of persistent ulcers, autograft donor site wounds, or chronic cutaneous affections such as eczema. Herein we describe the standard operating procedures for preparation and therapeutic deployment of the progenitor biological bandages within our translational musculoskeletal regenerative medicine program, as they are routinely used as adjuvants in our Burn Center to treat critically ailing patients.

Keywords Biological bandages, Burns, Cell therapy, Clinical cell banking, GMP manufacturing, Optimized protocols, Progenitor cells, Ulcers

Abbreviations

cATMP	Combined advanced therapy medicinal product
CDEA	Cultured dermal-epidermal autograft
CE	Certification mark of the European Economic Area

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CEA	Cultured epithelial autograft
cGMP	Current good manufacturing practices
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
D-PBS	Dulbecco's phosphate-buffered saline
FBS	Fetal bovine serum
GMP	Good manufacturing practices
ICU	Intensive care unit
IPC	In-process control
MCB	Master cell bank
PBB	Progenitor biological bandage
PCB	Parental cell bank
QRM	Quality risk management
SOP	Standard operating procedure
TBSA	Total body surface area
WCB	Working cell bank

1 Introduction

Severe cutaneous affections and wounds such as chronic ulcers and deep burn trauma fit in the categories of complex healing dynamics and current unmet needs in clinical settings around the globe. Indeed, traditional surgical techniques and pharmacotherapeutic management provide limited support toward full tissue structural integrity and functionality restoration of the skin barrier. Novel regenerative medicine products and protocols describing arrays of bioengineered skin substitutes and wound coverages have been demonstrably closing the gap between traditional care and the needs prompted by high variability of individual patient clinical evolutions [1–4]. In the context of severe burns, temporary cadaveric allografts or xenograft dressings and processed split- or full-thickness autografts enable reductions in morbidity and mortality rates, yet the outcomes remain limited. Creation of additional cutaneous effractions during healthy skin harvest for autografting, although sometimes necessary, may tilt the patients' health momentum negatively. Bioengineered constructs or biological combination products open a new dimension to wound control and tissue repair, potentially leading to eventual full recovery of structure and function, through restoration induction of cutaneous physiological homeostasis. Different commercially available allogenic or autologous cell-based solutions have been proposed (e.g., Apligraf®, Epicel®, OrCel®, ReCell®, TransCyte™), yet the biological starting materials used in such developments could benefit from further optimization, in view of therapeutic effect standardization and amelioration [5–16].

During the past three decades, proactive translational research and clinical developments have been driving vectors in the musculoskeletal regenerative medicine field in the Lausanne University Hospital (West of Switzerland). Extensive optimization of therapeutic cell source selection and bioprocessing thereof has been the basis for the establishment of a Fetal Transplantation Program and the development of novel biological bandages based on progenitor cell clinical applications (*see* [17]) [18–24]. Technical simplicity, ease of extensive cell banking, and high stability of progenitor cells allow for scalable, effective, and safe processes to be implemented in routine clinical practices. The optimal adequacy of human primary dermal progenitor fibroblasts with current good manufacturing practice (cGMP) requirements and the considerable inherent technical and clinical advantages of the considered cell types enabled the elaboration of an efficient and high therapeutic value solution for acute and chronic wounds. A single organ donation was demonstrated as potentially sufficient for the establishment of multitiered banked cryopreserved cellular material stocks in quantities equivalent to 39 billion therapeutic product units (*see* [17, 25]) [19–22, 26–30].

Cultured epithelial autografts (CEA) and cultured dermal-epidermal autografts (CDEA) are used in the Lausanne Burn Center in moderate to severe clinical cases [31]. The progenitor biological bandage (PBB), a moldable single-use bioengineered wound coverage, defined as a standardized transplant under Swiss law and more generally a combined advanced therapy medicinal product (cATMP), was initially developed for severe burn patients to reduce the need for donor site skin grafting and shorten production periods. Outstanding results in pediatric burn patient populations have led to the use of such bioengineered constructs as primary treatments in children and adult burn victims, as well as for the management of complicated ulcers, as PBBs promoted scar-free tissue repair [18, 19, 21, 24, 32]. Such bioresorbable constructs are obtained by direct off-the-freezer seeding of viable dermal progenitor fibroblasts on a CE marked lyophilized equine collagen sheet scaffold. Cellular materials originate from a non-enzymatically isolated progenitor fibroblast population, which has been culture-expanded in a tiered GMP biobanking program to optimize availability of therapeutic cells (*see* [17, 25]) [19–23]. Diversifications in the clinical indications and delivery methods for the progenitor fibroblasts have yielded excellent results for management of difficult ulcers, donor site wounds, and chronic affections such as eczema, psoriasis, or actinic dermatitis, whereas semisolid creams and gels have been evaluated as alternative delivery options for the active biological principles of interest.

Twenty years of clinical experience with the use of PBBs and retrospective studies have evidenced the safety and high therapeutic benefits for treatment of superficial to partial-thickness burns in

children. Absence of immunological rejection and rapid scarless healing efficiency were observed [18, 24]. Ongoing clinical trials in Asia (trial ID numbers NCT03624023 and NCT02737748) have substantiated the safety of application of the progenitor fibroblasts in various patient populations. In accordance with the Swiss regulatory context, prospective investigation of the safety and performance of PBBs is being devised within a marketing authorization process, in view of establishing a formal risk-benefit ratio and proceeding with clinical validation of the therapy based on appropriate objective endpoints (CHUV Priority Project *Bru_PBB*). In this work, we describe the GMP preparation and application of PBBs, as they are routinely used in our Burn Center to treat high TBSA (total body surface area) burn patients.

2 Materials

All considered materials, consumables, and product components should be sterile and GMP grade. Facilities and equipment are to be qualified and validated appropriately within applicable GMP guidelines. Waste disposal regulations are to be followed diligently.

2.1 Biological Starting Materials

Cryovials from a GMP production tier-2 working cell bank (WCB), stocked in the gas phase of liquid nitrogen, should serve as a source of therapeutic dermal progenitor fibroblasts. The cells should be derived from an organ donation under an established transplantation program, nonenzymatically isolated and serially culture-expanded under cGMP standards, as described in [17, 25]. Full traceability and safety testing should be documented for each production lot and serve for the final evaluation and clinical lot liberation. Individual cryovials from tier-2 WCB lots should contain 2×10^6 cells at Passage 7 or 8. Stability of the cellular material (e.g., karyotype evolution, cell surface marker evolution) should be assessed over storage time, and functional characteristics should be monitored over time as well (e.g., viability, stimulatory effect of fibroblasts in a defined cellular proliferation or migration model, biomarker level homogeneity).

2.2 Raw Materials

Raw materials are defined as materials which are present as an appreciable part of the final product when considering specific manufacturing lots. The following raw materials are required for the manufacture of PBB clinical lots.

1. KOLLAGEN *resorb*™ (RK 1209), RESORBA Medical GmbH, 2 mm × 9 cm × 12 cm, 5 sponges *per* pack, sterile, store at room temperature until use.
2. D-PBS buffer, pH 7.4, sterile, no calcium, no magnesium, store at room temperature until use.

2.3 Ancillary Materials

Ancillary materials are defined as materials which are used and subsequently removed during the manufacturing process, therefore not constituting an appreciable part of the final product. The following ancillary materials are required.

1. DMEM, GMP grade, high glucose, with pyruvate, with phenol red, store at 4 °C until use.
2. FBS, GMP grade, with certificate of animal origin and health, certificate of analysis, irradiation certificate, batch manufacturing record, store at −80 °C until use.
3. L-glutamine, 200 mM, store at −20 °C until use.

2.4 Contact Process Consumables

Contact process consumables come into direct contact with cell suspensions or the biological constructs during the manufacturing process. Store consumables at room temperature until use. The following contact process consumables are required.

1. Tissue culture Petri dishes, 15 cm diameter, sterile, uncoated culture surfaces.
2. Sterile scalpels.
3. Sterile surgical tweezers.
4. Aspiration pipets, sterile.
5. Serological pipets, sterile, 5, 10, and 25 mL, with cotton filters.
6. Sterile culture-grade conical centrifuge tubes, 15 mL, 50 mL.
7. Sterile culture-grade containers, 500 mL.

2.5 Noncontact Process Consumables

Noncontact process consumables do not come into direct contact with cell suspensions or the biological constructs during the manufacturing process. Store consumables at room temperature until use. The following noncontact process consumables are required.

1. T75 sterile cell culture flasks, 75 cm^2, uncoated culture surfaces, with filter screw caps.
2. Trypan blue solution, 0.4%, filter-sterilized.
3. Hemocytometer, Neubauer Improved.
4. Micropipette filter tips, 20, 200, and 1000 μL, sterile.
5. Microcentrifuge tubes, cell culture grade, sterile.
6. Denatured 70% ethanol, in vaporizing bottles.
7. Permanent markers, ethanol resistant.

2.6 Critical Process Facility and Equipment

The critical process facility comprises the production suite and storage unit, along with the installed equipment, instruments, and devices. Wherever applicable, equipment, instruments, and devices should be calibrated and appropriately qualified. The following infrastructure, equipment, instruments, and devices are required.

1. Cleanroom facility comprising the production suite, B grade, and the storage unit.
2. Biological safety cabinet, A grade, vertical laminar flow.
3. Complete sterile cleanroom personal clothing, suit, mask, hood, gloves, boots.
4. CO_2 incubators, temperature range 36.5 °C ± 1.5 °C, relative humidity range 80% ± 10%, CO_2 content range 5.0% ± 1.5%.
5. Liquid nitrogen Dewar storage tanks, level alarm fitted, with lock.
6. Ultralow-temperature freezer, temperature range −80 °C ± 5 °C.
7. Freezer, temperature range −20 °C ± 2 °C.
8. Refrigerator, temperature range 4 °C ± 2 °C.
9. Centrifuge, operational range 230 × *g* ± 10 × *g*, ambient temperature, swing-out buckets with adaptors, 15 mL and 50 mL tubes.
10. Optical inverted microscope, 40×, 100× and 400× optical magnification with phase contrast.
11. Water bath, temperature range 37 °C ± 2 °C, with distilled water.
12. Aspiration pump, handheld aspiration pipet mount, with sterile tubing.
13. Insulated isotherm transport container, sterile, with lock and key.
14. Motorized pipet for serological pipet manipulation.
15. Micropipettes, 20, 200, and 1000 μL, calibrated.
16. Cryotube racks, with star-foot adaptors.

3 Methods

See the Video 1 provided as Electronic Supplementary Material (available on the chapter's webpage at link.springer.com) for a film prepared in the Lausanne University Hospital depicting various steps of PBB production and clinical application in our Burn Center.

3.1 Generalities About Methods

1. Proper ethical and medical validations should be obtained before treatment of patients with progenitor biological bandages.
2. Applicable cGMP procedures and recommendations are always to be followed.
3. Ensure quality risk management (QRM) systems and in-process controls (IPC) are adequately implemented.

4. Legal retention samples (raw and starting materials, in-process control samples and production lot samples), reports, and records shall be constituted and stored appropriately along with full traceability of all manipulations, deviations, and corrective measures.
5. All preclinical manipulations requiring cell suspensions or the constructs to be exposed are to be conducted in the class A biological safety cabinet.
6. All materials and equipment entering the production area and biosafety cabinet are to be properly sanitized.
7. Necessary documents and labelling materials are to be prepared before processing operations begin.
8. All media, solutions, or buffers to be warmed at 37 °C are to be placed in a water bath that has been equilibrated at 37 °C for at least 1 h.
9. Procedures are to be carried out at room temperature unless otherwise specified.

3.2 Media Preparation

For complete medium preparation, proceed as follows.

1. Dilute FBS in DMEM, so that the final volumetric proportion of FBS is 10%, and the final volumetric proportion of DMEM is 90%.
2. Dilute L-glutamine 200 mM in DMEM, 10% FBS, so that the final volumetric proportion of the L-glutamine supplement is 1%. Store complete medium at 4 °C until use and use within 21 days after preparation.

3.3 Clinical Assessment and PBB Requirement Evaluation

Patient eligibility and treatment necessity must be evaluated based on the clinical case specificities by the medical team before considering use of progenitor biological bandages. Once the use of PBBs is validated, the adequate quantity of constructs is ordered from the GMP production unit. Validation of treatment initiation requires the following steps.

1. The Senior Medical Doctor evaluates the need for PBB construct application (e.g., $\geq$ second-degree burn lesions, $>$ 10–20% TBSA, extensive skin graft donor sites) (*see* **Note 1**).
2. Rule out contraindications for PBB application, and ensure no incompatibilities with the constructs are present during treatment. Perform bacteriological investigations (swab tests) of the wound beds to exclude colonization or infection if suspicions of such cases arise and are substantiated by several combined criteria (fever, specific odor, elevated inflammatory markers) (*see* **Note 2**).

3. Plan the therapeutic applications of constructs in accordance with the care schedule of the patient and the production capabilities of the GMP manufacturing unit. Ideal application occurs after showering, debridement, and disinfection of the wounds, in the operating theater.
4. Order the adequate quantity of constructs, and proceed with initial patient and wound care (*see* **Note 3**).
5. Organize the delivery of the PBB constructs with the GMP manufacturing unit according to the defined therapeutic calendar (*see* **Note 4**).

3.4 Thawing of Cryopreserved Progenitor Fibroblasts

Upon reception of the production order from the medical team in the clinic, the GMP production unit initiates cryopreserved vials of progenitor fibroblasts for direct seeding of the construct scaffolds. The following steps are performed.

1. Prepare all necessary materials for the defined number of PBBs to be manufactured, and transfer materials to the specific culture/production room.
2. Identify starting material lots to use and the quantity of tier-2 WCB vials to initiate (Fig. 1a) (*see* **Note 5**).
3. Remove vials from cryogenic storage, and update the corresponding storage logs.
4. Transfer the vials to the culture room on dry ice in a sterile container (*see* **Note 6**).
5. Transfer the cryovials to the water bath previously equilibrated at 37 °C, and allow the cell suspension to thaw, applying gentle rotations to the vials. Once the last frozen material has just thawed, transfer the cryovials to the laminar flow hood after thorough sanitation.
6. Homogenize the cell suspensions in each vial by gentle pipetting, ensuring that no air bubbles are formed by expulsion of air in the suspensions.
7. For each cryovial, aseptically transfer the thawed cell suspension to a properly labelled centrifuge tube containing 14 mL of warm (37 °C) complete medium, by dispensing the cell suspension dropwise into the medium. Discard cryovials and collect the original labels, if applicable.
8. Centrifuge the diluted cell suspensions at 230 × *g* for 10 min at ambient temperature.
9. Aseptically remove supernatants using an aspiration pipet and vacuum pump.
10. Resuspend cell pellets homogenously using 20 mL of warm complete medium.

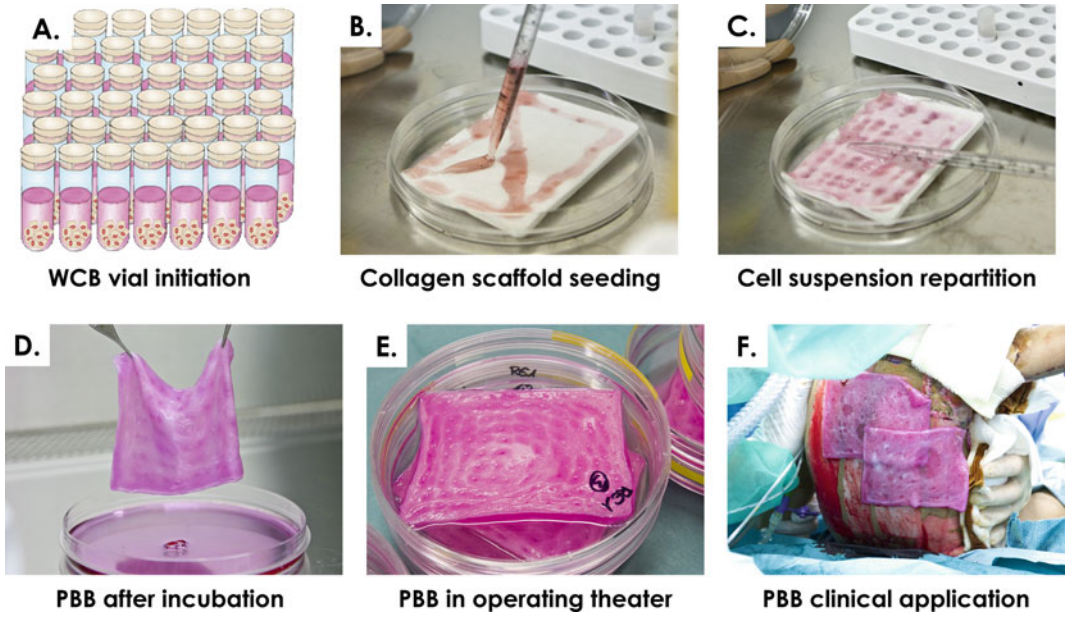

Fig. 1 Flowchart describing the different manufacturing steps necessary to produce progenitor biological bandages under GMP standards for clinical application on severe burn victims. (**a**) Working cell bank vials of dermal progenitor fibroblasts are identified in a tier-2 GMP production lot, based on patient needs, and are initiated. (**b**) Collagen scaffolds are preconditioned by regular indentations and are aseptically seeded with appropriate cell suspensions. (**c**) The cell suspensions are homogenously repartitioned over the surface of the constructs using the body of the serological pipet. (**d**) Aspect of PBBs after 24–72 h of incubation. The correct handling method is depicted here, in order to preserve construct structural integrity. (**e**) Aspect of PBBs after rinsing and monitored transport to the operating theater. Note the observable presence of the mechanical indentations applied during the cell-seeding phase. (**f**) Clinical application of PBBs on complex patient wounds after proper care and before standard gauze and bandage application

11. Pool cell suspensions in an appropriate sterile container, and homogenize the pool. Isolate and separately condition 3×0.5 mL of pooled suspension samples in microcentrifuge tubes for viability control, total and viable cell counts determination (*see* **Note 7**).
12. Determine total and viable cell counts by microscopic enumeration using hemocytometers and Trypan blue exclusion dye (1:1 dilution with the cell suspension). Verify that relative cell viability is $\geq$80% at this stage for the manufacturing process to continue.
13. Aseptically transfer cells to an appropriate sterile container, and dilute the cell suspension homogenously as needed using warm complete medium so that the final concentration becomes 10^5 total cells/mL.

3.5 PBB Scaffold Preconditioning and Cell Seeding

For effective integration of the cell suspension in the scaffold and adequate colonization thereof, the latter requires specific preconditioning, as follows.

1. Under the laminar flow, open the scaffold packaging, and aseptically transfer the scaffolds to individual Petri dishes (*see* **Note 8**).
2. Using sterile tweezers, make regular full-thickness indentations into the 9 cm × 12 cm membrane, in series of eight considering the long side and in series of four considering the short side of the membrane (*see* **Note 9**).
3. Using a serological pipet, homogenously distribute 5 mL of cell suspension (5×10^5 cells) on the construct membrane in a crosswise pattern (Fig. 1b).
4. Using the same pipet, adopt a slanted angle, and use the pipet body to evenly distribute the suspension over the whole preconditioned surface of the membrane, particularly around the indentations (Fig. 1c) (*see* **Note 10**).

3.6 PBB Construct Lot Preparation and Liberation

Optimal therapeutic results are obtained with viable cells at the time of the application to the patient (*see* **Note 11**). In order to allow cell survival, surface colonization, and optimal integration in the scaffold to form the construct, the latter requires a period of incubation after cell seeding. Proceed as follows.

1. When two series of biological constructs have been seeded, aseptically and gently add 25 mL of fresh warm (37 °C) complete medium to the edge of each culture dish, and gently transfer both series to the incubator set at 37 °C and 5% CO_2.
2. Maintain the dishes incubated and undisturbed for 24–48 h (*see* **Note 12**).
3. Remove dishes gently from the incubator.
4. Inspect the dishes and their contents both macroscopically and microscopically for obvious signs of contamination or observable defects (Fig. 1d) (*see* **Note 13**).
5. Remove the incubation medium and condition appropriate aliquots thereof for quality controls (sterility, bacterial, viral, fungal, endotoxin, and prion testing). Isolate small construct punches from each lot of constructs for quality controls.
6. Photographically record the appearance of each lot (macroscopic and microscopic).
7. Wash each construct twice and gently with 20 mL of warm (37 °C) D-PBS to eliminate serum traces and debris.
8. Add 4 mL of D-PBS to the surface of each construct for hydration maintenance during transport.

9. Seal the dishes and condition them in a labelled, double-layered sealed plastic bag. Place the package in the sterile isotherm transport container at ambient temperature, equipped with a lock and a temperature monitoring unit.
10. Establish lot documentation, and validate the release in a release report based on the quality control testing results (*see* **Note 14**).
11. Organize transport of the sealed container to the clinic (<2 h after conditioning).

3.7 Clinical PBB Construct Application

For clinical application of the PBB constructs to the patient, the medical team will proceed as follows.

1. Shower the patient.
2. Perform debridement and wound bed disinfection according to the standard clinical protocols.
3. Clean the traces of disinfecting agent from the wounds.
4. Photographically document the wound bed appearance before treatment application, including a ruler.
5. Open the container and packages, remove the PBB constructs from their dishes (Fig. 1e) using two forceps, and manipulate constructs by holding both upper corners (Fig. 1d).
6. Apply the constructs to the patient, overlapping them where necessary (Fig. 1f).
7. Once an individual PBB construct is in place, do not displace it or move it, to avoid rupture of the construct.
8. Evacuate trapped air bubbles from under the constructs very gently using the forceps or gloved fingertips.
9. Photographically document the wound bed appearance after treatment application has ended.
10. Once all the PBB constructs are placed, overlay them serially with Jelonet® gauze and cotton gauze before bandages are applied.

3.8 PBB Construct Exchanges and Course of Treatment

In accordance with the predefined therapeutic calendar and clinical evolution, ensure adequate quantities of PBB constructs are made available at the appropriate dates and times. For PBB construct exchanges, the medical team will proceed as follows every 2–4 days as necessary.

1. Open the outer layers of bandages until the PBB sheet remnants are apparent.
2. Photographically document the wound bed appearance with the remaining constructs, and then proceed to remove the construct remnants.

3. Perform sampling for bacteriology analysis in case of suspicion of wound colonization or infection, and shower the patient.
4. Photographically document the wound bed appearance without the constructs, including a ruler.
5. Apply necessary amounts of new PBB constructs.
6. Repeat the PBB applications if needed until sufficient skin closure is attained (*see* **Note 15**).

During the course of the treatment, the Senior Medical Doctor will evaluate the situation regularly, with great attention being paid to the following points.

1. Treatment duration with PBBs depends on the epithelialization and scar assessment evaluated by the Senior Medical Doctor (*see* **Note 16**).
2. Topical corticosteroids may be applied if necessary (in the case of tissue granulation) and according to the decision of the Senior Medical Doctor.
3. At any phase of the treatment, the presence of severe local infection should prompt immediate PBB treatment discontinuation and thorough showering the patient, according to the decision of the Senior Medical Doctor.
4. Applications of PBBs should be discontinued in case of absence of skin closure after 2 weeks of treatment, according to the decision of the Senior Medical Doctor.
5. All episodes of treatment with PBBs should be documented. The date, number of PBB constructs, and the zones of application should be recorded.
6. During PBB treatment, usual procedures should be followed as for all burn patients, comprising minimal hospitalization periods, protection of newly formed cutaneous structures, daily massages as soon as structurally supported by the skin, and application of silicone pressure garments, if needed.

3.9 Clinical Endpoints, Expected Therapeutic, and Adverse Effects

During the course of the treatment with PBBs, regular reassessment of the evolution of the wound bed by the medical team and general evaluation of the patient should lead to formal evaluation of the effect of the treatment. Primary and secondary objective clinical endpoints relative to safety and therapeutic performance such as the following may be used for this evaluation.

1. Days of treatment necessary to obtain reepithelialization, existence of a wound healing drive.
2. Length of hospitalization, in ICU and maintenance care.
3. Healing rates and evolutive TBSA percentages.

4. Quantity and surface of wounds requiring autografts.
5. Pain questionnaires.
6. Quantity of PBBs applied during the treatment.
7. Scar management and duration thereof (*see* **Note 17**).
8. Long-term assessment of skin functionality, expressed by mobility and contracture.
9. Long-term skin quality and sensory modifications (*see* **Note 18**).
10. Number and natures of infections.

With regard to side effects directly related to the application of PBBs, two decades of clinical use have not yielded any evidence or observations suggesting specific adverse reactions or events. Side effects inherent to the burn trauma itself may be observed (e.g., wound infections, pain, prolonged bleeding, pneumonia, urinary tract infections, punctiform keratitis, urticaria, upper respiratory tract infections). With regard to therapeutic performance and safety of application of the PBBs, the following observations have been made.

1. The healing outcomes of treatments with PBBs are at least as good as other standard treatments (e.g., AQUACEL® Ag + or Jelonet® coverages).
2. PBB treatment leads to total recovery of mobility (e.g., hands and fingers) and restoration of skin pigmentation.
3. Application and removal of PBBs are painless and do not inherently require anesthesia.
4. PBBs alleviate the risk of hypertrophic scar formation, retraction, and secondary breakdown of healed surfaces.
5. PBBs are microbiologically safe and pose no inherent risk of colonization or infection by virologic, bacterial, and fungoid agents.
6. No additional fixation devices (e.g., glue, staples, stitches, silicones) are needed, and ease of application on all anatomical sites is evident.
7. The use of progenitor cells alleviates the risk of immunological rejection of the treatment (*see* **Note 19**).
8. PBBs induce no observable inflammatory reaction, no secretion, and no allergy.
9. PBBs possess no inherent tumorigenic properties.

4 Notes

1. In the Lausanne Burn Center, indications of PBBs are limited for superficial to deep second-degree burn wounds. The constructs are applied directly after patient stabilization, cleaning, and debridement of the burn site. The therapeutic plan is defined taking into account the minimal 18- to 24-h notice period necessary for the manufacture of the PBBs (cell initiation and colonization of the collagen constructs under incubation).
2. PBBs should not be applied on colonized or infected wounds. Vitally unstable patients do not qualify for the treatment, nor do patients with documented allergies to products of equine or bovine origin. No data is available about the use of PBBs in pregnant and lactating women; therefore evaluation of the clinical situation is at the medical doctors' discretion. PBBs are single-use, sterile (except for the active progenitor fibroblasts), and manufactured under cGMP specifications and standards and should not be frozen or used if the packaging is damaged. It is recommended not to use Betadine® cream with PBBs.
3. Depending on patient health status and attained TBSA, the wounds are progressively treated. Patient wounds are initially covered with Jelonet® coverages. After the initial delay of 24–48 h for the first construct lots to be manufactured, the wounds are showered and cleaned before PBBs are applied.
4. The first PBB lots are usually applied 24–48 h after the patient is admitted. PBB exchanges are performed every 2–4 days (maximal continuous coverage of 4 days) after medical re-evaluation of the wounds and for a maximum of four serial applications in our current clinical protocols.
5. Preliminary experience was gathered with the use of a relative viable cell seeding density of 2.5×10^3 cells/cm^2 of the constructs, while the current protocol details the use of 4.5×10^3 cells/cm^2 or approximately 5×10^5 viable cells *per* 9 cm × 12 cm construct. Four constructs can therefore be manufactured with a vial containing 2×10^6 viable progenitor fibroblasts. During cell seeding, placing a small droplet of cell suspension on the surface of the culture dish allows for rapid assessment of preliminary cell adherence (used as control). For standardization of cell seeding, multichannel micropipettes or dedicated automatic dispensing devices may be considered.
6. Ensure the vials do not come into direct contact with the dry ice, which could negatively impact cell viability.

7. Centrifugation and resuspension of the cells enable rinsing of the DMSO and FBS-based cryopreservation medium. Use one aliquot for cell count determination and two aliquots to seed 2 × T75 culture flasks by dilution of the suspension in 10 mL of complete medium. Incubate the flasks to monitor cell behavior over 48 h. Sterility can be summarily assessed by culture monitoring. Perform viability controls for new cell production batches or after 6 months of storage.
8. The most porous or hydrophilic side of scaffolds should face up in the culture dishes. Beware of static electricity buildup, which can cause the scaffolds to stick to culture vessels or to instruments. Preliminary experience was gathered around the use of Baxter Tissue Fleece® (native equine collagen) as a scaffold. Current clinical protocols describe the use of Resorba KOLLAGEN *resorb*™, which was substituted due to a discontinuation in the Tissue Fleece® supply chain. The current scaffold is marketed as a medical device and is implantable, absorbable, pliable, hemostatic, and highly absorbent in its dry form and biocompatible, structurally stable, and elastic in the moist wound environment.
9. Up to ten scaffolds should be prepared simultaneously but not more, to avoid overcrowding of the hood workspace.
10. The progenitor fibroblasts adhere to the matrix and form a colonizing monolayer, spreading out from the indentations made in the sheets, after proper seeding and incubation. After clinical delivery, the cells probably exert their therapeutic effects by means of trophic paracrine modulation of regeneration and healing pathways in the neighboring wounded tissues (release of cytokines, growth factors, matrix proteins). Persistence of living therapeutic cells on and in the wounded tissue is very limited.
11. Limited clinical experience was gathered with the use of γ-irradiated progenitor fibroblasts, but the clinical protocol was soon reverted to using viable cells due to a relatively lower level of repair stimulation observed by the medical teams.
12. Urgent preparation of PBBs may take place on the same morning as the application is scheduled, in extreme cases requiring immediate action after initial debridement, showering, and disinfection. In such rare cases, add 25 mL of warm (37 °C) D-PBS buffer to the construct dishes before minimal incubation (>2 h).
13. Obvious signs of contamination warrant the destruction of the concerned lot. After 18 h of incubation, the cells should be attached to the surface of the constructs and growing as a monolayer. The cytocompatibility with the scaffold is assessed by cell viability (or adherence) and growth capacity on the

matrix. Microbiological quality controls performed on the medium and the final products should comprise mycoplasma and endotoxin detection.

14. The liberation of certain manufacturing lots may be necessary before the testing results are available to the production unit. In such cases, ensure adequate traceability is in place for each lot, to enable swift recalls in the case of a deviation with potential harmful impacts to the patient.
15. PBB constructs will stay in contact with the wound for 2–4 days in the case of burn wound treatment or for up to five days in case of graft donor site care. Current protocols limit the total number of new PBB applications to four.
16. In general, a PBB treatment duration of maximum two weeks until the autologous grafting (if necessary) should be considered.
17. Quantitative readouts of qualitative clinical evaluations such as the Vancouver scale should be used.
18. Quantitative readouts of clinical evaluations such as the erythema and melanin indexes should be used.
19. Progenitor cells are pre-immunocompetent and associated with a reduced capacity to evoke an immunological response in the patient due to the lack of post-thymic T-lymphocytes in the first 13 weeks of gestation.

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Validation of the Media Fill Method for Solid Tissue Processing in Good Manufacturing Practice–Compliant Cell Production

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Abstract

Over the past few years, a large number of clinical studies for advanced therapy medicinal products have been registered and/or conducted for treating various diseases around the world and many have generated very exciting outcomes. Media fill, the validation of the aseptic manufacturing process, is the simulation of medicinal product manufacturing using nutrient media. The purpose of this study is to explain the media fill procedure stepwise in the context of cellular therapy medicinal products. The aseptic preparation of patient individual cellular product is simulated by using tryptic soy broth as the growth medium, and sterile vials as primary packaging materials.

Key words Media fill, Method validation, Stem cells, GMP

1 Introduction

According to the European Regulation EC 1394/2007, the clinical use of advanced therapy medicinal products (ATMPs) requires the development of a process in compliance with the current good manufacturing practices (cGMP) [1]. Stem cell therapies (SCTs) are a fast-growing branch of ATMPs. Stem cells being isolated or collected from autologous or allogeneic human tissues and are ex vivo expanded, processed, and then administered to patients for treatments [2]. On the basis of cGMP regulatory steps, the safety of advanced therapies can be monitored and improved [3]. Ensuring microbiological safety in ATMPs is a major factor both for ATMPs manufacturers and regulatory agencies [4]. Although GMP are followed, sterilization cannot be guaranteed.

EudraLex, The Rules Governing Medicinal Products in the European Union, Volume 4, EU guided that validation of aseptic processing should include a process simulation test using a nutrient medium. Media fill (known as a "process simulation") is the part of an aseptic manufacturing process using a sterile microbiological growth medium, instead of the drug solution, to test whether the aseptic procedures are suitable for prevent contamination during

actual drug manufacture to ensure the safety of the drug. The guides also pointed that number of containers for media fills should at least equal the size of the product batch for small batches (fewer than 5000 units) and no contaminated units should be detected [5, 6].

In cellular therapy, cells were used as the drug. Thus, all the regulations about drugs should be applied for production of cellular therapy products to testing the weakness in process about microbial contamination as well as the operator's performance [1, 7–9].

Due to their nature, the process of cell therapy products are different from synthetic drug production. Therefore, the aim of this chapter is to describe a media fill test for cellular therapy products in good manufacturing practice–compliant production processes.

2 Materials

2.1 Materials and Reagents

- Soybean casein digest medium (tryptone soya broth—TSB): Aerobic bacteria, yeasts, and molds can grow in TSB medium. Guideline recommends the TSB in media fill process, instead of the final product for the method validation [10–12] (*see* **Note 1**).
- Sterile Vials (Fill-Ease™, DMF Number: 24938): Vial preparation is performed as plus 1 (one) more vial of the maximum number of vials prepared as the final product. In this study, since the maximum product output number is determined as three vials, four vials are prepared for the media fill test (*see* **Note 2**) (Fig. 1).
- Physiological saline solution (henceforth: saline): 0.9% saline is a mixture of sodium chloride in water and is widely used in medicine. As a result of our validation studies, it is decided to use saline in the final product.
- Microorganisms: According to European Pharmacopoeia, two types of bacteria—*Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922)—and one type of fungi—*Candida albicans* (ATCC 10231) strain—are used in this study (*see* **Note 3**).

Fig. 1 For small batches, the number of vials should be at least equal to the size of the product batch. Therefore, it is preferred to perform vial preparation as plus 1 (one) more vial of the maximum number of vials

2.2 Equipment and Apparatus

Equipment used in media fill process should be the same used in cell manufacturing (*see* **Note 4**). The following equipment is used for media fill trials.

- Biosafety cabinet (Thermo Scientific SAFE 2020 Class 2 Biological Safety Cabinet).
- Crimpers (BGB easy Grip Manual Crimper).
- Motorized Pipette Fillers (Thermo Scientific S1).
- Incubator (HeraTherm IMH60).

3 Methods

Process simulation steps and acceptance criteria should be clearly stated in the standard operation procedure (SOP); the procedure should be diligently followed and all steps should be recorded. The study is designed in triplicate (Fig. 1). The label with set information, number, and date is affixed on vials. During process simulation, environmental and personnel quality control tests should be carried out. Environmental and operator quality control results should also be in accordance with GMP.

3.1 Preparation of Process Simulation Group

The vial filling process is simulated for media fill trials. Sterile TSB is used for filling purpose. Primary packaging materials like sterile vials, rubber stopper, sealing cap, and equipment; filling method; and sealing of vials are the same as used in routine processes. TSB are transferred in the labeled vials of *process simulation group* as the same volume of the final product. Plugs of vials are placed and the caps are closed with a crimper (*see* **Note 5**).

3.2 Preparation of Positive and Negative Control Groups

The media fill is an experiment and therefore should include controls. A positive control for a media fill is a sealed product container of medium that is inoculated with a small number of microorganisms. A negative control may be prepared by preincubating the medium, or by aseptically transferring medium into a separate suitable sterile container and incubating the control simultaneously with the media fill test containers. All containers must be incubated in the same area and for the same duration of time (Fig. 2).

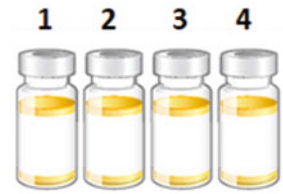

Fig. 2 Preparation of positive and negative control group. Vial number 1: negative control, vial number 2: *Staphylococcus aureus*, vial number 3: *Escherichia coli*, vial number 4: *Candida albicans*

3.3 Preparation of McFarland Concentration

McFarland standards provide a reference for standardization of bacterial suspensions used for susceptibility testing. The concentration of 0.5 McFarland is used for determination of microbiological contamination. Cultivated bacteria and mold colonies are selected by their colony type and inoculated in 4 ml TSB, then incubated at 35 °C for 2–6 h. The bacterial concentration of 10^8 colony forming units (CFU/ml) is diluted in saline by sequentially diluting to obtain 0.5 McFarland (i.e., less than 10^2) and transferring in the positive control labeled vials. Any microbial growth must be detected within 3 days for bacteria and up to 5 days for yeasts and molds. Plugs of vials are placed and the caps are closed with a crimper. The vials are incubated in the tubes inoculated with bacteria at 32.5 ± 2.5 °C for 3 days, then incubated in the tubes inoculated with molds at 22.5 ± 2.5 °C for 5 days (*see* **Note 6**).

3.4 Acceptance Criteria

The microorganisms to be tested should be identified by regulatory agencies which comply with the GMP regulations (European Pharmacopoeia—Current Edition, etc.). Initial qualification should be conducted in three successful consecutive simulations. The same process can be performed in the same manner as the initial simulation process and all authorized and qualified personnel should perform this procedure at least once a year. The contamination rate should be zero in 5000 U or less production. Each process should be confirmed yearly unless there is a significant change in the product, process, or facility. If there is a major change or changes in the product, process, or facility, revalidation is required and following the company's change control procedures is essential.

3.5 Data Analyses

The observation is done daily and the results are recorded. If there is no turbidity in the process simulation group at the end of the incubation period, there is no microbial growth (negative). In case of turbidity in the process simulation group, (positive) microorganisms should be subcultured and determined. The cause of the contamination is investigated. In this process, production is suspended. The accuracy of the test is checked, and a search for any false positive results is done. The test is repeated after the research and regulatory activity, and then it is decided whether to restart the process. If the acceptance criteria are met, it can continue its routine activities.

4 Notes

1. Guideline recommends the use of anaerobic medium such as fluid thioglycollate if the product is being filled in anaerobic conditions.
2. In case of different volumes or primary packaging, process simulation is performed separately for each packaging.

3. One or two of bacterial strains from house flora can be used as well.
4. Prior to setting up the MediaFill validation, it should be ensured that all equipment, utilities, and processes are validated and all instruments are calibrated.
5. It should be ensured that the medium touches the entire surface of the vial including the plug. The plug should be checked and the inappropriate plug should be considered positive.
6. Media inoculated with microorganisms should promote growth of microbes and uninoculated media should remain sterile. Media should promote growth within the stipulated period.

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Isolation, Culture, Cryopreservation, and Preparation of Umbilical Cord-Derived Mesenchymal Stem Cells as a Final Cellular Product Under Good Manufacturing Practices–Compliant Conditions

Nurullah Aydoğdu, Olga Nehir Öztel, and Erdal Karaöz

Abstract

Mesenchymal stem cells have gained popularity in cell-based therapies due to their regenerative capabilities, immunomodulation properties, and paracrine activity through trophic factors. It is of utmost importance to establish clinical-grade procedures for the preparation of the mesenchymal stem cells for clinical applications. Here, we describe detailed procedures for isolation, culture, cryopreservation, and preparation of mesenchymal stem cells derived from umbilical cord as a final product under good manufacturing practices–compliant conditions.

Key words Umbilical cord, Stem cells, Cell isolation, Culture, Cell therapy, GMP

1 Introduction

Stem cell-based therapies have emerged as a novel approach in the field of regenerative medicine for the treatment of various diseases in order to repair and/or replace the impaired cells and tissues [1]. Mesenchymal stem cells (MSCs) are well-characterized, self-renewable, multipotent adult stem cells that possess the ability to differentiate into various cell types of mesodermal origin [2]. MSCs can be isolated from different tissues, and can easily be expanded ex vivo and converted to mesodermal cell lineages [3]. One of the most attractive features of MSCs for clinical applications is their immunomodulation properties. They act as immune-conductors by producing and releasing various types of trophic factors in order to stimulate the neighboring parenchymal cells to start repairing the damaged tissue [4, 5]. These trophic factors possess different functions such as the enhancement of angiogenesis, stimulation of proliferation and differentiation, and modulation of the immune system [6].

Stem cell–based therapy is part of a broad term advanced therapy medicinal products (ATMPs) that refer to the clinical use

of medicinal products based on genes, cells, and tissues [7, 8]. The clinical use of ATMPs is regulated by the European Regulation EC 1394/2007 and the production of ATMPs must comply with good manufacturing practices (GMPs) which ensures the quality and purity of the final product through the control of all manufacturing processes and facilities [9]. GMP regulations include quality control assessments that are performed during the production as well as before the release of the cells for clinical use in order to assure the purity, quality, and safety of the cellular product. These assessments include cell number and cell viability assessment, characterization of cells, expression analysis of stemness genes, mycoplasma detection, analysis of telomerase activity, microbiological control, and bacterial endotoxin analysis. All methods for these analyses are validated and standard operating procedures (SOPs) are established based on these validation procedures [10, 11].

Here, we describe in detail the isolation, characterization, expansion, cryopreservation, and preparation of clinical grade umbilical cord-derived MSCs as a final product under GMP-compliant conditions.

2 Materials

Prepare all the solutions beforehand and warm in a water bath at 37 °C. All reagents and consumables must be sterile. Follow waste disposal regulations when disposing of waste materials.

2.1 Equipment

All equipment must be qualified for GMP compliance and maintained accordingly as required by EU-GMPs.

1. Cleanroom (GMP) facility (*see* **Note 1**).
2. Sterile cleanroom clothes (face masks, bones, hood, coveralls, gloves).
3. Laminar airflow biosafety cabinet (Class II) (Thermo Scientific, USA).
4. Airborne particle counter for biosafety cabinet (Lighthouse Worldwide Solutions, USA).
5. Optical inverted phase-contrast microscope (Olympus Life Science, USA).
6. Centrifuge (with swinging bucket rotor suitable for 15- and 50-ml tubes) (Thermo Scientific, USA).
7. Humidified CO_2 incubator (5% CO_2, 37 °C) (Heracell™, Thermo Scientific, USA).
8. 37 °C water bath (Stuart, UK).
9. Shaking incubator (Thermo Scientific, USA).
10. Liquid nitrogen tank (Taylor-Wharton, USA).

11. −86 °C freezer (Thermo Scientific, USA).
12. Cell freezing container (Mr. Frosty™, Thermo Scientific, USA).
13. Pipette controller (Thermo Scientific, USA).
14. Surgical instruments (forceps and surgical blades).
15. Glass vial crimper.

2.2 Reagents and Consumables

1. Disinfectant: Klercide™ 70/30 IPA (isopropyl alcohol)—Blended with WFI (water for injection) (Ecolab, USA).
2. Dulbecco's phosphate-buffered saline (DPBS), calcium and magnesium-free (Gibco, Germany).
3. Penicillin (10,000 units/ml)–Streptomycin (10 mg/ml) (Biological Industries).
4. 0.05% Trypsin–EDTA (Gibco, Germany).
5. Human AB Plasma (SeraCare, USA).
6. Collagenase Solution: Collagenase NB4 (Nordmark Biochemicals, Germany) dissolved in DPBS at a concentration of 0.45 U/ml. Filter-sterilize using a 10 ml syringe and a 0.22 μm syringe filter.
7. NutriFreez® D10 Cryopreservation Medium (Biological Industries).
8. Tissue Transfer Solution: DPBS containing 5% penicillin–streptomycin.
9. Thawing Solution: DPBS containing 10% Human AB Plasma.
10. Physiological Saline: 0.9% solution of sodium chloride.
11. Culture Medium: MSC NutriStem® XF Basal Medium (Biological Industries) supplemented with MSC NutriStem® XF Supplement Mix (Biological Industries), 2% Human AB Plasma, and 0.5% Penicillin–Streptomycin.
12. 225 cm^2 filter cap cell culture flasks (Corning Inc., USA).
13. 100 mm plastic petri dishes (Nest, China).
14. 100 mm glass petri dishes.
15. 15 ml and 50 ml conical tubes (Falcon™, Thermo Scientific, USA).
16. 10 ml syringes.
17. 0.22 μm syringe filters (Merck, Germany).
18. 2 ml and 5 ml cryogenic vials (Nest, China)
19. 5 ml, 10 ml, 25 ml, and 50 ml serological pipettes (Falcon™, Thermo Scientific, USA).
20. 10 ml and 20 ml glass vials (FILL-EASE™, Nucmedcor, USA).

3 Methods

After obtaining the informed consent from the donor in accordance with local laws and guidelines, transfer the tissue material (umbilical cord of a newborn, preferably the half of the cord close to the mother's side) within the Tissue Transfer Solution in a 15 ml conical tube to the facility (*see* **Note 2**). Wipe the conical tube with disinfectant and pass it into the Class B cleanroom via the airlock pass box. Wipe the tube with disinfectant again and then transfer it into the laminar airflow biosafety cabinet for the isolation procedure.

Carry out all procedures at room temperature, unless otherwise specified.

3.1 Isolation of Mesenchymal Stem Cells Derived from Human Umbilical Cord

Mesenchymal stem cells can be isolated from human umbilical cord either enzymatically by collagenase digestion or mechanically by cutting the tissue into small pieces. Both methods are described in detail below.

3.1.1 Enzymatic Isolation of Mesenchymal Stem Cells Derived from Human Umbilical Cord

1. Use forceps to transfer the umbilical cord to a 100 mm plastic Petri dish and wash three times with DPBS or physiological saline.
2. Take a sample from the Tissue Transfer solution for the microbiological tests (*see* **Note 3**).
3. Transfer the umbilical cord into a glass petri dish and cut it transversally into small pieces (~1 cm).
4. Open the small tissue pieces and remove the vessels of the cord using forceps and surgical blades.
5. Cut the tissue into smaller pieces (~2–3 mm) and transfer them into a 15 ml conical tube.
6. Add 5 ml of collagenase Solution.
7. Incubate in a shaking incubator at 37 °C for 1 h (*see* **Note 4**).
8. Centrifuge at 400 × *g* for 5 min.
9. Discard the supernatant and vortex the pellet.
10. Add 5 ml of the Culture Medium and resuspend the pellet.
11. Seed the cells and tissue pieces in a T175, T225, or T300 cell culture flask depending on the amount of the tissue obtained. Complete the flasks with the Culture Medium.
12. Incubate in a quarantine incubator (*see* **Note 5**).
13. Observe cell attachment every day under the microscope within the next 5–10 days.
14. Change the Culture Medium every 3–4 days.
15. Once cells are attached, perform the first subculturing. For the subculturing procedure, go to Subheading 3.2.

3.1.2 Mechanical Isolation (Explant Culture) of Mesenchymal Stem Cells Derived from Human Umbilical Cord

1. Use forceps to transfer the umbilical cord to a 100 mm plastic petri dish and wash three times with DPBS or physiological saline.
2. Take a sample from the Tissue Transfer Solution for the microbiological tests (*see* **Note 3**).
3. Transfer the umbilical cord into a glass petri dish and cut it transversally into small pieces (~1 cm).
4. Open the small tissue pieces and remove the vessels of the cord using forceps and surgical blades.
5. Cut the tissue into ~0.5 to 1 cm^3 pieces and place the tissue pieces on 100 mm plastic petri dishes. Incubate at room temperature for 5 min (*see* **Note 6**).
6. Add 10 ml Culture Medium onto the petri dishes slowly so as not to detach the tissue pieces from the surface.
7. Incubate in a quarantine incubator (*see* **Notes 5** and **7**).
8. Change medium completely every 5 days for maintenance.
9. Observe cell attachment every day under the microscope within the next 7–10 days (*see* **Note 8**).
10. Once the cells are attached, perform the first subculturing. For the subculturing procedure, go to Subheading 3.2.

3.2 Subculturing of Mesenchymal Stem Cells

1. Discard the old medium completely.
2. Wash the cell culture flask (or petri dishes for the first subculturing) with 6 ml DPBS.
3. Discard DPBS and add 6 ml trypsin–EDTA.
4. Move the flask to the incubator and incubate for 3–4 min in the incubator at 37 °C.
5. Check under the microscope if the cells are detached.
6. Transfer the cells to a 50 ml conical tube and add Human AB Plasma (*see* **Note 9**).
7. Collect the remaining cells in the flask by washing the flask with 6 ml DPBS.
8. Centrifuge at 400 × *g* for 5 min.
9. Discard the supernatant and finger-vortex the pellet.
10. Add 15–20 ml DPBS and centrifuge at 400 × *g* for 5 min. For final product preparation, go to Subheading 3.5.
11. Discard the supernatant and dissolve the pellet in 10 ml of the Culture Medium.
12. Take a small sample for cell number and viability analysis (*see* **Note 10**).
13. If cells are wished to be cryopreserved, go to Subheading 3.3.

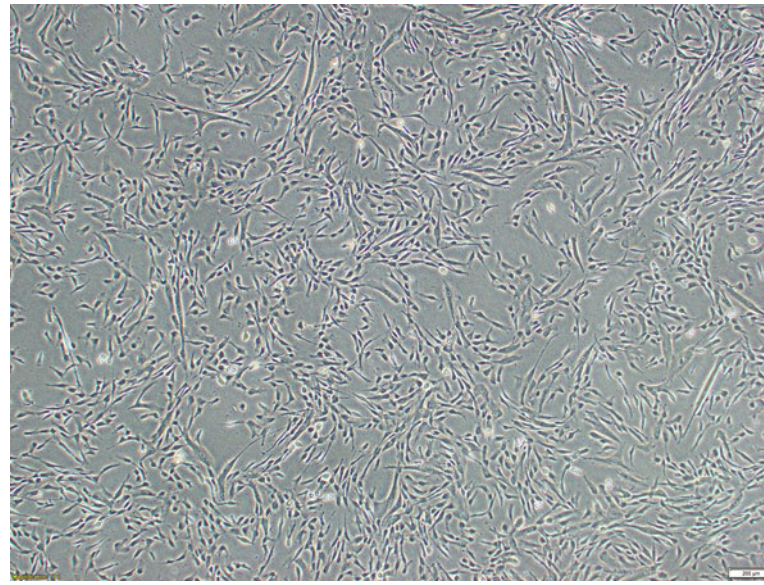

Fig. 1 Morphology of confluent mesenchymal stem cells at passage 3 isolated from the human umbilical cord. Scale bar: 200 μm

14. Seed cells into the flasks at a density of 4000 cells/cm^2 and place the flasks in the incubator (*see* **Note 11**).
15. Add 25 ml Culture Medium and move the flasks to the incubator.
16. Check the morphology of the cells and color of the medium every other day (Fig. 1). When the cell confluency is approximately 80%, subculture the flasks.

3.3 Cryopreservation of Subcultured Mesenchymal Stem Cells

1. Subculture cells as described in Subheading 3.2.
2. Centrifuge cells 400 × *g* for 5 min.
3. Resuspend cells in 15–20 ml DPBS and centrifuge 400 × *g* for 5 min.
4. Resuspend cells very slowly in NutriFreez® D10 Cryopreservation Medium at a concentration of 3×10^6 cells per ml.
5. Pipet 5 ml aliquots of resuspended cells into 5 ml cryogenic vials that have been labeled with the name, date, cell concentration, and passage number.
6. Place cryogenic vials inside a cell freezing container filled with isopropyl alcohol and place at −86 °C overnight (*see* **Note 12**).
7. Transfer the frozen cryogenic vials to a liquid nitrogen tank for storage.

3.4 Thawing Cryopreserved Mesenchymal Stem Cells

1. Remove frozen cryogenic vials from the liquid nitrogen tank and place them in a 37 °C water bath.
2. Rapidly thaw cells by swirling the cryogenic vials in the water bath (*see* **Note 13**).
3. Mix cells with an equal volume of Thawing Solution in a 50 ml conical tube.
4. Centrifuge cells 400 × *g* for 5 min.
5. Discard the supernatant and resuspend the pellet in 10 ml of Culture Medium. Take a small sample for cell number and viability analysis (*see* **Note 10**).
6. Seed cells into the flasks at a density of 4000 cells/cm^2 and add 25 ml Culture Medium.
7. Place the flasks in the incubator (*see* **Note 3**).

3.5 Preparation of Mesenchymal Stem Cells as a Final Product

1. Subculture cells as described in Subheading 3.2.
2. Wash cells with 15–20 ml DPBS and centrifuge at 400 × *g* for 5 min.
3. Wash cells with physiological saline and centrifuge at 400 × *g* for 5 min. Repeat this step once more.
4. Take a sample from the supernatant for microbiological tests. Discard the rest of the supernatant (*see* **Note 3**).
5. Resuspend cells in physiological saline and transfer the cells into a glass vial (*see* **Note 14**).
6. Take samples for cell count and cell viability assessments as well as for quality control tests (*see* **Notes 10** and **15**) (Fig. 2).
7. Transfer 1 × 10^6 cells into a 15 ml conical tube for replicate sampling.
8. Seal off the lid of the glass vial using a glass vial crimper. Then, label and package the glass vial.
9. For the replicate sample, resuspend cells in 1 ml NutriFreez® D10 Cryopreservation Medium in a 2 ml cryogenic vial.
10. Place the cryogenic vial inside a cell freezing container filled with isopropyl alcohol and place at −86 °C overnight.
11. Transfer the replicate sample to a liquid nitrogen tank for storage (*see* **Note 16**).

4 Notes

1. In a cleanroom, parameters such as temperature, humidity, and particle number in air are constantly controlled and monitored. Positive pressure air is supplied through high-efficiency particulate air (HEPA) filters. All equipment and surfaces are cleaned before and after any procedure in cleanrooms [12, 13].

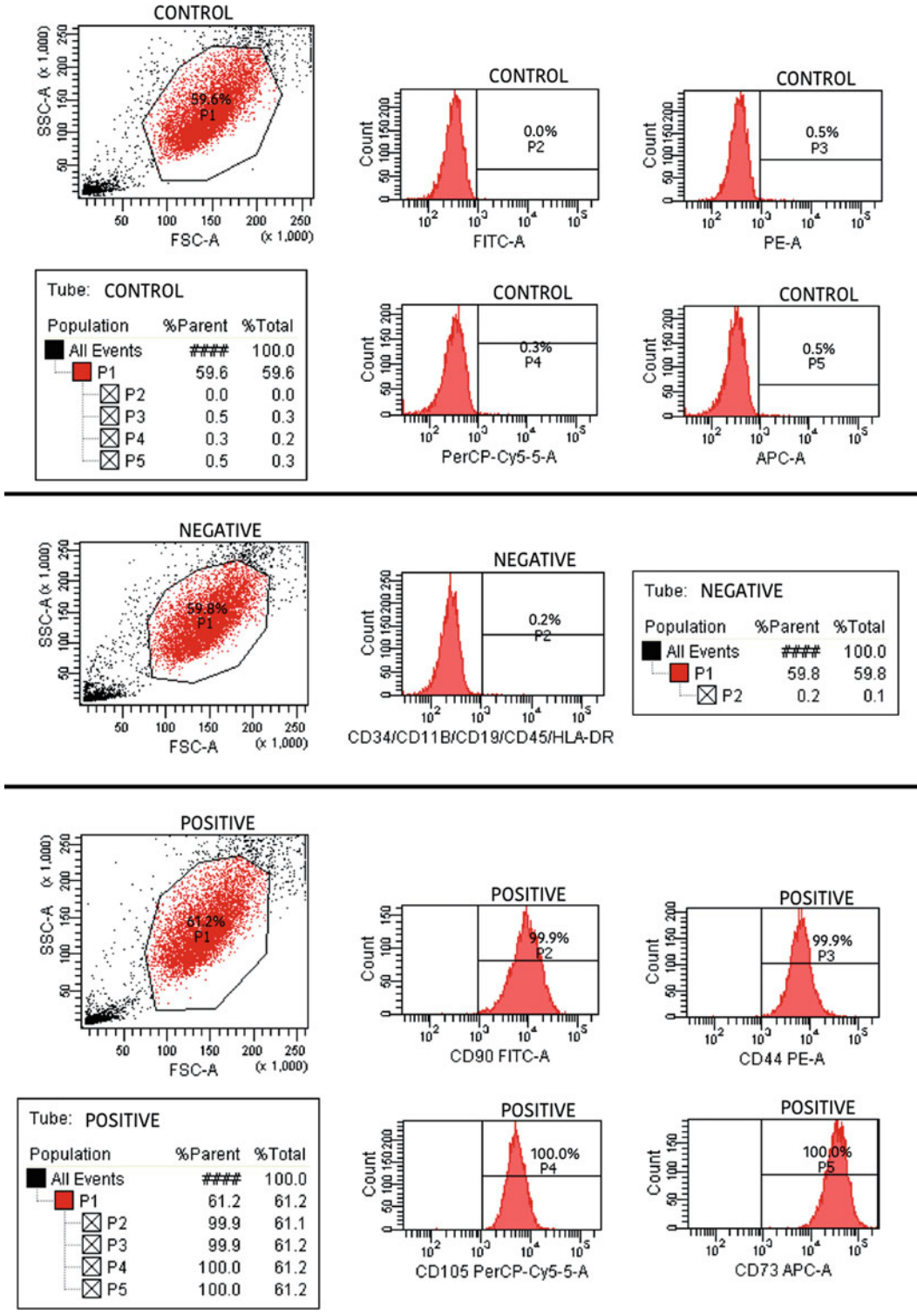

Fig. 2 Characterization of the mesenchymal stem cells at passage 4 derived from the human umbilical cord using flow cytometry analysis. Cells are positive for CD90, CD44, CD105, and CD73, and negative for CD34, CD11b, CD19, CD45, and HLA-DR

For GMP grade manufacturing, xeno-free reagents are preferred. If not possible, GMP grade, clinical-grade, or Pharma Grade reagents must be used [14].

2. Umbilical cords are accepted from the donors delivering infants by Cesarean sectioning after a full-term pregnancy with no complications during pregnancy. The following serological tests are run for the donor of the umbilical cord before mesenchymal stem cell isolation.

   (a) Blood type.
   (b) Anti-HIV 1/2.
   (c) Anti-HCV.
   (d) Anti-HBc Total.
   (e) HBsAg.
   (f) Anti-HBs.
   (g) VDRL.
   (h) Nucleic acid amplification tests (NAT) for HIV RNA, HCV RNA, and HBV DNA.

   The tissue is rejected and discarded if the donor tests are positive for HIV, Hepatitis C, or VDRL.

   For Hepatitis B, the tissue is accepted or rejected according to the following table.

Anti-HBs	Anti-HBc total	Action
+	+	Reject
+	–	Accept
–	+	Reject
–	–	Accept

3. We use BACT/ALERT® 3D automated microbial detection system (Organon Teknika, USA) to perform the microbiological tests for the detection of possible bacterial contamination. Solutions are injected into the pediatric culture and anaerobic culture bottles. The bottles are then placed in the BACT/ALERT 3D instrument and incubated for 7 days.

   In the isolation procedure of umbilical cord, the test result of the Tissue Transfer Solution is usually positive. In this case, a second microbiological test is performed using the first culture medium of the isolated mesenchymal stem cells. In case the second test result turns out positive, the process is canceled and isolated cells are discarded.

   In the thawing procedure, if cells are planned to be propagated for the final product preparation, then a microbiological test is performed from the culture medium of the flasks 1 day after cells have been seeded.

In the procedure of the final product preparation, the supernatant is used for the microbiological test in order to ensure that the cells that are to be administered are free of contamination.

4. It is helpful to check frequently to see if the tissue pieces are digested. Incubate in Collagenase Solution up to 3 h maximum if necessary.
5. A quarantine incubator is only used for the incubation of the isolated tissue in order to prevent cross-contamination until the microbiological tests turn out negative. Thereafter, the flasks are moved to a normal incubator.
6. Please the tissue pieces in 1 cm distances. Make sure that the tissue pieces dry out and are well attached to the surface of the petri dishes. Tissue pieces should not float when the culture medium is added.
7. After 72 h, check the dishes for contamination and cell attachment under the microscope.
8. Cells migrate from the tissue and attach onto the surface of the petri dishes. The cell confluency is expected to reach approximately 70% after 7–10 days.
9. Use 1 ml Human AB Plasma per 30 ml trypsin–EDTA.
10. We use Vi-CELL® XR Cell Viability Analyzer (Beckman Coulter Inc., USA) for cell count and viability analysis. Alternatively, cell counting chambers (e.g., Thoma) can also be used for this purpose.
11. After trypsinization of the cells at passage 3, we perform flow cytometry analysis, mycoplasma detection, gene expression analysis of stemness genes, and analysis of telomerase activity.

    Flow cytometry analysis is performed for the characterization of the mesenchymal stem cells using BD Stemflow™ hMSC Analysis Kit (BD Bioscience, USA). Mesenchymal stem cells are positive for CD90, CD105, CD73, and CD44, and negative for CD45, CD34, CD11b, HLA-DR, and CD19 [15].

    Mycoplasma detection is assessed by e-Myco™ Plus Mycoplasma PCR Detection Kit (LiliF Diagnostics, USA).

    Gene expression of stemness genes (BMP2, POU5F1, THY1, NES, LDHA, RUNX2, ICAM1, VCAM1, CD44, SOX2, ZFP42) and hTERT are carried out by quantitative real-time PCR.

    Telomerase activity is assessed using TeloTAGGG Telomerase PCR ELISA^{PLUS} Kit (Roche).

    Acceptance criteria are listed below.

Quality control test	Acceptance criteria
Cell viability	> 90%
Immunophenotyping	Positive markers: CD44 > 90%; CD73 > 90%; CD90 > 90%; CD105 > 90% Negative markers: CD11b < 2%; CD19 < 2%; CD34 < 2%; CD45 < 2%; HLA-DR < 2%
Endotoxin analysis	< 0.03125 EU/ml
Microbiological quality control	Negative
Mycoplasma analysis	Negative

12. We use Mr. Frosty™ Freezing Container (Thermo Fisher Scientific, USA) filled with isopropyl alcohol. This allows the cells to freeze down gradually at a cooling rate of −1 °C/min.
13. It is important to thaw cells very quickly since the thawing procedure is stressful to cryopreserved cells.
14. Cell number and volume of the final product are predetermined specifically for the patient's conditions by their physician based on the patient's body weight (as cell number per kg) and administration methods (e.g., intravenously, intramuscularly, or intratracheally).
15. In addition to cell number, cell viability, and microbiological tests, our quality control tests include flow cytometry analysis and bacterial endotoxin testing of the final product. Flow cytometry analysis is performed for the characterization of the mesenchymal stem cells. We use BD Stemflow™ hMSC Analysis Kit (BD Bioscience, USA). Mesenchymal stem cells are positive for CD90, CD105, CD73, and CD44, and negative for CD45, CD34, CD11b, HLA-DR, and CD19 [15]. A turbidimetric LAL method is used for the detection of bacterial endotoxins which are the lipopolysaccharides of the wall of gram-negative bacteria [16].
16. Replicate samples are stored in a liquid nitrogen tank for reference for a long period of time.

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Isolation, Culture, Cryopreservation, and Preparation of Skin-Derived Fibroblasts as a Final Cellular Product Under Good Manufacturing Practice–Compliant Conditions

Nurullah Aydoğdu, Olga Nehir Öztel, and Erdal Karaöz

Abstract

Cell-based therapies have become a popular approach in the field of regenerative medicine. Human fibroblast cells, one of the cell types widely used in clinical applications, have been used for skin regeneration and wound healing procedures. Furthermore, they are utilized for aesthetic purposes since fibroblasts lose their abilities such as collagen synthesis with age. Here, we describe detailed procedures for isolation, culture, cryopreservation, and preparation of fibroblasts derived from adult human skin as a final product under good manufacturing practice–compliant conditions.

Key words Fibroblasts, Cell isolation, Culture, Cell therapy, GMP

1 Introduction

Regenerative medicine is a translational field that deals with the process of engineering, replacing and repairing cells, tissues, or organs to restore their functions. It has been applied using different cell types and biomaterials in a number of fields including wound healing, hematopoietic reconstruction, and orthopaedic treatments [1]. Fibroblasts are the most abundant cells in the connective tissue of the skin and are responsible for the production and deposition of the extracellular matrix components like collagen, fibronectin, laminin, and proteoglycans, leading to the establishment of a three-dimensional microenvironment and allowing other cell types to migrate, settle, proliferate and perform their functions within the microenvironment [2, 3]. They also synthesize growth factors, cytokines, and proteases, and support skin regeneration [4]. They are used as autologous cells for the wound healing process and aesthetic outcome in clinical applications. Fibroblasts can be isolated from a skin biopsy of an adult or fetal donor, and easily be grown and propagated in cell culture. Therefore, they can be used autologously in clinical applications since their use avoids tissue rejection, prevents cross-infection, and lowers donor site

morbidity. In this chapter, we describe in detail the isolation, characterization, expansion, cryopreservation, and preparation of clinical grade skin-derived fibroblasts as a final product under GMP conditions.

2 Materials

Prepare all the solutions beforehand and warm in a water bath at 37 °C. All reagents and consumables must be sterile. Follow waste disposal regulations when disposing of waste materials.

2.1 Equipment

All equipment must be qualified for GMP compliance and maintained accordingly as required by EU-GMPs.

1. Cleanroom (GMP) facility (*see* **Note 1**).
2. Sterile cleanroom clothes (face masks, bones, hood, coveralls, gloves).
3. Laminar airflow biosafety cabinet (Class II) (Thermo Scientific, USA).
4. Airborne particle counter for biosafety cabinet (Lighthouse Worldwide Solutions, USA).
5. Optical inverted phase-contrast microscope (Olympus Life Science, USA).
6. Centrifuge (with swinging bucket rotor suitable for 15- and 50-ml tubes) (Thermo Scientific, USA).
7. Humidified CO_2 incubator (5% CO_2, 37 °C) (Heracell™, Thermo Scientific, USA).
8. 37 °C water bath (Stuart, UK).
9. Shaking incubator (Thermo Scientific, USA).
10. Liquid nitrogen tank (Taylor-Wharton, USA).
11. −86 °C freezer (Thermo Scientific, USA).
12. Cell freezing container (Mr. Frosty™, Thermo Scientific, USA).
13. Pipette controller (Thermo Scientific, USA).
14. Surgical instruments (forceps and surgical blades).
15. Glass vial crimper.

2.2 Reagents and Consumables

1. Disinfectant: Klercide™ 70/30 IPA (isopropyl alcohol)—Blended with WFI (water for injection) (Ecolab, USA).
2. Dulbecco's phosphate-buffered saline (DPBS), calcium and magnesium-free (Gibco, Germany).

3. Penicillin (10,000 units/ml)-Streptomycin (10 mg/ml) (Biological Industries).
4. 0.05% trypsin–EDTA (Gibco, Germany).
5. Human AB Plasma (SeraCare, USA).
6. Autologous serum: Serum obtained from the patient's blood.
7. Collagenase Solution: Collagenase NB4 (Nordmark Biochemicals, Germany) dissolved in DPBS at a concentration of 0.45 U/ml. Filter sterilize using a 10 ml syringe and a 0.22 μm syringe filter.
8. NutriFreez® D10 Cryopreservation Medium (Biological Industries).
9. Tissue Transfer Solution: DPBS containing 5% Penicillin–Streptomycin.
10. Thawing Solution: DPBS containing 10% Human AB Plasma.
11. Physiological Saline: 0.9% solution of sodium chloride.
12. Isolation Medium: Alpha-MEM (Biological Industries) supplemented with 10% autologous serum and 1% Penicillin–Streptomycin.
13. Culture Medium: MSC NutriStem® XF Basal Medium (Biological Industries) supplemented with MSC NutriStem® XF Supplement Mix (Biological Industries), 2% Human AB Plasma, and 0.5% Penicillin–Streptomycin.
14. 175 cm^2 filter cap cell culture flasks (Corning Inc., USA).
15. 100 mm plastic petri dishes (Nest, China).
16. 100 mm glass petri dishes.
17. 15 ml and 50 ml conical tubes (Falcon™, Thermo Scientific, USA).
18. 10 ml syringes.
19. 0.22 μm syringe filters (Merck, Germany).
20. 2 ml and 5 ml cryogenic vials (Nest, China)
21. 5 ml, 10 ml, 25 ml, and 50 ml serological pipettes (Falcon™, Thermo Scientific, USA).
22. 10 ml and 20 ml glass vials (FILL-EASE™, Nucmedcor, USA).

3 Methods

After obtaining the informed consent from the donor in accordance with local laws and guidelines, transfer the tissue material (punch biopsy of the skin of the patient, obtained ideally from the back of the ear) within the Tissue Transfer Solution in a 15 ml conical tube to the facility (*see* **Note 2**). Wipe the conical tube with disinfectant

and pass it into the Class B cleanroom via the airlock pass box. Wipe the tube with disinfectant again and then transfer it into the laminar airflow biosafety cabinet for the isolation procedure.

Carry out all procedures at room temperature, unless otherwise specified.

3.1 Enzymatic Isolation of Fibroblasts Derived from Human Skin

1. Use forceps to transfer the obtained skin tissue to a 100 mm plastic petri dish and wash three times with DPBS or physiological saline.
2. Take a sample from the Tissue Transfer Solution for the microbiological tests (*see* **Note 3**).
3. Transfer the skin tissue into a glass petri dish.
4. Cut the tissue into very small pieces (~1–2 mm) using forceps and surgical blades and transfer the tissue pieces into a 15 ml conical tube.
5. Add 5 ml of Collagenase Solution.
6. Incubate in a shaking incubator at 37 °C for 1 h (*see* **Note 4**).
7. Centrifuge at 400 × *g* for 5 min.
8. Discard the supernatant and vortex the pellet.
9. Add 5 ml of the Isolation Medium and resuspend the pellet.
10. Seed the cells and tissue pieces in a 175 cm^2 cell culture flask. Add 25 ml of the Isolation Medium.
11. Incubate in a quarantine incubator (*see* **Note 5**).
12. Observe cell attachment every day under the microscope within the next 7–10 days.
13. Change the Isolation Medium every 3 days.
14. Once cells are attached, perform the first subculturing. For the subculturing procedure, go to Subheading 3.2.

3.2 Subculturing of Skin-Derived Fibroblasts

1. Discard the old medium completely.
2. Wash the cell culture flask with 5 ml DPBS.
3. Discard DPBS and add 5 ml trypsin–EDTA.
4. Move the flask to the incubator and incubate for 3–4 min in the incubator at 37 °C.
5. Check under the microscope if the cells are detached.
6. Transfer the cells to a 50 ml conical tube and add Human AB Plasma (*see* **Note 6**).
7. Centrifuge at 400 × *g* for 5 min.
8. Discard the supernatant and finger-vortex the pellet.
9. Add 15–20 ml DPBS and centrifuge at 400 × *g* for 5 min. For final product preparation, go to Subheading 3.5.

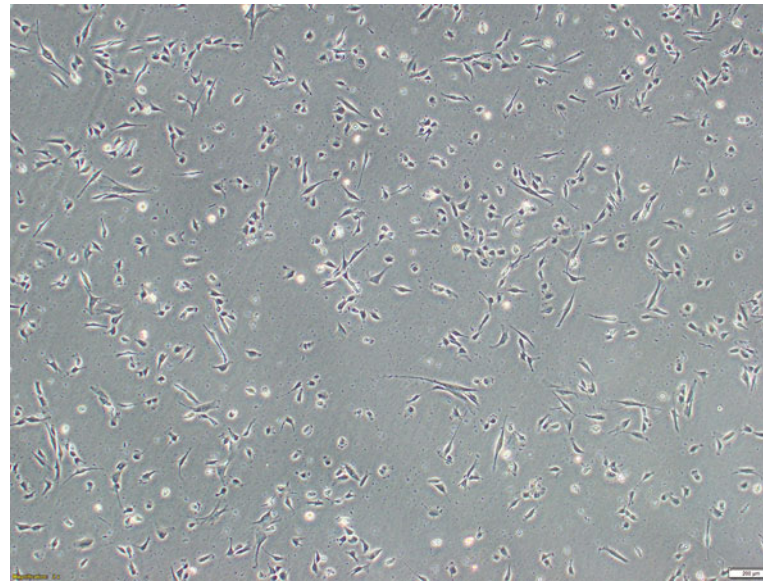

Fig. 1 Morphology of fibroblasts at passage 2 with spindle-like appearance isolated from adult human skin. Scale bar: 200 μm

10. Discard the supernatant and dissolve the pellet in 10 ml of the Culture Medium.
11. Take a small sample for cell number and viability analysis (*see* **Note 7**).
12. If cells are wished to be cryopreserved, go to Subheading 3.3.
13. Seed cells into the flasks at a density of 4000 cells/cm^2 and place the flasks in the incubator (*see* **Note 8**).
14. Add 20 ml Culture Medium and move the flasks to the incubator.
15. Check the morphology of the cells and color of the medium every other day (Fig. 1). When the cell confluency is approximately 80%, subculture the cell flasks.

3.3 Cryopreservation of Subcultured Fibroblasts

1. Subculture fibroblasts as described in Subheading 3.2.
2. Centrifuge cells at 400 × *g* for 5 min.
3. Resuspend cells in 15–20 ml DPBS and centrifuge 400 × *g* for 5 min.
4. Resuspend cells very slowly in NutriFreez® D10 Cryopreservation Medium at a concentration of 3 × 10^6 cells per ml.
5. Pipet 5 ml aliquots of resuspended cells into 5 ml cryogenic vials that have been labeled with the name, date, cell concentration, and passage number.

6. Place cryogenic vials inside a cell freezing container filled with isopropyl alcohol and place at −86 °C overnight (*see* **Note 9**).
7. Transfer the frozen cryogenic vials to a liquid nitrogen tank for storage.

3.4 Thawing Cryopreserved Fibroblasts

1. Remove frozen cryogenic vials from the liquid nitrogen tank and place them in a 37 °C water bath.
2. Rapidly thaw cells by swirling the cryogenic vials in the water bath (*see* **Note 10**).
3. Mix cells with an equal volume of Thawing Solution in a 50 ml conical tube.
4. Centrifuge cells at 400 × *g* for 5 min.
5. Discard the supernatant and resuspend the pellet in 10 ml of Culture Medium. Take a small sample for cell number and viability analysis (*see* **Note 7**).
6. Seed cells into the flasks at a density of 4000 cells/cm^2 and add 20 ml Culture Medium.
7. Place the flasks in the incubator (*see* **Note 3**).

3.5 Preparation of Fibroblasts as a Final Product

1. Subculture fibroblasts as described in Subheading 3.2.
2. Wash cells with 15–20 ml DPBS and centrifuge at 400 × *g* for 5 min.
3. Wash cells with physiological saline and centrifuge at 400 × *g* for 5 min. Repeat this step once more.
4. Take a sample from the supernatant for microbiological tests. Discard the rest of the supernatant (*see* **Note 3**).
5. Resuspend cells in physiological saline and transfer the cells into a glass vial (*see* **Note 11**).
6. Take samples for cell count and cell viability assessments as well as for quality control tests (*see* **Notes 7** and **12**) (Fig. 2).
7. Transfer 1 × 10^6 cells into a 15 ml conical tube for replicate sampling.
8. Seal off the lid of the glass vial using a glass vial crimper. Then, label and package the glass vial.
9. For the replicate sample, resuspend cells in 1 ml NutriFreez® D10 Cryopreservation Medium in a 2 ml cryogenic vial.
10. Place the cryogenic vial inside a cell freezing container filled with isopropyl alcohol and place at −86 °C overnight.
11. Transfer the replicate sample to a liquid nitrogen tank for storage (*see* **Note 13**).

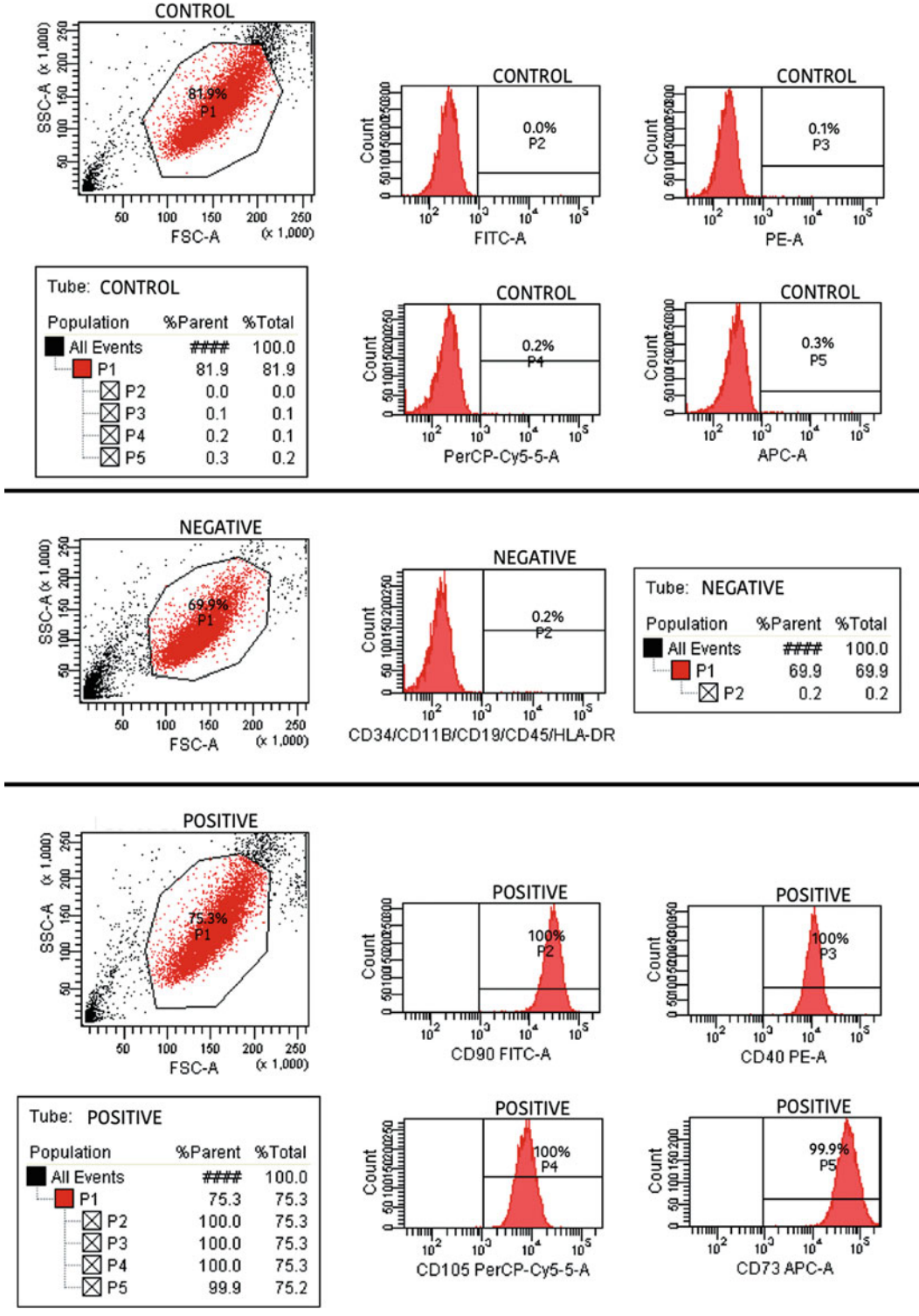

Fig. 2 Characterization of the fibroblasts at passage 3 derived from the human skin biopsy using flow cytometry analysis. Cells are positive for CD40, CD73, CD90, and CD105, and negative for CD11b, CD19, CD34, CD45, and HLA-DR

4 Notes

1. In a cleanroom, parameters such as temperature, humidity, and particle number in air are constantly controlled and monitored. Positive pressure air is supplied through high-efficiency particulate air (HEPA) filters. All equipment and surfaces are cleaned before and after any procedure in cleanrooms [5, 6].

   For GMP grade manufacturing, xeno-free reagents are preferred. If not possible, GMP grade, clinical-grade, or Pharma Grade reagents must be used [7].

2. The back of the ear of the patient is preferred for obtaining skin biopsy since this area is presumably less exposed to sun rays.

   The following serological tests are run for the patient before starting the isolation procedure.

   (a) Anti-HIV 1/2.

   (b) Anti-HCV.

   (c) Anti-HBc Total.

   (d) HBsAg.

   (e) Anti-HBs.

   (f) VDRL.

   (g) Nucleic acid amplification tests (NAT) for HIV RNA, HCV RNA, and HBV DNA.

   The tissue is rejected and discarded if the tests are positive for HIV, Hepatitis C, or VDRL.

   For Hepatitis B, the tissue is accepted or rejected according to the following table.

Anti-HBs	Anti-HBc total	Action
+	+	Reject
+	−	Accept
−	+	Reject
−	−	Accept

3. We use BACT/ALERT® 3D automated microbial detection system (Organon Teknika, USA) to perform the microbiological tests for the detection of possible bacterial contamination. Solutions are injected into the pediatric culture and anaerobic culture bottles. The bottles are then placed in the BACT/ALERT 3D instrument and incubated for 7 days.

   In the isolation procedure of the fibroblasts from the skin biopsy, the test result of the Tissue Transfer Solution is usually positive. In this case, a second microbiological test is performed

using the first culture medium of the isolated fibroblast cells. In case the second test result turns out positive, the process is canceled and isolated cells are discarded.

In the thawing procedure, if cells are planned to be propagated for the final product preparation, then a microbiological test is performed from the culture medium of the flasks 1 day after cells have been seeded.

In the procedure of the final product preparation, the supernatant is used for the microbiological test in order to ensure that the cells that are to be administered are free of contamination.

4. It is advisable to check frequently to see if the tissue pieces are digested. Incubate in Collagenase Solution up to 2 h maximum if necessary.
5. A quarantine incubator is only used for the incubation of the isolated tissue in order to prevent cross-contamination until the microbiological tests turn out negative. Thereafter, the flasks are moved to a normal incubator.
6. Use 1 ml Human AB Plasma per 30 ml trypsin–EDTA.
7. We use Vi-CELL® XR Cell Viability Analyzer (Beckman Coulter Inc., USA) for cell count and viability analysis. Alternatively, cell counting chambers (e.g., Thoma) can also be used for this purpose.
8. After trypsinization of the cells at passage 2, we perform flow cytometry analysis and mycoplasma detection.

   Flow cytometry analysis is performed for the characterization of the fibroblasts using BD Stemflow™ hMSC Analysis Kit (BD Bioscience, USA). Fibroblasts are positive for CD40, CD73, CD90, and CD105, and negative for CD11b, CD19, CD34, CD45, and HLA-DR [8].

   Mycoplasma detection is assessed by e-Myco™ Plus Mycoplasma PCR Detection Kit (LiliF Diagnostics, USA).

   Acceptance criteria are listed below.

Quality control test	Acceptance criteria
Cell viability	$> 90\%$
Immunophenotyping	Positive markers: CD40 $> 90\%$; CD73 $> 90\%$; CD90 $> 90\%$; CD105 $> 90\%$ Negative markers: CD11b $< 2\%$; CD19 $< 2\%$; CD34 $< 2\%$; CD45 $< 2\%$; HLA-DR $< 2\%$
Endotoxin analysis	< 0.03125 EU/ml
Microbiological quality control	Negative
Mycoplasma analysis	Negative

9. We use Mr. Frosty™ Freezing Container (Thermo Fisher Scientific, USA) filled with isopropyl alcohol. This allows the cells to freeze down gradually at a cooling rate of −1 °C/min.
10. It is important to thaw cells very quickly since the thawing procedure is stressful to cryopreserved cells.
11. Cell number and volume of the final product are determined as 50 million cells in 10 ml physiological saline. However, this can be adjusted by the patient's physician according to the patient's application protocol.
12. In addition to cell number, cell viability, and microbiological tests, our quality control tests include flow cytometry analysis and bacterial endotoxin testing of the final product. Flow cytometry analysis is performed for the characterization of the fibroblasts. We use BD Stemflow™ hMSC Analysis Kit (BD Bioscience, USA). Fibroblasts are positive for CD40, CD73, CD90, and CD105, and negative for CD11b, CD19, CD34, CD45, and HLA-DR [8]. A turbidimetric LAL method is used for the detection of bacterial endotoxins which are the lipopolysaccharides of the wall of gram-negative bacteria [9].
13. Replicate samples are stored in a liquid nitrogen tank for reference for a long period of time.

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The Isolation and Manufacture of GMP-Grade Bone Marrow Stromal Cells from Bone Specimens

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Abstract

Bone marrow stromal cells (BMSCs, also known as bone marrow mesenchymal stem cells) are a plastic-adherent heterogeneous cell population that contain inherent skeletal progenitors and a subset of multipotential skeletal stem cells (SSCs). Application of BMSCs in therapeutic protocols implies its isolation and expansion under good manufacturing practices (GMP). Here we describe the procedures we have found to successfully generate practical BMSCs numbers, with preserved biological potency.

Keywords Bone marrow stromal cells, Manufacture, GMP-grade, Clinical application

1 Introduction

Bone marrow stromal cells (BMSCs), also known as bone marrow mesenchymal stem cells [1, 2], are a plastic-adherent heterogeneous cell population that contain a subset of multipotential skeletal stem cells (SSCs), with the ability to form cartilage, bone, marrow adipocytes, and mielosuportive stroma [3–6]. Because of its inherent role as skeletal tissue progenitors, BMSCs have been the focus of extensive research envisioning their clinical application [7–11], especially for bone regeneration [12–15]. Now efforts are being made to effectively translate this potential to the bedside, what necessarily goes through the need to isolate and expand BMSCs in vitro, under good manufacturing practices (GMP), while maintaining their biological properties [16–19].

Nevertheless, despite many initiatives [19–27], there is no international consensus so far as to which adaptations should be made for large-scale BMSCs isolation and expansion, considering the different sources of bone marrow (aspirates vs bone specimens), the best cell culture medium (Alpha MEM vs DMEM; chemically defined medium vs supplemented with serum), and in vitro systems (bioreactors vs static multilayer bottles). Here we describe the procedures we stablished for GMP-grade BMSCs manufacturing, which we have found to successfully generate practical BMSC

numbers and to preserve its ability to form bone and reconstitute the bone marrow microenvironment when transplanted in vivo [28].

2 Materials

All reagents and materials used were purchased ready to use, unless otherwise stated. Those which go in direct contact with bone marrow samples and/or cells need to be certified as of clinical grade, IVD (in vitro diagnostic), to have been produced under good laboratory practices or have defined chemical purity standards (*see* **Note 1**).

2.1 Solutions

1. Isolation and expansion medium: α-minimum essential medium (α-MEM), 20% lot-selected fetal bovine serum (FBS, *see* **Notes 2** and **3**). Add 200 ml of FBS to 800 ml of α-MEM to prepare 1 l of culture medium. Prepare only the volume necessary for immediate use.
2. Phosphate-buffered saline (PBS).
3. Enzymatic digestion: TrypLE™ Express.
4. Stains for cell enumeration: Trypan Blue and Turk's solution.
5. Final washing solutions: 0.5% human serum albumin in Ringer's lactate and 5% human serum albumin in Ringer's lactate. Both solutions must be prepared at the time of use.
   (a) 0.5% human serum albumin in Ringer's lactate: Add 2.5 ml of human serum albumin to 497.5 ml of Ringer's lactate.
   (b) 5% human serum albumin in Ringer's lactate: Add 25 ml of human serum albumin to 475 ml of Ringer's lactate.
6. Cryopreservation medium: 5% dimethyl sulfoxide (DMSO) and 5% human serum albumin in 6% hydroxyethyl starch (HES). Once prepared, the solution must be used within 5 days. Mix 25 ml of human serum albumin and 25 ml of DMSO to 450 ml of HES to prepare 500 ml of the final solution.

2.2 Equipment and Supplies

1. Laminar flow hoods.
2. CO_2 incubators: set to 37 °C and 5% CO_2.
3. Centrifuge, with speed and temperature control.
4. Standard inverted phase-contrast microscope.
5. Ultrafreezer: set to −80 °C.
6. Liquid nitrogen tank.

7. Sterile labware: T-75 cm^2 flasks, two- and ten-layer cell factories, 10 ml and 20 ml pipettes, 50 ml centrifuge tubes, 70 μm cell strainers, 1.5 ml cryotubes, and P10, P20, and P200 filtered tips.
8. P10, P20, and P200 automatic pipettes.
9. Hand pipettor.
10. Neubauer chamber.
11. Mr. Frosty containers.

2.3 Bone Specimens

1. Trabecular bone specimens containing red marrow from healthy subjects (*see* **Notes 4** and **5**).

3 Methods

3.1 Preparation of Bone Marrow Cell Suspensions

1. Distribute the trabecular bone fragments in 50 ml tubes. Ideally, the sample should not exceed the 15 ml mark (*see* **Note 6**, Fig. 1a).
2. Add 15 ml of PBS per tube. Set the hand pipettor speed to high, and with a 10 ml pipette, vigorously mix up and down 10 times, to separate the marrow from the bone spicules (Fig. 1b). To avoid clogging the pipette during this step, always collect the PBS from the top. When mixing is done, wait 30 s for the bone spicules to settle at the bottom of the tube, and then collect the cell suspension and transfer to a new 50 ml tube (*see* **Note 7**).
3. Add another 15 ml of fresh PBS in the 50 ml tube containing the bone fragments and repeat **step 2** until the bones are visually clean of marrow (spicules will become white, Fig. 1c). After washing, bone spicules can be discarded.
4. Pass the collected bone marrow cell suspensions through a 70-μm cell strainer into new 50 ml tubes (as many as necessary, according to the sample volume).
5. Centrifuge at 300 × *g* and 4 °C for 5 min.
6. Discard the supernatant and add 20 ml of α-MEM supplemented with 20% FBS in one sample tube and resuspend the cell pellet (*see* **Note 8**). If working with more than one sample tube, collect the cell suspension from the first tube and subsequently transfer it to the other ones, to pool the cell pellets in just one tube.
7. Count the number of total nucleated cells in the final suspension. Take an aliquot of 20 μl of the marrow suspension and dilute in 180 μl (1:10 v/v) of Turk's solution for red blood cell lysis. Next, take 10 μl of this lysed sample and apply into a Neubauer chamber (*see* **Note 9**, Fig. 1d).

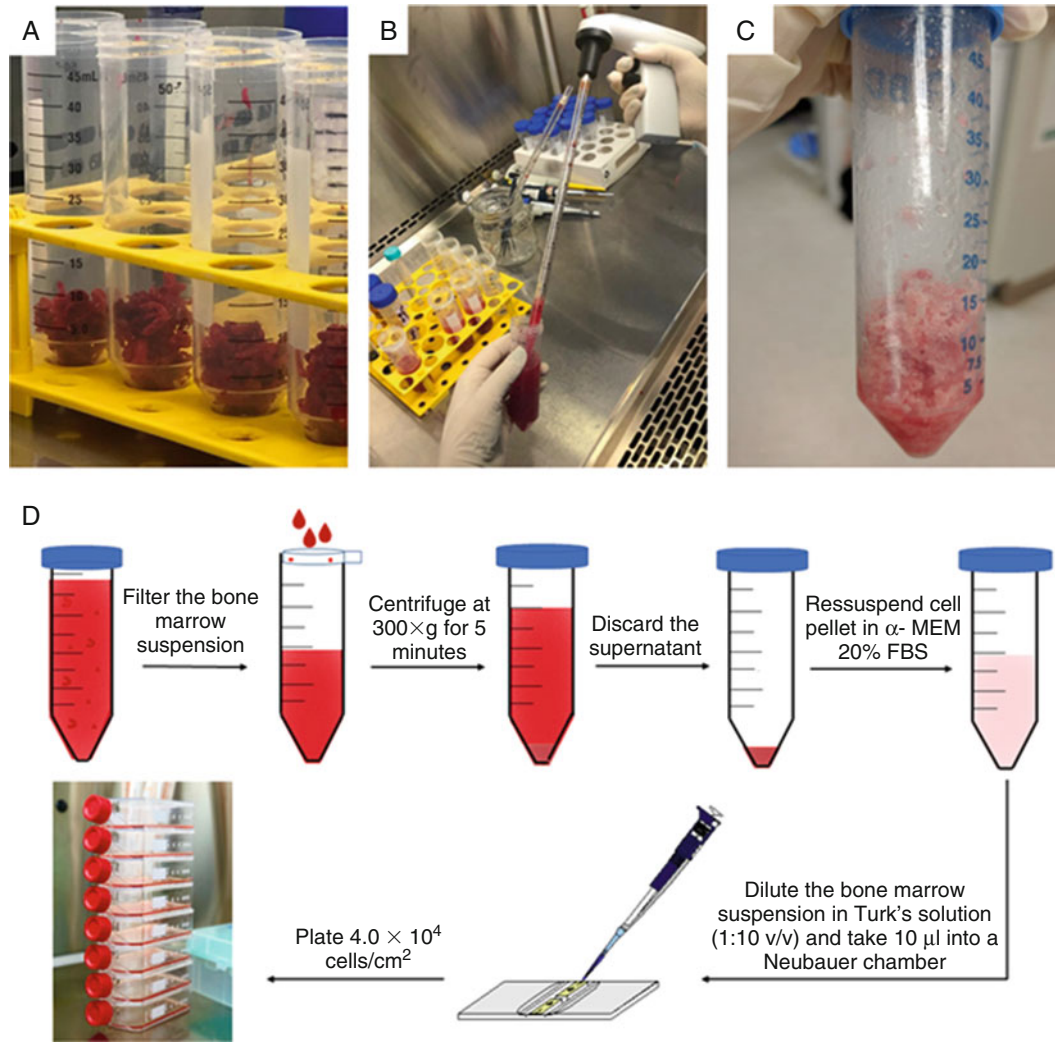

Fig. 1 BMSCs isolation steps. Trabecular bone fragments are distributed into 50 ml tubes (**a**) and washed with PBS to separate the bone marrow from the bone spicules (**b**, **c**). The obtained cell suspension is filtered through a 70-μm cell strainer and centrifuged (**d**). Finally, the cell pellet is resuspended in culture medium, the number of cells is determined, and 4.0×10^4 nucleated cells/cm^2 are plated per T-75 cm^2 flask (**d**)

3.2 BMSCs Isolation

1. Plate 3.0×10^6 nucleated cells (4.0×10^4/cm^2) from the bone marrow cell suspension per T-75 cm^2 flask in 10 ml of α-MEM supplemented with 20% FBS (Fig. 1d). Incubate the cells at 37 °C in a humidified atmosphere of 5% CO_2 (*see* **Note 10**).
2. After 3 days of incubation, remove the medium containing the unattached cells from each T-75 cm^2 flask with a 10 ml pipette and add 10 ml of PBS at room temperature. Wash the flasks vigorously and discard the PBS. Repeat this procedure three times.

3. Add 10 ml of fresh α-MEM supplemented with 20% FBS per flask and incubate the cells at 37 °C in a humidified atmosphere of 5% CO_2.
4. At every 3 days, until day 14 post plating, replace the medium.
5. On day 14, wash the T-75 cm^2 flasks three times with PBS, as described in **step 2**. Add 5 ml of TrypLE Express per flask and incubate at 37 °C for 8 min to detach the cells.
6. Add 3 ml of PBS per flask to inhibit TrypLE Express enzymatic activity. With a 10 ml pipette, homogenize the cell suspensions and transfer to 50 ml tubes. Use as many tubes as necessary.
7. Centrifuge at 300 × *g* and 4 °C for 5 min.
8. Discard the supernatant, resuspend, and homogenize the cell pellet in 20 ml of α-MEM supplemented with 20% FBS. If working with more than one tube, pool cell pellets in just one tube.
9. Determine the number of harvested BMSCs. Mix 10 μl of the cell suspension with 10 μl of Trypan Blue (1:2 v/v) and apply into a Neubauer chamber. Determine both the number of viable (unstained) and dead (blue) cells to calculate the percentual of viability. If viability is ≥70%, proceed to expansion (*see* **Note 11**).

3.3 BMSCs Expansion

1. Plate the BMSCs into two-layer cell factories at a density of 2.0×10^3 cells/cm^2. For each cell factory, add 2.5×10^6 BMSCs to 500 ml of α-MEM supplemented with 20% FBS and pour the suspension through the opening (Fig. 2a). To evenly distribute the cells over the layer's surfaces, gently tilt the cell factory forward on a flat work surface, hold it at 90°, and then lay it flat (Fig. 2b–e).
2. Incubate the cells at 37 °C in a humidified atmosphere of 5% CO_2.
3. Change the culture medium at every 3 days. Let the cells proliferate until the monolayers reach 70% confluence (*see* **Note 12**).
4. For cell harvesting, pour the spent media and add 80 ml of PBS to wash the cells. Repeat this procedure three times.
5. Add 120 ml of TrypLE Express per cell factory and incubate the cells at 37 °C for 8 min.
6. Add 40 ml of PBS to inhibit TrypLE. Tilt the cell factory 45° to the right, then aspirate the supernatant with a 20 ml pipette and transfer to 50 ml tubes.
7. Centrifuge at 300 × *g* and 8 °C for 5 min.
8. Discard the supernatants, homogenize, and pool the cell pellets in 20 ml of α-MEM supplemented with 20% FBS. Count the

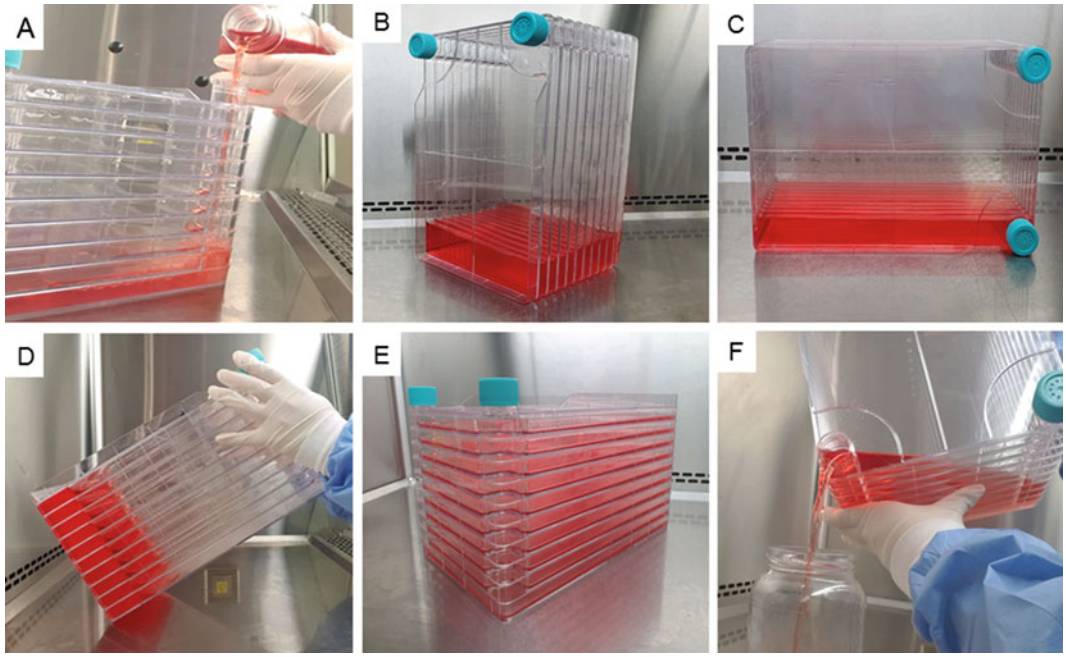

Fig. 2 Manipulation of cell factories. To evenly distribute a solution in multilayer cell factories, pour it through the large opening (**a**) and place the flask in the upright position (**b**) and then on its side (90°) for a few seconds (**c**), before turning it to the horizontal position (**d–e**). To discard the solution, lean the flask and pour the liquid into a sterile waste receptacle (**f**)

number of cells and determine the percentual of viability, as described in Subheading 3.2, **Step 9**. If viability is ≥70%, proceed to the second cycle of expansion (passage 2).

9. Plate the BMSCs into ten-layer cell factories at a density of 2.0×10^3 cells/cm^2. For each cell factory, add 1.2×10^7 BMSCs to 1,500 ml of α-MEM supplemented with 20% FBS, and pour the suspension through the large opening (Fig. 2a). To evenly distribute the cells over the layer's surfaces, gently tilt the cell factory forward on a flat work surface, hold it at 90°, and then lay it flat (Fig. 2b–e).
10. Incubate the cells at 37 °C in a humidified atmosphere of 5% CO_2.
11. Change the culture medium at every 3 days. Let the cells proliferate until the monolayers reach 70% confluence (*see* **Note 12**).
12. To harvest the cells, discard the medium and wash the cells with 500 ml of PBS. Repeat this procedure three times.
13. Add 500 ml of TrypLE Express per cell factory and incubate the cells at 37 °C for 8 min.

14. Add 150 ml of PBS to inhibit TrypLE. Tilt the cell factory 45° to the right, and then aspirate the supernatant with a 20 ml pipette and transfer to 50 ml tubes.
15. Centrifuge at 300 × *g* and 8 °C for 5 min.
16. Discard the supernatants and add 30 ml of ringer's lactate supplemented with 0.5% human serum albumin per tube. Homogenize and pool cell pellets into just one tube.
17. Centrifuge the cell suspension at 300 × *g* and 8 °C for 5 min. Discard the supernatant and resuspend the cell pellet in fresh 30 ml of ringer's lactate with 0.5% human serum albumin. Repeat this step four times, to clean up cells from expansion medium constituents.
18. After the final centrifugation, discard the supernatant and resuspend the cell pellet in 40 ml of ringer's lactate with 5% human serum albumin.
19. Determine the number of harvested BMSCs and the percentual of viability as in previous steps. If viability is ≥70%, cells can be prepared for cryopreservation (*see* **Note 13**).

3.4 Cryopreservation

1. Centrifuge the cells at 300 × *g* and 8 °C for 5 min. Resuspend the cell pellet in an appropriate volume of cold (4 °C) cryopreservation medium to reach a density of 5.0×10^6 BMSCs/ml.
2. Homogenize the cell suspension with a 10 ml pipette, and distribute 1 ml per cryotube.
3. Place the cryotubes in room temperature Mr. Frosty freezing containers, and immediately transfer the containers to a −80 °C ultrafreezer.
4. After 12 h, store the cryotubes in the vapor phase of a liquid nitrogen tank until use.

4 Notes

1. To avoid contaminations, do not aliquot reagents. Register the lot and/or serial numbers of all reagents and materials used in each step. This way, if any contamination occurs, all reagents and materials used in the procedures can be located and discarded.
2. The choice of FBS is a critical step in BMSCs isolation and expansion. Lots must be regularly tested to select the one which best allows cell attachment and proliferation. The best two assays to perform FBS lot selection for BMSCs are proliferation curves and the CFE assay. For details, see Refs. [28, 29]. Once chosen, perform all steps of cell isolation and expansion with the same FBS lot.

3. Although it is recommended that reagents from animal sources should be avoided when manufacturing cells for human use, evidence show that BMSCs isolated and expanded with FBS replacements have significant alterations in their biological properties [30]. Because we and others have shown that the final washing steps leave animal protein residuals below the accepted levels [19, 28], we prefer to use FBS, until a similar or better culture condition is defined.
4. The choice of the source of bone marrow must be evaluated at each case, depending on the strategy of cell application: autologous or allogeneic. Although we reason that autologous applications are always the preferable choice, in some instances, they are not the best or possible choice, such as in urgencies or when the patient has a genetic alteration which results in or contributes with the development of skeletal malformations. If BMSCs are being isolated for allogeneic strategies and/or cell banking, all the required tests, including serology, immunogenicity, and HLA compatibility tests, must be performed.
5. In our study, we tested the feasibility of trabecular bone discards from primary hip arthroplasties as the source of bone marrow for BMSCs isolation [28]. However, aspirates can be used instead, with some adaptations in the isolation step. We refer the reader, in this case, to Ref. [19].
6. Acetabular bone fragments from hip arthroplasties usually come triturated due to the bone reaming procedure. So, no additional fragmentation of the sample is necessary. If working with bigger pieces of bone, such as a femur head, it will be necessary to scrape the trabecular bone with scissors, steel blades, and/or pliers to turn it into smaller fragments. Bone samples should be processed immediately after harvesting or after a maximum of 12 h storage at 2–8 °C in α-MEM supplemented with 20% FBS.
7. While working with cells in suspension, whenever cells are not being directly manipulated, place the cell tubes on ice, to avoid cell death and/or adhesion to the plastic.
8. Red blood cells do not allow the formation of a cohesive cell pellet. Therefore, when discarding supernatants at this point, do not invert the tubes upside down.
9. An appropriate dilution of the cell-Turk's solution mixture must be achieved to allow cell counting. If the sample is not diluted enough, the cells in the Neubauer chamber will appear too crowded to count. If this happens, make an intermediary cell suspension by diluting with medium (e.g., 1:10 v/v), and, from this one, take a 20 μl sample to mix with 180 μl of Turk's solution. Just do not forget to take all dilutions in consideration when calculating the total number of cells in the original sample tube.

10. Plate at least five T-75 cm^2 flasks in this step to ensure the harvesting of the minimum number of BMSCs to start expansion in a two-layer cell factory.
11. We established 70% of cell viability at each passaging as a quality control parameter. Whenever we have an inferior viability, we discard the sample.
12. To estimate the degree of confluence in the cell factories, T-25 cm^2 flasks can be seeded simultaneously, at the same cell density as the cell factories, and kept as sentinels under the same culture conditions. To this, plate 2.0×10^3 cells/cm^2 into T-25 cm^2 flasks with α-MEM supplemented with 20% FBS.
13. We freeze the BMSCs products and maintain them on hold until all quality and functional control assays are performed, to certify that cells are free of contaminants and with biological properties preserved.

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Scalable Manufacturing of Human Hematopoietic Stem/Progenitor Cells Exploiting a Co-culture Platform with Mesenchymal Stromal Cells

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Abstract

In the context of hematopoietic cell transplantation, hematopoietic stem/progenitor cells (HSPC) from the umbilical cord blood (UCB) present several advantages compared to adult sources including higher proliferative capacity, abundant availability and ease of collection, non-risk and painless harvesting procedure, and lower risk of graft-versus-host disease. However, the therapeutic utility of UCB HSPC has been limited to pediatric patients due to the low cell frequency per unit of UCB. The development of efficient and cost-effective strategies to generate large numbers of functional UCB HSPC ex vivo would boost all current and future medical uses of these cells. Herein, we describe a scalable serum-free co-culture system for the expansion of UCB-derived $CD34^+$-enriched cells using microcarrier-immobilized human bone marrow-derived mesenchymal stromal cells as feeder cells.

Keywords Co-culture HSPC/MSC, Ex vivo expansion, Hematopoietic stem/progenitor cells, Stirred culture system

1 Introduction

A very rare population of hematopoietic stem cells (HSC) is responsible for generating all blood lineages. HSC are distinguished by their self-renewal capacity, multilineage differentiation ability, and capacity of generating long-term hematopoiesis when transplanted into immunocompromised hosts [1, 2]. Bone marrow (BM) transplantation is the gold standard clinical application for hematopoietic stem/progenitor cells (HSPC), being a lifesaving procedure for several malignant (e.g., leukemias) and non-malignant diseases. Umbilical cord blood (UCB) cells are an interesting alternative of HSPC to adult BM and mobilized peripheral blood (PB) counterparts as these cells can be obtained by a simple and non-invasive collection procedure, are associated to a reduced risk of infection transmission (e.g., blood-borne viruses), and can be immediately available for therapeutic use when fully characterized (i.e., human leukocyte antigen (HLA) typing) and

stored in cryopreservation banks [3–5]. Importantly, UCB cells allow a less stringent HLA matching between donor and recipient, which impacts the incidence risk of graft-versus-host disease (GVHD), a significant cause of morbidity and mortality upon allogeneic BM transplantation that results from the activation, proliferation, and effector cell function of donor T cells in combination with the release of proinflammatory cytokines, resulting in tissue damage [6]. Presently there are more than 700,000 HLA-characterized UCB units stored frozen in more than 50 UCB banks (public or private), with >45,000 UCB transplants performed to date [3, 4, 7, 8]. However, as a single UCB unit contains a limited number of cells, lower when compared to BM or PB [9, 10], the majority of UCB transplants have been limited to children with an average weight of 20–40 kg [11, 12]. One of the most effective approaches exploited to overcome cell dose limitations related with UCB relies on the transplantation of multiple UCB units, namely, double UCB transplantation that can use unmanipulated units only or, as alternative, involve the ex vivo manipulation of one of the grafts (reviewed in [3, 4, 13, 14]). In the last years, multiple strategies of ex vivo UCB manipulation exploring the proliferative and/or homing/engraftment capacity of HSPC have been pursued, and many of these are now being tested in early and late phase clinical trials (reviewed in [8, 14, 15]). Ex vivo expansion of UCB HSPC before transplantation, besides allowing the administration of higher cell doses, has been demonstrated to provide faster neutrophil and platelet engraftment, as well as reduced graft failure compared to transplantation settings where two unmanipulated UCB units were used [16, 17]. Therefore, the development of efficient and cost-effective strategies to generate large numbers of functional UCB HSPC while maintaining their function would be of major importance. In this context, controlled bioreactor systems are highly desirable for an efficient expansion of HSPC and a reliable alternative to standard tissue flasks. In the last years, different culture systems have been used to expand mononuclear cells (MNC) or HSPC such as spinner flasks [18–20], automated perfusion chambers [21], rotating wall vessels [22], roller bottles [23], and hollow fiber bioreactors [24, 25]. However, the results obtained were of limited clinical potential because of the low cell numbers obtained and the inclusion of animal-derived products in culture. In this chapter, we describe a protocol for the scalable expansion of UCB $CD34^+$-enriched cells (i.e., including HSPC) using BM-derived mesenchymal stromal cells (MSC) as feeder cells, to recapitulate physiological cues of the hematopoietic niche [26–28], under serum-free conditions.

2 Materials

2.1 Cells

1. Umbilical cord blood (UCB) units and bone marrow (BM) aspirates were obtained from healthy donors after written informed consent according to the Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells (Portuguese Law 22/2007, June 29), with the approval of the Ethics Committee of Hospital São Francisco Xavier, Lisboa, Centro Hospitalar Lisboa Ocidental, Portugal, and Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal, respectively.
2. Low-density UCB mononuclear cells (MNC) were isolated by Ficoll density gradient, according to Andrade et al. [29], and cryopreserved in a liquid/vapor phase nitrogen tank until further use.
3. BM mesenchymal stromal cells (BM MSC) were isolated from BM aspirates according to the protocol described by Dos Santos et al. [30] and cryopreserved in a liquid/vapor phase nitrogen tank until further use.
4. Murine stromal cell line-5 (MS-5, DSMZ) kept cryopreserved in a liquid/vapor phase nitrogen tank.

2.2 Solutions

1. Thawing medium for MSC: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 1% of Antibiotic-Antimycotic (A/A, 10,000 units/mL penicillin +10,000 μg/mL streptomycin and 25 μg/mL amphotericin B, (100×)). Store at 4 °C. All reagents from Thermo Fisher Scientific.
2. Thawing medium for hematopoietic stem/progenitor cells (HSPC): DMEM supplemented with 20% FBS, 1% of A/A, and 10 μg/mL endonuclease deoxyribonuclease I (DNase I, Sigma-Aldrich). Prepare before use.
3. Expansion medium for MSC: StemPro® MSC SFM XenoFree supplemented with 1% of GlutaMAX™ and 1% of A/A. Store at 4 °C. All reagents from Thermo Fisher Scientific.
4. Expansion medium for HSPC: QBSF-60® (Quality Biological Inc.) supplemented with 60 ng/mL stem cell factor (SCF), 55 ng/mL fms-related tyrosine kinase 3 ligand (Flt-3L), 5 ng/mL basic fibroblast growth factor (bFGF), 50 ng/mL thrombopoietin (TPO) (all from PeproTech), and 1% of A/A. Store at 4 °C.
5. Cell washing medium: DMEM supplemented with 10% FBS and 1% of A/A. Store at 4 °C.

6. Phosphate-buffered saline (PBS) solution. Prepare solution (1×) by dissolving PBS powder (Thermo Fisher Scientific) in 1 L of distilled water. Filter the solution using a 0.22 μm filter, and store at room temperature.
7. Deionized (DI) water. Store at room temperature.
8. TrypLE™ Select CTS™ cell dissociating reagent (Thermo Fisher Scientific). Store at room temperature.
9. Coating substrate: CELLstart™ CTS™ (Thermo Fisher Scientific). Store at 4 °C. Before use, prepare the working solution by diluting the necessary volume (1:100) in PBS.
10. CD34 MicroBead Kit (MACS Miltenyi Biotec).
11. Magnetic-activated cell sorting (MACS) buffer: PBS with 0.5% bovine serum albumin (BSA, Sigma-Aldrich) and 2 mM EDTA (Sigma-Aldrich).
12. Mitomycin C (Sigma-Aldrich) solution: Dissolve 0.5 μg/mL (for MSC) or 5 μg/mL (for MS-5) Mitomycin C in washing medium. Prepare before use.
13. Trypan blue stain 0.4% (Thermo Fisher Scientific). Store at room temperature.
14. Sigmacote® (Sigma-Aldrich).
15. MethoCult™ methylcellulose-based medium (STEMCELL Technologies). Store at −20 °C.
16. MyeloCult™ medium (STEMCELL Technologies). Store at −20 °C.
17. Medium for the cobblestone area-forming cell (CAFC) assay: MyeloCult™ medium supplemented with 350 ng/mL of hydrocortisone (STEMCELL Technologies). Prepare before use.
18. 1% (w/v) Paraformaldehyde (PFA) solution: Dissolve 1 g of PFA (Sigma-Aldrich) in 100 mL of PBS (*see* **Note 2**). Filter (0.22 μm) before use and maintain at 4 °C.
19. 1.5 μg/mL 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) solution (Sigma-Aldrich) in PBS, store at 4 °C. Prepare from 1 mg/mL stock solution in deionized (DI) water stored at −20 °C.
20. Far Red LIVE/DEAD™ Fixable Dead Cell Stain Kit (Thermo Fisher Scientific).
21. Mouse anti-human monoclonal antibodies: CD31-PE, CD73-PE, CD80-PE, CD90-PE, human leukocyte antigen HLA-DR-PE, CD105-PE, CD34 PerCP-Cy5.5 and CD34-PE, CD34 PerCP-Cy5, and CD45RA FITC (all from BD Biosciences).

2.3 Equipment and Supplies

1. Sterile labware not vendor specific: pipettes, polypropylene conical tubes, Eppendorf tubes, cell strainers (100 μm), and hemocytometer.
2. T-flasks 75: Falcon tissue culture treated flasks (T-75, BD Biosciences).
3. 24-well plates, Falcon tissue culture treated (BD Biosciences).
4. Fluorescence-activated cell sorting (FACS) tubes (BD Biosciences).
5. SoloHill® Plastic Microcarriers (Pall). Store at room temperature.
6. Lab scale.
7. Appropriate cell culture facilities.
8. Cell culture centrifuge.
9. Cell culture incubator with CO_2, temperature, and humidity control.
10. Inverted microscope equipped with ultraviolet (UV) light.
11. Temperature adjustable water bath set to 37 °C.
12. Thermomixer comfort (Eppendorf).
13. MidiMACS™ with LS columns (Miltenyi Biotec).
14. Bellco® spinner flask (Bellco) with 100 mL volume, equipped with 90° normal paddles and a magnetic stir bar.
15. StemSpan™ flat-bottom Spinner Flask with 50 mL volume, equipped with a unique agitator design including impeller blade and magnetic bar (Stem Cell Technologies).
16. Stirring plate (30–40 rpm).
17. Automatic analyzer YSI 7100 MBS (YSI Life Sciences).
18. FACSCalibur flow cytometer (BD Biosciences).

3 Methods

3.1 BM MSC Thawing and Expansion Under Static Conditions

1. Coat Falcon tissue culture treated flasks at least 30 min before cell thawing. Add diluted coating solution to culture plates at a final volume per surface area of 60 μL/cm^2. Incubate at 37 °C for 1 h. Before plating the cells, remove the excess of diluted coating solution.
2. Retrieve a cryogenic vial of MSC (approximately 1 mL) from the nitrogen tank, and quickly thaw in a 37 °C water bath.
3. Dilute the content of the cryogenic vial in MSC thawing medium (warmed to 37 °C) (1:5 dilution).
4. Centrifuge at 250 × *g* for 7 min, discard the supernatant, and resuspend the pellet in MSC expansion medium.

5. Count the cells, and plate on T-75 (10 mL of expansion medium) flasks precoated with coating solution, within a cell density range of (1–3) $\times$ 10^3 cells/cm^2.
6. Incubate cells at 37 °C, 5% CO_2 in a humidified atmosphere.
7. Refresh culture medium every 3 days.
8. Passage cells at 70–80% cell confluence.
9. Coat the new T-flasks at least 30 min before cell passaging according to **step 1**.
10. Remove the exhausted culture medium from the T-flasks, and add PBS (same volume as culture medium) to wash the cell layer. Remove PBS and add 4 mL TrypLE™ Select CTS™ (1×). Incubate at 37 °C for 7 min.
11. After complete cell detachment, recover the cell suspension to a polypropylene tube, and dilute it with twice the volume of washing medium. Wash the flasks once with expansion medium. Centrifuge at 250 $\times$ *g* for 7 min.
12. Discard the supernatant, and resuspend the pellet in MSC expansion medium. Determine cell number and viability using the trypan blue exclusion method. Mix cell suspension with 0.4% trypan blue stain (1:1). Viable (unstained cells) and dead cells (blue-stained cells) are identified and counted using a hemacytometer under the optical microscope.
13. Replate the cells on the new T-flasks (**step 9**) within a cell density range of (1–3) $\times$ 10^3 cells/cm^2 until reaching approximately (5–6) $\times$ 10^6 cells (*see* **Note 1**).

3.2 Expansion of BM MSC in the Spinner Flask

1. In order to prevent the microcarriers from sticking to the glass, the spinner flasks must be siliconized using Sigmacote (*see* **Note 2**). Add the necessary quantity of Sigmacote onto the vessels in order to wet the inner glass surfaces. Remove the excess of Sigmacote by pipetting, and allow the spinner flasks to air-dry inside the laminar flow hood. Pipet DI water to rinse the inner surfaces of the vessels, and repeat this procedure three times. Autoclave the vessels with DI water (121 °C, 20 min) until further use.
2. To start the MSC culture in the spinner flask, autoclave the Bellco® vessel with DI water (121 °C, 20 min), remove the excess water inside the spinner, and wash it once with expansion medium.
3. Weigh 1.6 g of plastic microcarriers in a 50 mL polypropylene tube.
4. Sterilize plastic microcarriers by autoclaving (121 °C, 20 min) in DI water.
5. Let the beads settle and wash once with PBS.

6. Prepare 10 mL of CELLstart™ CTS™ solution, and add to the microcarriers.
7. Place the tube in the Thermomixer for 1 h at 37 °C, with a cycle of 2 min agitated followed by 10 min non-agitated.
8. Wash the coated microcarriers once with PBS and MSC expansion medium.
9. Resuspend microcarriers in 25 mL of MSC expansion medium (*see* **Note 3**), and add these to the Bellco® spinner flask. Use more 5 mL of expansion medium to wash the conical tube containing the microcarriers, and collect any remains.
10. Add a total of 4×10^6 cells, previously expanded under static conditions in culture flasks, to the microcarriers inside the spinner flask, and finalize a total volume of 40 mL. This yields a culture with an initial MSC concentration around 1×10^5 cells/mL and 40 g/L of microcarriers.
11. Place the spinner flask inside the incubator with the lids slightly unscrewed to allow gas transfer, and set the agitation to 30 rpm for the first 24 h (*see* **Note 4**). From day 1 onward, set the agitation to 40 rpm.
12. At day 3 add approximately 40 mL of expansion medium for a final volume of 80 mL.
13. From day 4 until day 7, replace 25% of the medium daily.

3.3 Monitoring the MSC Culture in the Spinner Flask

3.3.1 Cell Count and Viability

1. Take daily duplicate samples of 0.5 mL from the homogeneous culture suspension (*see* **Note 5**).
2. Allow microcarriers to settle down, collect the supernatant (which will be used for metabolite analysis—*see* Subheading 3.3.2), and wash with PBS.
3. Remove PBS, add 1 mL of TrypLE™ Select CTS™ (1×) to each sample, and incubate in the Thermomixer for 7–8 min at 37 °C and 750–800 rpm (*see* **Note 6**).
4. Stop the enzymatic action by adding washing medium in a proportion of 1:3.
5. Separate the cells from the microcarriers through filtration using a cell strainer (100 μm).
6. Centrifuge at 250 × *g* for 7 min, discard the supernatant, and resuspend the pellet in PBS (0.5–1 mL).
7. Determine cell number and viability using the trypan blue exclusion method.

3.3.2 Metabolite Analysis

1. Collect 1 mL of supernatant to Eppendorf tubes (**step 2** from Subheading 3.3.1).
2. Centrifuge the samples for 10 min at 200 × *g*, and then transfer to another Eppendorf tubes. Store at −20 °C until analysis.

3. Analyze the samples in an automatic analyzer YSI 7100 MBS to determine the concentration of glucose (nutrient, *see* **Note 7**), lactate, and ammonia (metabolites) throughout culture.

3.3.3 Cell Distribution on Microcarriers

1. Every 2 days take 0.4 mL sample from the homogeneous culture suspension to a 24-well plate.
2. Let microcarriers to settle down, remove the supernatant, and wash twice with PBS.
3. Fix cells with 0.5 mL of 2% PFA solution for 20 min at room temperature.
4. Wash twice with PBS. Add 0.5 mL of 1.5 μg/mL DAPI solution, and incubate in the dark at room temperature for 5 min (*see* **Note 8**).
5. Wash three times with PBS, and keep it protected from light at 4 °C until observation (maximum 7 days). Observe using a microscope under UV light.

3.3.4 Immunophenotypic Analysis of Expanded MSC

1. Harvest MSC from the microcarriers (*see* **Note 9**) according to the method previously described in Subheading 3.3.1.
2. Centrifuge at 250 × *g* for 7 min.
3. Wash the cells twice with 1 mL of PBS and resuspend in PBS.
4. Split the cell suspension into the necessary FACS tubes.
5. Add the appropriate amounts of the antibodies (CD73-PE, CD90-PE, CD105-PE, CD45-PE, CD14-PE, CD19, HLA-DR-PE, CD34-PE) to each FACS tube, and incubate for 15 min at room temperature in the dark.
6. Add 2 mL of PBS to remove the excess of antibody, and centrifuge for 5 min at 160 × *g*.
7. Resuspend the cells, fix in 1% PFA, and store at 4 °C until analysis is performed.
8. Analyze the cells by flow cytometry to quantitatively determine the expression of each surface marker. Collect a minimum of 1×10^4 events for each sample and use.

3.4 Isolation of UCB-Derived CD34+ HSPC

1. Retrieve the necessary number of cryogenic vials of UCB MNC (approximately 1 mL/vial) from the nitrogen tank, and quickly thaw in a 37 °C water bath.
2. Dilute the content of the cryogenic vials in HSPC thawing medium (warmed to 37 °C) (1:10 dilution) (*see* **Note 10**).
3. Centrifuge at 250 × *g* for 5 min, discard the supernatant, and resuspend the pellet in 50 mL of cell washing medium.
4. Count the cells, centrifuge at 250 × *g* for 5 min, discard the supernatant, and resuspend the pellet in 300 μL/10^8 MNC in MACS buffer.

5. Enrich UCB cells for the $CD34^+$ HSPC phenotype using magnetic cell sorting according to manufacturer's instructions. Briefly incubate UCB MNC with magnetically labeled CD34 MicroBeads (30 min at 2–8 °C). Load the cell suspension onto a MACS® column, under a magnetic field. The magnetically labeled $CD34^+$ cells will be retained within the column, while the unlabeled cells will run through. Remove the column from the magnetic field, and the magnetically retained $CD34^+$ HSPC will be eluted as the positively selected cell fraction.
6. Count the cells, and resuspend 1×10^6 $CD34^+$ HSPC in 10 mL of HSPC expansion medium.

3.5 Ex Vivo Expansion of CD34+ HSPC Under Dynamic Conditions

1. Before performing co-cultures with UCB $CD34^+$-enriched cells, incubate MSC-containing microcarriers with the previously prepared Mitomycin C solution, to prevent stromal cell overgrowth, for 2.5 h at 37 °C (*see* **Note 11**).
2. Wash twice with PBS and add washing medium (*see* **Note 12**).
3. Count the cells, according to the method previously described in Subheading 3.3.1, and resuspend 2×10^6 MSC (adhered to the microcarriers) in 5 mL of HSPC expansion medium, and add these to the StemSpan™ spinner flask. Use an extra of 5 mL of HSPC expansion medium to wash the conical tube containing the microcarriers, and collect any remains.
4. Add 1×10^6 $CD34^+$ cells to the spinner flask already containing the Mitomycin-treated MSC immobilized on the plastic microcarriers (final proportion of 2 MSC:1 HSC).
5. Place the spinner flask inside the incubator with the lids slightly unscrewed to allow gas transfer, and during the first 12 h, set an agitation regimen of 5 min agitating at 40 rpm followed by 4 h non-agitated. After 24 h, set the agitation for 40 rpm.
6. On days 3 and 7, renew 50% of the medium volume.

3.6 Monitoring the HPSC Culture in the Spinner Flask

3.6.1 Cell Count and Viability

1. Take daily duplicate samples of 0.5 mL from the homogeneous culture suspension.
2. Allow microcarriers to settle down, and collect the supernatant to determine HSPC number and viability using the trypan blue exclusion method (*see* **Note 5**).
3. Centrifuge the supernatant for 10 min at $200 \times g$, and then transfer to another Eppendorf tubes. Store at −20 °C for metabolite analysis.

3.6.2 Colony-Forming Unit (CFU) Assay

1. At days 0 and 7 of the spinner flask culture, centrifuge 1,000 and 10,000 cells, respectively, at $250 \times g$ for 7 min.
2. Discard the supernatant, and resuspend the pellet in MethoCult™ methylcellulose-based medium.

3. Seed the cell suspension on 24-well plate in triplicates, and allow to form colonies for 14 days at 37 °C and 5% CO_2 (*see* **Note 13**).
4. Count and classify formed colonies (colony-forming unit granulocyte-macrophage (CFU-GM), colony-forming unit multilineage (CFU-Mix), and burst-forming unit-erythroid (BFU-E) progenitors) accordingly to the manufacturer's instructions, by visual inspection using bright-field microscopy.

3.6.3 Cobblestone Area-Forming Cell (CAFC) Assay

1. Prepare a growth-arrested feeder layer of MS-5 cells as previously described for BM MSC in 24-well plate in duplicates/condition.
2. At days 0 and 7 of the spinner flask culture, centrifuge 2,000 cells/mL at 250 × *g* for 7 min.
3. Discard the supernatant and resuspend the pellet in CAFC medium.
4. Seed the cell suspension on the top of the MS-5 feeder layers (in duplicate), and half-feed the cultures once a week.
5. After 14 days at 37 °C and 5% CO_2, visually inspect the wells using phase-contrast microscope for the presence of cobblestone areas of more than five tightly packed cells underneath the stromal layer [31].

3.6.4 Immunophenotypic Analysis of HSPC Expanded Under Dynamic Conditions

1. Collect 1 × 10^6 HSPC from the spinner flask according to the method previously described in Subheading 3.6.1.
2. Centrifuge at 250 × *g* for 7 min, discard the supernatant, and wash with 1 mL PBS.
3. Resuspend the pellet in 1 mL of PBS.
4. Add 1 μL of the reconstituted fluorescent reactive dye (from the Far Red LIVE/DEAD™ Fixable Dead Cell Stain Kit) to 1 mL of the cell suspension, and mix well.
5. Incubate at room temperature for 30 min, protected from light.
6. Wash the cells twice with 1 mL of PBS and resuspend in PBS.
7. Split the cell suspension into the necessary FACS tubes (*see* **Note 14**).
8. Add the appropriate amounts of the antibodies (CD34 PerCP-Cy5, CD90 PE and CD45RA FITC) to the FACS tube, and incubate for 15 min at room temperature in the dark. Add 2 mL of PBS to remove the excess of antibody, and centrifuge for 5 min at 160 × *g*.
9. Resuspend the cells, fix in 1% PFA, and store at 4 °C until analysis is performed.

10. Analyze the cells by flow cytometry to quantitatively determine the content of stem/progenitor cells ($34^+90^+45RA^-$). Collect a minimum of 1×10^4 events for each sample and use.

4 Notes

1. To initiate the expansion of MSC under dynamic conditions, there should be enough cells to inoculate spinner flask cultures and to perform the immunophenotype characterization according to Subheading 3.3.4. The phenotypic markers analyzed are part of the criteria suggested by the International Society for Cellular Therapy to define MSC for both scientific research and preclinical studies [32]. The expression of the MSC surface markers, before and after spinner flask cultures, should be identical.
2. Once glassware has been siliconized, it is not necessary to repeat treatment prior to each use. From our knowledge, vessels should be siliconized every 20 cultures.
3. In this step, beads should be resuspended in a larger volume to minimize the number of beads attached to the inner walls of the conical tube and pipette.
4. From our recent optimization studies, continuous agitation during the seeding step prevents microcarrier aggregation when compared to intermittent agitation [33].
5. A homogeneous sampling is essential for accurate cell number determination. Before taking 0.5 mL samples, it is important to assure an evenly mixed culture inside the spinner flask. For that purpose, a stirring plate may be used inside the laminar flow chamber to homogenize culture inside the spinner flask before sampling.
6. In the final days of culture, larger cell carrier aggregates are formed that require longer incubation times with TrypLE and higher agitation rates to be completely dissociated.
7. In MSC cultures, GlutaMAX (alanyl-L-glutamine dipeptide) is used as a glutamine substitute since it is more stable and does not spontaneously break down to form ammonia. Cells cleave the dipeptide bond to release L-glutamine as needed, and therefore it is not possible to determine glutamine consumption during culture.
8. The well plate should be protected from light. It is also advised to turn off the laminar flow chamber light while preparing the sample.

9. The sample volume should allow collecting enough cells to perform flow cytometry analysis. Estimate the necessary volume according to cell concentration in culture.
10. The addition of DNase I to the thawing medium minimizes the presence of free-floating DNA fragments and cell clumps during the thawing step of HSPC.
11. For the Mitomycin C treatment, use more cells than needed to start the co-culture spinner flasks because during this step, some MSC will die or detach from the microcarriers.
12. Co-cultures should be established within 24–48 h. Gently perform the washing steps.
13. Distribute the medium evenly across the surface of the well. If bubbles form, remove them with a sterile needle. Add approximately 2 mL of sterile water to an empty well to help maintaining humidity.
14. Account tubes for unstained cells, color compensation, and FMOs.

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GMP-Compatible, Xeno-Free Culture of Human Induced Mesenchymal Stem Cells

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Abstract

Mesenchymal stem cells (MSCs) have been used in therapies owing to their regenerative potential, paracrine regulatory effects, and immunomodulatory activity. To foster commercialization and implementation of stem cells treatments, researchers have recently derived MSCs from human induced pluripotent stem cells (iMSCs). For therapeutic applications, human iMSCs must be produced in xeno-free culture conditions and following procedures that are compatible with the principles of Good Manufacturing Practice.

Keywords Cell therapy, Clinical application, Good Manufacturing Practice, Human platelet lysate, Induced pluripotent stem cell, Mesenchymal stem cell, Regenerative medicine, Xeno-free culture

1 Introduction

Human mesenchymal stem cells (MSCs) can regulate immunological and inflammatory responses and boost tissue repair and healing [1]. Because of these properties, human MSCs have been used for the treatment of hematopoietic, cardiovascular, and autoimmune diseases and for the repair of traumatic bone and cartilage injuries [2], with numerous clinical trials conducted worldwide every year [3]. After transplantation, MSCs exert their therapeutic effects by transient release of trophic factors that can stimulate healing and regeneration [4]. This implies that large numbers of cells need to be administered to achieve therapeutic efficacy. Unfortunately, therapeutically functional human MSCs derived from adult tissues may not be available in sufficient numbers for every patient [5–8]. As a result of this, researchers have recently derived MSCs from human induced pluripotent stem cells (iMSCs) [9–13], which can be derived from every patient in virtually unlimited amounts [14]. Human iMSCs meet all criteria defining adult mesenchymal stem cells, including adherence to plastic, spindle morphology, surface expression profile, and multilineage commitment [9–13]. To achieve therapeutic doses of cells required for clinical applications [15, 16], human iMSCs must be cultured in vitro in xeno-free conditions and according to Good Manufacturing

Practice (GMP) guidelines. The use of animal components must be avoided because it carries the risk of zoonoses and xenogeneic immune reactions [17]. In addition, the cells must be cultured in conformity with guidelines recommended by agencies that control the manufacturing of safe and high-quality pharmaceutical products and medical devices [18].

In this chapter, a step-by-step protocol to culture and expand human iMSCs in xeno-free media supplemented with human platelet lysate or in commercially available GMP-compatible media is described. Compliance with this protocol and procedures is essential to effectively manufacture human iMSC lines for applications in clinical settings.

2 Materials

Prepare and store all reagents at recommended temperature. Before use, warm all reagents to room temperature or 37 °C if needed.

2.1 Cells

1. iMSCs are derived from human induced pluripotent stem cells using protocols previously described by de Peppo et al. [10]. Human induced pluripotent stem cells (iPSCs) are reprogrammed from patient cells after informed consent.

2.2 Xeno-Free Medium

1. Knockout-Dulbecco's Modified Eagle's Medium (KO-DMEM) (*see* **Note 1**).
2. GlutaMAX (*see* **Note 2**).
3. Minimum Essential Medium (MEM) nonessential amino acids (*see* **Note 3**).
4. Basic fibroblast growth factor (bFGF) (*see* **Note 4**).
5. Beta-mercaptoethanol (*see* **Note 5**).
6. Human platelet lysate (HPL) (*see* **Note 6**).
7. Heparin (*see* **Note 7**).

2.3 GMP-Compatible Medium

1. RoosterNourish™-MSC-CC (*see* **Note 8**).

2.4 General Cell Culture Reagents and Appliances

1. Dulbecco's phosphate-buffered saline (DPBS) without Ca^{++} and Mg^{++}.
2. 70% (v/v) ethanol solution in distilled water.
3. Laminar flow hood.
4. Incubator with CO_2, temperature, and humidity control.
5. Light microscope.
6. Delicate cleaning wipers.

7. Sterile tissue culture plasticware, typically T75, T150, or T300 cm^2 vented tissue culture flasks.
8. 15, 50 mL sterile centrifuge polypropylene tubes.
9. 1.5 mL sterile microcentrifuge tubes.
10. 5, 10, 25, and 50 mL sterile serological pipettes.
11. Motorized pipette controller.
12. P10, P100, P1000 tissue culture pipettes.
13. 10, 100, 1,000 μL sterile pipette tips.
14. Variable speed centrifuge.
15. Bottletop filters or similar filtering devices.
16. Fridges and freezers.
17. Water or dry bath (set a 37 °C).

2.5 Cell Passaging

1. Xeno-free cell dissociation reagent (*see* **Note 9**).
2. Hemocytometer (*see* **Note 10**).
3. 0.4% trypan blue staining solution.

2.6 Cell Storage

1. Cryovials.
2. Freezing solution (*see* **Note 11**).
3. Freezing containers for cryovials (*see* **Note 12**).
4. Liquid N_2 thank.

3 Methods

3.1 Before Starting

1. Wipe the hood and spray the reagent bottles and tissue culture appliances with 70% (v/v) ethanol.

3.2 Medium Preparation

1. Allow all reagents to reach room temperature before starting.
2. For xeno-free medium preparation, mix KO- DMEM with 10% (v/v) HPL (*see* **Note 13**), 11.25 ng/mL of heparin (*see* **Note 14**), MEM non-essential amino acids solution (at recommended concentration), GlutaMAX (at recommended concentration), 0.1 mM β-mercaptoethanol, and 1 ng/mL β-fibroblast growth factor using a sterile bottle. For RoosterNourish™-MSC-CC medium preparation, mix 500 mL of RoosterBasal™--MSC-CC with 10 mL of RoosterBooster™-MSC-CC as per manufacturer's instruction.
3. Filter the medium using bottletop filters if needed. Multiple filters may be required.
4. Make aliquots and store at 2–8 °C if needed. Use the medium within a week from preparation date.

5. Create a register describing all details of the medium prepared, including lot number of reagents and supplies, and expiration date.

3.3 Thawing Cells

1. Fill a polystyrene or plastic basket with dry ice.
2. Transfer the vial(s) containing cells from liquid N_2 to dry ice.
3. Place the cell vial(s) in a water or dry bath (set a 37 °C) until a small frozen portion is left.
4. Spray down the vial/s with 70% (v/v) ethanol before opening.
5. Pipette up and down the cell suspension and transfer it to sterile centrifuge polypropylene tubes containing culture medium at 37 °C (usually the content of 1 vial into 5–10 mL of culture medium).
6. Wash the vial/s once or twice with fresh culture medium to collect residual cells.
7. Let the cells accommodate for a few minutes.
8. Transfer a small aliquot of cell suspension to a sterile micro-centrifuge tube for counting and viability testing (*see* Subheading 3.4) (*see* **Note 15**).
9. Centrifuge the cell suspension down for 5 min at $200 \times g$ to eliminate any contaminant freezing solution.
10. Using an aspirating pipette, aspirate the supernatant from the centrifuge tube.
11. Resuspend the cell pellet in an adequate volume of culture medium to achieve the desired cell density for seeding.

3.4 Counting Cells

1. Mix the collected aliquot of cell suspension (*see* Subheading 3.4, **step 8**) with an equal volume of 0.4% trypan blue staining solution.
2. Pipette up and down to mix well. Vortex shortly if needed.
3. Add the required volume of cell suspension (usually 10–20 μL) between the hemocytometer and cover glass using a P20 tissue culture pipette.
4. Count live cells (not stained with trypan blue) using a light microscope. Alternatively, use an automated cell counter (*see* **Note 10**).

3.5 Plating Cells

1. Resuspend cells in fresh culture media and mix gently (*see* **Note 16**).
2. Plate cells into sterile vented tissue culture flasks at a density of 5,000–10,000 cells per cm^2 (*see* **Note 17**).
3. Gently shake the flasks to facilitate even distribution of the cells.
4. Incubate cells in a humified atmosphere at 37 °C and 5% CO_2.

5. Change media every 2 or 3 days.
6. Passage cells when they reach approximately 90% confluency (*see* Subheading 3.6).
7. Create a register describing all details of the plating procedure, including description of the reagents used, as well as cell density and date of seeding.

3.6 Passaging Cells

1. Wash cells twice with DPBS without Ca^{++} and Mg^{++}.
2. Add an adequate amount of xeno-free cell dissociation reagent to cover the cells (*see* **Note 18**).
3. Incubate the cells at 37 °C for about 5 min.
4. Following incubation, check if the cells have detached using a light microscope. If not, incubate for an additional minute and check again. When cells are completely detached, promptly dilute the dissociation reagent by adding a volume 3–5 times larger than that of fresh culture medium.
5. Using a sterile serological pipette, wash well the floor of the flask to flush the cells down and harvest.
6. Transfer the single cell suspension to 50 mL sterile centrifuge polypropylene tubes or sterile tissue culture bottles (*see* **Note 19**).
7. Using a light microscope, check that all cells have been collected. If there are residual cells attached, wash the flask again with fresh medium before continuing.
8. Pool all aliquots of cell suspension together and mix well using 5 or 10 mL sterile serological pipettes before counting (*see* Subheading 3.4), plating (*see* Subheading 3.5), or storing cells (*see* Subheading 3.7).
9. Create a register describing all details of the passaging procedure, including descriptions of reagents used, as well as cell density and date of passaging.

3.7 Storing Cells

1. Centrifuge single cell suspensions at 200 × *g* a room temperature for 5 min.
2. Aspirate the supernatant for sterility testing, suggestively mycoplasma and endotoxins testing.
3. Resuspend the pellet using DPBS without Ca^{++} and Mg^{++} to wash the cells, then centrifuge again at 200 × *g* a room temperature for 5 min. Repeat if needed.
4. Resuspend the pellet in xeno-free cryopreservation medium at a freezing concentration of 1,000,000–10,000,000 cells per mL of cryopreservation medium.
5. Aliquot cells into cryovials.

6. Freeze cells at negative 80 °C using freezing containers that provide a critical 1 °C per minute cooling rate.
7. Working on dry ice, transfer the cells to a liquid N_2 tank for long-term storage.

3.8 Cell Monitoring and Quality Control of Culture

1. At each passage, monitor the cell morphology and size, collect media aliquots for quality control and protein release analysis, and sample cells for backup and to confirm their mesenchymal phenotype and regenerative potential (*see* **Note 20**).

4 Notes

1. KO-DMEM is a basal medium optimized for the culture of induced pluripotent stem cells and their derivatives. KO-DMEM does not contain L-glutamine. In this chapter, it is described because it was initially used to derive and expand iMSCs in our laboratory [11]. Alternative defined culture media are recommended for GMP manufacturing of human iMSCs.
2. GlutaMAX is a high-performance L-glutamine supplement. It is added to the culture of highly dividing cells as additional source of nitrogen for the synthesis of proteins.
3. MEM nonessential amino acids are used as a supplement to increase cell growth and viability.
4. Basic FGF possesses broad mitogenic and cell survival activities. It is added to the media to promote cell proliferation and culture performance. Unpublished tests in our laboratory have observed a 5–10% increase in culture performance with 1 ng/mL of b-FGF.
5. Beta-mercaptoethanol is a potent reducing agent used in cell culture to avoid toxic level of oxygen radicals. In this chapter, it is recommended because it was initially used to derive and expand iMSCs in our laboratory [10, 13]. The degree to which its use improves iMSC culture performance remains to be determined.
6. In McGrath et al. [13], HPL was provided by the New York Blood Center, New York, NY. HPL is commercially available through a large number of different companies, hospitals, and blood banks. New HPL lots received from each provider should be tested to evaluate performance before use.
7. Heparin is an anticoagulant that is used to prevent gelation of HPL.
8. RoosterNourish™-MSC-CC is a standardized, xeno-free medium for the culture of human MSCs manufactured

conforming with current GMP principles. Alternative commercially available GMP-compatible media could be used as well. The performance of alternative media should be thoroughly tested before use.

9. In clinical grade iMSCs manufacturing, porcine trypsin should be replaced by commercially available xeno-free alternatives.
10. Different types of hemocytometers are available. Irrespective of the type used, it is critical to work with a single cell suspension to ensure accuracy of count. Automated cell counters are also available to achieve a more accurate and reproducible count.
11. Freezing solutions that conform with current GMP principles are commercially available. The use of defined freezing solutions is desirable to manufacture clinical grade iMSCs for therapies.
12. Conventional and alcohol-free containers are available and can be used interchangeably to freeze human iMSCs down to negative 80 °C.
13. Lower concentration of HPL could be used for culture of human iMSCs. The effect of source and concentration of HPL on the phenotype and expansion potential of iMSCs should be thoroughly tested before use.
14. If not derived from human sources, the use of heparin should be avoided in the manufacturing of iMSCs for clinical applications. Some providers distribute products derived from human platelet lysates that do not require the addition of heparin.
15. Post-thaw cell viability and recovery should be checked at each passage during culture.
16. Culture medium should cover the cells by 2–5 mm to facilitate oxygen diffusion to the cells. This corresponds to 0.2–0.5 mL per cm^2 of growth area.
17. Human iMSCs display small cell size and are typically seeded at densities between 5,000 and 10,000 cells per cm^2. The optimal cell density may vary depending on medium composition, donor, and cell passage. Donor variability is considerable when culturing iMSCs. Thus, protocols should be tested when culturing new lines for therapeutic applications.
18. Some cell dissociation reagents work best at 37 °C. If needed, warm the reagent using a water or dry bath before use.
19. Typically, at an initial density of 10,000 cells per cm^2, a T150 flask yields about 15,000,000–30,000,000 human iMSCs in about 3–4 days at early passage (P2-P3) and about 5,000,000–10,000,000 cells in about 5 days at late passage (P9-P10).
20. During culture and before use, cells must be tested for sterility, endotoxin, mycoplasma, identity, and mesenchymal

phenotype. Human iMSCs must meet all criteria defining MSCs, including adherence to plastic; expression of CD105, CD73, and CD90 but lack of expression of CD45, CD34, CD14, and HLA-DR surface molecules; and ability to differentiate into osteoblasts, chondroblasts, and adipocytes in vitro. These tests must be performed by trained personnel in certified laboratories using validated and approved methods.

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GMP-Grade Methods for Cardiac Progenitor Cells: Cell Bank Production and Quality Control

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Abstract

Cardiac explant-derived cells (cEDC), also referred as cardiac progenitors cells (CPC) (Barile et al., Cardiovasc Res 103(4):530–541, 2014; Barile et al., Cardiovasc Res 114(7):992–1005, 2018), represent promising candidates for the development of cell-based therapies, a novel and interesting treatment for cardioprotective strategy in heart failure (Kreke et al., Expert Rev Cardiovasc Ther 10(9):1185–1194, 2012). CPC have been tested in a preclinical setting for direct cell transplantation and tissue engineering or as a source for production of extracellular vesicles (EV) (Oh et al., J Cardiol 68(5):361–367, 2016; Barile et al., Eur Heart J 38(18):1372–1379, 2017; Rosen et al., J Am Coll Cardiol 64(9):922–937, 2014). CPC cultured as cardiospheres derived cells went through favorable Phase 1 and 2 studies demonstrating safety and possible efficacy (Makkar et al., Lancet 379(9819):895–904, 2012; Ishigami et al., Circ Res 120 (7):1162–1173, 2017; Ishigami et al., Circ Res 116 (4):653-664, 2015; Tarui et al., J Thorac Cardiovasc Surg 150(5):1198–1207, 1208 e1191–1192, 2015). In this context and in view of clinical applications, cells have to be prepared and released according to Good Manufacturing Practices (GMP) (EudraLex—volume 4—good manufacturing practice (GMP) guidelines—Part I—basic requirements for medicinal products. http://ec.europa.eu/health/documents/eudralex/vol-4; EudraLex—volume 4—good manufacturing practice (GMP) guidelines—Part IV—guidelines on good manufacturing practices specific to advanced therapy medicinal products. http://ec.europa.eu/health/documents/eudralex/vol-4). This chapter describes GMP-grade methods for production and testing of a CPC Master Cell Bank (MCB), consisting of frozen aliquots of cells that may be used either as a therapeutic product or as source for the manufacturing of Exo for clinical trials.

The MCB production method has been designed to isolate and expand CPC from human cardiac tissue in xeno-free conditions (Andriolo et al., Front Physiol 9:1169, 2018). The quality control (QC) methods have been implemented to assess the safety (sterility, endotoxin, mycoplasma, cell senescence, tumorigenicity) and identity/potency/purity (cell count and viability, RT-PCR, immunophenotype) of the cells (Andriolo et al., Front Physiol 9:1169, 2018).

Keywords Advanced therapy medicinal products, Biological/biotechnological products, Biopharmaceuticals, Cardiac explant-derived cells, Cardiac progenitors cells, Exosomes, Extracellular vesicles, Good manufacturing practices, Production methods, Quality control methods

1 Introduction

Cardiac explant-derived cells (cEDC), also referred as cardiac progenitors cells (CPC) [1, 2], represent promising candidates for the development of cell-based therapies for cardioprotection.

Several preclinical and clinical studies have shown the therapeutic potential of human cEDC in patient with heart disease [3–6].

Different approaches have been considered so far, relying on CPC alone as cell therapy product or in combination with other cell types and/or scaffolds (tissue engineered products), in autologous or allogeneic context [4, 7–10]. Moreover, recent evidence suggests that human CPC release EV that phenotypically recapitulate Exo [11]. Such vesicles inhibit cardiomyocyte apoptosis and promote angiogenesis in vitro, hence playing a fundamental role in the paracrine effect of CPC. In vivo, in a preclinical model of acute myocardial infarction in rats, CPC-derived Exo reduce tissue damage, inhibit cardiomyocyte apoptosis, and promote angiogenesis, improving post-infarction cardiac function [1, 5, 12, 13]. Key mediators of these effects have been identified in specific microRNAs such as miR-132 and miR-210 [1], as well as specific proteins such as the active form of pregnancy-associated plasma protein-A [2]. The therapeutic use of CPC-derived Exo may represent an alternative and innovative approach in respect to cellular therapies currently being tested in the cardiovascular field [5, 13].

In summary, CPC may be used for direct cell transplantation, for tissue engineering or as a source of extracellular vesicles (EV) such as Exo.

As a consequence, CPC may be considered as (1) advanced therapy medicinal products (ATMP) [14] or (2) cell substrates [15] for the production of innovative biopharmaceuticals, as therapeutic EV fall in this last class of medicinal products [16].

In both cases, in view of clinical applications CPC have to be manufactured according to Good Manufacturing Practices (GMP) [17, 18].

This chapter focuses on the GMP-grade xeno-free method developed in our facility [19] to produce a CPC Master Cell Bank (MCB) that may be used as ATMP or as cell source for EV product manufacturing. Figure 1 summarizes the MCB production flow, the In-Process Controls (IPC) performed during manufacturing and the quality control (QC) tests performed on the frozen cells. Despite the fact that normally a two-tier cell banking process (MCB and Working Cell Bank) is preferable, an approach based on MCB only is acceptable in early clinical stages, where thawing of few MCB vials per year is envisaged [15], as in first-in man clinical trials. To evaluate cell stability in culture, a Post Production Cell Bank (PPCB) is also generated, consisting of cells "at the limit of in vitro cell age used for production" [15].

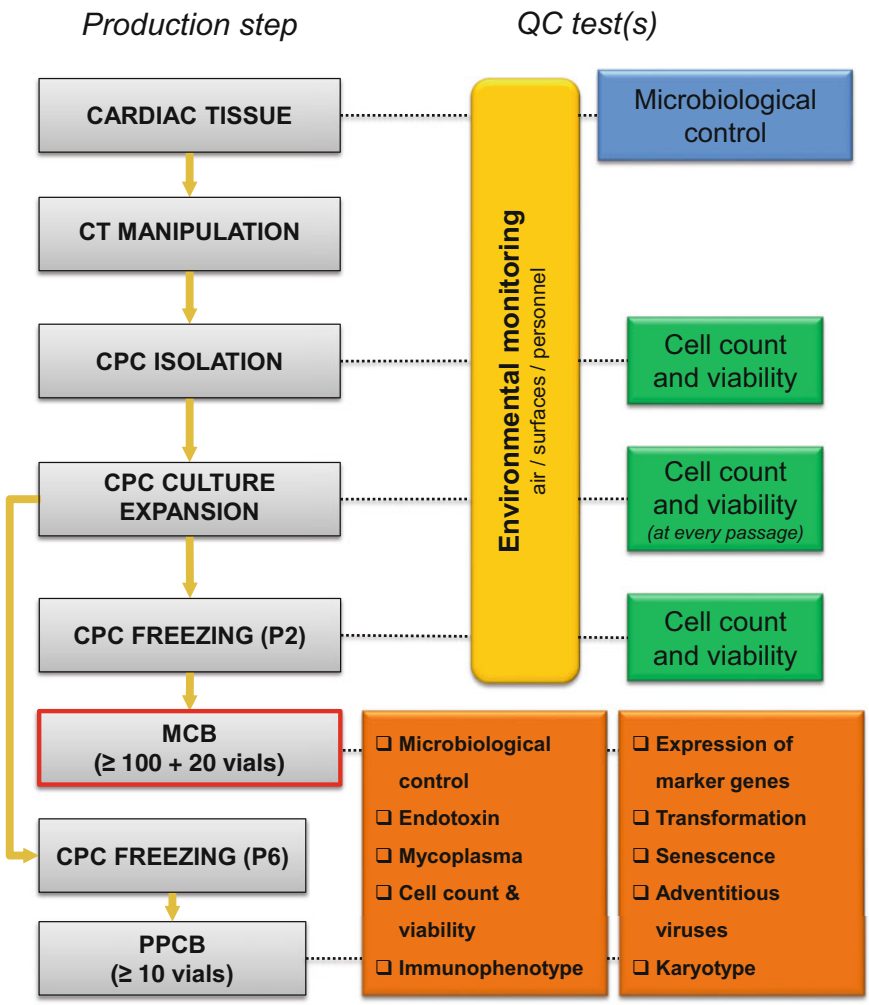

Fig. 1 Diagram of production steps with the corresponding QC activities. Legend for names: *CT* cardiac tissue, *CPC* cardiac progenitor cells, *MCB* master cell bank, *PPCB* Post Production Cell Bank. Legend for colors: blue = control of starting material; green = IPC; orange = release QC; yellow = environmental monitoring

This chapter also describes analytical methods implemented and performed in our cell factory as part of the release strategy for the CPC MCB, encompassing safety, identity/potency, and purity aspects [19]. The full QC panel for MCB and PPCB is reported in Table 1; for compendial assays, reference to the relevant European Pharmacopoeia (EP) [20] chapters is provided.

The MCB is released if all the QC results comply with the specifications.

Table 1
The full QC panel for MCB and PPCB

Test	Method	Specification
Microbiological control of cellular products	EP 2.6.27	No growth of microorganisms
Endotoxin assay	EP 2.6.14	<5.00 EU/mL[a]
Mycoplasma assay by NAT	EP 2.6.7	Negative
Expression study of marker genes	RT-PCR	Expression of GATA4, TBX18, TBX5 and MESP1
Cell count and viability	EP 2.7.29	≥80% total cells recovery ≥70% cell viability
Immunophenotype (identity assay)	EP 2.7.24	≥90% stained cells positive for CD90,CD105 and CD73 ≤10% stained cells positive for CD45/CD34/CD14/CD20/HLA-DR
In vitro transformation assay	WHO TRS 978, 2013 Annex 3	Under definition
In vitro senescence assay	β-galactosidase assay	Under definition
Mycoplasma assay by culture[b]	EP 2.6.7	Negative
Adventitious viruses[b]	EP 2.6.16	Negative
Karyotype[b]	WHO TRS 978, 2013 Annex 3	Normal diploid

[a]Specification in use for CPC tube used as EV source; for CPC as ATMP, the specification value must be changed according to EP 5.1.10, taking in the consideration the administration route and dose
[b]Outsourced assay

2 Materials

2.1 Master Cell Bank Production

High-quality and properly certified reagents and materials are mandatory for the whole process of GMP cell banking, according to regulatory expectations for ATMP and related products [21].

2.1.1 Cardiac Tissue Manipulation

1. Biological starting material: atrial appendage explant (*see* **Note 1**), in a sterile vessel containing cardioplegic solution [Plasma-Lyte A® solution (Baxter Healthcare, www.baxter.com) supplemented with mannitol (final concentration 0.3%), magnesium sulfate (0.2%), sodium bicarbonate (0.1%), lidocaine (0.01%), and potassium chloride (0.2%)].
2. Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS).
3. Petri dishes.

4. Single-use sterile scalpels and scissors.
5. Single-use sterile pipettes.

2.1.2 CPC Isolation and Culture

1. Tissue culture flasks 115 cm^2 with reclosable lids (TPP, www.tpp.ch), T-flasks (75–150 cm^2), CellBIND® HYPERFlask® (1720 cm^2), or HYPERStack®-12 (6000 cm^2) culture vessels (Corning, www.corning.com).
2. Cell culture medium (MSC-Brew GMP Medium Miltenyi Biotec GmbH, www.miltenyibiotec.com) (*see* **Note 2**).
3. CELLstart™ CTS™ (Gibco/Thermo Fisher Scientific, www.thermofisher.com) as adhesion substrate (*see* **Note 3**).
4. DPBS.
5. Sterile water.
6. TrypLE™ Select Enzyme (Gibco/Thermo Fisher Scientific, www.thermofisher.com) (*see* **Note 4**).
7. Single-use sterile pipettes.

2.1.3 CPC Freezing

1. Freezing medium: Cryostor® CS10 (BioLife Solutions, StemCell Technologies, www.stemcell.com) (*see* **Note 5**).
2. 1.8 mL cryovials.
3. Single-use sterile pipettes.

2.2 Master Cell Bank Quality Control

High-quality and properly certified reagents and materials are mandatory for QC assays.

2.2.1 Thawing of Cell Samples for QC

1. Cell culture medium (MSC-Brew GMP Medium Miltenyi Biotec GmbH).
2. 15 mL tubes.
3. Single-use sterile pipettes.

2.2.2 Microbiological Control of Cellular Product

1. BacT/ALERT FA PLUS (aerobic) (FA bottles) and BacT/ALERT FN PLUS (anaerobic) (FN bottles) (BioMérieux, www.biomerieux.com) (*see* **Note 6**).
2. Sterile syringes and needles.
3. Agar plates: Tryptic Soy Agar (TSA) or Columbia Blood Agar (CBA) and Sabouraud Dextrose Agar (SDA).

2.2.3 Endotoxin Assay

1. Endosafe® PTS™ cartridges—0.05 EU/mL sensitivity (Charles River, www.criver.com).
2. LAL Reagent Water.
3. Sterile and non-pyrogenic tubes and sterile and non-pyrogenic tips.

2.2.4 Mycoplasma Assay by NAT

1. TrypLE™ Select Enzyme.
2. 0.4% Trypan blue.
3. Universal Mycoplasma Detection Kit (ATCC, www.lgcstandards-atcc.org) containing lysis buffer, Universal master mix, Universal primers, Sample Lysis Tubes, Positive Control.
4. Calibrated Genomic Mycoplasma DNA (M. pneumoniae, ATCC or Bionique Testing Laboratories, www.bionique.com) as an Internal Control.
5. Single-use sterile pipettes.
6. Cell scrapers.
7. Sterile filter tips.
8. Polymerase chain reaction (PCR) tubes or strips with caps.
9. DNA ladder 100.
10. Agarose.
11. 1.5 mL tubes, PCR clean.
12. CellBIND® 6-well plates (Corning) or 6-well plates coated with adhesion substrate.
13. Cell culture medium.
14. 6× loading dye.
15. Tris Acetate-EDTA (TAE) buffer 1×.
16. SYBR Safe DNA Gel Stain (Invitrogen, www.thermofisher.com).
17. PCR water (certified nuclease-free).

2.2.5 Expression Analysis of Marker Genes

1. Single-use sterile pipettes.
2. Sterile filter tips.
3. Diethyl pyrocarbonate (DEPC)-treated water.
4. 1.5 mL tubes.
5. GeneJet RNA Purification Kit (Thermo Scientific, www.thermofisher.com).
6. Ethanol (96–100%).
7. Dithiothreitol (DTT) 2 mM.
8. Tris-EDTA buffer (TE) 1×.
9. DPBS.
10. Human Universal Total RNA, 1 μg/μL (Clontech, www.takarabio.com).
11. 0.2 mL PCR tubes with caps.
12. Kit GO Script RT System (Promega, worldwide.promega.com).
13. Mix 3.3 μM of each of the following primer pairs:

- GATA4: GCATCAACCGGCCGCTCATCA/GGTTCTTGGGCTTCCGTTTTCT
- TBX5: TACCACCACACCCATCAAC/ACACCAAGACAGGGACAGAC
- TBX18: GGGTTTGGAAGCCTTGGTGG/GGCAGAATAGTCAGCAGGGG
- MESP1: CTCTGTTGGAGACCTGGATG/CCTGCTTGCCTCAAAGTG
- GAPDH: ATGGGCAAGGTGAAGGTCGGAG/TCGCCCGACTTGATTTTGCAGG

14. GoTaq DNA Polymerase (Promega, worldwide.promega.com).
15. dNTP mix 10 mM (Thermo Scientific, www.thermofisher.com).
16. DNA ladder 100/Sharpmass.
17. Agarose.
18. Loading dye.
19. TAE buffer 1×.
20. SYBR Safe DNA Gel Stain (Invitrogen, www.thermofisher.com).

2.2.6 Cell Count and Viability

1. EVE™ Counting Slides (NanoEnTek, www.nanoentek.com).
2. 0.4% Trypan blue.
3. Sterile filter tips.
4. DPBS.

2.2.7 Identity Assay (Immunophenotype)

1. MSC Phenotyping Kit human (Miltenyi Biotec, www.miltenyibiotec.com) including MSC Phenotyping Cocktail (containing CD73-APC, CD90-FITC; CD105-PE, CD45-PerCP, CD34-PerCP, CD14-PerCP, CD20-PerCP) and Isotype Control Cocktail (containing the corresponding isotype controls).
2. Anti-HLA-DR-PerCP.
3. Staining buffer: DPBS, 0.5% Human Serum Albumin (HSA), 2 mM EDTA (*see* **Note 7**).
4. Flow cytometry tubes.
5. Flow cytometry running buffer (*see* **Note 8**).
6. Sterile filter tips.
7. Single-use sterile pipettes.

2.2.8 In Vitro Transformation Assay

1. CytoSelect™ 96-Well Cell Transformation Assay (Soft Agar Colony Formation; Cell Biolabs INC., www.cellbiolabs.com) (*see* **Note 9**).
2. CPC culture medium (MSC-Brew GMP Medium Miltenyi Biotec GmbH).
3. DPBS.
4. HeLa cell line (*see* **Note 10**).
5. HeLa culture medium.
6. Single-use sterile pipettes.
7. Sterile filter tips.

2.2.9 In Vitro Senescence Assay

1. FluoReporter lacZ Flow Cytometry Kit (Invitrogen, www.thermofisher.com).
2. TrypLE™ Select Enzyme.
3. Staining medium: DPBS, 4% FBS, 10 mM HEPES, pH 7.2.
4. Flow cytometry tubes.
5. Flow cytometry running buffer (*see* **Note 8**).
6. Sterile filter tips.
7. Single-use sterile pipettes.

3 Methods

3.1 Master Cell Bank Production

The whole production process is performed under sterile conditions in a GMP-certified classified area (Class A laminar flow hood in class B background).

3.1.1 Cardiac Tissue Manipulation

1. Transfer the atrial appendage explant in a Petri dish, perform 2–3 washings with DPBS, then isolate the cardiac muscle tissue (Fig. 2a), and mince it in small fragments (around 1 mm diameter) using sterile scalpels and scissors (Fig. 2b).
2. Transfer the fragments in a new Petri dish (Fig. 2c).
3. Add 1–3 mL of DPBS to wash the fragments, and then eliminate DPBS by aspiration; repeat this step twice.
4. Add 1–3 mL of TrypLE™ Select Enzyme and incubate for 5 min at room temperature.
5. Eliminate the enzyme solution by aspiration.
6. Add 1–3 mL of culture medium.

3.1.2 CPC Isolation and Culture

1. Coat 8–10 tissue culture flasks with reclosable lids with CELLstart™ CTS™ as adhesion substrate (*see* **Note 3**).

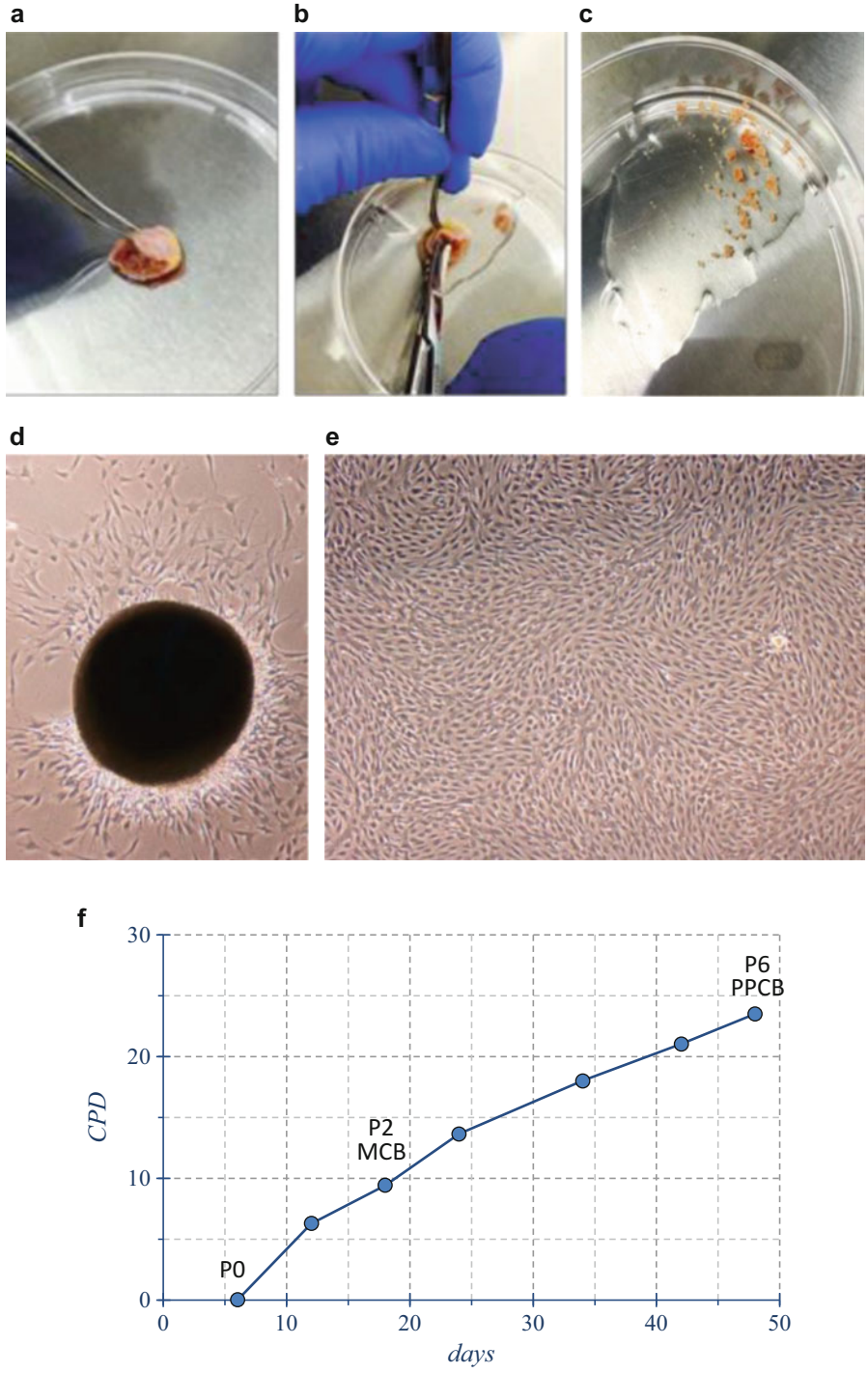

Fig. 2 Cardiac tissue manipulation and CPC expansion. (**a**–**c**) The atrial appendance is processed in order to obtained fragments of about 1 mm. (**d**) Representative images of CPC outgrowth from cardiac fragments and (**e**) CPC culture expansion at confluence. The Cumulative Population Doublings (CPD) over time is also shown (**f**) for the same representative experiment

2. Open the reclosable lids, and place the tissue fragments in the flasks, about 9 fragments/flask at distance of at least 3 cm each other.
3. Add culture medium (8–10 mL/flask), and put the flasks in incubator at 37 °C, 5% CO_2. Be careful in order to let the fragments adhere to the plastic.
4. Add fresh culture medium (about 1 mL/flask) every day for 7 days (final total volume 15–17 mL/flask). Be careful in order to let the fragments adhere to the plastic.
5. Regularly observe the flasks under the microscope, until detecting CPC outgrowth (passage 0, P0) (Fig. 2d).
6. Change the culture medium every 4–5 days being careful not to detach the fragments from the culture vessel.
7. At about 60–80% of cell confluence (cells outgrowing from adjacent fragments shouldn't come in contact), harvest P0 CPC using TrypLE™ Select Enzyme (*see* **Note 4**), and eliminate the tissue fragments (*see* **Note 11**).
8. Centrifuge the cell solution (10 min, 500 × *g* at room temperature), aspirate the supernatant, and suspend the cell pellet in an adequate volume of culture medium.
9. Determine cell count, and viability according to Subheading 3.2.6 (*see* **Note 12**).
10. Seed CPC at 8−10 × 10^4 cells/cm^2 in appropriate culture vessels (T-flasks, HYPERFlask® or HYPERStack®-12) in presence of culture medium (*see* **Note 13**). Incubate at 37 °C 5% CO_2.
11. At about 80% confluence (Fig. 2e), harvest P1 CPC using TrypLE™ Select Enzyme (*see* **Note 4**), and count them as described above. Seed cells as described above.
12. At about 80% confluence, harvest P2 CPC using TrypLE™ Select Enzyme (*see* **Note 4**) and count them as described above (*see* **Note 14**).
13. Maintain an aliquot of cells at P2 (about 5 × 10^6 cells) in culture until P6 for the generation of PPCB (*see* **Note 15**).
14. Freeze the remaining P2 CPC as MCB, according to Subheading 3.1.3.

3.1.3 Master Cell Bank Freezing

1. Label the required number of vials, and place them in a suitable rack at 4 °C until needed (*see* **Note 16**).
2. Put the freezing medium at 4 °C (*see* **Note 17**).
3. Centrifuge cells and remove the culture medium.
4. Suspend the cell pellet in freezing medium at the concentration of about 5 × 10^6 cells/mL (optimal range: 2–6 × 10^6 cells/

mL): gently add the solution (previous maintained at 4 °C) to the cells.

5. Transfer 1 mL aliquot in each cryovial, while maintain the cell suspension at 4 °C (*see* **Note 17**), then place the vials into a controlled-rate biological freezer (Biofreeze® BV45, Consarctic, www.consarctic.de), and proceed according to a suitable freezing program (*see* **Note 18**).
6. Store vials in a vapor-phase nitrogen tank.

3.1.4 Post Production Cell Bank Freezing

1. At about 80% confluence, harvest P6 CPC using TrypLE™ Select Enzyme (*see* **Note 4**), and count them as described above (*see* **Note 15**).
2. Proceed to preparation and freezing of vials as described in Subheading 3.1.3.

3.2 Master Cell Bank Quality Control

QC activities are performed in suitable GMP-certified labs, as detailed below.

3.2.1 Thawing of Cell Samples for QC Controls

1. Pre-heat the complete medium for 30′ at 37 °C.
2. Withdraw the desired number of vials of frozen CPC from the vapor-phase nitrogen tank.
3. Transport the vials to the laboratory in an insulated container.
4. Transfer the vials at 37 °C in a thermostat.
5. When warmed, transfer the vials under a laminar flow box, and disinfect them with isopropanol 70%.
6. Transfer the content of each vial into a 15-mL tube. Keep the vial(s) for later processing (**step 8**).
7. Gently dispense, dropwise, 8 mL of the pre-warmed medium into each tube.
8. Wash the vial with 1 mL of medium, and add it to the corresponding tube. Mix gently the cell suspension in the tube by 3 aspiration/dispensing cycles with the same pipette.
9. Centrifuge the cell suspension at 500 × *g* for 5 min at room temperature, discard the supernatant, and resuspend the cells in 1 mL of medium.
10. Count total and viable cells according to method in Subheading 3.2.6.

3.2.2 Microbiological Control of Cellular Product

This assay is performed to evaluate the sterility, by detecting fungal and/or bacterial contamination in the cellular products. It is designed to be performed on a cell suspension (on frozen MCB and PPCB) or a cell-free supernatant (*see* **Note 19**) provided that the assay is validated accordingly. It is carried out using an automated microbial detection system, the BacT/ALERT® 3D (BioMérieux, www.biomerieux.com) (*see* **Note 20**).

Procedure

The sample inoculum is performed in a clean room area (Class A in B background): microbial and particle contamination should be monitored according to GMP Annex 1 [22]. The subsequent steps (cultures, subcultures, and bacteria identification) are carried out in a standard QC microbiological laboratory.

1. Remove the FA and FN bottle caps and disinfect the rubber-septum.
2. Thaw the desired vials (*see* **Note 21**). With a syringe with needle remove the test sample volume to be inoculated in one bottle, remove the air completely and inoculate it in FN PLUS medium; repeat the same step for FA PLUS medium (*see* **Note 22**) and then for all the required bottles.
3. Mix the bottles.
4. Record all data on the dedicated forms or labels (*see* **Note 23**).
5. Transfer all samples outside the clean room area and load them into the Bact/Alert (bioMérieux) following the supplier's instructions.
6. Control daily the instrument for any growth alarm during the incubation time (*see* **Note 24**).
7. Evaluate the results: a sample is *negative* if no growth is detected (stable colorimetric signal), *positive* if growth is detected (increase of the signal). In case of positive sample, the microorganism must be isolated (*see* **Note 25**), and its genus and species must be identified (*see* **Note 26**).

3.2.3 Endotoxin Assay

This assay is performed to detect bacterial endotoxins in the final product. It is designed as a final product release test, to be performed on a frozen CPC suspension (MCB and PPCB). The method is performed using the Endosafe®-PTS™ system (Charles River, www.criver.com) in combination with FDA-licensed disposable test cartridges.

Procedure

The assay is performed in a standard QC laboratory. Sample manipulation is performed in a laminar flow box.

1. Insert the PTS endotoxin cartridge into the slot of the PTS reader following the manual instructions (*see* **Notes 27** and **28**).
2. Prepare at least 100 μL of the test sample diluted with LAL Reagent Water (*see* **Note 29**) according to validation data. Vortex the sample to prevent the endotoxin from sticking to the wall.
3. Load 25 μL of sample (*see* **Note 30**) into each of the four sample reservoirs (*see* **Note 31**).
4. Read and print the results. The assay detection limit is expressed as the sensitivity of the method (e.g., λ 0.05)

multiplied by the dilution factor (e.g., 100) of the validated sample (e.g., 5.00 EU/mL). The test results for samples below the detection limit are expressed as < detection limit (e.g., <5.00 EU/mL).

Acceptance Criteria

The assay is valid if:

- The spike recovery is between 50% and 200% (*see* **Note 32**).
- The percent Coefficient of Variation (CV%) for sample and Positive Control is less than 25%.

3.2.4 Mycoplasma Assay by NAT

According to EP [20], the Nucleic acid Amplification Technology (NAT)-based method can be used as a faster technique preliminary to a traditional method like the culture-based method, if it provides the same sensitivity and specificity.

The "Universal Mycoplasma Detection Kit" produced by ATCC (www.lgcstandards-atcc.org) uses the NAT technology to detect mycoplasma contaminants in the MCB and PPCB cultures.

The protocol setup in our lab is based on the manufacturer's indications, with the following modifications:

- Together with a sample of cells, a 20 mL sample from the medium is withdrawn; this sample (that will be mixed with the cells sample) is representative of the cell-free mycoplasma possibly present in the culture that would get lost if using the standard procedure
- A Calibrated Internal Control is run in parallel to test for the possible PCR inhibition by the samples. Fur such purpose, it is used at a concentration close to the Limit of Detection level (as determined during validation), so to have the best sensitivity in detecting any inhibitory activity.

Sample Preparation

In order to avoid contamination do not perform the following steps in post-PCR rooms, where PCR amplification products are manipulated.

The **steps 1** and **2** are carried out in a clean room, the remaining steps in a QC laboratory dedicated to cell biology testing or Pre-PCR steps.

1. Collect the cell supernatant (at least 20 mL of a pool made from equal aliquots from each culture vessel) at the end of the CPC manufacturing process, just before final cell detachment for cell freezing. Store the supernatant at −80 °C.
2. Thaw one vial of cells as described in Subheading 3.2.1, or withdraw a sample from the cells before starting the freezing process.
3. Seed the cells in 2 wells of a CellBIND® 6-well plate (around 7.5×10^4 cells in 5 mL medium/well), and culture from 50 to

70% confluence (avoid medium changes that could dilute mycoplasma).

4. Detach cells from one well (*see* **Note 33**), and perform a viable cell count by the Trypan Blue exclusion method (*see* Subheading 3.2.6) or an equivalent method.
5. Scrape the cells from the second well (*see* **Note 34**), and adjust the concentration to 10^4–10^5 cells/mL on the basis of the result obtained in **step 4** (*See* **Note 35**).
6. Thaw the supernatant from **step 1,** and dilute 1 mL of this cell suspension in 19 mL of cell supernatant.
7. Transfer the whole test sample (about 20 mL) to twenty 1.5 mL tubes.
8. Centrifuge at 18,000 × *g* for 3 min at 4 °C, and carefully remove and discard the supernatant.
9. Add 50 μL of lysis buffer to the pellet of one tube, resuspend, and transfer it along the whole series of the remaining tubes, in order to collect all material in one single tube.
10. Vortex for 10 s, and incubate for 15 min at 37 °C in a water bath.
11. Heat the sample at 95 °C for 10 min.
12. Centrifuge at 16,000 × *g* at 4 °C for 5 min and transfer the supernatant (lysate) to a new 1.5 mL tube.
13. Store the lysate at −80 °C (or use it immediately for PCR).

PCR Mix Preparation

In a lab dedicated to PCR mix preparation:

1. Thaw the universal PCR mix and primers; vortex and spin briefly the tubes.
2. Calculate the number of PCR reactions according to the following formula (*see* **Note 36**):

Number of test samples × 2 + 1 Positive Control + 1 Calibrated Internal control + 2 Negative Controls + 1 extra volume

3. Prepare the PCR mix in 1.5 mL tubes using the following volumes for each PCR reaction (*see* **Note 37**):

Universal Master Mix	20.0 μL
Universal primers	2.5 μL
Total volume/sample	22.5 μL

4. Move the PCR mix to the room dedicated to nucleic acid purification.

PCR Reaction Assembly

In a lab dedicated to nucleic acid purification:

1. Label the PCR tubes: 2 tubes for each test sample, A and B (e.g., 1A, 1B, 2A, 2B), Negative Control A and B, Calibrated Internal Control, Positive Control.
2. Thaw the Positive Control, the Calibrated Internal Control, and the samples; vortex and spin the tubes.
3. Add 22.5 μL of PCR mix in each tube.
4. Add 2.5 μL of each sample in duplicate to the corresponding sample tubes (i.e., two tubes/sample: 2.5 μL of sample in tube A and 2.5 μL in tube B), and close all the A tubes.
5. Add 2.5 μL H_2O to Negative Control tube A and close the tube (*see* **Note 38**).
6. Add 1 μL of a Calibrated Internal Control (10–20 copies/μL) (*see* **Note 39**) to sample B tubes.
7. Add 1 μL of a Calibrated Internal Control (10–20 copies/μL) to the Internal Control tube (+1.5 μL H_2O).
8. Add 2.5 μL of the positive control (plasmid DNA from the kit) to the Positive Control tube, and close the tube.
9. Add 2.5 μL of H_2O to the Negative Control tube B and close the tube.
10. Store the remaining lysates at −80 °C.
11. Place the tubes in a thermal cycler, and run the PCR using the following parameters:

	Temperature	Time	Cycles
Step 1	94 °C	1.5 min	1
Step 2	94 °C 70–60.5 °C[a] 72	30 s 30 s 45 s	20
Step 3	94 °C 60 °C 72 °C	30 s 30 s 45 s	12
Step 4	72 °C	45 s	1
Step 5	10 °C	Hold	1

[a]"Touch down" PCR step: −0.5 °C/cycle (*see* **Note 40**)

Agarose Gel Analysis

1. Prepare a 3% agarose gel in TAE buffer 1× (e.g., 3 g Agarose in 100 mL TAE buffer).
2. Add SyBR Safe DNA gel stain 10,000× (e.g., 10 μL in 100 mL TAE 1×).
3. Melt the solution on a heated stirrer or in a microwave oven (SyBR Safe DNA gel stain is resistant to microwaving).

4. Insert a comb in a gel electrophoresis tray, add the melted solution and wait until the polymerization is complete.
5. Insert the tray with the gel in the electrophoresis chamber, cover it completely with 1× TAE buffer, and gently remove the comb.
6. Add 2.5 μL of 100 bp DNA ladder to 2 μL of 6× loading dye, and load it in the first lane of the gel.
7. Add 10 μL of each sample to 2 μL of 6× loading dye, and load the resulting mix onto the gel, leaving an empty lane (if possible) between samples A and B.
8. Electrophorese at 80–110 V until tracking dye migrates 60–70% the length of the gel.
9. Take a picture through an Imaging Gel System (*see* **Note 41**), or view it with a UV illuminator.
10. Evaluate the results obtained with the samples amplified without the internal control:

    If no bands are visible in the 434–468 bp range, the sample is *negative*.

    If some bands are visible in the 434–468 bp range, the sample is *positive*.

Acceptance Criteria

The assay is valid if:

- The Positive Control exhibits a 464 bp band.
- The Calibrated Internal Control exhibits its specific length band.
- There are no visible bands (434–468-bp) in the negative control lanes.

Each sample result is acceptable if:

- The sample amplified with the Calibrated Internal Control exhibits the band of the specific length (*see* **Note 42**).

3.2.5 Expression Study of Marker Genes

The expression of transcription factors is evaluated by RT-PCR.

The genes GATA4 (Gene ID: 2626), TBX5 (ID 6910), TBX18 (ID 9096), and MESP1 (ID 55897) were selected according to literature data as CPC markers, representing mesodermal (TBX5, TBX18) and cardio-specific (GATA4, MESP1) transcription factors. As reverse transcription and amplification controls, primers on the GAPDH gene (ID 2597) are used.

Sample Collection and Preparation

The samples of cells should be taken from the cell suspension just before the freezing process.

1. Pellet 1 to 5 × 10^6 cells by centrifugation at 250 × *g*.
2. Resuspend pellet in max 1 mL of DPBS, and transfer in 1.5 mL tube.

3. Pellet again for 5′ at 250 × *g*.
4. Remove the supernatant. The pellet can be stored at ≤−70 °C before use.

RNA Extraction and Quantification

In a lab dedicated to nucleic acid extraction:

1. Calculate the required amount of lysis buffer by multiplying the number of samples (plus one for the extra volume) by 600 µL.
2. Add 20 µL DTT 2 mM per each mL of lysis buffer required.
3. Resuspend each sample in 600 µL of lysis buffer, and vortex for 10 s to mix thoroughly.
4. Add 360 µL of ethanol (96–100%), and mix the sample by pipetting.
5. Transfer up to 700 µL of lysate to the purification column inserted in a collection tube. Centrifuge the column for 1 min at ≥12,000 × *g*.
6. Discard the flow-through, and place the purification column back into the collection tube.
7. Repeat this step until all of the lysate has been transferred into the column and centrifuged.
8. Discard the collection tube containing the flow-through solution and place the purification column into a new 2 mL collection tube.
9. Add 700 µL of Wash Buffer 1 to the purification column, and centrifuge for 1 min at ≥12,000 × *g*. Discard the flow-through, and place the column back into the collection tube.
10. Add 600 µL of Wash Buffer 2 to the purification column, and centrifuge for 1 min at ≥12,000 × *g*. Discard the flow-through, and place the column back into the collection tube.
11. Add 250 µL of Wash Buffer 2 to the purification column, and centrifuge for 2 min at ≥12,000 × *g*.
12. Discard the collection tube containing the flow-through solution, and transfer the purification column to a sterile 1.5 mL RNase-free microcentrifuge tube.
13. Add 50 µL of nuclease-free water to the center of the purification column membrane. Centrifuge for 1 min at ≥12,000 × *g* to elute RNA.
14. Add another 50 µL of nuclease-free water, and centrifuge for 1 min at ≥12,000 × *g*.
15. Store the RNA at −80 °C, or proceed with the following quantification steps.
16. Prepare the High Positive Control (HPC) at 20 µg/mL, by diluting the Human Total RNA 1:50 in DEPC-water.

17. Prepare the Low Positive Control (LPC) at 5 μg/mL, by diluting the HPC 1:4 in DEPC-water.
18. Prepare the test RNA by diluting the sample 1:30 in DEPC-water.
19. Aliquot 50 μL of HPC, LPC, samples and blanks in triplicate in a UV-transparent, 96-well plate and measure OD260 and OD280 in a spectrophotometer like the Infinitive 200 PRO (Tecan, https://www.tecan.com).
20. Subtract the blank OD260 value from all the OD260 single measures to obtain the corrected OD for each well.
21. Multiply the corrected OD260 by 30 (the dilution factor) and 40 (the RNA quantity with 1 absorbance OD) to obtain the concentration in μg/mL.

    For example, if your corrected absorbance reading is 0.08, you would multiply $0.08 \times 30 \times 40 = 128\ \mu g/mL$
22. Calculate for each sample the mean quantity. A concentration of at least 160 μg/mL is needed to go on with the following steps.

RNA Retrotranscription

In a lab dedicated to mix assembly:

1. Mix 1 μL of oligo dT and 1 μL of Random Primers per each of the samples. The total number of PCR samples is calculated as:

   Number of RNAs to test × 2 + 2 negative controls + 1 extra volume
2. Bring the Mix tube (MIX1) in the lab dedicated to nucleic acid extraction.

In a lab dedicated to nucleic acid extraction:

1. Dispense 500 ng (max 3 μL, if less add some water to 3 μL) of each sample in two tubes, one for actual retrotranscription (RTplus) and the other for the no-RT (Retro-transcriptase) control (RTminus).
2. Dispense 3 μL of water in two tubes: one will be the negative control + RT and the other the negative control without RT.
3. Aliquot in all the tubes 2 μL of Mix1.
4. Heat in a 70 °C heat block for 5 min. Immediately chill in ice water for at least 5′.
5. Spin 10 s in a microcentrifuge. Store on ice.
6. Prepare the RTplus-MIX and the RTminus-MIX adding on ice the reagents listed in the table below. Each reagent volume must be multiplied by the total number of samples, calculated as:Number of RNAs to test + 1 negative control + 1 extra volume

	Volume per sample (μL)	
	RTplus-MIX	**RTminus-MIX**
Reaction buffer	4	4
$MgCl_2$	2	2
Nucleotide	1	1
RNasi inhibitor	0.5	0.5
RT	1	
DEPC-H_2O	6.5	7

7. Aliquot 15 μL of the two mixes to the corresponding tubes prepared at steps 1 and 2.
8. Incubate in a thermal cycler at the following temperatures/times:

	Temperature	Time	Cycles
Step 1	25 °C	5 min	1
Step 2	42 °C	60 min	1
Step 3	70 °C	15 min	1
Step 4	10 °C	Hold	1

9. Reactions can be stopped at this point for analysis of the cDNA or may be frozen for long-term storage.

Gene Amplification

In a lab dedicated to mix assembly:

1. Prepare the GAPDH-Mix (to amplify the RTplus and RTminus samples and controls). The total number of PCR samples is calculated as:

   Number of RT samples (with the 2 negative controls) × 2 + 1 extra volume

	Volume per sample (μL)
Green Master Mix 5×	5
dNTP mix 10 mM	0.5
Primer mix (Fw e Rev) 3.3 μM each	4
Taq	0.125
DEPC-H_2O	13.38

1. Prepare the four gene-specific mixes (to amplify the RTplus samples and controls). Use the same table above for volume reference, but changing the total number of PCR samples to multiply for, as shown here below:

   Number of RTplus samples (with the negative control) + 1 extra volume

   In a lab dedicated to nucleic acid extraction:

1. Add 20 μL of nuclease-free water to each cDNA.
2. Aliquot 2 μL of each cDNA in the following tubes:
   (a) 1 for each of the four test genes
   (b) 1 for the GAPDH control gene
3. Aliquot 23 μL of the PCR mixes in the corresponding tubes.
4. Close the tubes and bring them in a PCR/post-PCR lab.
5. Incubate in a thermal cycler at the following temperatures/times:

	Temperature	Time	Cycles
Step 1	95 °C	2 min	1
Step 2	95 °C 60 °C 72 °C	30 s 30 s 30 s	32 1 1
Step 3	10 °C	Hold	1

6. Store the tubes at 4 °C in a post-PCR lab.

Agarose Gel Analysis

1. Prepare a 2% agarose gel in TAE buffer 1× (e.g., 2 g Agarose in 100 mL TAE buffer).
2. Add SyBR Safe DNA gel stain 10,000× (e.g., 10 μL in 100 mL TAE 1×).
3. Melt the solution on a heated stirrer or in a microwave oven (SyBR Safe DNA gel stain is resistant to microwaving).
4. Insert a comb in a gel electrophoresis tray, add the melted solution, and wait until the polymerization is complete.
5. Insert the tray with the gel in the electrophoresis chamber, cover it completely with 1× TAE buffer and gently remove the comb.
6. Add 2.5 μL of 100 bp DNA ladder to 1 μL of 6× loading dye, and load it in the first lane of the gel.
7. Add 15 μL of each sample to 2.5 μL of 6× loading dye, and load the resulting mix onto the gel.

8. Electrophorese at 80–110 V until tracking dye migrates 70–80% the length of the gel.
9. Take a picture through an Imaging Gel System (*see* **Note 41** as an example), or view it with a UV illuminator.

Acceptance Criteria

The assay is valid if for the GAPDH gene:

- A band of 266 bp is observed in each lane containing the amplification of the RTplus samples.
- No bands are found in the lanes of the RTminus samples, and of the two (RTminus and RTplus) negative controls.

Each sample result is acceptable if:

- For each test gene, no bands are found in the lane of the RTplus negative control.
- A band of the expected length (see below) is observed:

GATA4	228 bp
TBX18	134 bp
TBX5	140 bp
MESP1	278 bp

3.2.6 Cell Count and Viability

CPC are counted by an automated system based on standard trypan blue technique (automated cell counter EVE, NanoEnTek, www.nanoentek.com). This instrument provides the number of total, viable and dead cells, and % of viability.

Procedure

1. Thaw a final product vial (containing frozen MCB or PPCB): put the vial in a bag, place in a 37 °C water bath until thawed, then disinfect the vial surface (*see* **Note 43**).
2. Mix immediately 10 µL of -CPC with 10 µL of trypan blue.
3. Load 10 µL of the sample mixture on EVE™ Cell counting slide.
4. Inserted on the EVE™ automated cell counter.
5. Repeat the procedure two times, and calculate the mean and Δ% of viable cell concentration and % of viability following the formula:

$$\Delta\% = \frac{\text{replicate} - \text{mean}}{\text{mean}} \times 100$$

Acceptance Criteria

The assay is valid if $-10 \leq \Delta\% \leq +10$

3.2.7 Identity Assay (Immunophenotype)

CPC express typical MSC markers such as CD73 (ecto 5′ nucleosidase), CD90 (Thy-1), and CD105 (endoglin) and lack expression for CD45 (pan-leukocyte marker), CD34 (expressed on primitive hematopoietic progenitors and endothelial cells), CD14 or CD11b (prominently expressed on monocytes and macrophages), CD79α or CD19 (markers of B cells, that also express CD20), and HLA-DR surface molecules. The aim of immunophenotype characterization is to establish the identity of CPC by flow cytometry analysis.

The assay is applied to the MCB and PPCB. The assay procedure has been set up in our lab according to indications provided by Miltenyi Biotec, whose monoclonal antibodies are used.

Procedure

1. Thaw a final product vial (containing frozen CPC): *see* Subheading 3.2.1.
2. Determine cell concentration (*see* Subheading 3.2.6).
3. Dispense up to 10^6 cells/tube (we use 0.25×10^6 cells/tube) into two flow cytometry tubes, identified as "ISO" and "MSC"; add staining buffer up to 1 mL total volume in each tube.
4. Centrifuge the tubes at 300 × *g*, 10 min at room temperature.
5. Aspirate supernatant completely, and resuspend each cell pellet in 100 μL of staining buffer.
6. Add 10 μL of MSC Phenotyping Cocktail and 10 μL of anti-HLA-DR-PerCP to the "MSC" tube; add 10 μL of Isotype Control Cocktail to the "ISO" tube.
7. Mix well (vortex 5 s) and incubate 10 min in the dark at 2–8 °C.
8. Add 1–2 mL of flow cytometry running buffer to each tube.
9. Centrifuge the tubes at 300 × *g*, 10 min at room temperature.
10. Completely aspirate the supernatant, and re-suspend each cell pellet in 200 μL flow cytometry running buffer.
11. Acquire samples on a flow cytometer using proper instrument settings and compensation, according to the manufacturer's indications.
12. Perform the analysis according to the following scheme. Example of results is shown in Fig. 3.

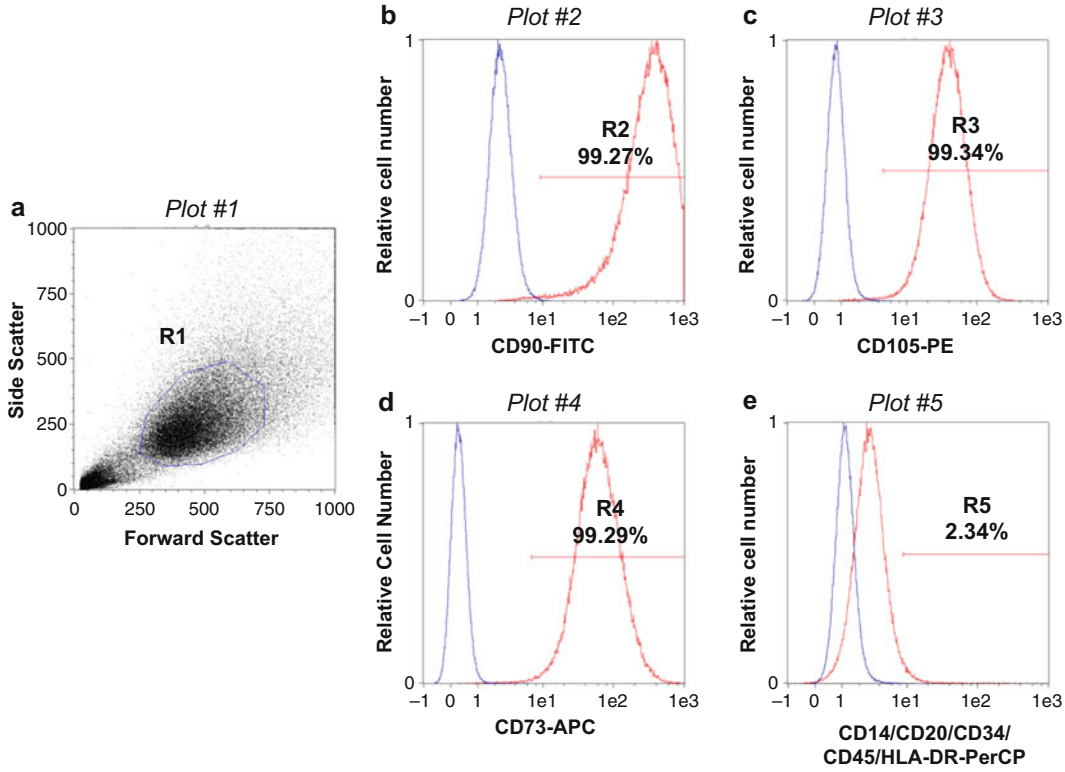

Fig. 3 Immunophenotype analysis, example of results. Events in R1 (**a**) represent cells. Plots (**b**) to (**e**) are overlaying histograms in which data from both "ISO" (blue peak) and "MSC" (red peak) tubes are represented. The percentages of cells positive for the following markers in the "MSC" tube are shown: CD90 (**b**, R2); CD105 (**c**, R3); CD73 (**d**, R4); CD14 or CD20 or CD34 or CD45 or HLA-DR (**e**, R5). R2, R3, R4, and R5 gates have been set to have 0.10% positive cells in the "ISO" tube

Plot #	Type	Parameters	Gated on	Notes
1	Dot plot "ISO"	Forward scatter vs. side scatter	–	Draw a region (R1) to exclude debris
2	Histogram "ISO"/"MSC" overlay	FITC fluorescence vs. relative cell number	R1	Draw a region (R2) between the right margin of the ISO histogram and the right border of the plot, to determine % $CD90^+$ cells

(continued)

Plot #	Type	Parameters	Gated on	Notes
3	Histogram "ISO"/ "MSC" overlay	PE fluorescence vs. relative cell number	R1	Draw a region (R3) between the right margin of the ISO histogram and the right border of the plot, to determine % $CD105^+$ cells
4	Histogram "ISO"/ "MSC" overlay	APC fluorescence vs. relative cell number	R1	Draw a region (R4) between the right margin of the ISO histogram and the right border of the plot, to determine % $CD73^+$ cells
5	Histogram "ISO"/ "MSC" overlay	PerCP fluorescence vs. relative cell number	R1	Draw a region (R5) between the right margin of the ISO histogram and the right border of the plot, to determine % CD45/CD34/ CD14 /CD20/ HLA-DR^+ cells

Acceptance Criteria

The test is considered valid if the cells positive for all the markers in the ISO tube are $\leq 5\%$.

3.2.8 In Vitro Transformation Assay

Transformed cells show reduced requirements for growth. In particular, anchorage-independent growth is one of the hallmarks of transformation, and it is considered the most accurate and stringent in vitro assay for detecting malignant transformation of cells. The soft agar colony formation assay is a common method to monitor anchorage-independent growth. The CytoSelect™ 96-Well Cell Transformation Assay is a kit that allow to perform the soft agar colony formation assay in a more quick and quantitative way since it requires 6–8 days of incubation instead of 3–4 weeks and does not involve manual counting of colonies, but cells growth is detected by a fluorescent dye.

This assay is performed on MCB and PPCB, and it has been set up in our laboratory following manufacturer instructions.

Preparation of Reagents

1. 1.2% Agar Solution: Place 0.24 g of Agar Powder in a sterile bottle, and then add 20 mL of sterile cell culture grade water. Microwave or boil until agar is completely dissolved.
2. 2× DMEM/20% FBS Medium: In a sterile tube, dilute the provided 5× DMEM in sterile cell culture grade water to 2× containing 20% FBS. For example, to prepare a 2.5 mL solution, add 1 mL of 5× DMEM, 0.5 mL of FBS and 1 mL of sterile cell culture grade water. Sterile filter the 2× media to 0.2 μm. (*See* **Note 44**).
3. CyQuant Working Solution: Immediately before use, prepare sufficient amount of the CyQuant Working Solution by diluting the CyQuant GR Dye 1:400 with DPBS. For example, add 10 μL to 4 mL of DPBS. Use the solution immediately; do not store the CyQuant Working Solution.

Preparation of Base Agar Layer

Following procedures must be run under sterile conditions.

1. Melt 1.2% Agar Solution in a microwave and cool to 37 °C in a water bath.
2. Warm 2× DMEM/20% FBS medium to 37 °C in a water bath. Allow at least 30 min for the temperature to equilibrate.
3. Mix equal volumes of 1.2% Agar Solution and 2× DMEM/20% FBS medium in a sterile; pre-warmed tube by inverting several times. Immediately transfer 50 μL of the mixture to each well of a 96-well sterile flat-bottom microplate. Gently tap the plate a few times to allow the agar solution to evenly cover the wells (*see* **Notes 45** and **46**).
4. Transfer the plate to 4 °C for 30 min to allow the base agar layer to solidify.
5. Prior to adding the Cell Agar Layer, allow the plate to warm up for 15 min at 37 °C.

Preparation of Cell Agar Layer

Samples should be assayed in triplicate.

1. Melt 1.2% Agar Solution in a microwave and cool to 37 °C in a water bath.
2. Warm 2× DMEM/20% FBS medium to 37 °C in a water bath. Allow at least 30 min for the temperature to equilibrate.
3. Thaw the vials containing frozen CPC and HeLa (*see* Subheading 3.2.1).
4. Determine cell concentration (*see* Subheading 3.2.6).
5. Harvest and resuspend cells in culture medium at 0.4–4 × 10^5 cells/mL (*see* **Note 47**); keep the cell suspension warm in a 37 °C water bath.

6. Mix equal volumes of 1.2% Agar Solution, 2× DMEM/20% FBS media, and cell suspension (1:1:1) in a sterile; pre-warmed tube by inverting several times. Immediately transfer 75 μL of the mixture to each well of the 96-well flat-bottom microplate already containing the solidified base agar layer (*see* **Note 48**).
7. Transfer the plate to 4 °C for 15 min to allow the cell agar layer to solidify.

Quantitation of Anchorage-Independent Growth

1. Add 100 μL of culture medium to each well.
2. Incubate the cells for 6–8 days at 37 °C and 5% CO_2. Examine the cell colony formation under a light microscope.
3. Remove culture medium by inverting the plate and blotting on paper towel. Gently tap several times.
4. Add 50 μL of Agar Solubilization Solution to each well of the 96-well plate. Incubate for 1 h at 37 °C.
5. Pipette each well 5–10 times to ensure complete agar solubilization.
6. Add 25 μL of 8× lysis buffer to each well. Pipette each well 5–10 times to ensure a homogeneous mixture.
7. Incubate the plate at room temperature for 15 min.
8. Transfer 10 μL of the mixture to a 96-well plate suitable for fluorescence measurement.
9. Add 90 μL of the CyQuant Working Solution to each well. Incubate 10 min at room temperature.
10. Read the plate in a 96-well fluorometer using a 485/520 nm filter set (*see* **Notes 49**, **50**, and **51** and the example of analysis shown in Fig. 4).

3.2.9 In Vitro Senescence Assay

Normal primary cells proliferate in culture for a limited number of population doublings prior to undergoing terminal growth arrest and acquiring a senescent phenotype. Senescent cells are characterized by an irreversible G1 growth arrest and show common biochemical markers such as expression of an acidic senescence-associated β-galactosidase (SA-β-Gal) activity.

Several protocols and commercial kits are available to measure SA-β-Gal activity. We choose to use the FluoReporter lacZ Flow Cytometry Kits that use the fluorogenic β-galactosidase substrate fluorescein di β-D-galactopyranoside (FDG) in conjunction with flow cytometry analysis. Nonfluorescent FDG is sequentially hydrolyzed by β-galactosidase, first to fluorescein mono galactoside (FMG) and then to highly fluorescent fluorescein. Low levels of β-galactosidase activity are readily detectable with FDG and moreover, because this flow cytometric technique is carried out on a cell-by-cell basis, it allows the detection of heterogeneous expression patterns.

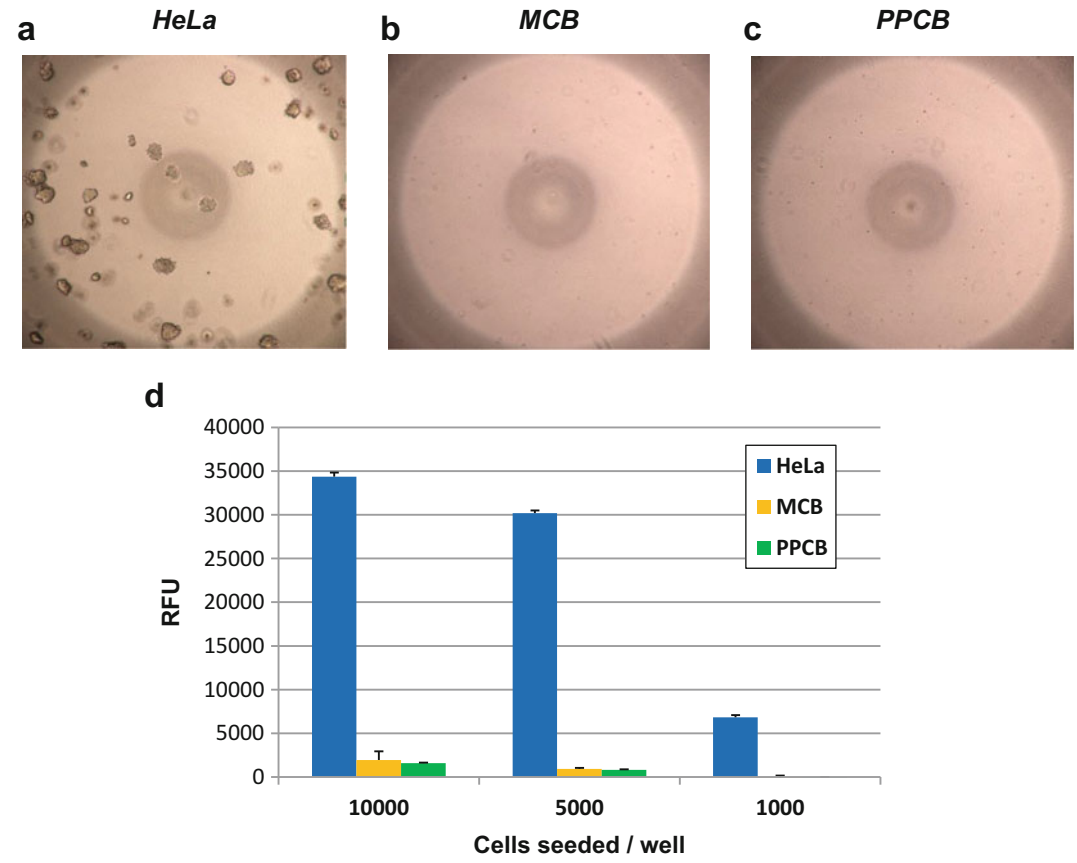

Fig. 4 In vitro transformation assay, example of results. HeLa cells (**a**), MCB (**b**), and PPCB (**c**) were plated 1000, 5000, and 10,000 cells/well, and the assay was performed following manufacturer instructions. Pictures of 1000 cells wells, taken after the 8 days incubation and before the quantitation of anchorage-independent growth, are shown. Fluorescence data of all the conditions (samples fluorescence minus blanks fluorescence) are shown in (**d**). RFU: relative fluorescence unit

This assay is performed on MCB and PPCB, and it has been set up in our laboratory following manufacturer instructions.

Preparing Cell Suspension

1. Thaw a final product vial (containing frozen CPC, *see* Subheading 3.2.1), plate cells, and culture them until they reach a maximum of 80% confluence (*see* **Note 52**).
2. Treat exponentially growing CPC with TrypLE™ Select Enzyme until they can be removed from the plate by gentle agitation. Inactivate TrypLE™ Select Enzyme by washing in staining medium (phosphate-buffered saline, 4% (v/v) fetal calf serum, 10 mM HEPES, pH 7.2) or tissue culture growth medium. Centrifuge the cell suspension and aspirate off the supernatant.

3. Resuspend cells in staining medium to ~10^7 cells/mL (*see* **Note 53**) and pipet 100 μL into an appropriate flow cytometer tubes. For each CPC sample, we set up two tubes (stained or not with FDG). Place cells on ice.

Loading Cells with FDG by Hypotonic Shock

1. Prepare a 2 mM working solution: Thaw the FDG reagent (Component A), warm it briefly at 37 °C, and vortex or shake to dissolve any crystals (*see* **Note 54**). Prepare the 2 mM FDG working solution by diluting the FDG reagent tenfold in deionized water. Prewarm the FDG working solution to 37 °C for 10 min prior to use.
2. Prepare staining medium with 1.5 μM (1 μg/mL) propidium iodide by diluting the propidium iodide reagent 100-fold in staining medium. Chill on ice.
3. Prewarm the flow cytometer tube containing 100 μL of cells in a 37 °C water bath for 10 min.
4. Start FDG loading by adding 100 μL of pre-warmed (37 °C) 2 mM FDG working solution only on stained tubes ("FDG" tubes). For the unstained controls ("US" tubes), add 100 μL of pre-warmed (37 °C) water. Mix rapidly and thoroughly. Return to the 37 °C water bath for exactly 1 min.
5. Stop the FDG loading at the end of 1 min by adding 1.8 mL ice-cold staining medium containing 1.5 μM propidium iodide (*see* **Note 55**). Treat also the unstained samples. Use ice-cold pipettes to aliquot the staining medium into the cells. Keep the cells on ice prior to flow cytometry analysis.

Flow Cytometer Set-Up and Calibration

1. Set up and calibrate the flow cytometer to detect fluorescein, propidium iodide, and forward scatter, according to standard procedures.
2. Using unstained cells, set the background autofluorescence compensation.
3. Perform the analysis according to the following scheme. Example of results is shown in Fig. 5.

Plot #	Type	Parameters	Gated on	Notes
1	Dot plot "US"	Forward scatter vs. side scatter	–	Draw a region (R1) to exclude debris
2	Dot plot "US"	PerCP fluorescence vs. side scatter	R1	Draw a region (R2) to exclude dead cells (propidium iodide positive)

(continued)

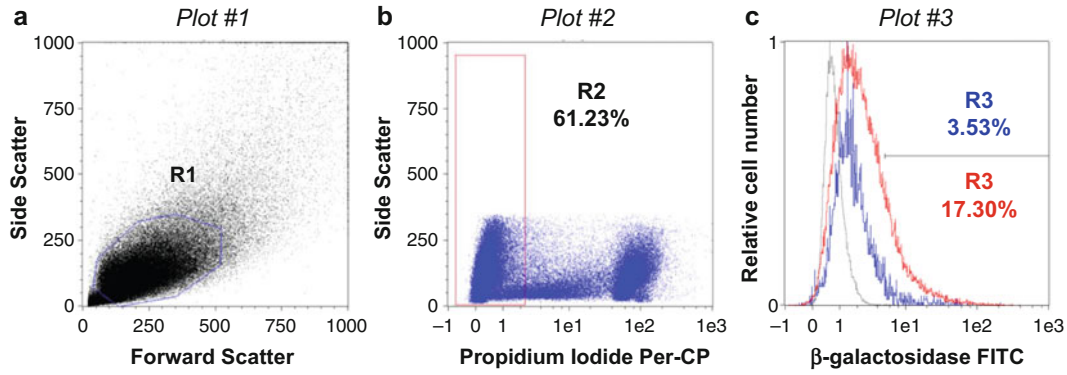

Fig. 5 In vitro senescence assay, example of a setup experiment. We measured β-galactosidase expression by two different CPC lines, one that was growing well in culture (blue) and one that was not growing (red). Events in Plot #1 R1 (**a**) represent cells. Events in Plot #2 R2 (**b**) represent viable cells. Plot #3 (**c**) is overlaying histogram in which data from unstained (grey peak), growing CPC cell line (blue peak) and not growing CPC cell line (red peak) are represented. The percentages of cells positive for β-galactosidase (R3) are shown for the two cell lines. Gate R3 is set to have 0.10% positive events in the unstained sample

Plot #	Type	Parameters	Gated on	Notes
3	Histogram "US"/"FDG" overlay	FITC fluorescence vs. relative cell number	R2	Draw a region (R3) between the right margin of the ISO histogram and the right border of the plot, to determine β-galactosidase positive cells

4 Notes

1. Atrial appendage explants are obtained from patients subjected to valve repair surgery who are not carriers of concomitant coronary artery disease. Human tissue collection is carried out in accordance with the Helsinki Declaration and specific authorization by the local Ethics Committee.
2. This medium was selected among several commercially available serum-free xeno-free alternatives, on the basis of its superior performance on CPC growth.
3. Flasks should be coated with CELLstart™ CTS™, Synthemax® II-SC or other adhesion substrates, provided that they are of suitable quality and properly certified. For the coating with CELLstart™ CTS™, flasks are incubated with the reagent diluted 1:50 in DPBS for 2 h at 37 °C. Before seeding the tissue

specimens or the cells, the coating solution is removed by aspiration.

4. Alternative enzymatic solutions may be used, provided that they are of suitable quality, non-animal derived, and properly certified. The procedure for the use of TrypLE™ Select Enzyme follows the manufacturer instructions.
5. Cryostor® CS10 is a serum-free, animal component-free, and defined cryopreservation medium containing 10% dimethyl sulfoxide (DMSO) as cryoprotective agent; it was selected on the basis of preliminary experiments proving high viability and recovery of frozen CPC. Other freezing media may be used, provided that they are of suitable quality, non-animal derived and properly certified.
6. These media were chosen because of the presence of an antibiotic-inhibitor in their composition. The medium fertility is therefore guaranteed even for test samples potentially containing antibiotics, such as patient-derived tissues.
7. Preparation: 8.9 mL DPBS +1 mL 5% HSA + 0.1 mL 200 mM EDTA. Storage: at 2–8 °C, max 1 month.
8. Depending on the instrument, different buffers can be used; we use MACSQuant Running Buffer for the MACSQuant Analyzer (Miltenyi Biotec, www.miltenyibiotec.com).
9. The soft agar colony formation assay is a technique widely used to evaluate cellular transformation in vitro and different protocols and kit can be used to perform this assay. The mentioned kit is only an example that we used in our lab for this purpose.
10. HeLa cells are used as positive control, i.e., cells able to grow and divide without binding to a substrate (anchorage-independent growth). Other tumor cell lines can be used as positive controls.
11. The time from tissue fragments seeding to P0 CPC harvest is variable among different donors (range 6–34 days, $n = 7$).
12. The number of CPC obtained from cardiac fragments culture is variable among different donors. Starting from about 8–10 culture flasks (115 cm^2) seeded with cardiac fragments for each lot ($n = 6$), the average cell yield at P0 was 5.12×10^6 cells (range 1.26×10^6 to 9.12×10^6).
13. Thanks to their negatively charged, highly hydrophilic CellBIND® surface (Corning), designed to facilitate cell attachment and spreading, HYPERFlasks® and HYPERStacks® do not require any coating.
14. The number of CPC harvested for the MCB generation is variable among different donors ($n = 6$); the average cell yield at P2 was 1.3×10^9 cells (range 4.8×10^8–2.3×10^9). An example of growth curve is shown in Fig. 2f.

15. For the PPCB, which is generated for QC purposes only, it should be enough to maintain in culture few flasks in view to have at least 50×10^6 cells at P6 (to freeze at least 10 vials, 5×10^6 cells/vial).
16. The number of vials is determined on the basis of the number of CPC available for freezing, considering 5×10^6 cells/vial. The MCB should consist of at least 120 vials (100 to be used as ATMP or as cell source for EV production, 20 for QC and as retention samples). The PPCB should consist of at least 10 vials for QC.
17. The freezing medium is kept at 4 °C on ice or using dedicated programmable devices such as ThermoStat™ C (Eppendorf, www.eppendorf.com). The same system should be used to maintain the cell suspension at 4 °C throughout the filling in vials.
18. Controlled-rate freezing is recommended to ensure maximum cell viability for a wide variety of cells, being aware that the profile should be optimized for each specific application, as different cell types and freezing media may have different requirements. The optimal freezing profile for CPC in Cryostor® CS10, 1 mL/vial, 5×10^6 cells/mL is summarized in the following table (the program starts after the freezing chamber reaches 4 °C).

Freezing step	Temperature	Time
1	0 °C	4 min
2	0 °C	Hold 10 min
3	−10 °C	10 min
4	−40 °C	1.5 min
5	−35 °C	1 min
6	−15 °C	3 min
7	−40 °C	30 min
8	−90 °C	30 min

19. The transport liquid (1–10 mL) is used instead of a cell suspension to test the sterility of the cardiac tissue.
20. Alternatively, other commercially available systems may be used, such as Bactec (Becton Dickinson, www.bd.com), according to the manufacturer's recommendations and upon proper validation.
21. The minimum amount to be tested depends on the total volume of the final cell product (as in EP 2.6.27):

Total product volume (V)	Inoculum volume/ bottle (FA or FN medium)	Total volume to be tested (FA + FN medium)
$V \geq 10$ mL	1% of total volume	2% of total volume
1 mL $\leq V < 10$ mL	100 µL	200 µL
$V < 1$ mL	Not applicable	Not applicable

For a product such as frozen CPC, consisting of multiple aliquots (frozen vials) the minimum volume to be tested determines the minimum number of aliquots to be tested.
Aliquot(s) should be representative of the whole batch: they must be homogenous (the cell suspension should be well resuspended during the pre-freezing phase) and, in case of several aliquots dedicated to microbiological assays, they have to be sampled among the first, middle, and final prepared aliquots.
The maximum product volume to be inoculated in every bottle must be determined during the method's validation.

22. Alternatively, the volume to be tested in both bottles may be drawn in a single syringe. In this case, completely remove the air, first transfer the inoculum to the FN bottle (half of the volume) and then to the FA bottle (all the remaining volume and air). This reduces air influx in the anaerobic bottle.
23. Forms or labels (in the latter case they can be stuck directly on the lab record book) must be sterile. Autoclaved paper or labels must be used and brought into the clean room in triple sterilized bags.
24. The incubation period at 36 ± 1 °C is usually of 7 days. This time can be changed during the method's validation for each specific product. At the end of the incubation period print the full record history from the instrument.
25. Use TSA or CBA plates for bacteria, incubating them under aerobic (from FA bottles) or anaerobic (from FN bottles) conditions. Use SDA plates for yeast and molds.

    Plate around 1 mL of the positive sample to obtain single colonies after 3 days at 32.5 °C.
26. A possible identification technique is based on the use of the VITEK® 2 system (bioMérieux). Briefly, a pure colony must be sub-cultured on the appropriate plate and condition (see note above), a Gram staining with cell morphology identification is performed, and, according to the result, the sample is loaded on an appropriate VITEK card for the microorganism classification.

27. Allow the cartridge to reach room temperature in the bag before use. During cartridge manipulation pay attention not to touch the reservoirs where the samples will be loaded: the cartridge has a rounded edge purposely designed to be touched.
28. The calibration code and the lot number required by the instrument are found in the cartridge's certificate of analysis.
29. Method setup and validation must be performed to define the optimal dilution of the final product. Different sample's dilutions can be tested with the inhibition/enhancement cartridge (Charles River).
30. Keep the pipette tilted and do not touch the well bottom with the pipette tip. Pipette slowly in order to avoid bubbles formation and squirts: they can cause non homogenous samples, interfere with light transmission and in the end produce false results.
31. Two out of 4 wells contain endotoxin spikes (0.5 EU/mL): they serve as positive control to assess for the presence of interfering substances in the sample.
32. The spike recovery (%) is calculated as: spike value/archived spike concentration × 100. The archived spike concentration is specific for each cartridge lot, and it is defined in the certificate of analysis.
33. Wash cells with 5 mL of DPBS, remove DPBS, add 1 mL TrypLE™ Select Enzyme, incubate for 5–7 min at 37 °C, add 1 mL of complete culture medium, and proceed with the cell counting.
34. TrypLE™ Select Enzyme may disrupt mycoplasma: do not treat the cell sample to be tested for mycoplasma with such agents.
35. This is the sample representative of cell-linked mycoplasma.
36. Example for 4 samples to be tested: 4 × 2 = 8 + 1 Positive Control +1 Internal Control +2 Negative Controls +1 excess reaction = 13.
37. Example for 13 reactions: Master Mix (20 μL × 13 = 260 μL); Primers (2.5 μL × 13 = 32.5 μL); Total 292.5 μL/13 tubes = 22.5 μL/tube.
38. Negative controls are in duplicate, such as the test samples: Negative Control A to test possible mix contamination, Negative Control B (Sentinel sample) to test operational contamination.
39. Use a calibrated and certified genomic DNA of one Mycoplasma strain among those suggested by EP [20].

40. The "Touch down" PCR is performed to reduce non-specific primer annealing and therefore increase specificity and sensitivity.
41. Using the Gel Doc EZ Imager/BioRad: place the gel on the purple tray (UV tray) and insert the tray into the instrument. Analyze with the Image Lab Software. Protocol setup: Application = Sybr safe; Exposure = Automatic/Faint Bands; Band Detection Sensitivity: High (Better for faint bands)
42. Some samples could not meet this criterion; the results from such samples are to be considered not valid, and the samples retested. If the problem persists, a dilution of the sample should be considered, even though such dilution would affect the assay sensitivity.
43. If the assay is applied during CPC culture, skip **step 1**, using the fresh cell suspension directly for **step 2**.
44. The provided DMEM might be substituted with a different medium, but with a 2× concentration.
45. Work quickly with the agar solution to avoid gelation. Also, try to avoid adding air bubbles to the well.
46. To avoid fast and uneven evaporation that leads to aberrant results, we suggest not using the wells on the plate edge, or filling the edge wells with medium to reduce evaporation.
47. In setup phase, seed cells at different concentrations (e.g., 4×10^5, 2×10^5 and 0.4×10^5 to have 1000, 5000 and 10,000 cells/well). This test, run on HeLa cells, was giving good results in term of signal with all the three concentrations.
48. Work quickly with the agar solution to avoid gelation, but gently pipette as not to disrupt the base layer integrity. Also, try to avoid adding air bubbles to the well. Always include negative control wells that contain no cells in the cell agar layer.
49. We use an Infinite F200 PRO plate reader (TECAN, www.tecan.com).
50. In the analysis, subtract blank wells value from sample values.
51. It is possible to make a cell dose curve to have a more quantitative calculation of anchorage-independent growth. See manufacturer instructions for details.
52. Keep cells as healthy as possible. Certain adherent cell types, such as 293 or NIH 3T3 cells, will have higher endogenous β-galactosidase activity if they are abused or allowed to grow to confluency.
53. The staining results are not critically dependent on cell concentration. Staining patterns are essentially the same using cell concentrations ranging from 10^5 to 5×10^7 cells/mL.

54. Do not keep the FDG solutions at elevated temperatures for extended periods, as spontaneous hydrolysis will occur.
55. This staining procedure relies on osmotic shock of the cells. For 1 min at 37 °C, FDG enters the cells by passive osmotic loading. The uptake is then stopped by rapid dilution into cold isotonic staining medium, thereby "freezing" the membrane and locking the substrate and product inside the cells. The nucleic acid counter stain propidium iodide can be used to selectively stain cells with compromised membranes.

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Suspension Culture of Human Induced Pluripotent Stem Cells in Single-Use Vertical-Wheel™ Bioreactors Using Aggregate and Microcarrier Culture Systems

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Abstract

Human induced pluripotent stem cells (hiPSCs) have the potential to be used in a variety of biomedical applications, including drug discovery and Regenerative Medicine. The success of these approaches is, however, limited by the difficulty of generating the large quantities of cells required in a reproducible and controlled system. Bioreactors, widely used for industrial manufacture of biological products, constitute a viable strategy for large-scale production of stem cell derivatives. In this chapter, we describe the expansion of hiPSCs using the Vertical-Wheel™ bioreactor, a novel bioreactor configuration specifically designed for the culture of shear-sensitive cells. We provide protocols for the expansion of hiPSCs in suspension, both as floating aggregates and using microcarriers for cell adhesion. These methods may be important for the establishment of a scalable culture of hiPSCs, allowing the manufacturing of industrial- or clinical-scale cell numbers.

Keywords Aggregates, Expansion, Human induced pluripotent stem cells, Microcarriers, Vertical-Wheel™ bioreactors

1 Introduction

Human induced pluripotent stem cells (hiPSCs) greatly impacted the Regenerative Medicine landscape, due to their capacity to generate all the cell types of the human body, associated with an unlimited self-renewal capacity in vitro [1]. These cells can be derived from patient-specific adult cells and are thus promising for applications such as disease modelling and drug testing [2], foreseeing a personalized medicine approach. Moreover, there are also ongoing clinical trials using hiPSCs for cell replacement therapies [3]. However, these applications require high cell numbers (e.g., ~10^9 cells per patient for cell transplantation [4]), and therefore, scalable methods for cell expansion are necessary. Planar culture platforms (tissue culture plates, T-flasks) are commonly used for routine hiPSC culture, yet they are not efficient for large-scale cell production [5]. Bioreactors have been explored as more robust

culture platforms, compatible with easier scale-up of the processes, while also allowing a more controlled and physiological culture environment. hiPSC culture in suspension can be performed as floating cell aggregates or using microcarriers to provide a surface for cell adhesion [6]. Microcarrier cultures provide an easier translation of adherent culture to suspension bioreactors, although most applications require complex separation processes for the removal of the microcarrier particles in the end of the culture and should ensure that the final products are free from microcarrier-derived particulates. While expansion as cell aggregates reduces the burden of downstream processing, the aggregates are more susceptible to diffusional limitations than microcarrier cultures. As such, both aggregate [6–8] and microcarrier cultures [9–11] are relevant for large-scale hiPSC expansion and have already been described in stirred tank bioreactors. These bioreactors have been widely used for the culture of microorganisms, mammalian cell lines, and, more recently, also stem cells. However, stem cell manufacturing has specific requirements that are different from other bioprocesses, as in the former the cells are the final product. In particular, the liquid mixing mechanism of stirred tank bioreactors does not result in a fully homogeneous shear stress profile throughout the bioreactor, with high shear "hot spots" close to the impeller [12]. For this reason, these bioreactors often require high stirring speeds to efficiently suspend cell aggregates or microcarriers, which may be detrimental for cell growth and viability. As such, it is important to deviate from traditional stirred tank bioreactors and to create novel systems focused on the quality of the final stem cell product.

Vertical-Wheel™ bioreactors (VWBRs) are a novel configuration, designed specifically for shear-sensitive mammalian cell culture bioprocessing. These bioreactors employ an innovative mixing technology, using a large vertical impeller which combines radial and axial agitation [13]. This mechanism, along with the U-shaped bottom of the vessel, leads to a more efficient and homogeneous mixing, allowing for suspension of microcarriers or cell aggregates with a reduced power input. Also, VWBRs are available in many different volume scales (from 0.1 to 80 L) while maintaining the same configuration of vessels and impellers, which allows the true scalability of the cell culture process. These advantages over conventional bioreactor setups have led to successful expansion of hiPSCs in VWBRs on microcarriers [14] and as floating aggregates [15]. Moreover, since VWBRs are single-use vessels, the transition to current Good Manufacturing Practices (cGMP) processes is facilitated, envisaging the clinical applications of derivatives of hiPSCs grown in the bioreactors.

This chapter describes protocols for the expansion of hiPSCs in VWBRs, using both microcarrier and aggregate culture systems. Although these processes are performed in a small scale, they can be

potentially scaled up to larger VWBR systems, for production of hiPSCs and derivatives to be used, prospectively, for pharmacological or Regenerative Medicine applications.

2 Materials

2.1 Laboratory Equipment

1. Laminar flow hood.
2. Cell culture CO_2 incubator.
3. Optical microscope with camera.
4. Centrifuge.
5. Water bath.
6. Micropipettes (0.5–10, 2–20, 20–200, 100–1,000 μL) and pipette tips.
7. Automatic pipettor and serological pipettes.
8. 6- and 24-well tissue culture plates (Corning).
9. 15 and 50 mL conical tubes (Corning).
10. 1.5 mL microcentrifuge tubes (Eppendorf).
11. Hemocytometer (Marienfeld).
12. PBS MINI 0.1 bioreactor vessels (PBS Biotech).
13. PBS MINI bioreactor base unit (PBS Biotech).

2.2 Culture Media and Solutions

1. Matrigel (Corning). Thaw the Matrigel bottle on ice overnight. Aliquot Matrigel using cold pipette tips and store the aliquots at −20 °C until the expiration date.
2. DMEM/F12 medium (Thermo Fisher Scientific): Dissolve 12 g of DMEM/F12 powder and 2.44 g of sodium bicarbonate in 1 L of water for injection (WFI). Add 10 mL of 10,000 units/mL penicillin and 10,000 μg/mL streptomycin (Pen Strep, Thermo Fisher Scientific). Filter using a sterile 0.22 μm filter and keep at 4 °C.
3. ROCK inhibitor Y-27632 (STEMCELL Technologies): Prepare a stock solution of 10 mM by diluting 5 mg of Y-27632 in 1560 μL of DMSO (Sigma-Aldrich). Use a working concentration of 10 μM.
4. Accutase (Sigma-Aldrich): Thaw the bottle at 4 °C overnight, divide in 40 mL aliquots and store at −20 °C until the expiration date. After thawed, individual aliquots can be stored up to 2 months at 4 °C.
5. 0.5 mM EDTA solution: Dissolve 1.8 g of sodium chloride in 1 L of DPBS. Add 1 mL of 0.5 M EDTA solution (Thermo Fisher Scientific). Filter using a sterile 0.22 μm filter and keep at 4 °C.

6. 0.4% Trypan blue stain solution (Thermo Fisher Scientific).
7. Ethanol 70% (V/V).
8. Dulbecco's phosphate buffered saline (DPBS) solution without calcium and magnesium (Thermo Fisher Scientific).

2.3 Dynamic Expansion of hiPSCs as Aggregates in PBS VWBRs

1. mTeSR1 medium (STEMCELL Technologies): Aseptically mix 400 mL of basal medium with 100 mL of 5× supplement and 2.5 mL of Pen Strep. mTeSR1 medium can be aliquoted and stored at −20 °C until the expiration date. After thawed, individual aliquots can be stored at 4 °C for up to 2 weeks.
2. (Optional) Dextran sulfate sodium salt, Mr. ~40,000 (Sigma-Aldrich): Prepare a stock solution of 100 mg/mL by diluting 1 g of dextran sulfate in 10 mL of WFI. Filter using a sterile 0.22 μm filter and keep at 4 °C.

2.4 Dynamic Expansion of hiPSCs on Microcarriers in PBS VWBRs

1. SoloHill Plastic microcarriers (Pall Corp.).
2. E8 medium (Thermo Fisher Scientific): Aseptically mix 490 mL of basal medium with 10 mL of 50× supplement and 2.5 mL of Pen Strep. E8 medium can be aliquoted and stored at −20 °C until the expiration date. After thawed, individual aliquots can be stored at 4 °C for up to 2 weeks.
3. Recombinant human vitronectin (VTN-N, Thermo Fisher).
4. Ultra-low attachment (ULA) 6-well culture plates (Corning).
5. Steriflip filter units, 100 μm pore size (Merck Millipore).
6. Rocking platform shaker.

3 Methods

3.1 Static Culture

Perform all steps under aseptic conditions. For aggregate culture, grow the cells in mTeSR1; use E8 for microcarrier culture.

1. Thaw a Matrigel aliquot on ice.
2. Prepare a 1:100 solution of Matrigel in DMEM/F12 and add 1 mL to each well of a 6-well tissue culture plate. Incubate the plate at room temperature for 2 h. If necessary, Matrigel-coated plates can be stored at 4 °C for up to 2 weeks.
3. Pre-warm 6.5 mL of culture medium.
4. Place a cryovial of cells in a water bath at 37 °C. When the content of the cryovial starts to thaw, transfer it to the laminar flow hood.
5. Collect 5 mL of culture medium with a serological pipette and add it dropwise to the cryovial. When the vial is full, aspirate its content and pipette dropwise again until the cell solution is fully thawed. Collect the cell solution in a 15 mL conical tube.

6. Centrifuge the 15 mL conical tube at 210 × *g* for 3 min. Meanwhile, remove the Matrigel solution from one well of a pre-coated culture plate and add 500 μL of pre-warmed culture medium.
7. Remove the supernatant from the 15 mL tube and add 1 mL of culture medium with a 1,000 μL pipette. Pipette up and down once or twice, until the cells are in suspension (*see* **Note 1**).
8. Pipette the cells slowly to the Matrigel-coated well. Shake the plate in a crosswise motion to distribute the cell clumps through the well (*see* **Note 2**).
9. Keep the cells in a humidified incubator at 37 °C and 5% CO_2. Replace the medium by 1.5 mL of pre-warmed culture medium every 24 h.
10. Once the cells achieve confluence (about every 2–4 days), passage them at a split ratio between 1:3 and 1:4 to new Matrigel-coated 6-well tissue culture plates. For passaging, pre-coat the necessary wells with Matrigel and pre-warm the necessary amount of culture medium (1.5 mL of medium will be required per well seeded). Wash the cells twice with 1 mL of 0.5 mM EDTA solution. After the second wash, add 1 mL of 0.5 mM EDTA solution and incubate for 5 min at room temperature. Meanwhile, remove the Matrigel solution from the pre-coated culture plates and add 500 μL of pre-warmed culture medium. Fully aspirate the EDTA solution and flush the cells with 1 mL of culture medium. Use a 1,000 μL pipette and flush each well no more than 3–4 times. Collect the cells in a conical tube, flush the remaining cells in the well with 1 mL of culture medium, and transfer to the conical tube. Add the appropriate volume of culture medium to the conical tube with the cells and distribute 1 mL/well of cell suspension to each Matrigel-coated well. Shake the plate in a crosswise motion to distribute the cell clumps through the well (*see* **Note 2**). Keep the cells in a humidified incubator at 37 °C and 5% CO_2. Replace the medium by 1.5 mL of pre-warmed culture medium every 24 h.

3.2 Dynamic Expansion of hiPSCs as Aggregates in PBS VWBRs

3.2.1 Cell Seeding and Bioreactor Culture

1. Start the protocol with hiPSCs cultured on Matrigel-coated plates at 80% confluence.
2. Add ROCK inhibitor stock solution (10 mM) to the culture medium at a dilution of 1:1,000, to a final concentration of 10 μM. Incubate the cells at 37 °C and 5% CO_2 for 1 h.
3. Aspirate the exhausted medium and wash the cells with 1 mL/well of DPBS.
4. Add 1 mL/well of Accutase and incubate at 37 °C for 7 min. (*See* **Note 3**).

5. Flush the cells with the Accutase solution and transfer them to a 50 mL conical tube. Flush the remaining cells in the well again with 1 mL/well of mTeSR1 medium, transferring them to the same conical tube.
6. Centrifuge the 50 mL conical tube at 210 × *g* for 3 min.
7. Discard the supernatant and resuspend the pellet in an appropriate volume of mTeSR1 medium (e.g., 3–5 mL) with 10 μM of ROCK inhibitor.
8. Perform a cell count, using a hemocytometer and the trypan blue dye exclusion test, under an optical microscope. Calculate the cell suspension volume necessary to inoculate the bioreactor. A seeding density of 250,000 cells/mL and a minimum working volume of 60 mL is recommended. Under these conditions, 1.5×10^7 cells will be necessary per bioreactor.
9. Transfer the cell suspension to a PBS MINI 0.1 vessel (*see* **Note 4**). Add mTeSR1 supplemented with 10 μM of ROCK inhibitor to the bioreactor until the final volume of 60 mL is reached.
10. Close the PBS MINI 0.1 vessel and place it on a PBS MINI base unit inside the incubator at 37 °C and 5% CO_2. Set the agitation to 30 rpm.
11. After 48 h, stop the agitation and let the aggregates settle. Remove 80% of exhausted medium (48 mL, if the working volume is 60 mL), without harvesting aggregates, and replace with the same volume of pre-warmed mTeSR1 medium without ROCK inhibitor.
12. Replace 80% of exhausted medium by pre-warmed mTeSR1 medium every day.

3.2.2 Sampling and Cell Counts

1. During the culture, take two daily samples of 700 μL from the PBS MINI 0.1 vessel in order to verify the quality and number of cells. Keep the bioreactor under dynamic conditions during sampling, to ensure homogeneity. Collect the samples in a 24-well plate.
2. Inspect the samples visually under an optical microscope, and collect photos of at least 100 aggregates for later measurement (*see* **Note 5**).
3. Transfer each aggregate sample to a separate 15 mL conical tube. Collect the maximum number of aggregates possible.
4. Centrifuge the 15 mL conical tubes at 210 × *g* for 3 min.
5. Remove the culture medium supernatant. Keep the supernatant in a 1.5 mL microcentrifuge tube for glucose/lactate analysis (*see* **Note 6**).
6. Add 700 μL of Accutase to each 15 mL tube and incubate at 37 °C for 7 min. Pipette up and down 5–10 times to help with the dissociation into single cells. Add additional 700 μL of culture medium into the dissociated cells.

7. Centrifuge at 210 × *g* for 3 min. Remove the supernatant and resuspend in medium (e.g., 500 μL, *see* **Note 7**).
8. Perform a cell count using a hemocytometer and the trypan blue dye exclusion test, under an optical microscope.

3.2.3 Cell Harvest at the End of Culture

1. If the cells will be replated after bioreactor culture, add an appropriate amount of ROCK inhibitor stock solution (10 mM) to the medium, to achieve a final concentration of 10 μM, and incubate at 37 °C and 5% CO_2 for 1 h before starting the harvest procedure.
2. Collect the bioreactor contents in 50 mL conical tubes. Wait for the aggregates to settle down and remove the supernatant.
3. Wash with 10 mL of sterile DPBS once. Wait for the aggregates to settle down and remove the DPBS.
4. Add 10 mL Accutase and incubate for 7–12 min at 37 °C. Mix occasionally to help cell dissociation.
5. Pipette up and down 5–10 times to help with the dissociation into single cells. Add 10 mL of culture medium into the dissociated cells.
6. Centrifuge at 210 × *g* for 3 min. Remove the supernatant and resuspend in culture medium. If the cells will be replated, add ROCK inhibitor stock solution (10 mM) to the medium, to achieve a final concentration of 10 μM.
7. Perform pluripotency assays in order to verify the quality of the cells (e.g., flow cytometry, immunocytochemistry, and qRT-PCR to assess pluripotency marker expression; embryoid body assay and directed differentiation toward different cell lineages to assess trilineage differentiation potential; karyotyping to assess genomic stability).

3.3 Dynamic Expansion of hiPSCs on Microcarriers in PBS VWBRs

3.3.1 Microcarrier Preparation

1. Weigh Plastic microcarriers in a 50 mL conical tube in order to provide a concentration of 20 g microcarriers/L of culture medium. For a working volume of 80 mL, 1.6 g of microcarriers will be required per bioreactor.
2. Sterilize the microcarriers using ethanol 70%. As a reference, use 40 mL of ethanol for 1.6 g of microcarriers. Incubate for 1 h at room temperature, using a rocking platform shaker.
3. After sterilization, let the microcarriers settle down and remove the ethanol solution. Wash 3 times with 10 mL of sterile DPBS.
4. Prepare the microcarrier coating solution using recombinant vitronectin. A coating concentration of 0.5 μg VTN-N/cm^2 of microcarriers is recommended. For the previously mentioned recommended conditions, and for Plastic microcarriers with 360 cm^2 of surface area per gram, add 576 μL of VTN-N to 32 mL of PBS to prepare the coating solution. Then add the

32 mL of coating solution to the pre-sterilized microcarriers. Incubate for 2 h at room temperature on a rocking platform shaker.

3.3.2 Cell Seeding and Bioreactor Culture

1. Remove the exhausted culture medium from a culture plate with 80% confluent hiPSCs.
2. Wash the cells twice with 1 mL of 0.5 mM EDTA solution per well. After the second wash, add 1.5 mL of 0.5 mM EDTA solution and incubate for 5 min at room temperature.
3. Fully aspirate the EDTA solution and flush the cells with E8 medium supplemented with 10 μM ROCK inhibitor. Use a 1,000 μL pipette and flush each well no more than 3–4 times. Collect the cell suspension in a 50 mL conical tube. Repeat for all the wells.
4. Perform a cell count, using a hemocytometer and the trypan blue dye exclusion test, under an optical microscope (*see* **Note 8**).
5. Calculate the cell suspension volume necessary to seed the microcarriers with 2.5×10^4 cells/cm^2 of microcarrier area. In the recommended conditions, 1.44×10^7 cells will be necessary per bioreactor (*see* **Note 9**).
6. Remove the coating solution from the microcarriers prepared in Subheading 3.3.1. Add the appropriate volume of cell suspension calculated in **step 5** to the tube with the microcarriers.
7. Transfer the suspension to an ultra-low attachment (ULA) 6-well plate (split the suspension into different wells, according to the total volume; *see* **Note 10**). Incubate for 1 h at 37 °C/5% CO_2 to allow the cells to adhere, mixing the plate (manually) every 15 min.
8. Transfer the cell-microcarrier suspension to a PBS MINI 0.1 vessel. Wash the ULA plate with E8 supplemented with 10 μM of ROCK inhibitor to recover all the cells and microcarriers and transfer into the bioreactor.
9. Add E8 supplemented with 10 μM of ROCK inhibitor to the bioreactor until the final volume of 80 mL is reached.
10. Close the PBS MINI 0.1 vessel and place it on a PBS MINI base unit inside the incubator at 37 °C and 5% CO_2. Start the agitation. The culture should be agitated continuously at low speeds (10–14 rpm) during the first 48 h.
11. After 48 h increase the agitation to 22 rpm. Agitation should be adjusted to the minimum value which allows complete suspension of the microcarriers ("just suspended speed"), typically between 22 and 26 rpm for a 9-day culture.

3.3.3 Sampling and Cell Counts

1. During the culture, take two daily samples of 700 μL from the PBS MINI 0.1 vessel in order to verify the quality and number of cells. Keep the bioreactor under dynamic conditions during sampling, to ensure homogeneity. Collect the samples in a 24-well plate.
2. Inspect the samples visually under an optical microscope and take photos, if necessary.
3. Let the microcarriers settle down and remove the culture medium supernatant. Keep the supernatant in a 1.5 mL microcentrifuge tube for glucose/lactate analysis (*see* **Note 6**).
4. Wash the cells/microcarriers with DPBS once.
5. Add 700 μL of Accutase and incubate at 37 °C for 10 min. Inspect the samples under the microscope for complete cell detachment from the microcarriers. If necessary, pipette up and down 5–10 times to help with the detachment and dissociation into single cells. Add additional 700 μL of culture medium into the dissociated cells.
6. Using a 100 μm cell strainer, separate the microcarriers from the cell suspension. Collect the cell suspension in a centrifuge tube.
7. Centrifuge at 210 × *g* for 3 min. Remove the supernatant and resuspend in medium (e.g., 500 μL, *see* **Note 7**).
8. Perform a cell count using a hemocytometer and the trypan blue dye exclusion test, under an optical microscope.

3.3.4 Cell Harvest at the End of Culture

1. If the cells will be replated after bioreactor culture, add an appropriate amount of ROCK inhibitor stock solution (10 mM) to the medium, to achieve a final concentration of 10 μM, and incubate at 37 °C and 5% CO_2 for 1 h before starting the protocol.
2. Collect the bioreactor contents in 50 mL conical tubes. Wait for the microcarriers to settle down and remove the supernatant.
3. Wash the contents of each 50 mL conical tube with 10 mL sterile DPBS once. Wait for the microcarriers to settle down and remove the DPBS.
4. Add 10 mL Accutase to each 50 mL conical tube and incubate for 10 min at 37 °C. Mix occasionally to help cell dissociation.
5. Pipette up and down 5–10 times to help with the detachment and dissociation into single cells. Add additional 10 mL of culture medium into the dissociated cells.
6. Filter the cell suspension using a Steriflip filter unit (100 μm pore size). Use one unit per 50 mL tube, to prevent clogging.

7. Centrifuge at 210 × *g* for 5 min. Remove the supernatant and resuspend in culture medium. If the cells will be replated, add ROCK inhibitor stock solution (10 mM) to the medium, to achieve a final concentration of 10 μM.
8. Perform pluripotency assays in order to verify the quality of the cells (e.g., flow cytometry, immunocytochemistry, and qRT-PCR to assess pluripotency marker expression; embryoid body assay and directed differentiation toward different cell lineages to assess trilineage differentiation potential; karyotyping to assess genomic stability).

4 Notes

1. The pellet must be broken so that the cells remain in suspension as small clumps. Avoid pipetting the solution excessively, in order not to singularize the cells.
2. If the cells appear singularized after visualization under the optical microscope, add 10 μM of ROCK inhibitor to the culture medium to improve cell survival. After 24 h, replace the medium with 1.5 mL of pre-warmed culture medium without ROCK inhibitor. Note that it is not advised to use ROCK inhibitor *routinely* for hiPSC passaging.
3. The incubation time required depends on the cell line. Incubation may be stopped when cells seem loose and start detaching by tapping the bottom of the plate.
4. Cell expansion may be improved by the supplementation of this medium with dextran sulfate at 100 μg/mL [15, 16]. After 48 h, the medium is replaced with mTeSR1 without dextran sulfate.
5. Use the FIJI software [17, 18] to measure the area (A) of each aggregate. Assuming the aggregates to be spherical, the diameter of each aggregate is computed as $d = \sqrt{\frac{4A}{\pi}}$.
6. For measurement of glucose/lactate concentration, remove cell debris by centrifuging the culture medium supernatant at 360 × *g* for 10 min and analyze using appropriate equipment (e.g., YSI 7100MBS).
7. Adjust the volume as per the expected number of cells in the bioreactor. For the first days of culture, 500 μL is enough for a clear counting in the hemocytometer. For the late-culture stage, use higher volumes of medium.
8. When 0.5 mM EDTA is used for cell detachment, cells remain as small clumps and not as single cells. Since it may be difficult to have an accurate counting with cell clumps, a 100 μL sample of cell suspension can harvested to a conical tube and incubated with 400 μL of Accutase for 7–10 min in a water bath at 37 °C. This diluted sample will contain a single cell suspension that

can be used for cell counts using a hemocytometer. Note that this additional dilution (1:5) has to be taken into consideration when calculating the cell number.

9. If using a PBS MINI 0.5, a minimum working volume of 300 mL is recommended. In this case, the microcarrier surface area (maintaining the microcarrier concentration) will be 2,160 cm^2 and 5.4×10^7 cells will be necessary.
10. This step aims to promote cell-microcarrier interactions. Ideally, the microcarriers should cover the whole surface area of each well with approximately 2–3 layers of beads in height. The number of wells where the suspension is placed can be adjusted to achieve these conditions and lead to a homogenous initial cell adhesion to the microcarrier beads.

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Addressing Manufacturing Challenges for Commercialization of iPSC-Based Therapies

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Abstract

The development of reprogramming technology to generate human induced pluripotent stem cells (iPSCs) has tremendously influenced the field of regenerative medicine and clinical therapeutics where curative cell replacement therapies can be used in the treatment of devastating diseases such as Parkinson's disease (PD) and diabetes. In order to commercialize these therapies to treat a large number of individuals, it is important to demonstrate the safety and efficacy of these therapies and ensure that the manufacturing process for iPSC-derived functional cells can be industrialized at an affordable cost. However, there are a number of manufacturing obstacles that need to be addressed in order to meet this vision. It is important to note that the manufacturing process for generation of iPSC-derived specialized cells is relatively long and fairly complex and requires differentiation of high-quality iPSCs into specialized cells in a controlled manner. In this chapter, we have summarized our efforts to address the main challenges present in the industrialization of iPSC-derived cell therapy products with focus on the development of a current Good Manufacturing Practice (cGMP)-compliant iPSC manufacturing process, a comprehensive iPSC characterization platform, long-term stability of cGMP compliant iPSCs, and innovative technologies to address some of the scale-up challenges in establishment of iPSC processing in 3D computer-controlled bioreactors.

Keywords 3D computer-controlled bioreactors, Cell therapy, cGMP, Commercialization, Induced pluripotent stem cells, Process development

1 Introduction: Industrialization of Human Pluripotent Stem Cell-Based Therapies

Reprogramming of human somatic cells into induced pluripotent stem cells (iPSC) offers huge potential for cell replacement therapy, basic research, disease modeling, and drug development. The iPSC-based therapy is a quickly growing field that largely builds on the scientific understanding and technologies developed for embryonic stem cells (ESC) [1–4]. The remarkable discovery of generating iPSCs from somatic tissue has dramatically impacted the field of drug discovery, toxicity testing, in-a-dish disease modeling, gene therapy, and gene editing during the last decade [5–10]. This is mostly due to the capacity of iPSCs to expand in vitro and their differentiation potentials (i.e., differentiation into any cell type in

the body), making them a promising source for curative clinical therapies [11].

Several clinical trials have been initiated using iPSC-derived cells. In 2016, Cynata Therapeutics initiated allogeneic iPSC-derived mesenchymal stem cell called CYP-001 for the treatment of steroid-resistant acute graft versus host disease/GvHD [12]. Also, iPSC-derived dopaminergic progenitors were transplanted into human patients for the treatment of Parkinson's disease at Kyoto University in 2017 [13]. More recently, Fate Therapeutics Investigational New Drug (IND) application was cleared to use an NK cell-based cancer immunotherapy for the treatment of advanced solid tumors with the cells derived from a clonal master characterized iPSC line [14]. The demand for high-quality tissue and pluripotent stem cell-based therapeutics are increasing as the incidence of degenerative disorders is growing significantly, in addition to inefficiencies with existing treatments and limited tissue availability and access to functional primary human somatic cells [15].

Despite the advancements in the field of iPSCs, there are major challenges that need addressing before iPSC-based therapies become readily available and manufacturing processes are industrialized. In particular, establishment of a robust directed differentiated process starting from high-quality cells, manufactured using a robust and cGMP-compliant process, still remains a major challenge in enabling clinical utility of iPSC-based therapies. Inherent difficulties in achieving high-quality cGMP-grade pluripotent stem cells and their progenies are a major obstacle in cell-based therapy and should be overcome before these cell types can be used to treat diseases [16].

One initial obstacle was that the reprogramming process was carried out using retroviruses which could raise safety concerns associated with permanent integration of the transgenes into the iPSC genome. This could significantly undermine clinical relevance of these iPSCs for therapeutic applications. Additionally, these cells were mostly generated and expanded using a feeder-layer system, which can result in high lot-to-lot variability, regulatory and safety concerns, as well as impact on the scalability of the process. Recently, alternative integration-free reprogramming methods have been used for iPSC generation. For instance, expression of non-integrating plasmid DNA carrying appropriate transcription factors (Oct4, Sox2, Klf4, c-Myc, and Lin28) into somatic cells, such as $CD34^+$ cells derived from newborn umbilical cord blood or adult peripheral blood mononuclear cells, has been used to successfully reprogram cells [17]. Although integration-free methods seem to be more promising, the reprogramming and expansion processes have been practically inefficient and technically challenging. Thus, the development of a robust, reproducible, and cGMP-compliant manufacturing process for the generation of clinical-

grade iPSCs in order to produce therapeutically relevant cellular products is critical. Appropriate documentation and controls on tissue sourcing, manufacturing unit operations, testing, and storage of the iPSCs and their differentiated products are critical aspects of the process to be addressed. In this chapter, we summarize our approach toward development of human iPSC manufacturing process under cGMP, which could serve as a guide for the development of stem cell-based therapies. In particular, we highlight some of the best practices in the development of a robust and reproducible manufacturing process to meet cGMP design considerations. We further underline the need to establish appropriate analytical methods and characterization assays to evaluate the critical quality attributes of the intermediate and final products as well as monitor the status of the cells over the course of manufacturing process. Moreover, we report the long-term stability of iPSCs manufactured using our cGMP-compliant process. Finally, we demonstrate the steps taken to industrialize the manufacturing of iPSC-based therapies through the establishment of iPSC expansion and directed differentiation protocols using 3D computer-controlled bioreactors [15, 16, 18–20].

2 Development of a Human iPSC Manufacturing Process Under Current Good Manufacturing Practices

Generation and development of a cGMP-compliant process for production of high-quality human iPSCs is an important first step toward the manufacturing of clinically relevant cell therapy products. Initially, there were efforts put toward transitioning PSCs generated under non-cGMP conditions to be cGMP-compliant by employing additional testing. However, this approach may be limited due to lack of proper documentation to trace the raw materials and reagents used in the process, presence of xenogeneic agents and feeder cells in the manufacturing process, and/or in some cases lack of donor consent and/or issues with meeting donor eligibility criteria [16]. In order to develop a high-quality cGMP iPSC bank, it is important to incorporate the proper controls, documentation, and testing from tissue sourcing to cell expansion and banking. By inclusion of the proper testing strategy with good understanding of the critical quality attributes (CQAs) and respective critical process parameters (CPPs), a robust, reproducible, and cost-effective manufacturing platform can be developed. For the iPSC manufacturing process, a sample of CPPs and their impact on iPSC generation is listed in Table 1.

In order to establish a robust and reproducible cGMP-compliant iPSC manufacturing process, we took a step-by-step approach to establish a cGMP-compliant iPSC manufacturing

Table 1
A sample of critical process parameters (CPPs) and their impact on iPSC generation

Unit operation	Critical process parameters	Impact on iPSC generation
Tissue handling/cell isolation	Controlled thawing parameters	Lowered cell viability and recovery
	Cell-to-bead concentration (i.e., magnetic separation of $CD34^+$)	Not able to recover sufficient number of cells
Reprogramming of cells	Concentration of genetic construct	Lower reprogramming efficiency
	Electroporation program used	Lower reprogramming efficiency and lower cell recovery
iPSC expansion	Cell seeding density	Elongated lag phase, impact on cell quality, variable growth kinetics
	Cell passaging reagents	Loss of quality and viability
iPSC cryopreservation	Controlled freezing profile	Loss of quality and lower recovery of cells
	Cell concentration	
	DMSO exposure time	

process. This included (1) establishing an iPSC generation process using a non-integrating, episomal-based technology, (2) performing process optimization and protocol development based on maintaining the CQAs, and (3) transferring the developed manufacturing process into a cGMP cell therapy manufacturing suite. This approach has resulted in the manufacturing of several iPSC banks at Lonza, for cell and gene therapy customers producing clinical products. An overview of the process is shown in Fig. 1.

2.1 Establishing an iPSC Generation Process Using a Non-integrating, Episomal-Based Technology

2.1.1 Tissue Acquisition

The process of tissue acquisition and receiving starting material for iPSC generation should follow appropriate guidelines that include defining the tissue requirements, working with a tissue recovery agency, establishing forms and standard operating procedures for recovering tissue, and determining donor eligibility. The process of tissue acquisition must be built upon strict adherence to the FDA regulations concerning the acquisition of human donor tissue (FDA 21 CFR 1271 human cells, tissues, and cellular and tissue-based products). A fundamental component of tissue sourcing is to obtain informed consent from the donor which includes (1) the intended use of the donated tissue; (2) the benefits, if any, to the donor or others; (3) the risks and potential discomfort of the donation; (4) confidentiality of donor information; and, (5) a statement demonstrating voluntary participation of the donor in the program. Following donor consent, it is also important to have a well-characterized starting material as this may potentially impact the clinical utility of the iPSC-derived product. The first major step in the characterization of the starting material is pre-acquisition testing, which determines the eligibility of the donor to provide tissue and involves reviewing of medical records, performing a

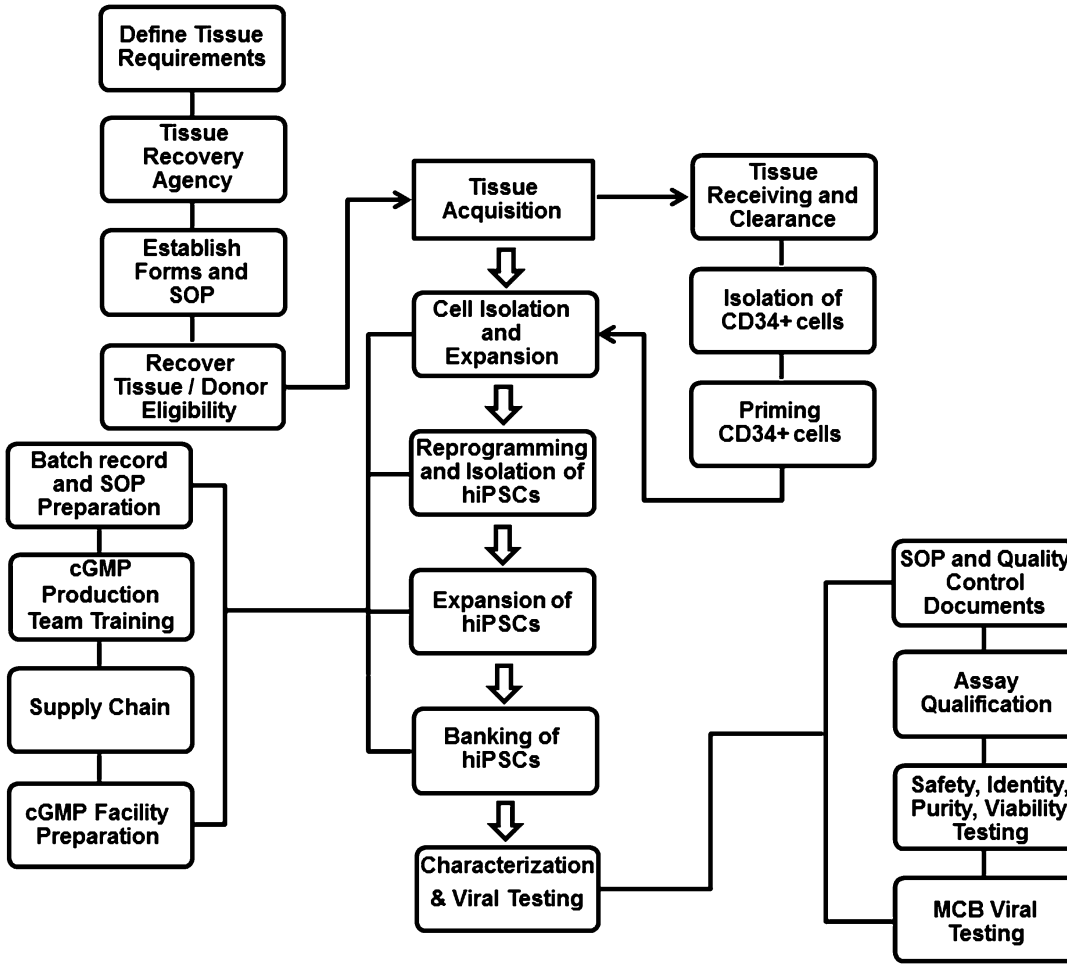

Fig. 1 An overview of the manufacturing of human induced pluripotent stem cells under cGMPs. A tissue acquisition program was established, focusing on defining donor and tissue requirements, working with a tissue recovery agency, establishing forms and standard operating procedures, recovering tissue, and donor eligibility determination. The manufacturing started with the isolation of $CD34^+$ cells from a fresh cord blood unit and continued to priming, expansion, and then reprogramming of $CD34^+$ cells. After generation of iPSCs and expansion, the cells were banked and eventually tested. Every step of the manufacturing process was documented and performed according to the batch records and standard procedures. Following characterization of the final bank, the results were reviewed by quality assurance group to release the GMP iPSC lot [16]

medical exam, and clinical screening for relevant communicable disease agent or diseases (RDCADs). In addition, there may be additional donor characteristics required to generate iPSCs for certain cell therapy products [21].

Following Good Tissue Practices (GTP), as outlined in FDA 21 CFR 1271 and 210/211, the tissue should be recovered aseptically and transported to the manufacturing site. The tissue is then stored at appropriate temperature under monitoring until the appropriate documentation has been reviewed and approved by

the quality assurance team at the manufacturing site. Upon document approval, the tissue is transferred to the cGMP manufacturing suite to initiate the process. For platform processes designed at Lonza, we have chosen to use cord blood-derived $CD34^+$ cells as the starting material because of several practical and technical considerations as summarized by Rao and colleagues [22]. The $CD34^+$ cells are isolated from the cord blood units using a magnetic bead-based separation process that results in greater than 80% $CD34^+$ cells. These cells are then culture primed for 4 days prior to the introduction of the reprogramming factors [16].

2.1.2 Reprogramming and Cell Culture

During the past 12 years, many efforts have been made to optimize the original Yamanaka reprogramming procedure and improve the safety and efficiency of iPSC reprogramming. Toward these aims, several groups applied different reprogramming factors such as non-integrating viral vectors, synthetic RNAs, recombinant proteins, and small molecules [23]. Particularly, identifying and applying chemical compounds such as RepSox, Kenpaullone, BIX01294, Bayk8644, RG108, CHIR99021, and tranylcypromine [7, 24–27] have shown great promise to move away from gene-based cellular reprogramming. Although successful reprogramming of mouse cells into iPSCs using only small molecules was demonstrated [28], gene-free reprogramming of human cells to iPSCs has not been achieved so far. More research is needed to recognize chemical substitutes for OCT4 in human somatic cell reprogramming. An all-chemical reprogramming method of generating iPSCs will greatly enhance the field of stem cell biology and regenerative medicine. In the meantime, to address the safety concerns surrounding the use of integrating viral reprogramming methods [29, 30], non-integrating, episomal-based methods (initially developed by Cheng and colleagues, 2011) have been utilized [17]. This technology works based on the single transfection of two plasmids: pEB-C5 (i.e., a polycistronic episomal reprogramming plasmid containing five genes encoding the transcription factors Oct4, Sox2, Klf4, c-Myc, and Lin28) and pEB-Tg (i.e., an episomal plasmid expressing SV40 Large-T antigen demonstrated to increase reprogramming efficiency) [17].

We have used this reprogramming method at Lonza to generate iPSC banks for our customers. As mentioned in the previous section, isolated $CD34^+$ cells are placed in culture and primed for 4 days prior to introduction of the reprogramming factors. Following priming, the cells are electroporated using our cGMP-compliant Lonza Nucleofector system and placed back into culture (in hPSC-specific expansion medium-Lonza L7 medium). In the next step, multiple iPSC colonies are selected and subcultured in tissue culture flasks in a feeder-free culture system [16].

2.2 Performing Process Optimization and Protocol Development Based on Meeting Critical Quality Attributes of the Target Cells

As mentioned previously, the iPSC manufacturing process can consist of the following defined unit operations: tissue handling/cell isolation, inclusion of a priming step (if required for the cell type), electroporation of the reprogramming factors into the cells, cell culture and expansion/passaging, and cell harvest and cryopreservation. For each of these unit operations, there are a number of process parameters that should be optimized to allow for robust iPSC generation. In our case, we evaluated and optimized a number of process parameters (for instance, priming condition, cell number and nucleofection conditions, cell culture system, cryopreservation) to ensure that we could meet the cGMP manufacturing process and target quality attributes. Conventionally, PSCs were isolated and cultured on a feeder cell layer that provides the cells with the appropriate substrate and soluble factors for their expansion and phenotypic maintenance. The inclusion of feeder cells in the cell culture system of iPSCs has a number of issues that include possible introduction of contagions that cannot be effectively screened for, introduction of xeno-containing cells (i.e., murine embryonic fibroblast feeders), and variability in the quality of the feeders that can introduce variability into the manufacturing process. While a number of PSC media and matrices were commercially available to remove the need for feeders, we found that these "rich" media and matrices did not allow for the generation of high-quality iPSCs from $CD34^+$ cells that met our CQAs. In particular, we found the existing commercially available media could impact the downstream directed differentiation process for generation of iPSC-derived products. Therefore, we opted to develop a proprietary matrix in-house, L7 hPSC matrix, that was paired with our defined, xeno- and serum-free medium (L7 hPSC Medium), allowing generation and expansion of our iPSCs and unbiased directed differentiation [16, 31, 32].

When we switched to L7 cell culture system, we found that there was a significantly lowered reprogramming efficiency of $CD34^+$ to iPSCs compared to a feeder-dependent system. Therefore, we evaluated the impact of environmental conditions, reagents, and culture conditions on the reprogramming efficiency. From these development studies, we further modified the reprogramming process to occur under hypoxic culture conditions (3–5% O_2) and included a selective inhibitor of TGF-β type I receptor ALK5 (A83-01) and Alhydrogel (i.e., an aluminum hydroxide wet gel suspension) as reprogramming enhancers. All these modifications significantly improved the efficiency of integration-free reprogramming under defined and feeder-free conditions [16].

In order to maintain the proliferative capacity and quality of iPSCs in culture, it is important to passage cells when they are in their exponential growth phase and exhibiting limited spontaneous differentiation. The iPSC cultures are typically initiated as small

clumps of cells, expanded as intact colonies with defined edges, and don't experience contact inhibition as observed with other adult stem cells. This results in cells packing tightly in their colonies that can form multiple layers, resulting in different cellular gene expression between cells at the periphery of the colonies from those within. Therefore, it is important to properly control excessive amount of cell packing/layering, which could result in cell death as well as increased spontaneous differentiation. To avoid this, it is important to employ a passaging system that allows cells to be passaged at defined intervals (either time-dependent or scored to minimize subjectivity), as small clumps within a defined inoculation density. For the manufacturing of cGMP-compliant iPSCs, Lonza developed a passaging method to allow passaging of the cells in the form of cell aggregates/clumps within a certain time range. A chemically defined, sodium citrate-based, non-enzymatic solution was developed for the serial subculture of iPSCs, thereby allowing operators to generate more consistent clumps at passage [32]. It is important to note that iPSCs are typically expanded as clumps to maintain the cellular microenvironment that enables higher cell survival and a reduced lag phase and minimizes the risk of karyotypic abnormalities compared to single cell passaging (without additional additives). However, it has been shown that iPSCs can be adapted to single-cell culture and minimize these aforementioned risks with the addition of an inhibitor of the rho-associated protein kinase signaling pathways [33]. By inhibiting these pathways, cells can be passaged as single cells, at more defined cell densities, that can further increase the reproducibility and robustness of the iPSC manufacturing process.

The final manufacturing unit operation is the harvesting and cryopreserving of the culture-expanded iPSCs. The cryopreservation protocol can influence the cells, and it is important to optimize the cryopreservation protocol to ensure that the CQAs of the manufactured iPSCs are maintained. Conventionally, cells are cryopreserved in DMSO-containing cryopreservation medium, and the length of cell exposure to DMSO can have an impact on the recovery, viability, and phenotype of cells post-cryopreservation. The length of cell exposure to DMSO is dependent on the time it takes for pre-formulated cells (i.e., in DMSO-containing medium but not at the final concentration) to be counted and how long it takes to fill all cryovials prior to CRF loading/operation. In addition to evaluating the cryopreservation medium suitable for iPSC banking, we characterized the cryopreservation media exposure time and defined a maximum time that cells could be held in DMSO-containing medium. This influenced the design of this final unit operation in order to ensure that appropriate number of cryovials are filled by the required maximum hold time.

From this process development work, we were able to reproducibly generate high-quality iPSCs from $CD34^+$ cells isolated

from multiple donors (more than ten different people) using episomal plasmids and confirmed their phenotype using the appropriate cell-based assays (e.g., surface markers by flow cytometry testing, karyotyping, polymerase chain reaction (PCR), and embryoid body formation) [16].

2.3 Technology Transfer of the Optimized Manufacturing Process to the cGMP Manufacturing Suite

Upon the establishment of a robust and reproducible iPSC manufacturing process during process development phase, we started the technology transfer stage, and the process was transferred to cGMP manufacturing. Several major steps were taken during the technology transfer phase and prior to the start of cGMP manufacturing runs including (1) raw material handling and supply chain considerations, (2) training runs to train manufacturing personnel and develop manufacturing batch records (MBRs), (3) engineering runs for reproducing the process in the manufacturing facility with approved/near-approved MBRs and SOPs, and (4) aseptic process simulation (APS) runs to ensure that the process and manipulations performed maintain sterility. In addition, several other manufacturing challenges were evaluated including instrumentation validation, facility preparation, sample submission strategy, warehousing, and storage.

Evaluation of raw material handling and supply chain considerations plays a critical role in establishing a cGMP manufacturing process. This includes several steps occurring simultaneously prior to and/or throughout the iPSC process technology transfer. The major activities include establishing a complete list of raw materials as well as establishing relevant quality control and bioassay testing, primary and secondary vendor qualifications, raw material part setup, and inventory management (including storage conditions, tracking expiration, reorder points, and material flow to and within GMP manufacturing suites) [34]. During the process development/optimization studies, it is important to generate an up-to-date bill of material (BOM) to evaluate the criticality of the materials for the process and assess vendors' qualifications based on the available regulatory requirements and guidelines (e.g., US regulatory requirements). Furthermore, vendor evaluation and approval can be performed by supply chain and quality assurance (QA) teams who evaluate the vendor based on a written vendor questionnaire, verification of specific tests shown on the certificate of analysis (CoA), relevant material shipment documents, and site audits [16]. A series of critical activities and events are required as part of supply chain activities including setting up client/project-specific part numbers in the system applications and products (SAPs) database. This enables capturing of all relevant information, including the CoA, material origin information, and shipping conditions, and ultimately the information will be included on the raw material specification sheet (RMSS). The RMSS has to be reviewed and approved by the subject matter expert and QA group. A specific

part number assigned to each raw material in SAP provides full visibility throughout the period of manufacturing process and facilitates the handling, ordering, tracking, and availability of the materials from suppliers to the warehouse and from the warehouse to the cGMP suite.

Training runs (usually two) provide the opportunity to train cell therapy technicians on the developed iPSC manufacturing process prior to execution of the process in the cGMP manufacturing suite. In addition, during the training runs, MBRs and SOPs for both the manufacturing process and analytical methods are developed. The training runs can be carried out in a process development/research laboratory using the defined and cGMP-compatible process and cell culture system established at the end of the process development/optimization activities. Training runs are followed by engineering runs in which the final iPSC manufacturing process is re-evaluated in a cGMP manufacturing suite using approved (or nearly approved) production documents. A minimum of two engineering runs are carried out in the cGMP manufacturing suite, with products (including intermediate products and final products) being tested and evaluated using cGMP testing protocols, and provide an opportunity to identify any potential gaps throughout the entire manufacturing process [16]. Importantly, assays and testing methods need to be qualified or validated during the tech transfer phase depending on the clinical trial phase of application and the intent of the assay [16]. Additionally, as mentioned previously, the cell manufacturing process needs to be in compliance with FDA regulations and additional country-specific guidelines if the iPSC-derived cells may be distributed internationally. After engineering runs, and prior to cGMP manufacturing runs, APS runs (typically a minimum of three APS runs) are carried out. The APS runs are required to demonstrate adequate aseptic handling during the manufacturing process and ensure that all of the manipulation steps performed in the manufacturing suites maintain sterility. Finally, prior to the start of the cGMP manufacturing runs, the production documents and MBRs are finalized, and all attempts should be made to ensure the manufacturing process executed during GMP runs does not deviate from the process performed during engineering runs.

For the cGMP iPSC manufacturing run performed at Lonza, the fresh tissue was sourced from one donor, packaged, and shipped to the manufacturing site according to our validated procedures. When received, the cGMP-compliant tissue was quarantined, while initial documents associated with the tissue, including the tissue collection date, shipping and handling conditions, and tissue volume/size, were being reviewed. The manufacturing process was initiated within specific tissue shipping and receiving time window established during process development stage. Once the manufacturing process was initiated, samples of iPSCs were

cryopreserved at different passages to perform critical in-process testing (e.g., karyotype test or plasmid clearance test). At the end of the process, the cells were harvested and cryopreserved and then tested for release based on pre-defined analytical test methods [16].

3 Analytical Methods and Characterization iPSC During the Manufacturing Process

The implementation of the appropriate assays for in-process and release testing is critical in order to track the CQAs of the product as it is being manufactured and at the end of the process. These assays include cell count and viability, flow cytometry for measuring identify and purity, PCR for tracking expression of key genes, embryoid formation for assessing germ layer differentiation, short tandem repeats (STR) for matching patient or donor identity, and compendial assays that include additional safety tests—plasmid clearance, sterility, mycoplasma, endotoxin, and adventitious viral testing. These assays are typically developed and optimized during the process development phase. Depending on the target criteria for release of iPSC bank as well as existence of appropriate specification and/or historical data for assay qualification, a decision needs to be made to set an assay as a release assay (i.e., indicating safety, identity, or purity) or for information only (FIO) assay (e.g., embryoid formation for iPSCs). Prior to qualification (if applicable) and even for the assays that are used for characterization or in-process monitoring, it is important to have confidence in the reported assay results, and thus optimization should be done. For instance, for the flow cytometry assay used for evaluation of pluripotency markers of the iPSCs, we performed extensive optimization studies focusing on optimization of the flow cytometry protocol (e.g., antibody titration, specificity of antibodies, blocking steps), implementation of appropriate gating strategy, setup of appropriate flow panels, and establishing the proper reference banks and controls. If the assay is going to be used for decision-making purposes (i.e., go/no-go decision point) or release of the product, then it needs to be qualified (in the USA, basing qualification on the FDA Guidance For Industry: Analytical Procedures and Methods Validation for Drugs and Biologics and ICH Q2 R1) [35, 36]. Depending on the purpose of assays (e.g., identity versus potency), the assay qualification may cover evaluating the accuracy and range of the assay, linearity and precision of the reported results, lower and upper limit of quantification, specificity of the reagent (i.e., antibody), and intra-/inter-assay precision. For the iPSC manufacturing process, the list of release assays is listed in Table 2 that have all been qualified at Lonza [19].

Table 2
Assays used to characterize human iPSCs manufactured under cGMP condition (i.e., quality control (QC) testing and release assays) [19]

Assay	Objective	Evaluation criteria	Category	Tested iPSC line
Assay release				
Pluripotency markers	Identity and purity	SSEA-4 > 70%, Tra-1-60 > 70%, Tra-1-81 > 70%, Oct3/4 > 70%; purity: CD34 < 5%	Release assay	All lines
Karyotype analysis	Safety	46, XX or 46, XY	Release assay	All lines
Mycoplasma testing	Safety	Negative	Release assay	All lines
Sterility testing	Safety	Negative	Release assay	All lines
Endotoxin testing	Safety	Standard QC release (<0.5 EU/mL)	Release assay	All lines
Vector clearance	Safety	No trace of episomal plasmid DNA detected	Release assay	All lines
STR genotyping	Purity and identity	STR profiles of starting population and iPSC line are identical	Release assay	Al lines
Cell count & viability	Viability	% viability >50; minimum cell number/vial	Release assay	All lines
Viral panel testing	Safety	Standard MCB release panel	Release assay	LiPSC-GR1.1
Characterization assays				
EB formation	Identity and potency	Detection of at least one marker per germ layer	FIO[a]	All lines
Gene Array analysis	Identity	Clustering with established hPSCs	FIO[a]	All lines
Colony morphology	Identity and purity	Characteristic morphology of culture/colonies; lack of spontaneously differentiated cells	FIO[a]	All lines
Post-thaw plating	Thawing efficiency and viability	20+ colonies/vial (after 7 days or 50% confluency)	FIO[a]	All lines
HLA typing	Identity	HLA-A, B, C, DRB1, and DQB1 Type	FIO[a]	All lines
CGH + SNP microarray	Identity	Amplifications and/or deletions of specific genes	FIO[a]	LiPSC-GR1.1 and ER2.1
Whole genome sequencing	Identity	HiSeq X human whole genome sequence	FIO[a]	LiPSC-GR1.1 and ER2.1

[a]For information only (FIO)

Besides the release testing panel, as the true value of iPSCs is the differentiations of these cells into mature, functional cells in the body, we also performed directed differentiation studies to demonstrate that the human iPSC lines were able to differentiate into specialized cells from all three embryonic lineages with morphological and cellular characteristics of cardiomyocytes, definitive endoderm (DE), and neural stem cells (NSCs). Two of our cGMP-compliant iPSC lines were successfully expanded and differentiated into cardiomyocytes, neural stem cells (NSCs), and definitive endoderm [15, 16].

4 Long-Term Stability of iPSC Master Cell Banks (MCBs) and Working Cell Banks (WCBs)

Long-term storage of iPSCs in LN2 carries a risk of bacterial, viral, and mycoplasma infection. In addition, long-term storage may cause a loss of critical quality attributes (CQA) including potency, purity, identity, and sterility. Owing to these concerns, we have recently reported the effect of long-term cryopreservation on the CQAs of human iPSC lines, sterility, genomic stability, and changes in telomerase activity and telomere length [20]. Human iPSC lines were directly differentiated into cells from the three germ layers and also expanded in 2D and 3D culture conditions to evaluate their long-term expansion potential in feeder-free culture environments. Thawed cells successfully attached to the L7™ matrix and formed typical iPSC colonies that expressed pluripotency markers over 15 passages. Furthermore, post-thaw cells showed negative mycoplasma and sterility testing as well as a normal karyotype. These cells maintained both their 2D and 3D proliferation potential after 5 years of cryopreservation without loss of pluripotency, telomerase activity, or acquiring karyotypic abnormalities. Our results illustrated, for the first time, a more in-depth understanding of the impact of long-term cryopreservation on quality and stability of human iPSC banks. This is an important step in establishing a reliable, long-term source of starting materials for clinical and commercial manufacturing of iPSC-derived cell therapy products.

5 Scale-Up of iPSC Expansion and Differentiation in 3D Bioreactors

In this chapter, we have discussed the steps taken to develop a cGMP-compliant iPSC manufacturing process using a 2D cell culture system including tissue culture flasks. However, the banks generated with this process yield around 1.0×10^9 for the MCB and WCB each. For allogeneic cell replacement therapies, this number of cells may not be sufficient to generate appropriate

clinical quantities of iPSC-derived specialized cells. Indeed, it is estimated that 10^9 cells would be required per patient in heart failure [37], and with the market for heart failure potentially requiring thousands of doses a year, 10^{11}–10^{14} cells may be required annually. In order to generate this large amount of clinically relevant cells, a scalable manufacturing process is required that can reduce the labor and manufacturing footprint, enable process monitoring and control of CPPs, and lead to a more homogenous cell product. It is important to note that iPSCs can expand both as adherent cells (in 2D culture), in 3D suspension as free-floating aggregates of cells [18, 38, 39], and in 3D suspension on microcarrier [40]. We and others have employed stirred tank bioreactors (STRs) for expansion of iPSCs and directed differentiation into specialized cells (e.g., cardiomyocytes). In addition to scalability and ease of implementing process control techniques, the culture of iPSCs as aggregates in STRs is favorable as no attachment substrate is required, the downstream processing may be relatively simple, and the majority of directed differentiation protocols for first-to-clinic therapies (e.g., heart failure, type 1 diabetes) utilize aggregated iPSCs as their starting material. When using STRs there are two main design aspects that must be taken into considerations: (1) the oxygen mass transfer and (2) the impact of liquid shear/hydrodynamics on the cells in culture. This is especially important when cell cultures are scaled up from small scale (e.g., below 1 L) to 3 L and above. Oxygen supply to cultures is important as it can affect not only the growth kinetics but also the quality and differentiation potential of iPSCs. The specific oxygen consumption rate of mammalian cells has been reported to be between 1.7×10^{-17} and 1.7×10^{-16} mol O_2/cell/s [41]. Typically, in small-scale bioreactors, the oxygen is supplied through the headspace, and diffusion is limited by the dissolution of oxygen into the culture medium at the gas-liquid interface (i.e., interfacial surface area). In 1989 [42], Aunin's and colleagues developed a correlation for the volumetric mass transfer coefficient ($k_L a$) for surface oxygenation that incorporated the geometry of the impeller, shear stress, and medium properties and is given by:

$$k_L a = 1.08 \times N_{RE}^{0.78} \left[\frac{D_{O_2} a}{D_T}\right].$$

where N_{RE} is the impeller Reynolds number, D_{O_2} is the oxygen diffusion coefficient, a is the mass transfer interfacial area, and D_t is the tank diameter. At small scale (i.e., <1 L), the oxygen demands of the culture system can be satisfied by headspace aeration alone. However, as the working volume of the bioreactor increases, the surface area-to-volume ratio (i.e., the aspect ratio) decreases. As an example, when scaling up from the BioBLU 3c to 50c (Eppendorf) with a working volume of 3 and 50 L, respectively, the aspect ratio

would decrease from 2.27 to 0.71 m^{-1}. Therefore, as the scale of bioreactors increases, the area available for oxygen transfer through the interfacial surface of the medium in the bioreactor decreases, resulting in decreased mass transfer of oxygen into the bulk liquid medium. In order to increase the available oxygen in the bulk medium, one can (1) increase the oxygen concentration in the headspace, (2) sparge oxygen into the culture, and/or (3) increase the agitation rate of the impeller. Using sparging, the oxygen supply occurs via small bubbles introduced into the cell culture medium, whose diameter needs to be controlled in order to minimize the chance of entrapping cells and damaging them (i.e., through carrying them to the surface and bursting) [43]. Sparging may also cause foaming in protein-rich medium (i.e., human serum or albumin-containing medium) that can be mitigated with the addition of anti-foam, although the use of anti-foam itself would need to be evaluated for its impact on cell quality. As a rule of thumb, it is favorable to increase the oxygen concentration in the headspace first and then supplement the oxygen supply to the culture with sparging. Increasing the agitation rate is another option; however as the agitation increases, the shear stress experienced by the cells could be detrimental and could cause excessive cell damage.

For scaling up STR cultures, relatively simple numerical equations have been used to calculate important hydrodynamic parameters. However, one caveat of using these equations is that they have been designed to scale up much larger bioreactor systems and have inherent inaccuracies when scaling up from relatively small-scale vessels (i.e., < 50 L) to larger vessels. Additionally, as these equations rely heavily on specific geometry of the bioreactors and impeller design, further inaccuracies can result when bioreactor configurations are not similar between small-scale and large-scale vessels. As the hydrodynamics within the bioreactors have a major impact on the phenotype and differentiation of iPSCs, the inaccuracy present in the equations impedes the ability to properly scale from lower to higher volume. In order to address this issue, we have employed computational fluid dynamics (CFD) modeling where we are able to capture the geometry of the bioreactors and simulate the hydrodynamic environment with this given geometry. There are a number of parameters that are important to consider when scaling up the bioreactor protocols, such as maximum shear stress, power-to-volume ratio, and maximum energy dissipation rate. Conventionally, stem cell cultures have been scaled up based on maximum shear stress, which is found near the tip of the impeller. In this regard, the cells are typically only experiencing this maximum shear stress for a fraction of their time in the bioreactor which does not capture the average hydrodynamic forces experienced by the cells. Additionally, with cells expanding as aggregates, it is important to maintain the size of the aggregates

for a number of reasons. As aggregate size increases, the concentration gradient of oxygen, nutrients, and metabolites within the aggregate can increase. This can result in differential gene expression between cells within the aggregate, as well as necrosis at the center of the aggregate if oxygen/nutrients are not able to get to the cells. These diffusion limitations can also exacerbate concentration gradients of small molecules/cytokines within an aggregate that can negatively impact the efficiency of directed differentiation. Based on a recent publication and our experience, we found that the average energy dissipation rate is a critical scale-up parameter to maintain comparable aggregate size distribution between small-scale and larger-scale bioreactors [18]. We scaled up iPSC cultures from 125 mL spinner flasks to a 3 L computer-controlled bioreactor using the average energy dissipation rate and found that we had comparable growth kinetics and average aggregate size while maintaining their pluripotency (Fig. 2). These aggregates could then be directly differentiated into cardiomyocytes using a published multi-step induction protocol with a defined medium [44]. This system

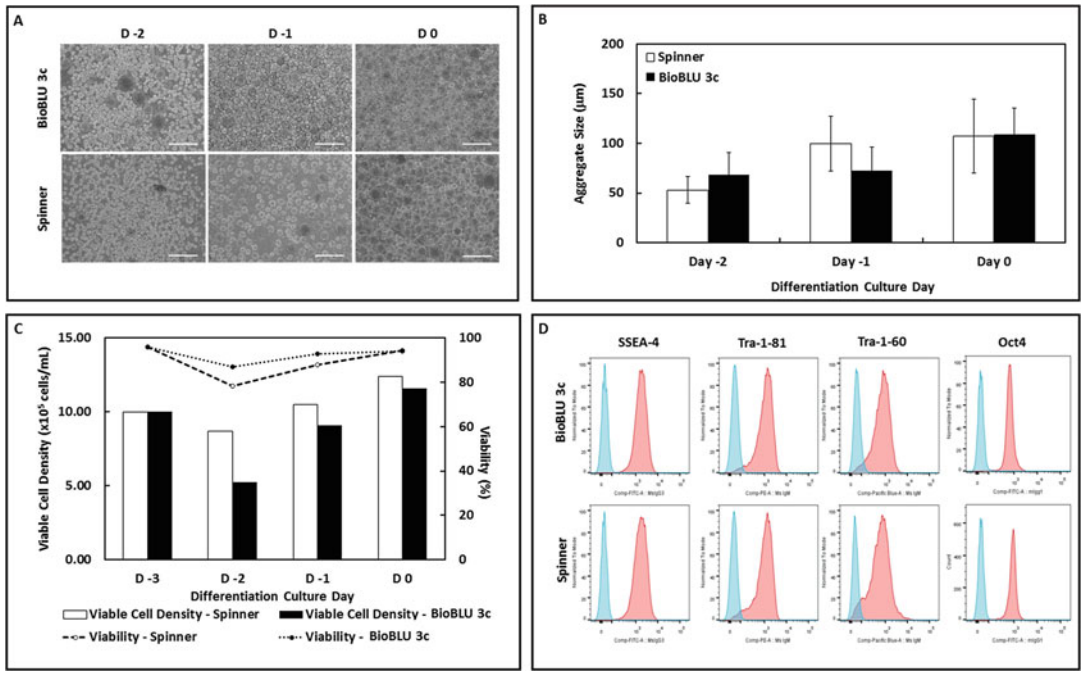

Fig. 2 Expression of human iPSCs in the BioBLU 3c and spinner flask. (**a**) Representative phase contrast aggregate images were taken during iPSC expansion. iPSCs formed aggregates which grew from day −3 to 0 in both the spinner flask and bioreactor. (**b**) The average aggregate size was shown to be similar between the BioBLU 3c and spinner flask. (**c**) Viable cell density and percent viability during expansion showed similar growth kinetics. (**d**) Flow cytometry analysis of undifferentiated iPSC on day 0 of the cardiomyocyte induction process showed high expression of key pluripotency markers SSEA-4, Tra-1-81, Tra-1-60, and Oct4 in both spinner flask and bioreactor (pink, target; blue, isotype). Scale bars represent 200 μm. iPSC, induced pluripotent stem cells [18]

can be easily translated into cGMP manufacturing process, and the use of CFD modeling enables us to realize the future demand of clinically relevant iPSC-derived cells. This is a major step forward in development of scalable manufacturing processes for expansion and directed differentiation of iPSCs in 3D conditions in computer-controlled bioreactors.

In conclusion, we have summarized some of the major steps we have taken in development of high-quality and fully characterized iPSCs that can be used in the generation of clinical quantities of iPSC-derived products using commercially viable manufacturing methods. In the first step, we developed a robust, manufacturing process, enabling the generation of high-quality iPSCs under cGMP condition. We highlighted some of the major steps that we have taken in the development of our proprietary cGMP-compatible process and showed how this further enabled us to meet the pre-defined CQAs and allowed generation of high-quality iPSC lines that were able to differentiate into all three germ layers. We also discussed the development of a scalable method to generate clinically relevant numbers of iPSC derivatives in 3D suspension bioreactor using a novel scale-up approach (i.e., CFD modeling). This allows us to further scale up the process to meet clinical and commercial demands. Finally, we demonstrated the long-term stability of cGMP iPSC lines, which is an important step in manufacturing of a reliable source of initial materials for clinical and commercial use of iPSC-derived products.

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Standard Operating Procedure for the Good Manufacturing Practice-Compliant Production of Human Endometrial Stem Cells for Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is the most common cause of neurological diseases. Although, there are some effective medications with regulatory approval for treating MS, they are only partially effective and cannot promote repairing of tissue damage directly which occurs in the central nervous system. Therefore, there is an essential need to develop novel therapeutic approaches for neuroprotection or repairing damaged tissue in MS. Accordingly, cell-based therapies as a novel therapeutic strategy have opened a new horizon in treatment of MS. Each setting in cell therapy has potential benefits. Human endometrial stem cells as an invaluable source for cell therapy have introduced treatment for MS. In this respect, good manufacturing practice (GMP) has a pivotal role in clinical production of stem cells. This chapter tries to describe the protocol of GMP-grade endometrial stem cells for treatment of MS.

Keywords Endometrial cells, Mesenchymal stem cells, Multiple sclerosis, Therapeutic use

Abbreviations

ATMP	Advanced therapy medicinal products
CBMPs	Cell-based medicinal products
CD	Cluster of differentiation
cGMP	Current good manufacturing practice
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
FBS	Fetal bovine serum
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HSCs	Hematopoietic stem cells

HTLV	Human T-lymphotropic virus
MS	Multiple sclerosis
MSCs	Mesenchymal stem cells
PBS	Phosphate-buffered saline
QMS	Quality management system
RPR	Rapid plasma reagin
SOP	Standard operating procedure

1 Introduction

The scope of cell-based therapies and regenerative medicine has opened new frontiers in medical sciences with the possibility of regenerating damaged cells and treatment of various degenerative diseases including MS [1–4]. MS is a long-lasting neurodegenerative and autoimmune disease in which the protective sheath (myelin) of nerve fibers (in brain and spinal cord) is damaged [5, 6]. Typically, there is a wide range of treatments for MS including pharmacological, physical, and cell-based therapies [7–10]. In accordance with several investigations, the most abundant type of cells which are used for treatment of MS is autologous hematopoietic stem cells (HSC) [11–14]. Additionally, mesenchymal stem cells (MSCs) with the immunologic and neurologic impacts can be used as a potential stem cell source for treatment of MS [15–18]. Among the various MSC sources, endometrium is a highly regenerative tissue with rhythmic cycles of growth and differentiation [3, 19, 20]. Based on previous studies, endometrial stem cells as a group of important sources for cell therapy in neurodegenerative diseases can react to neuro-differentiation signaling molecules and reprogram neuronal cells [21–23]. Therefore, it seems that they can be applied for MS treatment. Herein, the protocol of clinical manufacturing of human endometrial stem cells according to GMP standards is introduced.

2 Materials

2.1 Human Endometrial Tissue Procurement

(a) Tissue container with ice.

(b) Endometrium specimen biopsy of women without endometriosis (women with MS disease) (*see* **Notes 1** and **2**).

2.2 Endometrial Tissue Harvesting, Endometrial Stem Cell Isolation, Culture, Subculture, Cryopreservation, and Banking

2.2.1 Raw Materials

All reagents used in this protocol should be purchased from companies that guarantee their compliance production. The material and reagents are:

1. Pipette tips: crystalline, yellow, and blue (TPP, Switzerland).
2. 35 and 100 mm Petri dish (TPP, Switzerland).
3. Falcon tubes 15 ml, 50 ml (TPP, Switzerland).
4. PluriStrainer® 70 μm (pluriSelect, Germany).
5. Falcon cell strainer 40 μm (Corning, Falcon®, Germany).
6. Filter cap cell culture flasks (75 and 25 cm^2) (TPP, Switzerland).
7. Motorized pipette (Orange Scientific, Belgium).
8. 0.2 μm sterile and endotoxin-free syringe filter (Orange Scientific, Belgium).
9. 70% ethanol (Merck, Germany).
10. Cryovial (Greiner Bio-One, Germany).
11. Penicillin/Streptomycin (Pen/Strep) (ATOCEL, Australia).
12. Collagenase I (Thermo Fisher Scientific, Gibco™, USA).
13. Amphotericin B solution (Sigma-Aldrich, USA).
14. Phosphate-buffered saline (PBS) (CliniMACS®, Miltenyi Biotec, Germany).
15. Dulbecco's modified Eagle's medium-F12 (DMEM-F12) (PAA, Austria).
16. Fetal bovine serum (FBS) (Pharma Grade, Australian origin, and gamma irradiated, PAA, Austria) (*see* **Note 3**).
17. TrypLE™ Select (recombinant trypsin-like substitute) (Life Technologies, USA) (*see* **Note 4**).
18. Hank's balanced salt solution (HBSS) (PAA, Austria).
19. CryoSure-dimethyl sulfoxide (DMSO) USP grade (WAK-Chemie, Germany).
20. Trypan blue solution 0.4% (Invitrogen, USA).
21. Ficoll-Hypaque (GE Healthcare, England).

2.2.2 Equipment

1. Clean room (GMP) facility (*see* **Notes 5** and **6**) (Fig. 1).
2. Sterile clean room clothing (face masks, hood, powder-free gloves, boots, and coveralls).
3. Biological safety cabinet (laminar vertical airflow cabinet).
4. Weighing balance (Sartorius, Germany).
5. Centrifuge (swing-out rotor with buckets for 50 and 15 ml tubes) (Eppendorf, Germany).

Fig. 1 Clean room as a GMP-compliant facility with controlled temperature, humidity, air pressure, and air particles can reduce contaminations in the manufacturing biomedical products process

6. Optical inverted microscope equipped with phase-contrast option (Nikon, Japan).
7. CO_2 incubator (set at 5% CO_2, 37 °C, and 95% relative humidity) (Memmert, Germany).
8. 37 °C water bath (Memmert, Germany).
9. Ultralow temperature freezer (New Brunswick, Eppendorf, USA).
10. Liquid nitrogen tank (Statebourne Cryogenics, UK).
11. Pre-cooled Mr. Frosty freezing container (NalgeneTM cryo, Thermo Scientific, USA).
12. NucleoCounter® NC-100TM (Chemometec, Denmark).
13. Sterile stainless steel surgical tray.

3 Methods

3.1 Human Endometrial Tissue Procurement

Study of protocol and use of the human specimens was approved by the Ethics Committee at Tehran University of Medical Sciences, Tehran, Iran (approval ID: IR.TUMS.VCR.REC.1398.423). After describing the procedures and aims of the study, a written informed consent was obtained from donor in compliance with regulations concerning the use of human tissues.

The whole process including tissue harvesting, tissue disruption, and cell separation and other processes should be done in a laminar vertical airflow cabinet which is set up in a GMP (clean room) facility and compatible with the conforming quality assurance guidelines.

3.2 Controls Prior to Endometrial Tissue Processing

1. According to donor eligibility, medical history and full physical examination of the donor should be assessed [24, 25].
2. The biopsy should not be obtained from:
   (a) Women with cancer, hyperplasia, etc. (all of the non-endometrial benign pathological conditions)
   (b) Women who have received exogenous hormones prior to the date of the biopsy
   (c) Women with any pathological condition in the uterus
   (d) Women with infectious pathology (excluded through the HIV, HCV, and HBV tests)
3. Sample of the women's blood should be obtained for serological tests including HBV antibody, HCV antibody, HIV-1, HIV-2, HTLV-1/HTLV-2, and RPR for syphilis and nucleic acid tests for HIV and HCV [26].
4. After procurement, transfer tissues to the cGMP facility (*see* **Notes 5** and **6**).
5. All accompanying documents such as written informed consent, medical history, and physical examination documents.
6. At the arrival time, for appropriate labeling, probable leakage, the volume of the transfer medium, and maternal blood sample, the container should be checked, and record all information based on the documentation SOPs (*see* **Note 6**).
7. Put the container at 4 °C until the processing time.
8. According to organization-specific SOPs, all documents, labeling, and coding requirements should be provided prior to processing (*see* **Note 6**).
9. Environmental parameters must be within their predetermined range and be documented according to SOPs (*see* **Note 6**).
10. All maintenance programs must be fully implemented and documented perfectly (*see* **Note 6**).
11. All equipment such as laminar vertical airflow cabinet and CO_2 incubator and also working place must be sterilized according to the specific SOPs (*see* **Note 6**).
12. Turn on the laminar airflow cabinet for 15 min before starting medium preparation time and processing.
13. Prepare all culture medium, enzymes, and reagents before processing.

3.3 Endometrial Tissue Preparation and Endometrial Stem Cell Isolation

1. Human endometrial tissues were obtained from five women after hysteroscopy for non-endometrial benign pathological condition. Full-thickness endometrium, with size of $1 \times 1 \times 1$ cm^3, is collected in pre-warmed Hank's media containing 1% Pen/Strep and 1 μg/ml amphotericin B and 5% FBS, stored at 4 °C, and processed within 2–18 h (*see* **Notes 2** and **7**).
2. Transfer the human endometrial tissue to the clean room (GMP) facility (see **Notes 5** and **6**).
3. Place the specimen in a sterile 100 mm Petri dish.
4. Wash the specimens with fresh, pre-warmed Hank's media containing 1% Pen/Strep and 1 μg/ml amphotericin B (*see* **Note 7**).
5. Scrape myometrium from the endometrium and discard blood and mucus.
6. Transfer the specimen to another sterile Petri dish with isolation media (containing DMEM/F12 + FBS 5% + antibiotic1%), and slice it into small pieces (1–2 mm^2) using tweezers and scissors.
7. Transfer the specimen to another sterile Petri dish, and cut it into 1–2 mm pieces with a sharp scalpel (avoid letting the specimen dry out).
8. Transfer the specimen pieces into sterile 15 ml Falcon tubes with sterile forceps.
9. Add collagenase I (3 mg/ml: 3 mg collagenase is dissolved in 1 ml Hank's media), and incubate at 37 °C (cell incubator or water bath) for 30–45 min. Shake the Falcon tube every 15 min.
10. Add pre-warmed sterile isolation media (equal volume of collagenase I media) to neutralized collagenase I when cells are separated from the specimen (it maybe takes 2 h based on the size of the specimen).
11. To remove glandular epithelial components, pass cells once through a 70 μm cell strainer, and then pass it through a 40 μm cell strainer twice.
12. Centrifuge the cells that pass through the strainer at $300 \times g$ for 5 min (the centrifugation step may be performed or excluded).
13. Add the passed cells gradually (1:1 sediment/Ficoll [100 mg/ml]) on Ficoll in a 15 ml Falcon tube at room temperature.
14. Centrifuge at $400 \times g$ for 20 min. Then, throw away sediment containing red blood cells, and take the turbid interface phase of Ficoll and the cell culture media.

15. To the interface phase, add 4 ml pre-warmed PBS and centrifuge at 100 × *g* for 10 min.
16. Throw away the supernatant, add 1 ml plating media into the sediment, and then transfer them to T25 culture flasks. Add an additional 2.5 ml of plating media containing DMEM-F12, 15% FBS, and 1% Pen/Strep. Incubate for 24 h in the cell incubator. The frequency of cells adhering to the flask can increase by a total volume of 3.5 ml cell culture media in T25 culture flasks for the first day. Note that larger volumes may result in fewer cells adhering to the flask.
17. The next day, add 3 ml more of plating media slowly, and keep it in the cell incubator for 1 week. Do not shake it or stir it each day.
18. Wash cells with pre-warmed PBS and discard PBS when cells are 90% confluent. Add 0.25% trypsin-EDTA (1 ml). Next, incubate in the cell incubator for 5 min, and then transfer the cells to a 15 ml Falcon after the cells dissociate from the flask, and add 1 ml DMEM-F12 supplemented with 10% FBS and 1% Pen/Strep. Centrifuge at 300 × *g* for 10 min.
19. Add cell sediment to other T25 or T75 culture flasks, and use DMEM-F12 supplemented with 10% FBS and 1% Pen/Strep, respectively, for the rest of cell passages.

3.4 Endometrial Stem Cell Culture and Subcultures

1. The next day of cell isolation, pull out the flasks from the CO_2 incubator, and observe the macroscopic appearance of the culture medium:
   (a) If the medium is dense and yellow, there is the suspect of a contamination. In this case the procedure is stopped, and sampling for microbiological control is needed.
   (b) If the appearance is clear and the color is red or dark red, go on with this procedure.
2. Remove the half of media and exchange with new plating media and keep it in the CO_2 incubator for 3 days.
3. On the third day, remove the total media, and wash with PBS+ (PBS containing calcium and magnesium) to remove red blood cells and non-adherent cells as stromal matrix debris (*see* **Notes 8** and **9**).
4. Renew the medium every 3 days.
5. When cells reach 90% approximately 85–90% confluency, discard the media, and wash them twice with pre-warmed PBS (*see* **Note 8**).
6. Collect and discard PBS.
7. Add TrypLE™ (1 ml) (*see* **Note 4**).
8. Incubate in the CO_2 incubator for 5–10 min.

9. Check it for cell detachment during incubation period.
10. After the cells dissociate from the flask, transfer the cells to a 15 ml Falcon.
11. Add 1 ml DMEM-F12 supplanted with 10% FBS and 1% Pen/Strep.
12. Centrifuge at 300 × *g* for 10 min.
13. Transfer tubes into a safety cabinet and remove the supernatant gently and add 1 ml DMEM-F12 supplemented with 10% FBS and 1% Pen/Strep.
14. By pipette flow, resuspend cell pellet in culture medium softly.
15. Use trypan blue 0.4% solution (1:1 dilution) and the hemocytometer for counting resuspended cells, and estimate cell viability and purity. Confirm the mentioned parameters by using NucleoCounter.
16. Record all parameters according to the documentation SOPs (*see* **Note 6**).
17. Add resuspended cells into other T25 culture flasks (100,000 cells/ml) or T75 culture flasks (300,000 cells/ml), and for the rest of cell passages, use 5 and 9 ml DMEM-F12 supplemented with 10% FBS Pharma Grade, 1% Pen/Strep, respectively.
18. Put the flask in the CO_2 incubator (37 °C, 5% CO_2, humidified) (*see* **Note 10**).
19. Store reagents at 4 °C.

3.5 Characterization of Cultured Endometrial Stem Cell

1. For various batches of the cells at the same number of subcultures, karyotyping should be conducted, and also before releasing for clinical transplantation, the karyotype must be normal.
2. Immunophenotypic characterization of endometrial stem cells should be accomplished to determine cell surface markers (CD markers) [27] by flow cytometry (Fig. 2). The expression of CD105, CD90, and CD146 and the lack of expression of CD34 and CD31 as positive and negative measures for the identification of human endometrial stem cells were suggested. Flow cytometry can be used for detection of expression of these markers at third passages.
3. In vitro differentiation into osteoblasts (osteogenic differentiation), adipocytes (adipogenic differentiation), and chondroblasts (chondrogenic differentiation) should be evaluated using Alizarin Red, Oil Red O, and Alcian Blue staining, respectively.

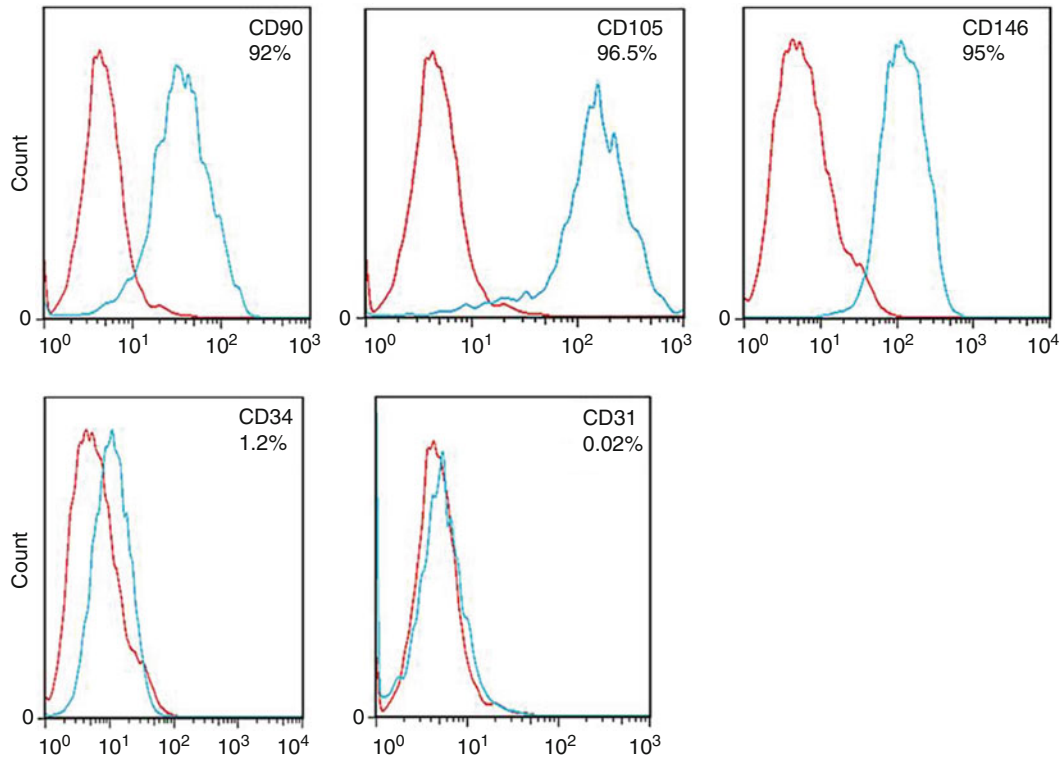

Fig. 2 Flow cytometry analysis for human endometrial stem cells after third passage

3.6 Cryopreservation and Banking

Freezing of cells for cell banking can be performed in confluency of 80–90% (Fig. 3).

1. Prepare complete medium if necessary.
2. Warm complete medium, PBS, and Trypsin-EDTA or TrypLE™ in the water bath at 37 °C.
3. Transfer the cultures under the biohazard hood.
4. Aspirate and discard the supernatants.
5. Add the PBS wash solution (5 ml for T25 flasks, 10 ml for T75 flasks, and 20 ml for T150 flasks and gently shaking for 30s).
6. Add Trypsin-EDTA solution into the cells (2 ml for T25 flasks, 4 ml for T75 flasks, and 8 ml for T150 flasks).
7. Incubate the cells in the CO_2 incubator for 15 min to allow cell detachment.
8. After inactivation of enzyme, transfer resuspended cells to 15 ml tubes, and centrifuge for 7 min at 300 × *g*, at room temperature.
9. In the biological safety cabinet, open the centrifuged tubes, carefully discard the supernatants, gently shake the pellets, and count the cells.

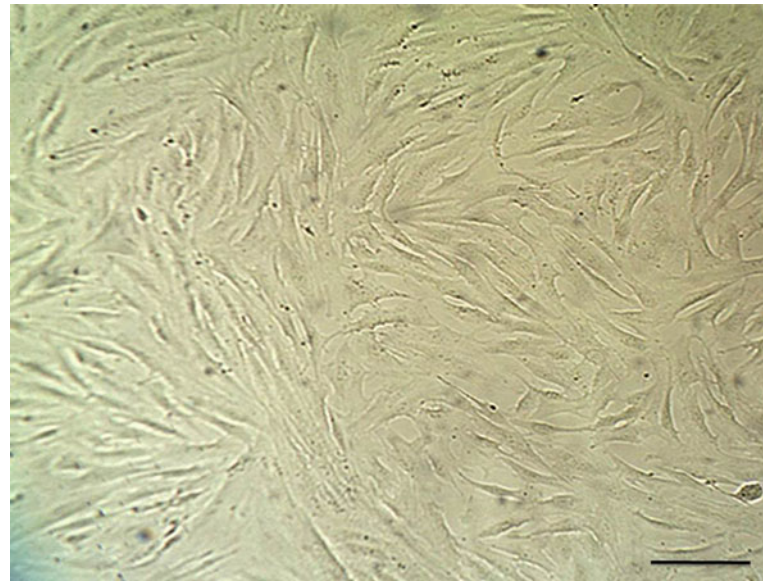

Fig. 3 Phase-contrast microscope morphological observation of human endometrial stem cells after third passage with 90% confluency. Scale bar: 100 μm

10. Set the number of cryovials to use for freezing: 1 cryovial up to 3×10^6 _0.5×10^6 cells. Cryovials as final product containers should be sterile, while their components should be non-pyrogenic, non-cytotoxic, DNA-free, DNase-free, and RNase-free.
11. Place the empty cryovials in a refrigerated rack.
12. Prepare the cell freezing mixture in a tube: 0.1 ml of DMSO and 0.9 ml of FBS (mixture for one cryovial) (*see* **Notes 11** and **12**).
13. Resuspend, filter, and place the mixture in the refrigerated rack.
14. Carefully discard the supernatant of the centrifuged tubes, and gently resuspend the cell pellet.
15. Add 1.6 ml of the freezing mix to each pellet, drop by drop, using a sterile pipette while gently shaking the tubes with the other hand, and gently resuspend and transfer into the cryovials.
16. Close the cryovials, and put them in a storage box that enables a slow decrease of the temperature (about 1 °C per minute).
17. Exit the clean room and place the container into a freezer set at a 75 °C temperature.
18. After about 24 h place the cryovials in the vapor phase of a liquid nitrogen tank.

19. Put a label on the outer container or cryovials, which must contain:
    (a) Identification of the manufacturer
    (b) Identification of the product
    (c) Indication of the use (i.e., autologous use only)
    (d) Batch number
    (e) Identification code of the patient
    (f) Expiration date
    (g) Signature of the person in charge

4 Notes

1. Endometrium biopsy is isolated during secretory phase of endometrium on 14th to 28th day from 30- to 45-year-old women (without endometriosis and with MS disease). These women had not taken exogenous hormones for 3 months before specimen collection.
2. The biopsy should be delivered to the laboratory within 2–18 h [28].
3. For GMP-grade cellular products manufacturing, using xeno-free supplements are suggested. Alternatively, in the absence of xeno-free supplements, it is recommended to use GMP-grade, clinical-grade, and Pharma Grade reagents. In this context, using FBS Pharma Grade which is covered in the European Medicines Agency (EMA) guidelines (to help medicine developers for applying marketing authorization applications) is favorable.
4. TrypLE™ as an animal origin-free recombinant fungal trypsin-like protease can be selected and provided for an efficient dissociation of various mammalian adherent cells and also should be manufactured at a cGMP-compliant facility registered with the US Food and Drug Administration [29, 30].
5. Advanced therapy medicinal products (ATMP) are based on the cell and gene products which include gene therapy medicinal products and somatic cell therapy medicinal product (tissue-engineered product) [31]. In Europe, it is necessary that, GMP facility (cell factory) allowed to produce ATMP products with the competent authority [32]. Moreover, for achieving reasonable safety, all manufacturers should conduct a quality management system (QMS) (facilities, standard operating procedures (SOPs), personnel, etc.) and should be compliant with the cGMP guideline principles.

6. For clinical purposes using cell-based medicinal products (CBMPs), a high level of protection in quality management and quality assurance structure such as cGMP is required [33]. cGMP involves all of cell manufacturing processes such as facilities, instruments, equipment maintenance, human resources, documentation, standard operating procedures (SOPs), reporting, environmental control and monitoring, validation, labeling, storage requirements, risk management, and training [34].
7. Use pre-warmed media (37 °C) for all steps [35, 36].
8. Use PBS+ at the first time of medium refreshment [37]. Calcium and magnesium in PBS+ help in the attachment of cultured cells [38].
9. Do not use PBS+ for cell passage or cell isolation [39, 40].
10. An incubator with a separate CO_2 supply for preparing some backup flasks for each sample is required [38, 41].
11. Due to deposition of ice in the extracellular environment, the freezing procedure should be done slowly. In fact, rapid freezing leads to ice crystal formation within the cells, resulting in the rupture of the plasma membrane at the time of thawing [42]. As the DMSO makes cell membranes permeable, the freezing mixture encloses also FBS at high concentration in order to maintain cell integrity. CryoSure-DMSO USP grade is sterile, endotoxin-free, pyrogen-free, and also free of mycoplasma [43].
12. DMSO in the cryopreservation protocol should be used at lower concentrations and also be removed before clinical transplantation because the DMSO can cause various adverse effects and toxic reactions on cells [44, 45].

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GMP-Compliant Production of Human Placenta-Derived Mesenchymal Stem Cells

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Abstract

Mesenchymal stem cells are one of the most attractive sources for stem cell research and therapy. Their safety and efficacy have been demonstrated in many clinical trials. Because of their low immunogenicity and immunomodulatory properties, allogenic MSCs have been transplanted in different clinical studies. MSCs could be in different adult- and fetal-derived tissues including pregnancy products. Placenta-derived mesenchymal stem cells (PLMSCs) that can be harvested without using any invasive procedures from a discarding tissue are one of the important types of mesenchymal stem cells for therapeutic applications. Stem cell manufacturing for therapeutic applications should be in compliance with the basic principles of good manufacturing practice (GMP). Herein, the current chapter is to describe GMP-compliant production of human PLMSCs, which are suitable for clinical applications.

Keywords Cell culture, Cell therapy, Clinical application, GMP, Mesenchymal stem cells, Placenta

Abbreviations

CBMPs	Cell-based medicinal products
DMEM-LG	Dulbecco's modified Eagle's medium-low glucose
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GMP	Good manufacturing practice
MNCs	Mononuclear cells
MSCs	Mesenchymal stem cells
PBS	Phosphate-buffered saline
Pen/Strep	Penicillin/streptomycin
PLMSCs	Placenta-derived mesenchymal stem cells
SOP	Standard operating procedure
WHO	World Health Organization

1 Introduction

Mesenchymal stem cells (MSCs) as multipotent cells with self-renewal and differentiation capacity are being used progressively in clinical trials due to their diverse functional properties [1–5]. MSCs are widely popular for therapeutic use because of their low immunogenicity, simple manufacturing procedure, and less ethical considerations than other counterparts [6–8]. Moreover, they can be expanded in vitro and isolated from various sources such as the bone marrow, adipose tissue, peripheral blood, umbilical cord, placenta, fetal tissues, muscle, and lung [9–11]. Placenta, which can be harvested in large quantities without using any invasive procedures from discarding pregnancy products, is an attractive source of MSCs for clinical applications. Placenta-derived MSCs (PLMSCs) have been employed for the treatment of a variety of disorders, e.g., liver, neurological, and cardiac diseases [12–14]. For clinical application, like other cell-based medicinal products (CBMPs), PLMSCs should be manufactured under the principles of good manufacturing practice (GMP) [15–17]. Adhering to the GMP principles ensures that the product meets preset criteria and the manufacturing process is reproducible [18–20]. Hereupon, the current chapter covers the manufacturing of GMP-compliant human PLMSCs for clinical applications.

2 Materials

2.1 Human Placenta Tissue Procurement

1. Human placenta (collected from a healthy mother during the routine cesarean section).
2. Sterile tissue bag.
3. Cool box with ice.
4. Temperature date logger (TESTO, Germany).

2.2 Placenta Tissue Harvesting, PLMSCs Manufacturing, and Banking

1. 70% ethanol (kimia alcohol zanjan, Iran).
2. Injectable normal saline (IPH Co., Iran).
3. Phosphate-buffered saline (PBS) (CliniMACS®, Miltenyi Biotec, Germany).
4. Collagenase NB6 GMP grade (SERVA Electrophoresis, Germany).
5. Ficoll-Paque PREMIUM (GE Healthcare, USA).
6. Dulbecco's modified Eagle's medium-low glucose 1 g/100 ml (DMEM-LG) (Biowest, USA).
7. Fetal Bovine Serum (FBS) Biopharm—EDQM certified (Biowest, USA)—(*see* **Note 1**).
8. Trypan blue solution 0.4% (Invitrogen, USA).

9. TrypLE™ Select (Thermo Fisher Scientific, USA) (*see* **Note 2**).
10. CryoSure-dimethyl sulfoxide (DMSO) USP grade (WAK-Chemie, Germany).
11. StemPro™ Adipogenesis Differentiation Kit (Thermo Fisher Scientific, USA).
12. StemPro™ Osteogenesis Differentiation Kit (Thermo Fisher Scientific, USA).
13. NucleoCassette™ (Chemometec, Denmark).
14. Clean room (GMP) facility (*see* **Note 3**).
15. Biological safety cabinet (Esco, Singapore).
16. Inverted microscope with phase-contrast (Nikon, Japan).
17. Shaking water bath (Memmert, Germany).
18. Weighing balance (Sartorius, Germany).
19. NucleoCounter® NC-100™ (Chemometec, Denmark).
20. Refrigerated centrifuge (swing-out rotor with buckets for 50 and 15 ml tubes) (Hettich, Germany).
21. CO_2 incubator (set at 5% CO_2, 37 °C, and 95% relative humidity) (Memmert, Germany).
22. Ultralow temperature freezer (New Brunswick Eppendorf, USA).
23. Liquid nitrogen tank (Statebourne Cryogenics, UK).
24. Motorized pipette (Orange Scientific, Belgium).
25. Sterile clean room clothing (face masks, hood, powder-free gloves, boots, and coveralls).
26. 100 × 15 mm petri dish (SPL, Korea).
27. Sterile serological pipettes 5, 10, and 25 ml (SPL, Korea).
28. Sterile scissors, tweezers, and surgical tray.
29. Sterile conical tubes 15 and 50 ml (SPL, Korea).
30. Filter cap cell culture flasks (300, 175, 75, and 25 cm^2) (TPP, Switzerland).
31. Hemocytometer and cover glass.
32. Pipette tips: crystalline, yellow, and blue (TPP, Switzerland).
33. 0.2 μm sterile syringe filter (Corning, USA).
34. Falcon® 100 μm cell strainer (Corning, USA).
35. Cryovial 2 ml (Corning, USA).
36. Mr. Frosty freezing container (Nalgene™, Thermo Fisher Scientific, USA).

3 Methods

3.1 Human Placenta Tissue Procurement

The human term placenta must be collected during the routine cesarean section after obtaining full informed consent from the donor (healthy mother) based on ethical committee approval. A detailed donor evaluation procedure should be performed through checking the medical history, laboratory tests, and physical examination (*see* **Note 4**).

The placenta tissue should be aseptically retrieved in operating room, double-packed, immersed in injectable normal saline, labeled, and transported in cool box (contains ice pack and data logger) to the GMP facility (*see* **Note 5**).

3.2 Controls Prior to Placenta Tissue Processing

All steps of the tissue processing should be performed in a clean room using appropriate aseptic techniques by a qualified person. Clean room should be regularly cleaned and monitored to ensure continuous compliance with GMP (Fig. 1). According to the GMP, aseptic processing with open procedures should be performed in a class A area (biosafety cabinet) with class B background (*see* **Note 6**).

1. All donor-related documents (which are provided based on the organization's SOPs) such as physical examination and laboratory test documents, past medical history, and written informed consent should be checked.
2. After receiving the cool box, tissue bag should be transferred to refrigerator and stored at 4 °C until the processing time. Data logger should be checked to ensure maintenance of appropriate temperature (1–10 °C) during transportation.
3. Before starting tissue processing, the biosafety cabinet should be turned on, wiped with 70% ethanol, and ventilated for 15 min to allow the cabinet for purgation.
4. The environmental conditions of the processing room should be monitored; environmental parameters (like particle counts) should be checked and recorded based on the SOPs and should be in the predetermined range.
5. To avoid unnecessary traffic in clean area, all required instruments and materials should be transferred to the clean room (production site).
6. All maintenance programs should be documented utterly.
7. All equipment including CO_2 incubator and biosafety cabinet should be sanitized and disinfected in accordance with the particular SOPs.

Fig. 1 Clean room maintenance and monitoring. (**a**, **b**) Routine maintenance of grade (**a**) room, (**c**) nonviable particle counting with laser particle counter, (**d**) viable particle monitoring with active air sampler

3.3 Placenta Tissue Processing and MSCs Isolation

1. Record the beginning time of the processing in appropriate form (based on the SOP; *see* **Note 6**).
2. Spray 70% ethanol on the outside of tissue bag, and transfer it to clean room facility.
3. Remove the outer layer of tissue bag, and transfer the inner layer into the biosafety cabinet which is located in grade B clean room. Open the inner layer under the biosafety cabinet, and aseptically transfer the tissue to a sterile surgical tray.
4. Collect a sample of the transfer medium to perform microbiological tests.
5. Record the weight of the placenta tissue.
6. Rinse it thoroughly with normal saline to remove all excess blood.
7. Put it on another sterile surgical tray.
8. Remove the placenta membrane (amnion and chorion), and collect the remaining placenta tissue (chorionic villi with mesenchymal core).

9. Put the tissue specimen on 100 × 15 mm petri dish, and cut it into small fragments with sterile scissors and tweezers.
10. Based on the volume of the dissected samples, transfer them to one or multiple 50 ml conical tubes.
11. Add sterile enzymatic solution containing 1 mg/ml collagenase NB6 and CliniMACS® PBS for digestion. The volume of enzymatic solution should be equal to the sample volume.
12. Place the tubes (containing samples) in 37 °C shaking water bath for at least 1 h. Check the sample in regular interval, and remove it after complete digestion.
13. Add equal volume of cooled CliniMACS® PBS to digested tissue.
14. Pass the tissue digest once through a 100 μm cell strainer.
15. Centrifuge it at 300 × *g* for 5 min at 4 °C.
16. Discard the supernatant, and layer the cell suspension gradually on Ficoll (3:7 cell suspension/Ficoll) in 15 ml conical tubes.
17. Centrifuge at 450 × *g* for 20 min (room temperature).
18. Then collect the mononuclear cells (MNCs) layer, and throw away sediment (contain red blood cells).
19. Add CliniMACS® PBS and wash the MNCs twice at 100 g for 10 min.
20. Discard the supernatant, resuspend the cell pellet in culture medium, and count the MNCs using an appropriate method (such as NucleoCounter® device).
21. Approximately each 10–15 × 10^6 MNCs is seeded into a T75 culture flask.
22. Use DMEM-LG supplemented with 10% FBS Biopharm as complete culture medium.
23. Place the culture flasks in incubator (37 °C, 5% CO_2, and 95% humidity) (*see* **Note 7**).

A pictorial summary of aforementioned procedures has been demonstrated in Fig. 2.

3.4 PLMSCs Manufacturing

1. After 24 h cell, check the flasks for any sign of contamination.
2. Spindle-shaped PLMSCs should be visible under inverted microscope after 48–72 h (Fig. 3). At this time, under biosafety cabinet, discard culture medium, and wash the flask with pre-warmed CliniMACS® PBS to remove red blood cells and non-adherent cells. Then add fresh culture medium (the cell culture medium should be changed twice per week).
3. When PLMSCs reach approximately 85–90% confluency (Fig. 3), remove the media and wash them with pre-warmed PBS.

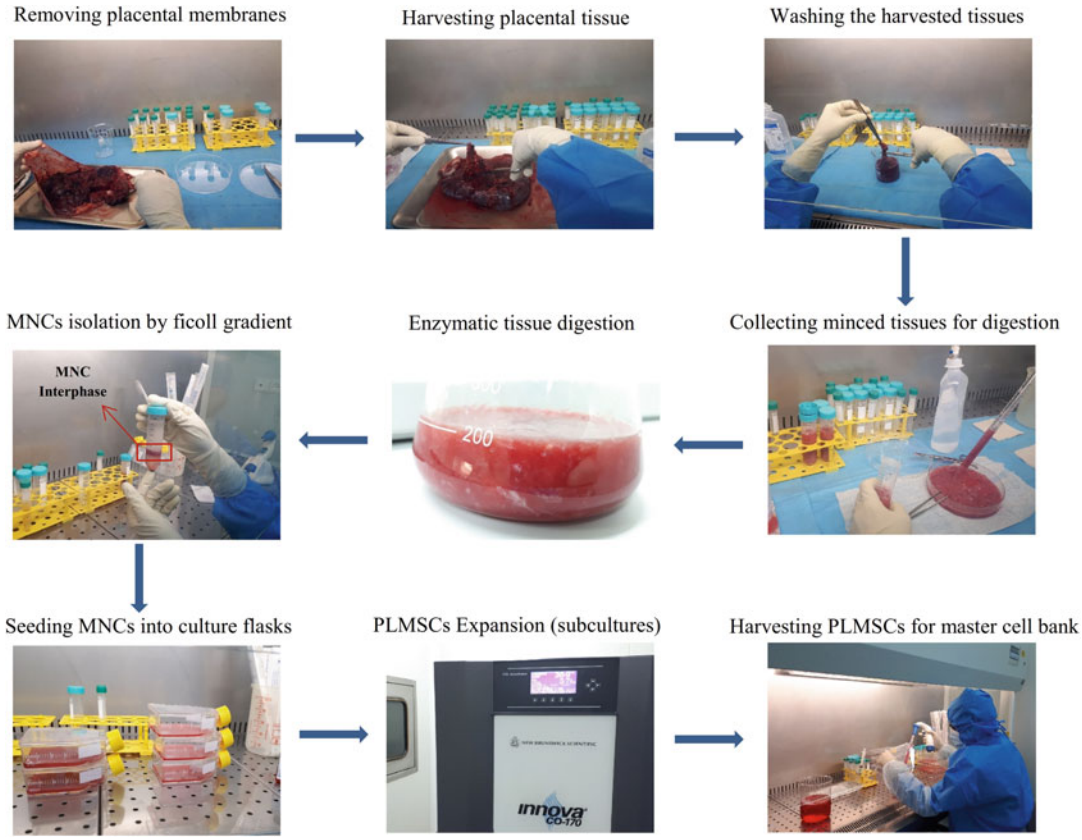

Fig. 2 PLMSCs' manufacturing process in our GMP facility

4. Then, add TrypLE™ Select (2–3 ml for 75 cm^2 flask and for other flasks, adjust the volume according to their surface) for cell dissociation.
5. Incubate the flasks in the CO_2 incubator for 5–10 min, and check them for cell detachment.
6. After completion of cell dissociation, add equal volume of PBS, and transfer the cell suspension to 50 ml conical tube(s).
7. Centrifuge at 300 × *g* for 5 min.
8. Discard the supernatant gently, and resuspend the cell pellet in appropriate amount of complete culture medium.
9. Use trypan blue 0.4% solution (1:1 dilution) and the hemocytometer to count PLMSCs, and measure the cell viability.
10. Record all parameters according to the prepared SOP (*see* **Note 6**).
11. Subculture the PLMSCS at a density of 10^4 cells/cm^2 in the new flasks.

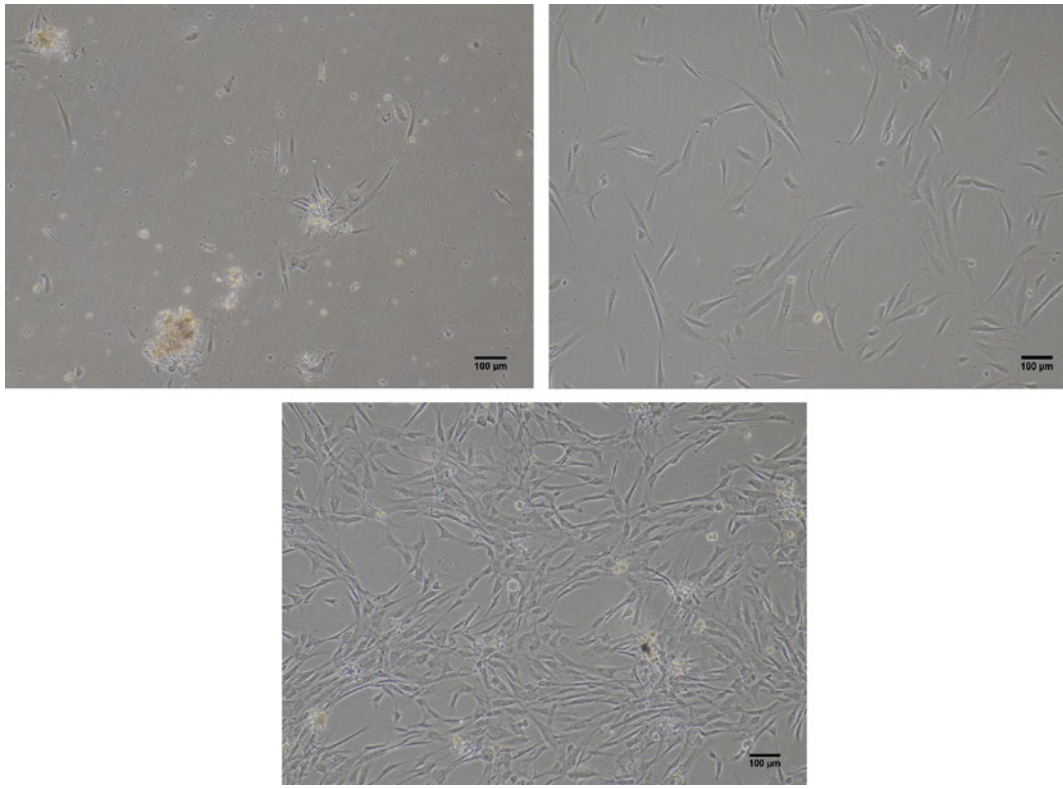

Fig. 3 Microscopic appearance of PLMSCs at primary culture (3rd to 12th days)

12. Put the flasks in the CO_2 incubator (37 °C, 5% CO_2, humidified), and continue the cell expansion procedure up to yielding the sufficient number of PLMSCs for making a master cell bank (usually 2–3 subcultures). During cell expansion and before cell banking, different samples should be collected for microbiological studies. Mycoplasma contamination (PCR method) and endotoxin (LAL Gel clot) should be checked before cryopreservation.

3.5 Characterization of PLMSCs

1. Check the karyotype of PLMSCs at different subcultures including the first subculture and working cell bank (before using for clinical application, karyotype must be normal) (Fig. 4a).
2. Check the immunophenotypes (CD markers) using flow cytometry (*see* **Note 8**) (Fig. 5).
3. Check in vitro differentiation capacity of cells into adipocytes (adipogenic differentiation) and osteoblasts (osteogenic differentiation), and confirm it by Oil Red O and Alizarin Red staining, respectively. To evaluate the differentiation capacity, commercially available media was used (Fig. 5).

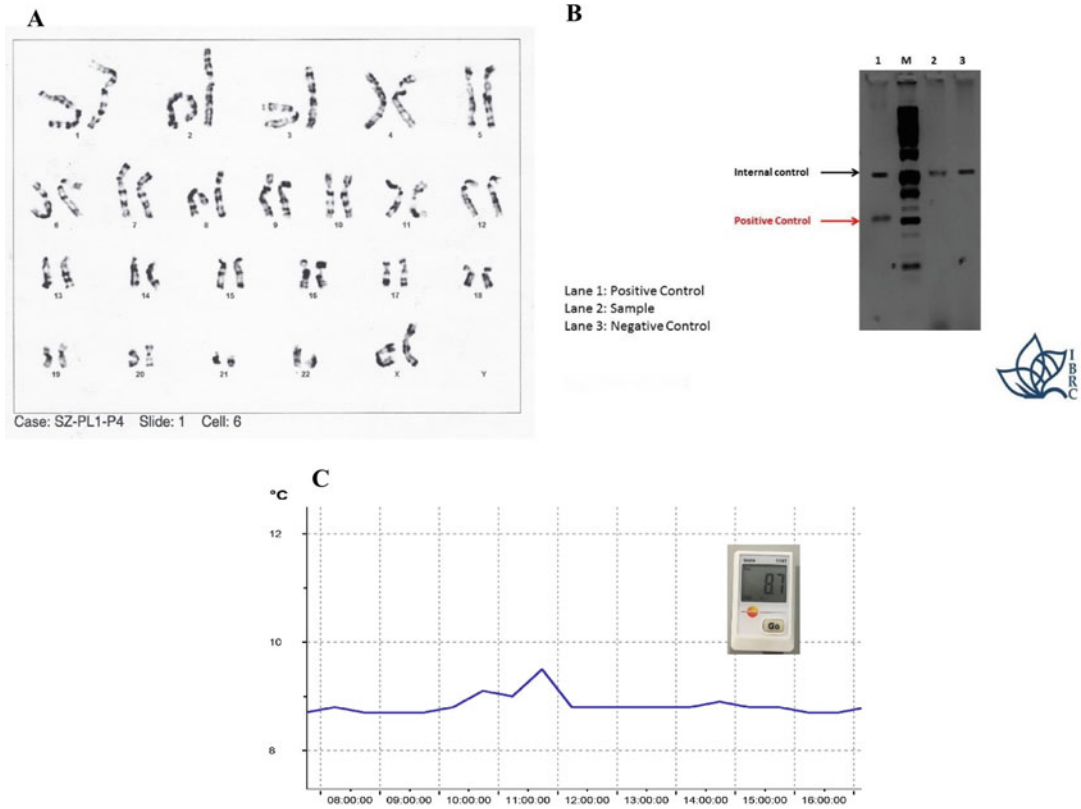

Fig. 4 Some qualification assays. (**a**) Karyotyping, (**b**) PCR-based mycoplasma test, (**c**) verification of transportation temperature with data logger

3.6 Cryopreservation and Banking

Freezing for cell banking can be accomplished in 80–90% confluency.

1. Harvest the PLMSCs using TrypLE™ Select (as described in Subheading 3.4).
2. Check cell count and viability by using NucleoCounter device.
3. Prepare the freezing media by adding 10% DMSO to complete culture medium (*see* **Note 9**).
4. Suspend the PLMSCs in 1.5 ml freezing media, and divide them into 2 ml cryovials (about 10×10^6 cells/vial) (*see* **Note 10**).
5. Close the cryovials, and put them in pre-cooled Mr. Frosty freezing container (*see* **Note 11**).
6. Place the container into an ultralow temperature freezer (−80 °C), and wait at least overnight.
7. Then remove the cryovials, and put them in the vapor phase of a liquid nitrogen tank.

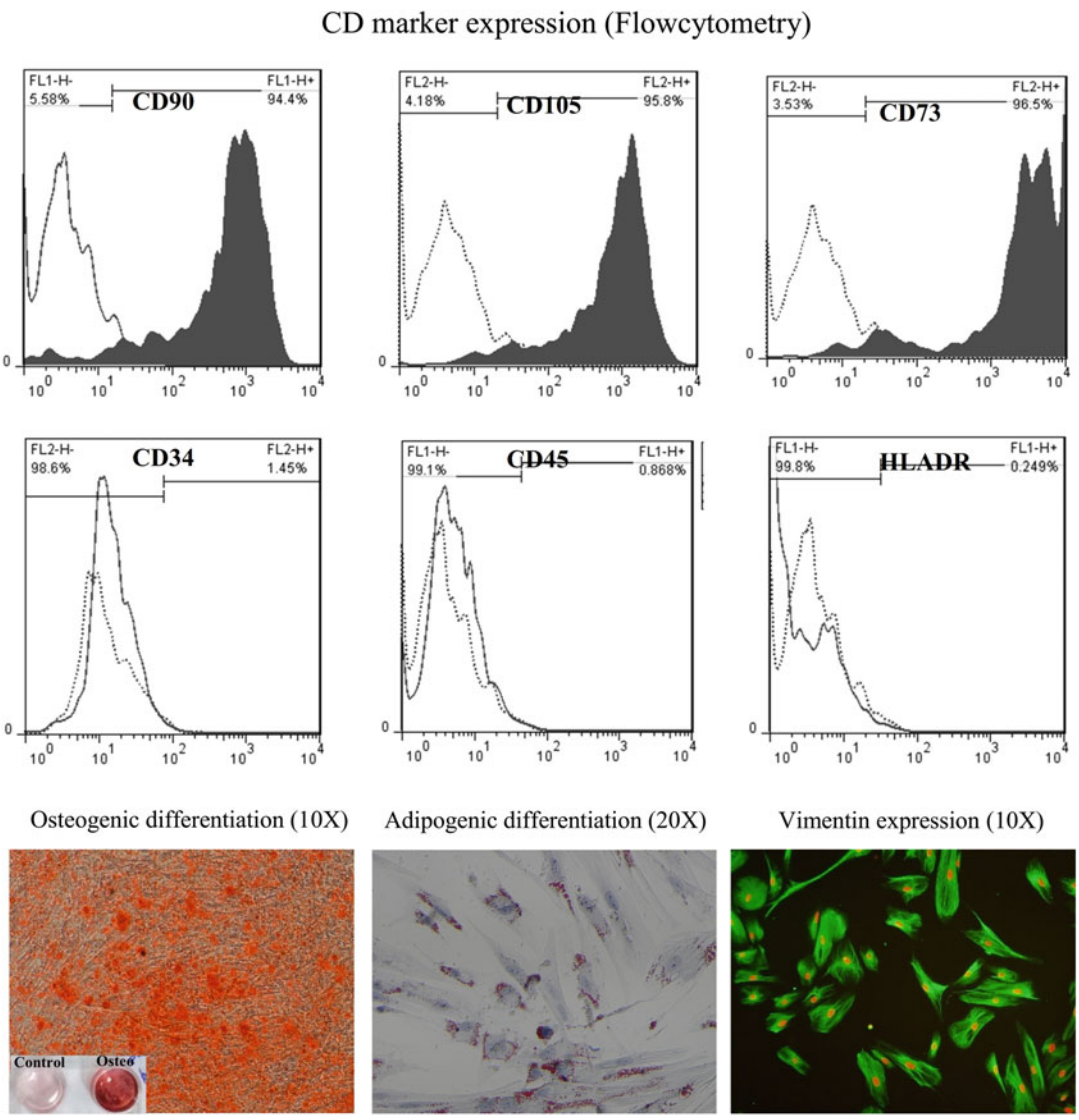

Fig. 5 PLMSCs' characterization

4 Notes

1. For GMP-grade production of cellular products, using a qualified FBS (with full traceability) is strongly recommended. The European Directorate for the Quality of Medicine & HealthCare (EDQM) lists the TSE-certified FBS in its website (which updated periodically). In the current protocol, FBS Biopharm (from Biowest Company) has been used for serum supplementation. This FBS is EDQM certified and has been suggested by the company for biopharmaceutical manufacturing and other

applications where the highest standard of product quality and documentation is required.

2. TrypLE™ Select, as an animal origin-free recombinant enzyme which is manufactured according to GMP principles, can dissociate a wide range of adherent mammalian cells [21, 22, 24].
3. Clean room facility as enclosed spaces with controlled temperature, humidity, air pressure, and air particles is crucial for conducting GMP-based and clinical-grade production [21, 22, 25].
4. Sample of the donor's blood must be collected to conduct serological tests including HBsAg, HBcAb, HCV antibody, HIV-1 and HIV-2 Ab, HTLV 1/HTLV-2 Ab, EBV Ab, CMV Ab, Toxoplasma Ab, and RPR for syphilis. If it is not possible to retest the donor after 180 days, the nucleic acid tests for HIV, HBV, and HCV should be performed [21–23, 26].
5. All data about the tissue and donor, e.g., age and weight of the tissue, must be written on a proper label and placed on the cool box.
6. Application of cell-based medicinal products (CBMPs) for therapeutic objects needs a high level of assurance in quality management including standard operating procedures (SOPs), environmental control and monitoring, equipment maintenance, validation, labeling, storage requirements, documentation, risk management, reporting, and training [21–23].
7. It is suggested to prepare some backup flask for each sample in another incubator with separate CO_2 source.
8. The expression of CD105, CD73, and CD90 and the lack of expression of hematopoietic markers, i.e., CD34, CD45, and CD14 or CD11b, CD79a or CD19, and HLA-DR, as positive and negative measures for the identification of PLMSCs were suggested [27–29].
9. Use lower concentrations of DMSO (5–10%) to reduce different adverse effects and toxic reactions on cells [21, 22, 30].
10. Cryovials must be sterile with non-pyrogenic, non-cytotoxic, DNA-free, DNase-free, and RNase-free components [21, 22].
11. Cell freezing proceedings must be accomplished rapidly.

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GMP-Compliant Human Schwann Cell Manufacturing for Clinical Application

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Abstract

Schwann cells as glial cells in the peripheral nervous system can participate in neurons protection and forming myelin. Additionally, they are important for nerve pulse conduction supporting along axons. On the other hand, it was demonstrated that they are promising cells for the treatment of demyelinating disorders and also central nervous system damages. Herein, for therapeutic application, Schwann cells should be manufactured based on good manufacturing practice standards to achieve safe and effective clinical products. In this respect, the current chapter tries to introduce a standard protocol for manufacturing of human GMP-compliant Schwann cells for clinical application.

Keywords Clinical application, GMP, Peripheral nervous system, Quality assurance, Schwann cells

Abbreviations

CNS	Central nervous system
DMEM-LG	Dulbecco's modified eagle medium-low glucose
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
GMP	Good manufacturing practice
PBS	Phosphate-buffered saline
PNS	Peripheral nervous system
QA	Quality assurance
SCs	Schwann cells
SOP	Standard operating procedures

1 Introduction

The nervous system as a complicated system has two main parts: (1) central nervous system (CNS) and (2) peripheral nervous system (PNS). CNS and PNS can control and regulate sensory and motor activities. More precisely, CNS and PNS are composed of

neurons and glial cells (glia). Neurons are electrically sensitive cells which are responsible for transmitting nervous information around the body. Glial cells can contribute neurons supporting and protection along with forming myelin as a protecting coat around nerve fibers. Schwann cells (SCs) as the glial and myelinating cells within the PNS have key roles in supporting nerve pulses conduction along axons [1–4]. They are easily accessible and can be expanded in vitro [5]. Moreover, SCs have revealed a great promising result for cell therapy in demyelinating disorders and central nervous system damages such as spinal cord injuries [6–10]. In this respect, manufacturing of SCs is important basic step in their clinical application procedure which requires strict control based on good manufacturing practice (GMP) principles [8, 9]. Indeed, GMP is a professional quality assurance (QA) system which contribute safe and reasonable biomedical products producing. Additionally, it covers all aspects of manufacturing containing the equipment, materials, staff training, and environment [11–15]. Accordingly, in this chapter standard protocol for manufacturing of human SCs will be described and discussed based on GMP principles.

2 Materials

2.1 Human Peripheral Nerve Procurement

1. Selected donors (brain death or amputation).
2. Tissue container.
3. Cool box with ice.
4. RPMI-1640 medium (ICN, USA).

2.2 Human Schwann Cell Isolation, Culture, Subculture, Cryopreservation, and Banking

1. 70% ethanol (Kimia Alcohol Zanjan, Iran).
2. Phosphate-buffered saline (PBS) (CliniMACS®, Miltenyi Biotec, Germany).
3. Collagenase NB6 GMP grade (SERVA Electrophoresis, Germany).
4. Dulbecco's Modified Eagle Medium-low glucose 1 g/100 ml (DMEM-LG) (Biowest, USA).
5. Fetal Bovine Serum (FBS) Biopharm-EDQM certified (Biowest, USA)—(*see* **Note 1**).
6. Transferrin (Sigma, USA).
7. Insulin (Sigma, USA).
8. Trypan blue solution 0.4% (Invitrogen, USA).
9. TrypLE™ Select (Thermo Fisher Scientific, USA) (*see* **Note 2**).
10. CryoSure-dimethyl sulfoxide (DMSO) USP grade (WAK-Chemie, Germany).
11. Clean room (GMP) facility (*see* **Note 3**) (Fig. 1).

Fig. 1 Clean room (GMP) facility. Clean room is environmentally controlled aria which is required for manufacturing of biomedical products in accordance with GMP principles

12. Sterile clean room clothing (face masks, hood, powder-free gloves, boots, and coveralls).
13. Biological safety cabinet (Esco, Singapore).
14. Sterile scissors, forceps, and surgical tray.
15. 10 cm petri dish (SPL, Korea).
16. Sterile Serological Pipettes 5 and 10 ml (SPL, Korea).
17. 0.2 μm sterile syringe filter (Corning, USA).
18. Sterile conical tubes 15, 50 ml (SPL, Korea).
19. Falcon® 100 μm Cell Strainer (Corning, USA).
20. Filter cap cell culture flasks (75, and 25 cm^2) (TPP, Switzerland).
21. Water bath (Memmert, Germany).
22. Inverted microscope with phase contrast (Nikon, Japan).
23. Weighing balance (Sartorius, Germany).
24. Hemocytometer and cover glass.
25. NucleoCassette™ (Chemometec, Denmark).
26. NucleoCounter® NC-100TM (Chemometec, Denmark).
27. Pipette tips: crystalline, yellow, and blue (TPP, Switzerland).
28. Motorized pipette (Orange Scientific, Belgium).
29. CO_2 incubator (set at 5% CO_2, 37 °C, and 95% relative humidity) (Memmert, Germany).
30. Liquid nitrogen tank (Statebourne Cryogenics, UK).

31. Refrigerated Centrifuge (swing-out rotor with buckets for 50 and 15 ml tubes) (Hettich, Germany).
32. Ultralow-temperature freezer (New Brunswick Eppendorf, USA).
33. Mr. Frosty freezing container (Nalgene™, Thermo Scientific, USA).
34. Cryovial 2 ml (Corning, USA).

3 Methods

3.1 Peripheral Nerve Samples Preparation

1. After getting informed consent, under the aseptic and sterile condition in the operating room, harvest peripheral nerves (sural nerves) from brain death donors or amputated limbs.
2. Wash samples with PBS.
3. Place samples in tissue container including RPMI-1640 medium and transfer it to GMP facility(clean room) by a sterile cool box with ice (*see* **Note 4**).

3.2 Controls Prior to Processing and Schwann Cell Isolation

1. Provide and check donor-related documents based on the organization's SOPs such as laboratory tests documents, past medical history, and written informed consent (*see* **Note 5**).
2. Check the donors' blood samples for HBsAg, HBcAb, HCVAb, HIV1, 2 Ab, HTLV1, 2 Ab, and RPR.
3. Keep harvested samples at 4 °C until the processing time.
4. Check the environmental parameters based on the SOPs (*see* **Note 5**).
5. Transfer all required instruments and materials to the clean room before starting the cell isolation process.
6. Turn on the biosafety cabinet before starting the cell isolation process, wipe with 70% ethanol, and ventilate for purgation (at least 15 min).
7. Sanitize and sterilize CO_2 incubator and biosafety cabinet due to the particular SOPs (*see* **Note 5**).

3.3 Human Schwann Cell Isolation and Culture

1. Record the start time of the cell isolation process according to the SOPs (*see* **Note 5**).
2. Spray 70% ethanol on the outside of the tissue container, and transfer it to the biosafety cabinet.
3. Transfer the peripheral nerve samples to a sterile 10 cm petri dish on the sterile surgical tray (Fig. 2a).
4. Obtain a sample of the transfer medium for performing microbiological tests (*see* **Note 6**).

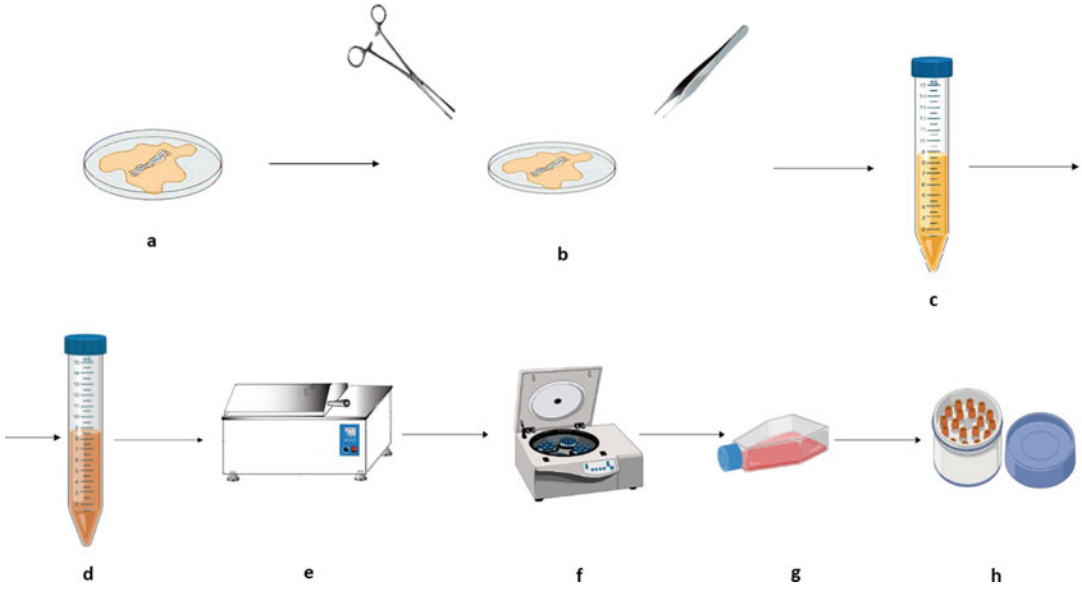

Fig. 2 Human peripheral nerve-derived Schwann cell Isolation, culture, subculture, and cryopreservation. (**a**) Peripheral nerve samples in a sterile 10 cm petri dish. (**b**) Cutting nerve fascicles into 1–2 mm pieces. (**c**) Piece in falcon conical tubes. (**d**) Adding sterile enzymatic solution containing collagenase and PBS for digestion. (**e**) Incubating the sample in 37 °C water bath and agitate every 10 min. (**f**) Centrifuging the cell suspension. (**g**) Seeding the cells in the culture flask. (**h**) Cryopreservation of cells

5. Cut nerve fascicles, remove adjacent connective tissue, and after that cut it into 1–2 mm pieces (Fig. 2b).
6. Transfer sample to 50-ml falcon conical tubes (Fig. 2c).
7. Add sterile enzymatic solution containing 0.125% collagenase and PBS for digestion (Fig. 2d).
8. Incubate the sample in 37 °C water bath, and agitate it every 10 min until the sample is fully digested (Fig. 2e).
9. Neutralize collagenase activity by adding DMEM with 10% FBS.
10. Centrifuge the suspension at 400 × *g* for 10 min (Fig. 2f).
11. Discard supernatant and re-suspend cell pellet in culture medium.
12. Filter it through a 100 μm cell strainer and centrifuge at 300 × *g* for 10 min.
13. Re-suspend cells in culture medium including DMEM, transferrin, and insulin (10 μg/ml) (do not use the serum for 48 h to prevent fibroblast contamination (fasting method)).
14. Use trypan blue 0.4% solution and the hemocytometer to count isolated SCs, and measure the number of viable cells/ml.

15. Confirm cell count and viability by using the NucleoCounter device.
16. Record all data according to the SOPs (*see* **Note 5**).
17. Seed the SCs in the T75 flasks (Fig. 2g).
18. Place the flasks in the CO_2 incubator (37 °C, 5% CO_2, humidified).
19. Check the flasks after 24 h for any signs of contamination (*see* **Note 7**).
20. After 48 h change the culture medium by complete culture medium (DMEM with 10% of FBS).
21. Check the morphology of SCs after 48 to 72 h (spindle oval shape cytoplasm and a prominent nucleus should be visible under the inverted microscope).
22. Renew the culture medium every 3 days.

3.4 Schwann Cell Subculture

1. When SCs are in approximately 85–90% confluency, remove the medium and wash them with pre-warmed PBS.
2. Add TrypLE™ select for cell dissociation.
3. Put flasks in the CO_2 incubator for 5–10 min.
4. Check cell detachment under inverted microscope (*see* **Note 8**).
5. Neutralize TrypLE™ select activity by adding DMEM with 10% of FBS.
6. Transfer cell suspension into 15 ml falcon conical tubes under biosafety cabinet.
7. Centrifuge at 300 × *g* for 5 min.
8. Remove the supernatant gently.
9. Re-suspend cell pellet in culture medium softly.
10. Count re-suspended cells, and measure cell viability by using NucleoCounter.
11. Transfer around 10^5 cells per cm^2 into the T75 flasks.
12. Add complete culture medium (DMEM supplemented with 10% FBS).
13. Place the flasks in the CO_2 incubator (37 °C, 5% CO_2, humidified).

3.5 Characterization of Schwann Cells

1. Check the karyotype of SCs at different subcultures (*see* **Note 9**).
2. Check the immunophenotypic markers by immunostaining (*see* **Note 10**) (Fig. 3).

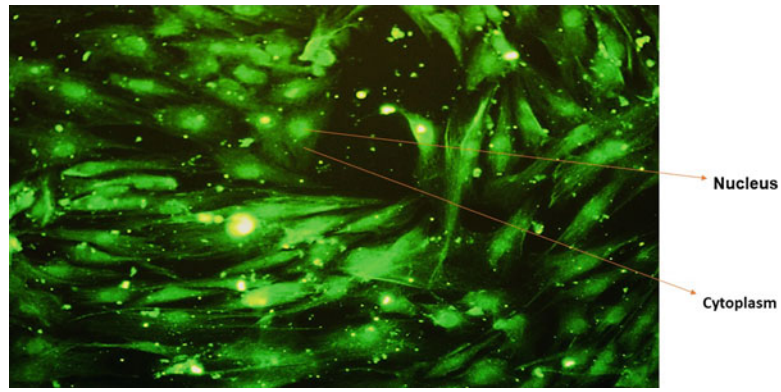

Fig. 3 S100 Staining of Cultivated Schwann Cells. Schwann cell morphology based on S100 staining in both cytoplasm and nucleus under inverted microscope (40×)

3.6 Cryopreservation and Banking for Clinical Transplantation

1. Provide freezing medium (40% DMEM, 50% serum, and 10% DMSO) (*see* **Note 11**).
2. Attach specific labels on cryovials (*see* **Notes 12** and **13**).
3. Harvest the SCs (similar to the steps which described in Subheading 3.4).
4. Count cells, and measure cell viability by using NucleoCounter.
5. Suspend the SCs in 1.5 ml freezing medium and transfer them into cryovials (*see* **Note 14**).
6. Record the number of cryovials and cell counts based on the SOPs (*see* **Note 5**).
7. Place cryovials in pre-cooled freezing container (Fig. 2h).
8. Put freezing container into the ultralow-temperature freezer (−80 °C) (for overnight).
9. After overnight transfer cryovials into the vapor phase of liquid nitrogen tank (−140 °C or −180 °C).
10. Record the list of stocked cell products according to the SOPs (*see* **Note 5**).

4 Notes

1. The use of qualified FBS (with traceable standards) is highly recommended to produce GMP grade cell-based products. The European Directorate confirms FBS Biopharm (from Biowest Company) for the Quality of Medicine & Healthcare (EDQM) for biopharmaceutical and biomedical manufacturing.

2. TrypLE™ Select as a GMP grade animal protein-free product can be used for passaging a various range of mammalian adherent cells for clinical grade production [16, 17].
3. Clean room facility as environmentally controlled spaces is required for managing clinical-grade production in accordance with GMP principles [18, 19].
4. During transportation data logger can be used to ensure maintenance of proper temperature (4 °C).
5. Standard operating procedure (SOP) is defined in more details as written guidelines to achieve the uniform performance of a particular function which it is in line with the goal of GMP principles [20–22].
6. Microbiological testing of harvested samples is vital for the production process to avoid probable contaminations.
7. Signs of contamination include opacity and changing the natural color of the culture medium.
8. Detached cell morphology changes to round-shaped, and they are free-floating in the enzyme solution [16, 17].
9. Before releasing cells for clinical transplantation, it should be ensured that the cell karyotype is normal [16].
10. S100 and P75 antibody staining can evaluate the immunoreactivity and purity of cultured SC [8, 9].
11. Since DMSO can be cytotoxic, it is recommended to use lower concentrations of DMSO in the cryopreservation protocol and eliminate it before transplantation [16, 23].
12. Sterile, non-pyrogenic, non-cytotoxic, DNase-free, and RNase-free cryovials must be used for clinical production [16, 17].
13. Labeling, coding, and tracing procedures must be conducted in accordance with the GMP principles [16, 24].
14. To prevent undesired effects of DMSO, the freezing and thawing process must be performed quickly.

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GMP-Compliant Adenoviral Vectors for Gene Therapy

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Abstract

Recently, gene therapy as one of the most promising treatments can apply genes for incurable diseases treatment. In this context, vectors as gene delivery systems play a pivotal role in gene therapy procedure. Hereupon, viral vectors have been increasingly introduced as a hyper-efficient tools for gene therapy. Adenoviral vectors as one of the most common groups which are used in gene therapy have a high ability for humans. Indeed, they are not integrated into host genome. In other words, they can be adapted for direct transduction of recombinant proteins into targeted cells. Moreover, they have large packaging capacity and high levels of efficiency and expression. In accordance with translational pathways from the basic to the clinic, recombinant adenoviral vectors packaging must be managed under good manufacturing practice (GMP) principles before applying in clinical trials. Therein, in this chapter standard methods for manufacturing of GMP-compliant Adenoviral vectors for gene therapy have been introduced.

Keywords Adenovirus, Clinical application, Gene therapy, GMP, Viral vector

Abbreviations

AAV	Adeno-associated virus
Ad	Adenovirus
AIDS	Acquired immune deficiency syndrome
BSL2	Biosafety level 2
CF	Cystic fibrosis
CPE	Cytopathic effect
FBS	Fetal bovine serum
GMP	Good manufacturing practice
LB	Luria broth
MEM	Minimum essential medium
PBS	Phosphate-buffered saline
pfu	Plaque-forming unit
QA	Quality assurance
RCA	Replication-competent adenovirus
RT	Room temperature

1 Introduction

A wide range of diseases, such as cancers, AIDS, cardio vascular diseases, cystic fibrosis (CF), diabetes, and hemophilia, are of worldwide great concerns. In accordance with published studies, gene therapy as one of the most promising treatments of the twenty-first century is a suitable candidate which can apply genes to treat such diseases. Until now, only a few successes of clinical trials related to gene therapies have been reported. Accordingly, numerous experiments are still needed in order to meet the expectations that gene therapy would emerge [1–4]. Vectors as gene delivery systems play a pivotal role in the progress of gene therapy procedures by delivering genes to the targeted cells [5, 6]. Vectors are divided into viral and non-viral types. Based on several studies, viral vectors have been increasingly introduced as a hyper-efficient tools for gene therapy [6–9]. Commonly they can be derived from adenovirus (Ad), adeno-associated virus (AAV), lentivirus, and retrovirus [10]. Ad vectors supply a multifaceted system for transferring mammalian genes and have a high capability for gene therapy. Indeed, they are not integrated into host genome. Further, they are distinctly used in vaccine developments and various gene therapy settings (notably for immunotherapy in gene-based cancer therapy) [11–13]. On the other hand, they can be adapted for direct transduction of recombinant proteins into targeted cells in addition to transduction of nucleic acids. Moreover, they have large packaging capacity, high levels of efficiency to infect cell types, and high levels of expression [10, 14–16]. Since it is important to follow GMP principles during translation of basic knowledges into clinical applications, recombinant Ad vector packaging (Fig. 1) must be fabricated under GMP principles before applying in clinical trials [17–19]. In fact, GMP compliant products are manufactured and controlled based on quality assurance (QA) standards [20–24]. Herein, the purpose of this chapter is introducing methods for manufacturing of GMP-compliant Ad vectors for gene therapy.

2 Materials

2.1 Reagents

1. Agarose (UltraPure, Invitrogen, USA) (1% w/v) (*see* **Note 1**).
2. Bacteria BJ5183 cells (*RecA*-proficient) (Addgene, USA).
3. Bacteria DH5α (*RecA*-deficient, Invitrogen, USA).
4. Buffer-saturated phenol (Thermo fisher, USA).
5. Cell lines, low-passage 293 cells (Microbix, Canada).
6. A549 cells (ATCC, USA).
7. 293N3S cells (Microbix, Canada).

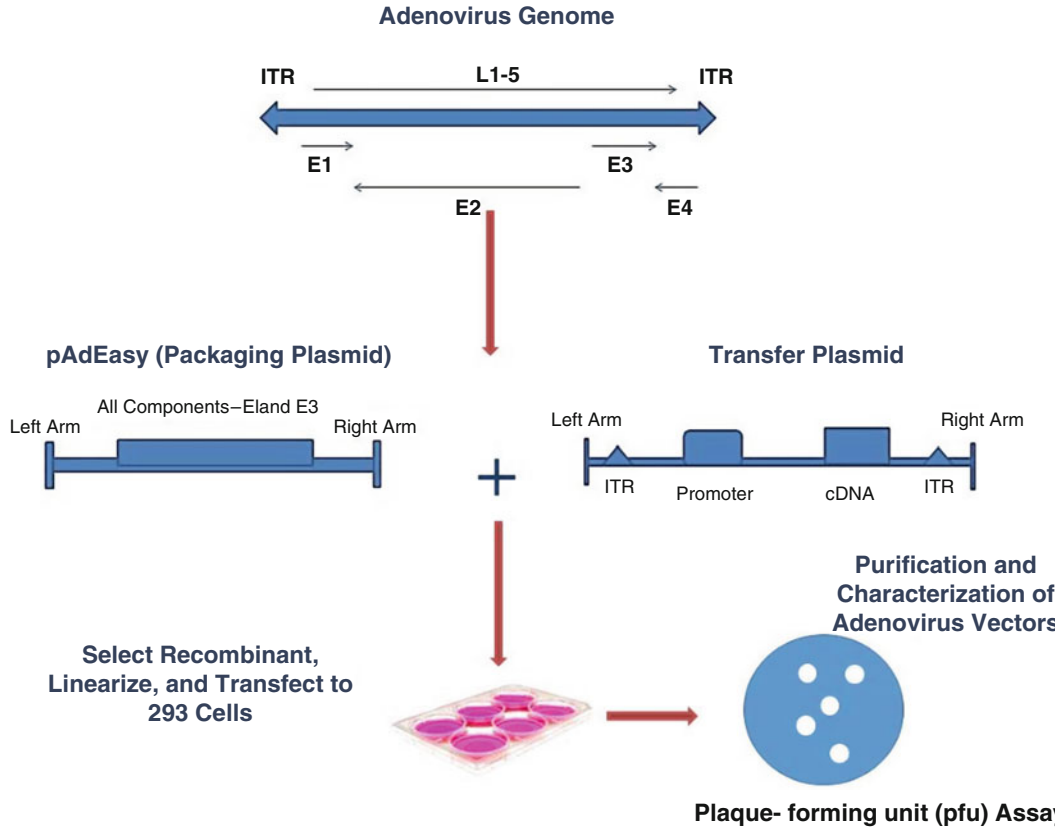

Fig. 1 Adenoviral vector packaging methods. The adenovirus genome is a double-stranded DNA including multiple spliced transcripts. Inverted terminal repeats (ITRs) are placed at either end of the genome. Genes are divided into early (E1–4) and late (L1–5) transcripts. Two genes (E1 and E3) are deleted in recombinant adenovirus. Adenoviral vector constructs have left and right arms for facilitating homologous recombination of the transgene into the plasmid. Recombinant plasmids should be verified for size and individual patterns of restriction digestion to discover that the transgene has been inserted. After verification, the recombinant plasmid should be linearized with Pac I (restriction enzyme) to produce a linear dsDNA construct. 293 cells can be transfected with the linearized construct, and about 7–10 days later virus can be harvested. Purified recombinant adenovirus vectors should undergo conventional quality checks to control its potency and purity prior to apply in gene therapy

8. Cesium chloride gradient solutions (Sigma-Aldrich, USA) (1.25 and 1.35 g/ml).
9. Chloroform: isoamyl alcohol (24:1 v/v) (Sigma Aldrich, USA).
10. 2× Citric saline (Medicago, Sweden).
11. 270 mM KCl (Medicago, Sweden).
12. 30 mM sodium citrate (Medicago, Sweden).
13. 500 ml of Minimum Essential Medium (MEM) (Sigma-Aldrich, USA).
14. 500 ml of 10% FBS (Sigma-Aldrich, USA).

15. 2 mM GlutaMAX (Invitrogen, USA).
16. 1× antibiotic-antimycotic (Invitrogen, USA).
17. Dialysis buffer (10 mM Tris–HCl at pH 8.0) (Thermo fisher, USA).
18. DNase I (Sigma-Aldrich, USA).
19. Ethidium bromide (Sigma-Aldrich, USA).
20. Isopropanol (Sigma-Aldrich, USA).
21. Kanamycin (25 g/ml) (Thermo fisher, USA).
22. Luria broth (LB) medium (Thermo fisher, USA).
23. $MgCl_2$ (2 M) (Sigma-Aldrich, USA).
24. 2 mM GlutaMAX (Invitrogen, USA).
25. 1× antibiotic-antimycotic (Invitrogen, USA).
26. 2× antibiotic/antimycotic (Invitrogen, USA).
27. Phosphate-buffered saline (PBS) (CliniMACS®, Miltenyi Biotec, Germany).
28. Plasmids (shuttle and backbone plasmids).
29. *Pac*I restriction endonuclease (New England BioLabs, USA).
30. *Pme*I restriction endonucleases (New England BioLabs, USA).
31. RNase A (10 mg/ml) (Sigma-Aldrich, USA).
32. NaCl (5 M) (Golhateb, Iran).
33. Sodium deoxycholate (5% w/v) (Sigma-Aldrich, USA).
34. SDS–proteinase K solution (Thermo fisher, USA).
35. SDS (0.1% w/v)-TE (Thermo fisher, USA).
36. Sucrose (Sigma-Aldrich, USA) (40% w/v).
37. SuperFect transfection reagent (QIAGEN, Germany).
38. Tris–HCl (10 mM) (Sigma-Aldrich, USA).
39. 1 mM EDTA (Sigma-Aldrich, USA).
40. 1× Trypsin-EDTA (Sigma-Aldrich, USA).
41. 70% ethanol (Merck, Germany).
42. Penicillin-Streptomycin (Pen-Strep) (ATOCEL, Australia).
43. Biosafety level 2 (BSL2) facility (*see* **Note 2**) (Fig. 2).

2.2 Laboratory Supplies

1. Sterile clothing (face masks, hood, powder-free gloves, cover glass, and etc.).
2. Laminar vertical airflow cabinet.
3. Weighing balance (Sartorius, Germany).
4. Centrifuge (Eppendorf, Germany).
5. Optical inverted microscope equipped with phase-contrast option (Nikon, Japan).

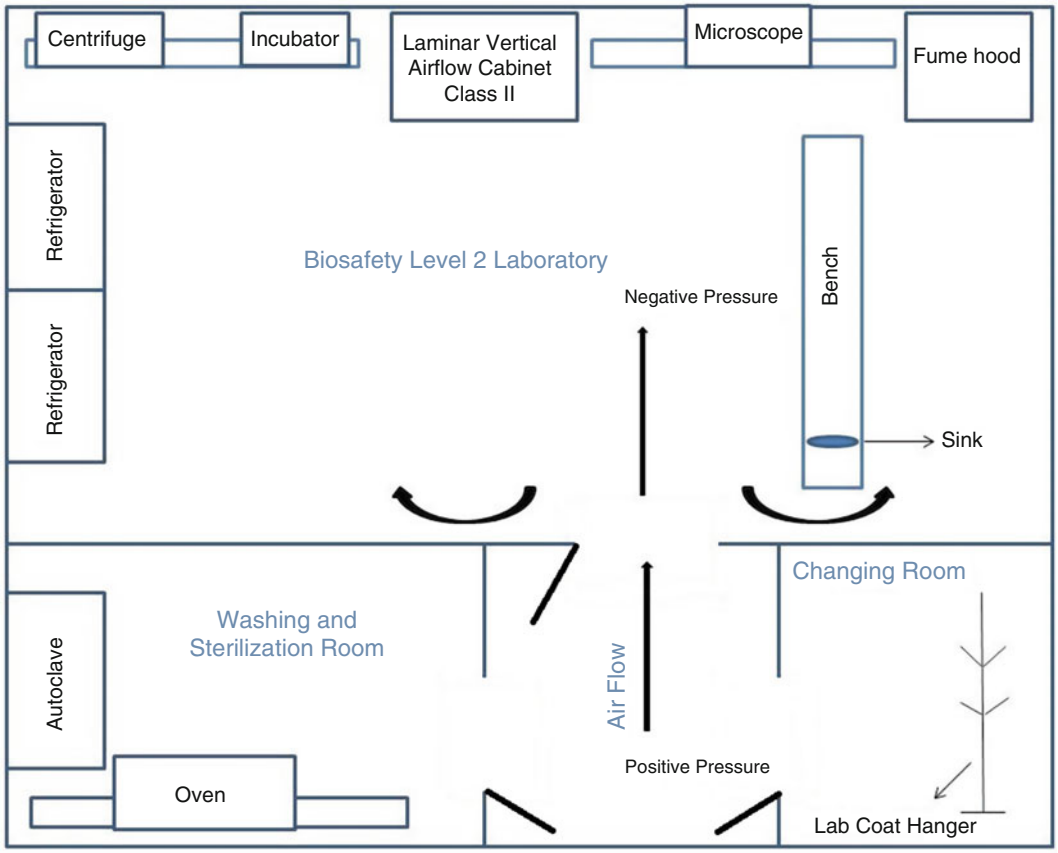

Fig. 2 Biosafety level 2 facility. BSL2 is proper for operations which require agents with the moderate potential danger to personnel and the environment. In this level of biosafety, it is necessary for laboratory personnel to wash their hands during entering and exiting the lab and using lab coat, safety glasses, and gloves. They must be trained for handling pathogenic agents. The procedures which may be created infectious aerosols or splashes should be conducted in laminar vertical airflow cabinet (class II) or other physical containment equipment and access to the laboratory should be restricted during the work in progress. Sterilizing and changing rooms should be placed outside the BSL2 laboratory. Biohazard warning signs must be posted at the entrance. The BSL2 laboratory has negative air flow [25]

6. Water bath (Memmert, Germany).
7. Ultralow temperature freezer (New Brunswick Eppendorf, USA).
8. Sterile stainless steel surgical tray.
9. Motorized pipette (Orange Scientific, Belgium).
10. Pipette (TPP, Switzerland).
11. 0.2 μm sterile and endotoxin-free syringe filter (Orange Scientific, Belgium).
12. Falcon tubes 15, 50 ml (TPP, Switzerland).
13. Hemocytometer (VWR International, USA).

14. Incubator (37 °C, 5% CO_2) (Eppendorf, Germany).
15. Magnetic stirrer (five position, Bellco Glass).
16. Microcentrifuge tubes (1.5 ml) (Eppendorf, Germany).
17. Pasteur pipettes, sterile cotton-plugged (Sigma-Aldrich, USA).
18. Petri dishes containing 1.5% agar (Fisher Scientific, USA).
19. Quick-Seal (Beckman).
20. Slide-A-Lyzer dialysis cassettes (Pierce, USA).
21. Polypropylene tubes (13 ml capped))Parsetylene kish, Iran).
22. Polystyrene tubes (5 ml))Parsetylene kish, Iran).
23. Spinner flasks with the impeller assembly (250 ml) (*Sigma*-Aldrich, Iran).
24. Syringe (3 cc))Pars syringe, Iran).
25. Ultracentrifuge (Beckman, USA).
26. 70.1.Ti rotor (Beckman, USA).
27. SW 41 Ti swinging bucket rotor (Beckman, USA).
28. Ultra-Clear (14 × 89 mm) ultracentrifuge tubes (Beckman, USA).

3 Methods

Ad vector manipulation (Fig. 3) should be accomplished according to GMP principles in a laminar vertical airflow cabinet which is located at BSL2 laboratory (*see* **Note 2**). Moreover, it is important to applying sterile equipment and reagents and recording all data based on the SOPs (*see* **Note 3**).

3.1 Producing Infectious Plasmids

1. Produce a shuttle vector which carries the interest transgene to perform standard cloning procedures.
2. Use 1 μl of PmeI in a total volume of 20 μl for digesting 2 μg of the shuttle vector (overnight at 37 °C).
3. In a 15 ml capped polypropylene tube, add 3 μl of the digested shuttle vector and 3.3 μl of supercoiled pAdEasy (0.1 μg/μl).
4. For negative control, add 3 μl of digested shuttle vector without supercoiled pAdEasy to another tube (ice-cold).
5. Thaw two aliquots of BJ5183 competent cells (0.2-ml).
6. Add BJ5183 cells to the DNA and incubate on ice (25 min).
7. Incubate the tubes at 42 °C (90 s).
8. Then, incubate them on ice (2 min).
9. Add 1 ml of LB in each tube and incubate at 37 °C with shaking (20 g, 25 min).

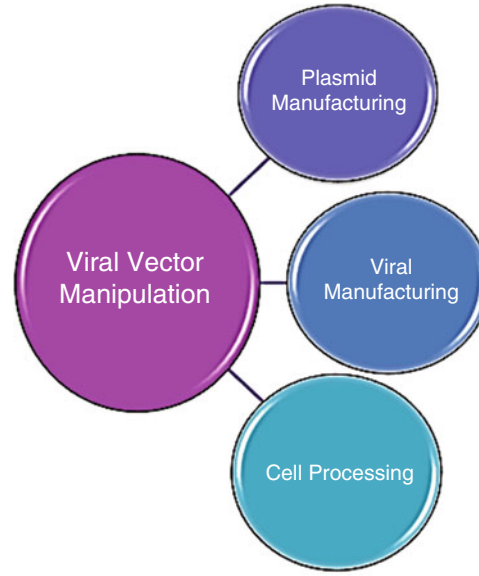

Fig. 3 Viral vector manipulation. In vector manipulation, cells are transfected with plasmids to produce the viral vectors. Viral particles accumulate in the cytoplasm and total yield can be increased by cell lysing. Finally, harvested vector concentrated, purified, titrated, characterized, and stored

10. Transfer the bacteria to 1.5 ml microcentrifuge tubes.
11. Centrifuge it at 8,500 × *g* for 1 min (at room temperature (RT)).
12. Discard 1 ml of supernatant and resuspend the pellet in a total volume of 0.2 ml.
13. Coat the surface of two petri dishes (including 1.5% solid agar in LB supplemented with 25 μg/ml kanamycin) by bacterial suspension (0.1 ml) to form colonies (*see* **Note 4**).
14. Select 4–8 small colonies and purge the DNA of plasmid by means of small-scale alkali lysis.
15. Suspend the purified DNA in a 0.1× TE (total volume of 25 μl).
16. Repeat **steps 6–13** with 5 μl of DNA and 100 μl of DH5α.
17. Choose two colonies from each plate and purge the DNA by small-scale alkali lysis.

3.2 Cell Procurement

3.2.1 Cell Culture for Transfection

1. Spray 70% ethanol on the surface of laminar vertical airflow cabinet and all of the requirement instruments.
2. Put 150 mm dishes of 293 or A549 cells under the laminar vertical airflow cabinet to subculture of adherent cells.
3. Remove medium from dishes.

4. Rinse monolayer of 293 cells with 5 ml of citric saline (1×) twice (for monolayer of A549 cells use 2 ml of trypsin-EDTA).
5. Incubate the dishes at 22 °C until starting cell detachment (5–10 min).
6. For detaching all cells, tap the sides of the dishes.
7. Resuspend the cells in complete medium including FBS.
8. Distribute it into new dishes and finally place the plates in the incubator (37 °C, 5% CO_2).

3.2.2 293 Cells Suspension Preparation

1. Transfer six confluent 293 cells dishes (150 mm) to spinner flask.
2. Increase the total volume in the flask to 1 l.
3. Transfer 2 ml of suspension cells to a 15 ml polystyrene conical tube (each 1–2 days).
4. Add 2 ml of citric saline (2×) and vortex vigorously (10 s).
5. Incubate the cells at 37 °C (15 min, then strongly vortex them (10 s).
6. Count cells using hemocytometer.
7. Record all data based on the SOPs (*see* **Note 3**).

3.3 Transfection for Rescue of Ad Vectors

1. Encode the recombinant Ad genome in the digested plasmid (10 μg) using PacI (2 μl in a total volume of 50 μl) at 37 °C (overnight).
2. Mix PacI-digested DNA (40 μl) with MEM (360 μl) and SuperFect reagent (16 μl) in a 5 ml polystyrene tube and vortex vigorously (10 s) and allow to form DNA-SuperFect complexes at RT (15 min).
3. Add MEM (2.4 ml) to each tube which contain the DNA-SuperFect complexes.
4. Rinse four plates containing seeded 293 cells twice with PBS (2 ml).
5. Transfer the plasmid complex suspension (0.7 ml) to each plates.
6. Place the cells in incubator (37 °C, 5% CO_2) (3 h).
7. Around 2.5 h later, melt 1% (w/v) agarose solution using microwave oven (*see* **Note 5**).
8. Equilibrate the temperature (to 42 °C) in a water bath.
9. Equilibrate maintenance medium (2×) to 37 °C.
10. Three hours later, remove the transfectant from the cells.
11. Rinse the monolayer using PBS.
12. Mix the agarose solution and 2× maintenance medium (with equal volumes).

13. Add 3 ml of this mixture to each 35-mm petri dish of 293 cells.
14. Leave at 22 °C to solidify (~15 min).
15. Put the cells in the incubator until plaques form (~7–12 days).
16. Select the best isolated plaques.
17. Remove agarose over each plaque using a sterile cotton-plugged Pasteur pipette.
18. Transfer each plug in a vial including 4% sucrose-PBS.
19. Vortex it and store at −80 °C.

3.4 Screening of Plaque Isolated Ad Vector

1. For ensuring that the resulted virus is from a single clone, again, add 100 μl of plasmid complex suspension to two plates of 293 cells (~90% confluence).
2. Put the cells in the incubator until virus adsorbing (1 h).
3. Shake the dishes every 10–15 min (gently).
4. After that, add maintenance medium (2 ml) to each plate.
5. Return them to the incubator.
6. Evaluate plates to examine cytopathic effect (CPE) (cells with rounded morphology or detached from the plate).
7. Use one plate for screening the recombinant Ad structure.
8. Store the contents of another plate for vector expansion.
9. To analyze the plaque-isolated Ad vector, after reaching complete CPE, place the plates in laminar vertical airflow cabinet until the detached cells rest at the bottom of the plate (10 min).
10. Remove the medium (gently) and resuspend the remaining cells in SDS–proteinase K solution (0.2 ml).
11. Transfer sample to a microcentrifuge tube.
12. Incubate the lysate at 37 °C (overnight).
13. Add TE (0.3 ml) to the lysate.
14. Extract sample using buffer-saturated phenol (0.5 ml), followed by of chloroform: isoamyl alcohol (0.5 ml).
15. Add 5 M NaCl (0.1 ml) and isopropanol (0.5 ml).
16. Pellet the DNA through centrifugation (20,000 × *g*, 10 min) at 4 °C.
17. Resuspend the DNA in TE (20–50 μl).
18. Digest sample with proper restriction enzymes (5–10 μl).
19. Check the pattern of resulted banding using electrophoresis (on a 0.8% agarose gel and staining with ethidium bromide).
20. To expand plaque-isolated Ad vector, after reaching complete CPE scrape the cells from the second dish into the medium and transfer them to a 4 ml vial.

21. Add sucrose PBS (40% to a final concentration of 4%).
22. Vortex and store at −80 °C until using.
23. Infect petri dish (150 mm) of 293 cells with inoculum (1 ml).
24. Return cells to the incubator and leave it until the virus adsorb (1 h).
25. Rock the dishes every 10–15 min.
26. After that, add maintenance medium (2 ml) to each plate.
27. Return them to the incubator.
28. Check plates for CPE.
29. After reaching complete CPE, remove the cells and medium to a 50 ml conical tube.
30. Add 40% sucrose PBS (1/10 volume to a final concentration of 4%).
31. Store inoculum at −80 °C.
32. Warm the inoculum in water bath (22 °C) (if necessary, increase the volume of the inoculum using MEM).

3.5 Ad Vectors Preparation

3.5.1 Preparation Using Adherent 293 Cells

1. Remove medium of 30 dishes (10 at time) including ~90% confluent 293 cells (150-mm) and replace with inoculum (1 ml).
2. Leave sample in a 37 °C, 5% CO_2 incubator until the virus be adsorbed (1 h).
3. Rock the plates every 10–15 min.
4. Add maintenance medium (20 ml) to every dish.
5. Return the cells to the incubator.
6. Check cells every day for CPE (~2–3 days).
7. Transfer medium and cells to 500 ml polypropylene bottles (two bottles).
8. Rinse groups of 10dishes using the same pipette twice with PBS (10 ml).
9. Centrifuge cells (650 × *g*, 20 min) at 4 °C.
10. Discard the medium and retain the cell pellets.
11. Resuspend the cells in 4% sucrose PBS (3 ml) and transfer suspension to a 50 ml conical tube.

3.5.2 Preparation Using Suspension Adapted 293 Cells

1. Distribute 293 cultured cells (3 l) to eight 500 ml bottles.
2. Centrifuge them (650 × *g* for 20 min) at RT.
3. Discard the medium to sterile 1 l Bottles.
4. Retain spent medium (1 l) and add it to the 3 l flask.
5. Place the flask in the incubator.

6. Use the spent medium for resuspending the cell pellets (final volume of 40 ml).
7. Transfer the suspension to 250 ml spinner flask.
8. Rinse the bottles using 10 ml of spent medium (twice).
9. Transfer sample to the spinner flask.
10. Add the thawed inoculum (total volume about 100 ml).
11. Place the flask in the incubator.
12. Agitate the cells (2 h).
13. Transfer the cells to the 3 l suspension flask (including spent medium (1 l)).
14. Rinse the 250 ml spinner flask using 250 ml of fresh maintenance (twice).
15. Transfer it to the 3 l spinner flask.
16. Add fresh maintenance medium (500 ml to final volume of 2 l).
17. Transfer the cell suspension (2 ml) to 35 mm petri dish.
18. Put it in the incubator (until cells reattach to the plate).
19. 2–3 days after reaching complete CPE, transfer the suspension to 500 ml bottles.
20. Centrifuge sample (650 × *g*, 20 min) at 4 °C.
21. Discard the supernatant and save pellets.
22. Resuspend the cells pellets in 4% sucrose PBS (3 ml).
23. Transfer it to 50 ml conical tube.
24. Rinse the bottles using 2 ml of 4% Sucrose PBS (Once).
25. Repeat the last part using 4–5 ml of 4% Sucrose PBS (cells with a total volume of 15 ml can be used immediately to purify vector or can be stored at −80 °C).

3.6 Purification of Vector

1. Thaw pellets at 22 °C.
2. Add 5% deoxycholate (1.5 ml) to the pellet.
3. Incubate it at 22 °C (30 min with frequent inversion) (until the lysate be in thick and highly viscous consistency).
4. Add 2 M $MgCl_2$ (0.3 ml), 10 mg/ml RNase A (0.15 ml), and 10 mg/ml DNase I (0.15 ml).
5. Incubate for 30–60 min at 37 °C (with occasional inversion).
6. Until the viscosity of the lysate be reached near to water, centrifuge (1,000 × *g* for 10 min) at 22 °C.
7. Provide CsCl step gradients in Ultra-Clear ultracentrifuge tubes (two tubes per virus).
8. Add 1.35 g/ml CsCl (2 ml) to each tube.

9. Centrifuge the samples use slow acceleration and deceleration profiles (30 × *g* for 5 min to 130,000 × *g* for 1 h) at 10 °C.
10. Pierce the tube about 1 cm below the virus using a 3 cc syringe.
11. Turn the bevel until it is paralleled to the band and can slowly remove (lower the syringe needle as the band lowers).
12. Fill the Quick-Seal tube including a virus to the base of the neck using 1.35 g/ml CsCl.
13. For sealing the Quick-Seal tubes, use a heat sealer.
14. Centrifuge (130,000 × *g*, overnight) with maximal acceleration and deceleration at 10 °C.
15. Penetrate the top of the sealed tube about 1 cm below the virus to form an air inlet with a 3 cc syringe.
16. Turn the bevel until it is paralleled to the band and can slowly remove (lower the syringe needle as the band lowers).
17. Add the Ad into a prepared dialysis cassette by injection.
18. Discard the air bubble using the syringe.
19. Dialyze the Ad vector at 4 °C (24 h) against 2volome of dialysis buffer (500 ml).
20. Remove the vector from the dialysis cassette.
21. Add 40% sucrose PBS to the vector and the dialysis buffer (to a final concentration of 4%).
22. Store purified vector in small aliquots (~100–200 μl) at −80 °C (can be stored for 1 year) and the buffer at −20 °C.

3.7 Characterization of Purified vectors

Ad vectors can be characterized by:

1. Assessing the genetic structure based on electrophoresis.
2. Determining titer by plaque-forming unit (pfu) assay (*see* **Note 6**).
3. Checking for contamination with replication-competent adenovirus (RCA) (*see* **Note 7**).

4 Notes

1. Sterilize prepared agarose using autoclave (15 min at 121 °C), store it at RT, and melt it in microwave oven before using.
2. A collection of biocontainment cautions which are needed to disassemble hazardous biological agents in an enclose laboratory is known as biosafety level. BSL2 is proper for operations which require agents with the moderate potential danger to personnel and the environment. In this level of biosafety, it is necessary for laboratory personnel to wash their hands during entering and exiting the lab. Additionally, they must be trained

for handling pathogenic agents and guided by advanced training experts. On the other hand, the procedures which may be created infectious aerosols or splashes should be conducted in laminar vertical airflow cabinet or other physical containment equipment and during these procedures access to the laboratory should be restricted [25].

3. An essential aspect of QA systems is to operate all steps (from sampling to analyzing the result) based on detailed and specific Standard Operating Procedures (SOPs) [26].
4. Use a plate as control, no colonies should form on the negative control plate. In addition, if "background" colonies appear, recombinant colonies are substantially smaller than those produced by bacteria.
5. To avoid solidification of the agarose, this step must be done quickly, but gently to avoid disturbing the monolayer.
6. Plaque assay is one of the most essential procedures for determining the quantity of infectious virus. It is determined the number of pfu in a virus sample based on microbiological methods [27].
7. The RCAs is a safety concern for biologics based on recombinant adenoviruses; therefore RCA test is required for clinical material dissemination [28, 29].

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Use of Multipotent Mesenchymal Stromal Cells, Fibrin, and Scaffolds in the Production of Clinical Grade Bone Tissue Engineering Products

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Abstract

Tissue engineering products (TEP) are a new type of medicines resulting from the combination of cells, scaffolds, and/or signalling factors, which can be used for the regeneration of damaged tissues thus opening new avenues for the treatment of complex conditions. However, such combination of biologically active elements, particularly living cells, poses an unprecedented challenge for their production under pharmaceutical standards.

In the methods presented here, we formulated two types of TEP based on the use of multipotent mesenchymal stromal cells with osteogenic potential combined with osteoinductive and osteoconductive bony particles from tissue bank embedded in a fibrin hydrogel that, altogether, can induce the generation of new tissue while adapting to the diverse architecture of bony defects. In agreement with pharmaceutical quality and regulatory requirements, procedures presented herein can be performed in compliance with current good manufacturing practices and be readily implemented in straightforward facilities at hospitals and academic institutions.

Keywords Cell-based therapy, Tissue engineering product, Multipotent mesenchymal stromal cells, Regenerative medicine, Scaffold, Bone, Fibrin glue, Good manufacturing practice

1 Introduction

Advanced therapy medicines (ATM) are a new type of drugs based on the use of genes, tissues, and cells or their combination with medical devices, offering ground-breaking new opportunities for the treatment of unmet clinical conditions [1]. Although the application of ATM in regenerative medicine has already resulted in a number of clinical trials and also a few marketed products [2, 3], their development is challenging [4]. This is due to the nature of the active ingredients and the major role of nonindustrial academic laboratories that need to adapt their facilities, methodologies, and personnel to pharmaceutical production environments [5]. In this context, we have successfully developed several ATM with multipotent mesenchymal stromal cells (MSC) for clinical use in

orthopedic conditions including gonarthrosis [6, 7], pseudoarthrosis [8], spinal fusion, and osteonecrosis. This type of progenitor cells holds osteogenic potential by both paracrine signalling and commitment to the bone lineage [9, 10] and holds an excellent safety profile [11].

Production of combined advanced therapies represents a challenge to developers, who need to define adequate production bioprocesses and suitable quality control panels to ensure that the drug products comply consistently with expected specifications. The methods described herein allow for the production of combined advanced therapy products using either cells in suspension or adhered to the surface of bony scaffolds for use in bone regeneration in compliance with good manufacturing practices. In particular, two formulations are presented, according to the use of osteogenic cells either loaded onto the surface of bony scaffolds or in suspension.

2 Materials

Prepare all solutions under sterile conditions. Customized kits are gamma-irradiated at 25–30 kGy prior to use. The traceability, integrity, and sterility of reagents and materials should be preserved at all times. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Equipment

Biological safety cabinet.

Orbital shaker.

Scale.

Tube connector.

Tube sealer.

Stopwatch.

Centrifuge.

Thawing system for bags.

Fridge.

2.2 Reagents/Solutions

Dulbecco's Modified Eagle's Medium-High Glucose (DMEM-HG).

Human serum (hSer) from pools of at least three healthy donors.

0.05% trypsin-ethylenediaminetetraacetic acid (EDTA).

Plasmalyte 148.

20% (w/v) human serum albumin (HSA).

TissuCol Duo.

Decellularized bone matrix from tissue bank.

Washing solution: 2% (w/v) HSA in Plasmalyte 148.

Thawing solution: 10% (w/v) HSA in Plasmalyte 148.

2.3 Kits and Consumables

The design of kits can vary according to the needs of each individual laboratory and in accordance to appropriate risk analysis to avoid contaminations. We propose designs that ensure a close system for most of the process as depicted in Figures 1 and 2. In order to prepare such kits, the next items are required:

2-pathway sterile connector.

Sterile Y-connector.

Transfer set (TS).

Syringes of different sizes.

Needles.

Transfer bags (TB) of different sizes.

Gas-permeable bags.

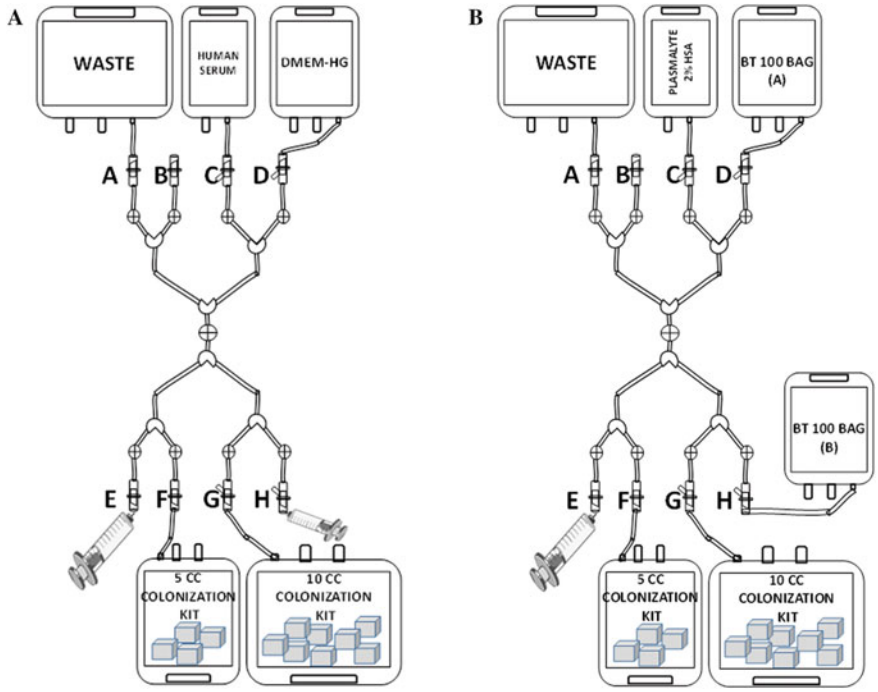

Fig. 1 Customized kits. Schematic representation of the connections used in the basic kit for colonization (**a**) and washing (**b**). Sterile connection of tubing sets, bags and syringes allows the closure of processing systems therefore mitigating risks of contamination. This is a simple and versatile approach that can suit the needs of any specific bioprocess. Customized kits are conveniently packed, labelled and irradiated before use

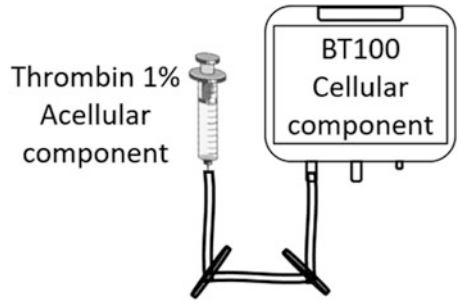

Fig. 2 Schematic of final TEP setup with cells in suspension. The acellular component consists of diluted thrombin whereas the cellular component consists of cells in fibrinogen. The mixture of both solutions results in the clotting of fibrin and the engulfment of cells

Abbocath.

Clamp.

Sterile twirl bag.

3 Methods

Cells, reagents, and materials must be handled in biological safety cabinets in class B environmental conditions or using an isolator unless otherwise specified. In order to avoid contaminations, we opted for using closed systems in all critical steps. This is achieved by generating customized kits with appropriate bags, syringes, and tubing.

3.1 Human Multipotent Mesenchymal Stromal Cells

We have tested these methods using clinical grade MSC derived from Wharton's jelly (WJ-MSC) and bone marrow (BM-MSC) after ex vivo expansion up to sufficient numbers, as reported elsewhere [7, 12–14]. We believe that MSC from other sources or even other type of osteogenic cells may be used instead with similar outcomes. Although MSC used in either one of the protocols can be freshly prepared or thawed from cryopreserved vials, we recommend the use of freshly expanded cells for colonization (Subheading 3.2) and thawed cells for suspension (Subheading 3.3). If MSC have been cryopreserved, we strongly suggest to follow the protocol described next in Subheading 3.1.1.

3.1.1 Cell Thawing

1. Place the bag/cryotubes in a dry thawer system pre-warmed at 37 °C. Check the cellular suspension periodically, and, immediately before the complete thaw of the cellular suspension occurs, proceed with the next step.

Table 1
Conditions for the addition of the thawing solution

Step	Thawing solution % (v/v)	Time (min)
1	25	2
2	25	2
3	50	2

2. Transfer the cellular suspension to a TB100, and measure the exact volume using a syringe. If a bag is used, connect it to a sterile Y-connector, and place a 20 mL syringe containing a TS and the TB100 in the free ends. In case of using cryotubes, use a 20 mL syringe coupled to a needle or an Abbocath catheter to collect the cell suspension. Then, connect the syringe to the TB100.
3. During the process of cell thawing, a 1:2 dilution of the cellular suspension is performed using syringes with different volumes of *thawing solution*. Depending on the total volume of cells transferred, determine the volume of *thawing solution* that will be added to the TB100 at each step, according to Table 1. Use cold accumulators to keep the cells at a temperature within the range of 2–8 °C for the entire process.
4. Proceed with cell washing and concentration.

3.1.2 Cell Washing and Concentration

1. Connect the TB100 from the previous step to a sterile Y-connector, and add a 50 mL syringe and a TB150 containing the *washing solution* to the free ends. Add *washing solution* to the TB100 up to a final volume of 100 mL.
2. Homogenize well, and take an aliquot of 0.6 mL for cell counting and cell viability assessment. Typically, the acceptance criterion for cell recovery and viability is $\geq$70% in both tests.
3. Centrifuge for 10 min at 400 × *g*, 20 °C, and low break to avoid disruption of the pellet.
4. Determine the volume of supernatant that must be removed to obtain a cellular suspension of 1×10^6 viable hMSC/cc matrix (for protocol described in Subheading 3.2) or 1.2×10^7 viable hMSC/mL (for protocol described in Subheading 3.3). An example of such calculations is shown in the next equations.

$$\text{Resuspension volume} = \frac{\text{Cellular concentration}\left(\text{viable}\,\frac{\text{hMSC}}{\text{mL}}\right) \times \text{Sample volume}}{1.2\text{E}7\ \text{viable hMSC/mL}}$$

$$\text{Removed volume of supernatant} = \text{Sample volume} - \text{Resuspension volume}$$

5. *(Optional)* Take an aliquot of the removed supernatant for quality control testing (i.e., cell recovery and viability, cell surface marker expression, and endotoxin levels).

3.2 Manufacture of Combined Products with Colonization

This section describes the methodology for the colonization of hydrated bone matrix from tissue bank with human MSC.

3.2.1 Colonization of Bony Scaffolds

1. Hydrate bone matrices using Plasmalyte 148 for at least 24 h, and leave them in gas-permeable bags at 2–8 °C until use (no more than 6 months). We typically prepare bags of 5 cc and 10 cc bone matrix formats and perform sterility tests to ensure their suitability for production.
2. Before use, saline solution is removed by using a syringe.
3. Create a basic colonization kit as shown in Fig. 1a. First, place a 6 mL of hSer in port C and 60 mL DMEM-HG medium bag in port D. Additionally, place the bags with bone matrix in port F and G and the syringe containing 1×10^6 viable hMSC/cc matrix in port H.
4. Supplement DMEM-HG medium with hSer, seal the serum bag and discard it. Then, connect a TB100 to port C. Next, inoculate the cell suspension to the supplemented medium. Seal the syringe from port H and discard. Remove the saline solution from the two bags, and dispense it in the TB100 in port C. Then seal the bag and discard it. Finally, connect a syringe in port E, dispense the cell suspension to the conditioned bone matrices (40 mL/10 cc bag or 20 mL, for the 5 cc bag), and seal them.
5. Place the bags containing bone matrix horizontally on an orbital shaker. Ensure that the matrix is distributed evenly over the surface of the bag and immersed in the medium. Handle the bags gently to prevent the bone particles from moving abruptly, as this could negatively affect cell viability due to shear stress.
6. The colonization process alternates cycles of shaking and cell sedimentation to increment the colonization rate, as follows: 5 × (10 min shaking +20 min static) + 10 min shaking +45–60 min static (total accumulated time: 205–220 min (*see* **Notes 1** and **2**).

3.2.2 Incubation (Optional)

1. If needed, an additional incubation step of no more than 4 days can be performed before conditioning of the final product. To do this, wash each bag with DMEM-HG, and then condition them with DMEM-HG supplemented with 10% hSer.
2. Incubate bags at 35–38.5 °C, 2.5–5% CO_2, and 95% relative humidity for no more than 4 days, and proceed to the next section (*see* **Note 2**).

3.2.3 Washing

1. Create a new kit as follows (Fig. 1b). First, prepare fresh *washing solution* and connect it to port B. Next, connect two TB100, labelled A and B, to ports C and D, and the bags with the colonized bone matrix to ports F and G. Collect the supernatant from the two colonization bags in TB100 A, seal the bag, label it, and store it for further quality control testing. Using a syringe, add the needed volume of *washing solution* to the colonized matrices (40 mL/10 cc bag or 20 mL/5 cc bag) (*see* **Note 3**).
2. Shake bags gently on an orbital shaker maintaining them horizontally for 5 min at 15 rpm and room temperature (RT). Remove the washing solution from the two colonization bags, and dispense it in a TB2000 in port A. Repeat the washing step three times per bag, seal the bag, label it, and store it for further quality control testing.

3.2.4 Final Conditioning

1. Add *washing solution* to the colonized bone matrices (40 mL/10 cc bag or 20 mL/5 cc bag), collect the *washing solution* in TB100 B after washing, seal it, label it, and store it for further quality control testing.
2. Again, add another volume of *washing solution* to the colonized bone matrices according to the format size (40 mL in the 10 cc bag and 20 mL in the 5 cc bag), and seal it after washing.
3. Store the final product (bag containing 10 cc of colonized bone particles) overnight at 4 °C. The bag containing 5 cc of colonized bone particles will be used for quality control testing (i.e., cell count, viability and cell surface marker expression, endotoxin levels, sterility, presence of *Mycoplasma* or Gram staining).
4. If results demonstrated that the final product complies with established specifications, proceed to package it in a sterile twirl bag. Wrap again with second twirl bag.

3.3 Combined Products with Cells in Suspension

This section describes the methodology for the production of a TEP that combines (1) a cellular component consisting of allogeneic ex vivo expanded hMSC (in a saline solution supplemented with HSA and fibrinogen) and (2) a noncellular component (consisting of thrombin 1% (w/v) in saline solution).

3.3.1 Preparation of the Cellular Component

1. If the cellular concentration is out of the range $1.2 \times 10^7 \pm 20\%$ hMSC/mL, it is required to adjust cell dose. To do this, calculate the volume of *washing solution* that needs to be added. After sterile addition of the volume needed, take a new aliquot to confirm cell count and viability.
2. Using a 10 mL syringe connected to a TS, measure the volume of cellular suspension in the TB100 and adjust it to 5 mL.
3. Add a sterile Y-connector to the TB100 and connect one TS keeping the "luer female" termination at one end and a 50 mL syringe with a TS in the other end (*see* **Note 4**).
4. Unwrap the TissuCol Duo, and ensure that both components (fibrinogen and thrombin) are completely thawed (*see* **Note 5**). Take the syringe containing fibrinogen, open it, and lock it with the female luer connection to the TB100 (*see* **Note 6**).
5. Using the 50 mL syringe, remove as much air as possible. Then, inject 5 mL of fibrinogen to the TB100, homogenize gently, and aspirate again to remove any fibrinogen left in the syringe. At this point, a 1:2 dilution of the cellular suspension is performed.
6. Connect a new syringe with TS, and take an aliquot of 3.5 mL for quality control (i.e., cell count, viability and surface marker expression, endotoxin levels, sterility, presence of *Mycoplasma* or Gram staining). At this point, acceptance criteria are defined as $3 \times 10^7 \pm 20\%$ of live cells and cell viability $\geq 70\%$.

3.3.2 Preparation of 1% (v/v) Thrombin Solution

1. Place a punch with tubing in the input port of a 500 mL bag of Plasmalyte 148.
2. Connect a 10 mL syringe with TS and extract 5 mL of Plasmalyte 148. Seal the tube and discard the syringe containing 5 mL of saline solution.
3. Connect one TS to the plasmalyte bag keeping tubing of less than 10 cm length.
4. Take the syringe containing thrombin from the TissuCol Duo kit, open it, and placed it at the TS connected to the plasmalyte bag (*see* **Note 6**).
5. Inject 5 mL of thrombin into the plasmalyte bag, homogenize the solution, and aspirate again to remove any thrombin left in the syringe. Seal the bag and discard the syringe.
6. Extract two samples of 2% and 4% of the volume to check sterility.
7. Connect the 1% thrombin solution to a sterile Y-connector, and add one 5 mL syringe with TS and one 20 mL syringe with TS to the free ends. Use the 5 mL syringe to transfer the

thrombin solution to the 20 mL syringe. 3 mL of 1% thrombin/10 mL of final product must be transferred.

8. Identify properly the 20 mL syringe containing 1% thrombin solution, and seal the tube keeping a length of 3–4 cm.

3.3.3 Preparation of the Final Product, Storage, and Release

1. Perform a sterile connection between the TB100 containing the cellular component and the 20 mL syringe containing the thrombin solution (acellular component).
2. Add two clamps to prevent the cellular and acellular components from mixing (Fig. 2).
3. Place the final product in a twirl bag and seal it. Repeat this step.
4. Once finished, the final product can be stored up to 24 h at 2–8 °C. Cold temperature must be guaranteed also during transport to the hospital and delivery in the surgery room.

3.4 Analytical Methods

Attributes of medicinal products need to be demonstrated before clinical use [4, 5]. Although the present chapter focuses on the production of combined tissue engineering products, please note that a number of techniques can be used for the release of such medicines.

3.4.1 Product Release

In all cases, levels of endotoxins and presence of bacterial, fungi, and *Mycoplasma* must be determined. Additionally, cell dose can be assessed by a number of methods. For instance, in colonized bone TEP, cell count and viability can be performed by enumerating cells in the supernatant and then subtracted from the inoculum, so the percentage of colonization can be determined, as shown in previous works [8, 15, 16]. Another very rapid method is the determination of ATP content using commercial kits such as Cell Titer-Glo®, resulting in values that correlate with cell viability.

3.4.2 Further Analyses

For research purposes or in-depth validation of the production process, further characterization of the final product can be performed by imaging techniques, such as specific staining and confocal or electron microscopy and analyses of nucleic molecules [8].

4 Notes

1. During the dynamic cycle, shaking must be gentle (≈15 rpm), and during the static cycle, the shaker must be stopped and the bag in a horizontal position to maintain bony particles in contact with the medium.

2. Place the bags horizontally to ensure that bone particles are covered by the culture medium. Avoid direct contact of the bags and the surface of the incubator by using a plastic rack.
3. Leave a volume of air equivalent to half that of the liquid, so the air bubble occupies the upper surface of the bag. The bone matrix should be distributed evenly over the entire surface of the bag and immersed in the washing solution.
4. Leave the maximum possible volume of air in the syringe and enough tubing to perform a new sterile connection (5–6 cm).
5. One hour before starting the conditioning of the final product, thaw the two components of TissuCol Duo at room temperature.
6. Please note that the system is open at this point. Pay attention to avoid any contamination according to your own risk analysis [13, 17].

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Quality Management Systems (QMSs) of Human-Based Tissue and Cell Product Manufacturing Facilities

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Abstract

In the broadest sense, a quality management system (QMS) runs by continuous interaction of elements based on processes, procedures, policies, guidelines, and resources that are compiled to guide an organization under the scope of its operational mission, vision, and objectives. QMSs in the biopharmaceutical sector defines a written and applied set of rules which aids in improvement of the quality of biopharmaceutical process engineering, while primarily assuring human tissue- and cell-based starting material and end product safety and minimizing the risk of human medicinal product recall, in the most cost-effective ways possible. This chapter aims to outline the crucial position of a QMS under the scope of good practices (GxPs) in the biopharmaceutical industry, as regards human tissue- and cell-based products.

Key words Advanced therapy medicinal products, Biopharmaceutical, Biotechnology, Current Good Manufacturing Practice (cGMP), Current Good Tissue Practice (cGTP), Good practices (GxPs), Human medicinal products, Human tissue- and cell-based products, Process engineering, Quality management system (QMS), Traceability

1 Introduction

Before, human tissue- and cell-based products intended for clinical use were processed in small-scale laboratories with uncontrolled air and particle conditions which posed high risk of contamination on both the products and the personnel. As rules for the quality and safety of such products were gradually settled, human-based tissue and cell product manufacture to trended toward high standard procedures and stringent traceability systems. Therefore, the need has risen in such facilities for designing an appropriate quality management system (QMS) which defines the parameters that cover the key personnel, planning and installation, and environmental compatibility and traceability.

In the broadest sense, a QMS runs by continuous interaction of elements based on processes, procedures, policies, guidelines, and resources that are compiled to guide an organization under the scope of its operational mission, vision, and objectives. QMS in the biopharmaceutical sector defines a written and applied set of rules which aids in improvement of the quality of biopharmaceutical

process engineering, while ensuring starting material and end product safety and minimizing the risk of human medicinal product recall, in the most cost-effective ways possible. Biopharmaceuticals cover a large scale of medicinal products intended for human use, from minimally manipulated human tissue- and cell-based products manufactured on a small scale to substantially manipulated and genetically engineered advanced therapy medicinal products manufactured on an industrial scale, either intended for autologous or allogeneic use. Small-scale manufacturing usually involves small- and medium-sized enterprises (SMEs) whereas industrial-scale manufacturing generally involves big pharmaceutical companies.

A QMS is not necessarily a prerequisite for small-scale manufacturing. However, it is the interface of an organization's roadmap to attaining corporate identity. When it comes to manufacturing biopharmaceuticals, a QMS may be a vital tool in terms of assuring and updating process controls and end user assurance according to the current advances in biotechnology. This chapter aims to outline the crucial position of a QMS under the scope of current good practices (cGxPs) ruled by competent authorities to govern the biopharmaceutical industry.

2 The Leading Current Good Practices (cGxPs) in Biopharmaceutical Manufacturing

The term "current" in good practices (GxPs) refers to keeping up-to-date with novel technologies and procedures used in processing high-tech products. In the ever-evolving scientific advances of human tissue- and cell-based technologies, knowledge of how the human organism operates gains depth by the day [1]. In parallel, the use of current know-how, equipment, and systems is essential in especially eliminating the risk of cross-contamination and mix-ups in tracking and tracing during the manufacturing life span, and meeting the most recent biopharmaceutical product safety and quality standards.

GxPs define the minimum requirements for running a QMS in a manufacturing facility. National and regional competent authorities issue legislative GxP documents to be able to standardize the minimal obligations of a proper QMS.

QMS in biopharmaceutical manufacturing must firstly adhere to current Good Manufacturing Practice (cGMP). cGMP rules are defined in the national and regional guidelines issued by competent authorities across the world (Table 1). When the starting material is an organ part, tissue, cell of a human donor, there is also a need to follow by current Good Tissue Practice (cGTP) rules as a prerequisite [8]. A number of countries and regions have been issuing legislative documents in order to specifically address cGTP rules. Some examples of cGTP legislative documents are listed in Table 2.

Table 1
A list of some national and regional competent authorities, and their cGMP guidelines

Competent authority	Title of guideline	References
U.S. Food and Drug Administration (FDA)	Current Good Manufacturing Practice for finished pharmaceuticals	[2]
World Health Organization (WHO)	A WHO guide to Good Manufacturing Practice (GMP) requirements	[3]
European Medicines Agency (EMA)	Good Manufacturing Practice	[4]
European Commission	EudraLex—Volume 4—Good Manufacturing Practice (GMP) guidelines on Good Manufacturing Practice specific to advanced therapy medicinal products	[5]
Turkish Medicines and Medical Devices Agency (TİTCK)	Directive regarding manufacturing sites of human medicinal products; Human medicinal product manufacturing sites—Good Manufacturing Practice (GMP) guideline	[6, 7]

Table 2
A list of some national and regional competent authorities, and their cGTP guidelines

Competent authority	Title of guideline	References
European Directorate for the Quality of Medicines & HealthCare of the Council of Europe (EDQM)	The guide to the quality and safety of tissues and cells for human application	[9]
U.S. Food and Drug Administration (FDA)	CFR—Code of Federal Regulations Title 21 Chapter I Subchapter L Part 1271 human cells, tissues, and cellular- and tissue-based products Subpart D current good tissue practice; Guidance for industry current good tissue practice (CGTP) and additional requirements for manufacturers of human cells, tissues, and cellular and tissue-based products (HCT/Ps)	[10, 11]
European Commission (EC)	Directive 2004/23/EC of The European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells; Commission Directive 2006/17/EC of 8 February 2006 implementing Directive 2004/23/EC of the European Parliament and of the Council as regards certain technical requirements for the donation, procurement, and testing of human tissues and cells; Commission Directive 2006/86/EC of 24 October 2006	[12–14]

(continued)

Table 2
(continued)

Competent authority	Title of guideline	References
	implementing Directive 2004/23/EC of the European Parliament and of the Council as regards traceability requirements, notification of serious adverse reactions and events and certain technical requirements for the coding, processing, preservation, storage, and distribution of human tissues and cells	
Turkish Ministry of Health (MoH), and Turkish Medicines and Medical Devices Agency (TİTCK)	Directive regarding the quality of human tissues and cells, and related centers; Public communique related to the marketing authorization of human-based tissue and cell products, and to manufacturing, exporting, importing storage, and distribution centers	[15, 16]
National Pharmaceutical Control Bureau Ministry of Health Malaysia	Good tissue practice guideline	[17]

3 The Essentials of a Proper Biopharmaceutical Quality Management System (QMS)

3.1 Key Personnel

Key personnel working at a human-based tissue and cell product manufacturing facility must be qualified for the position that is allocated to them and personnel numbers have to be sufficient. Duties and responsibilities of the personnel are documented in open, current, and written format. Each incoming personnel undergoes a basic education before starting their duties. As procedures are updated in accordance with novel scientific advances, personnel is immediately subjected to an education module related to the expected advanced activities of the facility.

Personnel must be equipped with the following upon the completion of each education module.

- Performance sufficiency and qualification.
- Equipped knowledge in the relevant scientific/technical processes and principles.
- Full control over the organization chart, QMS, and healthcare and safety rules of the facility.
- Widespread knowledge of the universal ethical, legal, and administrative framework related to their duties and responsibilities.

3.2 Quality Procedures

3.2.1 Quality Assurance (QA)

Quality assurance (QA) is a crucial element of a QMS. QA gives the end user—in this case the clinicians—the guarantee that each released biopharmaceutical product is safe, with no risk of the spread of transmissible diseases or contamination, and qualified to perform the intended mode of action to prevent, repair, restore, or regenerate a given pathology, with expected adverse events or reactions, or no adverse events or reactions on the treated patient. QA goes hand in hand with stringent quality control (QC) procedures.

3.2.2 Quality Control (QC)

A proper QMS must design, operate, conduct, monitor, and validate appropriate QC procedures in the safety of the starting material—donor eligibility assessment, the procurement of biological and chemical manufacturing material, the environmental controls of the facilities and the equipment, the critical steps of the engineered processes, in each batch release [1, 2, 4–17]. The fundamental QC tests can be listed as but are not limited to cell viability and count, microbiological contamination, bioburden testing, environmental controls, and trace element and excessive hazardous substance detection and quantification. Installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ), where necessary, are the three major monitorization components of the calibration and maintenance of properly functioning equipment and hand tools [18, 19].

Live cells are fragile and cannot be sterilized, as they will lose viability and die out. To circumvent the sterilization steps, manufacturing operations must be carried out in an aseptic environment [1]. Usually, quality control test results are not always completed before the release of the end product to the clinic. There is, hence, a need to perform immediate tests which would aid predictable safety outcomes before the product reaches the patient. However, such tests may also demand more time than that of the release procedures. Therefore, the premises must be designed and maintained periodically, beforehand, in order to avoid any possible risks of contamination. Routine particle, temperature and humidity, pressure and ventilation controls as well as HEPA filter, freezer alarm and chemical residue controls alongside cleaning procedures in terms of disinfection and sanitation of the equipment and the clean room suits are the major preventive actions [18].

Guarding the safety of the personnel is equally important as designing and maintaining aseptic conditions. Key personnel is tested yearly or every other year for any emerging or current contagious or serious pathologies that would cause hazard to themselves in the working environment. Each personnel assigned to work at a human-based tissue and cell product manufacturing facility is well-equipped with appropriate knowledge in biotechnological advances and is specifically and fully trained for following

safety and quality procedures taking place throughout the whole life span of a manufacturing cycle. The use of any hazardous chemical or biological substances used during maintenance and manufacturing that would cause toxicity, sensitivity, or irritability on the personnel is monitored by risk management procedures.

Each batch of consumables, reagents, and chemical or biological substances used in manufacturing procedures have to be quarantined, tested, screened, and approved for safety and quality before being released to the clean room facilities. Substances free of viral contamination, free of transmissible spongiform encephalopathy (TSE) contamination, preferably produced under GMP conditions, intended for human use can be released for processing after identity, purity, sterility, and quantification of endotoxins are defined; human and animal origin reagents are preferably substituted; antibiotics should be avoided due to the possible risk of anaphylactic shock on the recipient patients; final residues of reagents should be quantified beforehand; and risk assessment of any residual agents should be performed [18].

In some cases, some competent authorities may accept the specifications written on the certificates of analysis (COAs). However, it is best to test the batch within the facilities and cross-check with the results specified in the COAs. If the safety results do not match those specified on the COAs, the supplier must be contacted immediately, recall procedures must be initiated and a new batch must duly be ordered.

3.3 Process Validation

The effectiveness of an engineered process is defined by process validation. Key aspects in process validation include at least the following.

- Materials used during processing,
- Human-based tissue/cell specifications,
- Critical manufacturing steps,
- QA/QC,
- Residual substance analyses,
- Sampling plan,
- Packaging and labeling materials,
- Release criteria,
- End product specifications, including storage and distribution conditions and shelf-life, where necessary.

Revalidation is also necessary in cases such as the type of antibiotic, the concentration of and exposure limits to a reagent used for processing, temperature changes during processing and/or storage, and any causes of contamination and cross-contamination.

Fig. 1 The fundamental steps to which risk management applies in human tissue- and cell-based product manufacture

3.4 Risk Management

An effective QMS provides a proactive and systematic mechanism to at least, but not limited to, identify, analyze, and evaluate potential process and product risks during the development, processing, preservation, and distribution of tissues and cells [20]. In the setting of human-based tissue and cell product manufacture, QMS starts from donation and continues as far as product release, and distribution to the end user (Fig. 1).

3.4.1 Risk Analysis and Risk Assessment

Key steps in risk analysis and assessment are hazard identification as to what could go wrong, severity and probability assessment as to how likely is an event to happen and how bad it would be, classification and prioritization evaluation as to the identification of the most serious risks and to where the effort should be focused, and risk mitigation and acceptance category management as to how we can reduce the level of such risks and if the residual risk level can be acceptable [20]. A proper risk management plan has to be in place in order to appropriately maintain quality and safety in the timeliest manner. Key aspects can be listed as follows.

- Inter-personnel communication with the aim to increase product quality and safety.
- Information mining.
- Data analysis.
- Detection and investigation of problems jeopardizing the products and quality standards.

This can be conducted by corrective and preventive actions (CAPAs) which include the following.

- Elimination of the detected incompatibility.
- Focusing on the preliminary issue.
- Preventing repetitions.
- Solving the major problems.

3.4.2 Corrective and Preventive Actions (CAPAs)

Corrective actions can simply be defined as actions taken toward probable incompatibilities which have not yet taken place, whereas preventive actions are those taken toward incompatibilities which have already occurred. Another way to put it is that removal of the first occurring problem is a corrective action while removal of probabilities with possible cause to a similar problem is a preventive action.

One corrective action alone may not be sufficient to resolve an incompatibility. For analysis of the underlying core reasons, there is a necessity to investigate, manifest and resolve all fundamental causes which have led to the CAPAs.

3.5 Donor Eligibility Assessment

Donor eligibility assessment is especially vital for assurance of the safety of the end product. Once the donor tissues and cells reach the manufacturing facility, they are kept under quarantine until all QC procedures are completed and the tissues and cells are found appropriate to initiate manufacturing procedures.

Donor eligibility assessment rests on two major pillars: donor history and serology reports which cover medical and social evaluation of the donors. Donor history contains information on the potential donor's lifestyle (e.g., alcohol abuse, use of drugs, and other intoxications), travel information, sexually transmissible diseases, associating diseases which would affect donation [18, 19]. Donor history is sought from an individual such as the physician responsible of treating the donor, and family members, and friends who have known the donor well, in cases where the donor is deceased [13]. A living donor can give the information themselves and the physician responsible of treating the donor can also be consulted. If no evidence of donor history can be found, the harvested tissues and cells are rejected. Written informed consent including an explanation, in understandable terms, of all the reasonable risks and potential harm, both for the donor and recipient, as well as all the tests to be performed are collected from living donors [18].

Globally, the minimum testing criteria for all donors are the results of the nucleic acid amplification technique (NAT) for anti-HIV1,2 (Human Immunodeficiency Virus) for HIV1 and HIV2; HBsAg and anti-HBc for Hepatitis B; and HCV antibody for hepatitis, transmissible spongiform encephalopathy (TSE), and syphilis [1, 8, 9, 11–14]. Donor eligibility criteria may differ from one country or region to the other [8–17, 21]. Nevertheless, any suspected or proven data on sexually transmissible diseases is a matter of contraindication; hence, the donor tissue is rejected [18].

3.6 Coding, Traceability, and Product Labeling

A codification system is necessary to ensure traceability coding, donor eligibility, procurement, processing, preservation, storage, transport, distribution, or disposal to QA/QC [18]. In Europe, a single European code (SEC) is allocated to all donated material, to ensure traceability [22].

Traceability starts with a unique identification (ID) number belonging to the specific donor. The unique donor ID is assigned at the time of procurement of the raw tissue and cell material all the way up to the time of release of the processed end product. The donor ID is traced with either corresponding or added numbers

and digits that are uniquely linked to it. This is obtained by the use of product labeling procedures.

Product labeling starts as early as when the starting material reaches the facility, continues throughout the manufacturing procedures and finalizes at the time of release of the end product to the clinic. This is set in place in order to track and trace the donor eligibility assessment of the starting material up until it ends up with the appropriately safe and qualified end product and to send the end product to the appropriate intended end user [18]. Product labeling can be carried out in handwritten and/or electronic format. The labeling, which provides a unique identity to the end product, is also crucial for patient follow-up, for any occurring adverse events or reactions.

3.7 Storage and Distribution

Storage areas are usually isolated and continuously temperature-controlled rooms with controlled personnel passages. Here, consumables, reagents, chemical and biological materials, and donor tissues and cells which are admitted as starting materials are kept, each at a separate section. Storage rooms must have quarantine and release divisions separated with clearly defined boundaries along with storage areas with temperatures starting from −196 °C and −86 °C, and going as high as ambient temperature.

Donor tissues and cells are kept in quarantine until they are assessed and found eligible for manufacture. This includes donor eligibility assessment, risk assessment of possible microorganism contamination, and physical examination for starting material integrity. Quarantined as well as in-process and end product donor cells and tissues can be easily separated from each other by appropriate sectioning rules. Consumables, reagents, and chemical and biological materials are kept in quarantine until safety results are obtained. Starting materials and others found in concordance with the acceptance criteria can be released for processing while those not found in concordance are duly rejected. Separate sections for quarantine, release, and rejection must be well-defined in the storage areas [18]. Once released for use, such substances are ordered with the first-in–first-out principle to provide the use of all substances within the limit of their expiry dates.

Shipping containers must be adequately designed and utilized to protect the released end product from any risks of contamination, sharp rises and falls in temperature, and any other harmful interference until it reaches the end user. Temperature can be monitored by the use of data loggers [16].

End products shipped by a third party must always carry the label defining that tissues and cells are in the content of the package and therefore the package must be handled with care. Packages of cellular products must have a warning for no irradiation. If the end product has been detected positive for a relevant infectious disease, the warning of a biological hazard must be written on the package.

End products intended for autologous transplantation must include a warning for autologous use only. If there are special storage conditions such as an avoidance to freeze the end product, this must also be stated on the package [12, 15, 18].

3.8 Biovigilance, Product Recalls, and End User Complaints

Biovigilance can be summarized as the reporting of an adverse event or reaction suspected to have been caused by a defect of safety or quality in the tissue- or cell-based product in a recipient [18]. Biovigilance is best practiced through a well-defined codification system which addresses the traceability of tissues and at each step from the donor to the recipient and back. The traceability of tissues and cells requires coding and documentation, in written or electronic format, and most importantly, adverse event or reaction reporting from end users (Fig. 2).

For which ever reason should a tissue- or cell-based product be found unsuitable for transplantation, recall procedures are initiated by the manufacturer and/or the third-party transporting company, where available. All relevant tissues and cells and origin donors must also be tracked and traced for inclusion to the recall. All hospitals to which such tissues and cells were dispatched for transplantation and any recipients to which cells, tissues or organs were transplanted are notified [18]. Recalled tissues and cells are marked and stored separately while CAPAs are taken. A product is found unsuitable if and when (1) an event occurs during the manufacturing process that may affect the safety, purity, or potency of a product that has been distributed; (2) there has been an error during product testing or labeling that may affect the safety, purity, or potency of a product that has been distributed; (3) the final product specifications were not met and

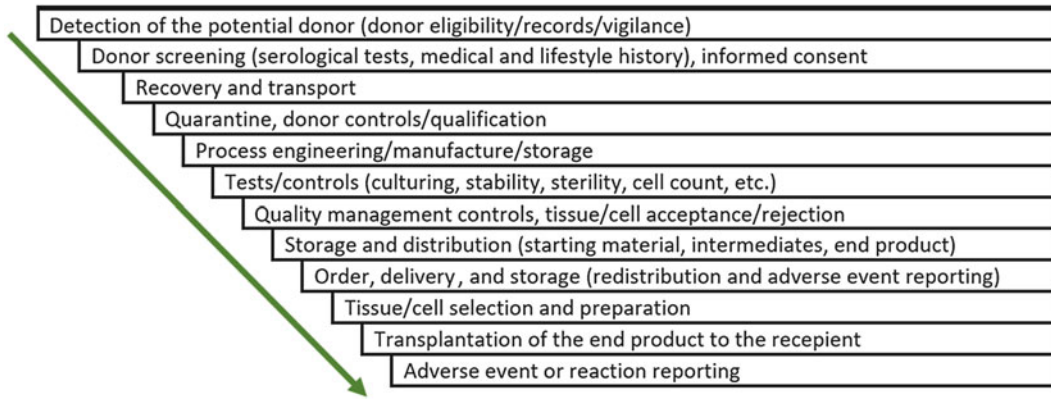

Fig. 2 Main steps in biovigilance and traceability. The most critical parameter of human tissue- and cell-based manufacturing is traceability. It is an obligation to monitor where from and to tissues/cells are traveling, how they are manufactured and tested, and by which end user(s) the end product(s) have been received

the product was distributed; and (4) the QA/QC procedures were not followed and the product was distributed [19]. Such products decided not to be suitable for human application after recall are rejected, and can be used for other purposes such as for further research [4].

Once a complaint regarding a human-based tissue/cell product is communicated, the detected or suspected defect shall also be investigated for a possible similar effect on other batches manufactured from the same donor origin. Complaint records are subject to periodic review in order to identify any recurring events of risk so that CAPA procedures are appropriately implemented.

4 Strategic Planning in Aid of a High-Performance QMS

A well-established QMS rests on four main pillars: (1) planning, (2) QA, (3) QC, and (4) quality improvement (Fig. 3). A proper QMS must ensure that procedures regarding related GxP requirements are established, maintained, and documented in a clear language, which is easily accessible to the key personnel. Required procedures include those for receiving, examining, investigating, evaluating, validating, and documenting information; definition of the purchasing control program including supplier management, and the CAPA program; as well as the mission and vision of an establishment along with the duties and responsibilities of the functioning key personnel [19].

Strategic planning is especially important to maintain continuous communication between the relevant departments of an establishment. In a human-based tissue and cell product manufacturing

Fig. 3 The four main pillars of a well-established QMS

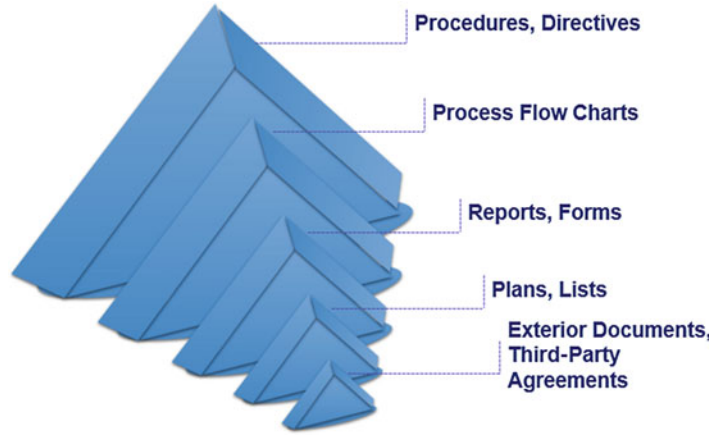

Fig. 4 The hierarchy of the documents organized under the Quality Management System (QMS) documentation ladder

site, the departments can be typically listed as, but not limited to, the following: (1) clinical department, (2) finance department, (3) administrative department, and (4) operations department. Ideally, strategic planning may be done for the projection of a 2- to 5-year period.

5 The QMS Documentation Ladder

All required procedures can be organized in a QMS ladder (Fig. 4), which can easily be updated according to the evolving scientific knowledge and increasing competency.

6 Notes

When allogeneic tissues and cells are used as starting materials, minimizing the risk of communicable diseases is crucial. This may not always be the case for autologous tissues and cells as starting material; however, the material passes hands while exiting the clinic and entering the laboratory, then back from the laboratory to the clinic after it has been processed, again from one hand to the other. We would also have to keep in mind that even autologous, products manufactured from cell types such as chimeric antigen receptor (CAR)-T cells may also cause a potential risk due to the transfection during substantial manipulation of the cells in processing [1]. Allogeneic or autologous, donor infection and contamination during processing, adverse events and reactions on the recipient, and

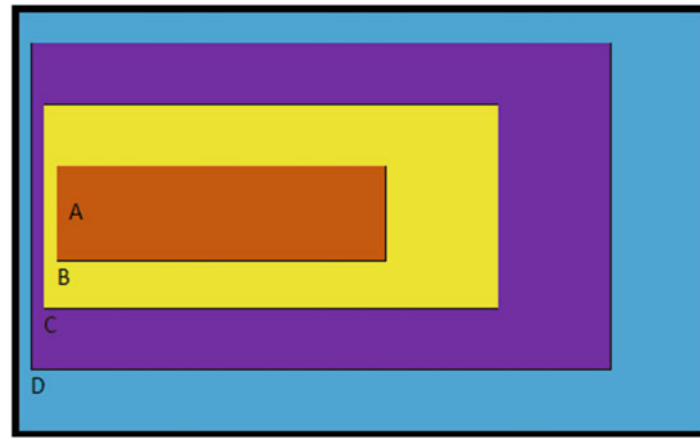

Fig. 5 A representation of the onion layer design of a clean room. Pressure increases as particle concentration decreases, from class D to A: +10–15 Pa (class D; air change per hour: 5–10 times), +20–30 Pa (class C; air change per hour: 10–20 times), +40–45 Pa (class B; air change per hour: 20–40 times). There is no direct passage from class D to A. Differential single direction personnel and material passage is performed from class D to C and class C to B with class A laminar flow, respectively. End products and residues, with separate exits, are transferred from class B directly to the outside

malignant transformation potential are still major concerns of competent authorities around the world.

Although human donor-based, cellular therapies are classified as human medicinal products, and they differ from the conventional medicines in the sense that each batch release is equivalent to one donor per manufacture. In this manner, it is not so easy to reach high sample sizes as it would be for serial manufacturing. Additionally, one end user, a patient, has to be matched with the appropriate donor in order to start manufacturing a cellular therapy product [23]. Here, traceability is crucial for assurance of safe products released to the clinics. Not only traceability but also the fact that a proper and uniform QMS which would cover both serial and custom manufacturing procedures at the same manufacturing facility must be operated.

The layout of the facilities must include a reception area, human-based tissue and cell processing suites, a packaging and labeling area, storage rooms, a distribution area, QC laboratories, an archive, and administrative offices [18]. Only authorized personnel can have access to such working areas.

The layout of clean rooms are generally designed by principle of onion layers (Fig. 5). The maximum accepted airborne particle concentration is reduced from the outside areas going in [2, 4, 7, 10, 15, 18] (Table 3). Four grades can be distinguished: (a) Grade A: Normally such conditions are provided by a laminar air flow work station. Laminar air flow systems should provide a

Table 3
The maximum accepted airborne particle concentrations, at rest and in operation

	At rest		In operation	
Class	**0.5 μm**	**5 μm**	**0.5 μm**	**5 μm**
A	3520	20	3520	20
B	3520	29	352,000	2900
C	352,000	2900	3,520,000	29,000
D	3,520,000	29,000	Unspecified	Unspecified

Table 4
Average recommended limits for microbial contamination during environmental controls in a clean room setting

Class	Air sampling cfu/m^2	Sedimentation method (90 mm RODAC plates) cfu/4 h	Pressure method (55 mm RODAC plates) cfu/RODAC	Fingerprint method (hands inside gloves) cfu/glove
A	<1	<1	<1	<1
B	10	5	5	5
C	100	50	25	–
D	200	100	50	–

homogeneous air speed in a range of 0.36–0.54 m/s (guidance value) at the working position in open clean room applications. The maintenance of laminarity should be demonstrated and validated. A unidirectional air flow and lower velocities may be used in closed isolators and glove boxes; (b) Grade B: This is the background environment for the grade A zone if tissues or cells are processed according to GMP rules. The risk assessment tool for defining the air quality can be used to select the background; (c) Grade C and D: Clean areas for carrying out less critical stages according to documented risk assessment in the processing activities [18].

Average recommended limits for microbial contamination during environmental controls are given in Table 4 [18]. Sinks, hand sanitizers and desks are present in airlocks between classes D and C whereas only hand sanitizers are present in airlocks between classes C and B/A. For proof of compliance with expected storage conditions, critical parameters such as temperature, humidity, and potential risks of contamination must continuously be controlled, tracked, and recorded.

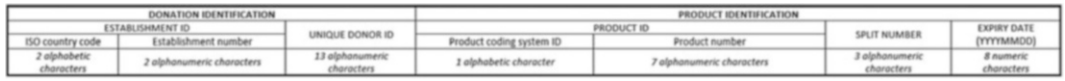

Fig. 6 The Single European Code (SEC) barcoding system

The SEC shall incorporate at least [22] (a) donation identification, (1) unique ID number and (2) identification of the tissue establishment; (b) product identification, (1) product code (basic nomenclature), (2) split number (if applicable), and (3) Expiry date (Fig. 6).

The biopharmaceutical QMS documentation ladder is designed to cover key aspects such as, but not limited to, the following,

- Equipment and premises.
- Personnel.
- Environmental controls.
- Materials and reagents.
- Process engineering.
- Process and quality control validation.
- Change control procedures.
- Labeling controls.
- Storage and distribution conditions.
- Donor eligibility assessment.
- Avoidance of pooling.
- Traceability and coding.
- End-user complaints.
- Adverse event and adverse reaction reporting.

Key aspects do, occasionally, vary from one country or region to the other. There is a need to follow current advances in international laws and regulations governing human-based tissue and cell product QMSs.

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