ISO/TC 276 N 564

Date: 2019-07-30

ISO/WD 24651:2019(E)

ISO/TC 276/WG 2

Secretariat: DIN

Biotechnology — Biobanking — General requirements for biobanking of human mesenchymal stromal cells derived from bone marrow

Élément introductif — Élément central — Titre de la partie

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NP stage

Document type: International Standard
Document subtype:
Document stage: (20) Preparatory
Document language: E

STD Version 2.9p

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Foreword

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This document was prepared by Technical Committee ISO/TC 276, Biotechnology.

Any feedback or questions on this document should be directed to the user’s national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Mesenchymal stromal cells (Mesenchymal stromal cells) are multipotent cells that are characterized by differentiate into mesodermal cell lineages. This cell population has been associated with tissue regeneration processes [1], [2], [3]; regulation for quiescence, self-renewal and maintenance of primitive phenotypes of others cell populatios [4], [5] and regulation of the immune system [6], [7], [8].

Several clinical trials have shown the efficiency of these cells in the treatment of hematological diseases, cardiovascular disorders, damage to the musculoskeletal, nervous and digestive systems. [9], [10], [11].

Mesenchymal stromal cells were initially characterized by Alexander Friedenstein in the 60's, who isolated from the bone marrow and described as cells with fibroblastoid morphology [20]. In 2006, International Society Cellular Therapy (SITC), proposed three criteria for defining mesenchymal stromal cells: (i) adherence to plastic substrates *in vitro*, (ii) expression of antigens such as CD73, CD90 and CD105 in the absence of proteins hematopoietic as CD11b, CD14, CD19, CD34, CD45, HLA-DR and (iii) *in vitro* differentiation capacity into osteoblasts, adipocytes and chondrocytes [12].

At present, mesenchymal stromal cells are defined as a heterogeneous cell population with functional properties whose depend on the tissue of origin, clinical characteristics of the donors, expansion protocols (culture medium), number of passes, among other aspects [4], [13], [14]. Therefore, it is important to consider evaluating relevant aspects related to its morphology, phenotype and function during its characterization.

Biotechnology — Biobanking — General requirements for biobanking of human mesenchymal stromal cells derived from bone marrow

1 Scope

This document provides requirements for collection, preparation, isolation, culture, expansion, cryopreservation and characterization of human mesenchymal stromal cells isolated from bone marrow for use in research.

This document is applicable to all organizations performing biobanking with human mesenchymal stromal cells used for research.

Biobank users, regulatory authorities, organizations and schemes using peer-assessment, accreditation bodies, and others can also use this document.

This document does not apply to cell therapy protocols, tissue engineering or therapeutic use with mesenchymal stromal cells isolated from human bone marrow.

NOTE International, national and/or regional regulations and/or requirements can also apply to specific topics covered in this document.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 14155:2011, *Clinical investigation of medical devices for human subjects — Good clinical practice*

ISO 20387, *Biotechnology — Biobanking — General requirements for biobanking*

ISO/DIS 21973, *Biotechnology — General requirements for transportation of cells for therapeutic use*

International Council for Standardization in Hematology (ICSH) protocols [31]

3 Terms and definitions

For the purposes of this document, the following terms and definitions and those of ISO 20387 apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at http://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
mesenchymal stromal cells
heterogeneous population of cells present in multiple tissues (e.g., bone marrow, umbilical cord, placenta and adipose tissue) with paracrine secretion, immunomodulation, angiogenic and differentiation properties that are demonstrated using a matrix of functional assays

3.2
multipotent cells
cells that have the capacity of self-renewal by dividing, and which can develop into multiple specialized cell types present in a specific tissue or organ

Note to entry 1: Most adult stem cells are multipotent stem cells.

3.3
flow cytometry
method of measuring the number of cells in a sample, the percentage of live cells in a sample, and certain characteristics of cells, such as size, shape, and the presence of tumor markers on the cell surface

Note 1 to entry: The measurements are based on how the light-sensitive dye reacts to the light.

3.4
informed consent
permission to perform healthcare activities, voluntarily given by a subject of care having consent competence or by a subject of care proxy, after having been informed about the purpose and the possible results of the healthcare activities

Note 1 to entry: Informed consent is documented by means of a written, signed and dated informed consent form.

[SOURCE: ISO 13940:2016, 11.2.6]

3.5
bone marrow
soft, sponge-like tissue in the center of most bones which produces white blood cells, red blood cells, and platelets

3.6
self-renewal
attribute to divide mitotically and replace itself while retaining potency

3.7
senescence
ageing, or loss of regenerative capacity, of cells *in vivo*

4 Symbols and abbreviated terms

Abbreviation	Term
Agg	aggrecan
ALP	alkaline phosphatase
AP2	adipocyte protein-2
BM-mesenchymal stromal cells	bone marrow mesenchymal stromal cells
CD	clusters of differentiation
CEBPα	CCAAT / enhancer-binding protein alpha
CFSE	carboxyfluorescein succinimidyl ester
Col10	Collagen type X
COL2A1	Collagen type 2A1
DMEM	
DMSO	Dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
HBsAg	Hepatitis B Surface Antigen
HIV	human immunodeficiency virus
IFN-γ	interferon-gamma
LPL	lipoprotein lipase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
OCN	osteocalcin
OPN	osteopondin
PDT	population doubling time
PPARy	peroxisome proliferator-activated receptor gamma
RunX2	runt related transcription factor 2-
SOX9	Sry-related HMG box 9
VDRL	

5 General requirements

5.1 General

The biobank shall work according to the requirements described in ISO 20387. Additional requirements for the biobank are indicated in this document. ISO/DTR 22758 can be used as additional reference for the implementation of ISO 20387.

The biobank shall use validated and/or verified methods and procedures for activities pertaining to mesenchymal stromal cells according to ISO 20387:2018, 7.9.2 and 7.9.3 at all stages of the biological material life cycle.

The donor's health, tissue harvest conditions as well as culture methods can influence the properties of mesenchymal stromal cells. Workflow steps cannot always be controlled. Thus, their impact on the properties of mesenchymal stromal cells for biobanking shall be investigated, and mitigation measures shall be established to enable the required quality control. In these cases, risk assessment is recommended.

The authenticity and properties of mesenchymal stromal cells shall be investigated throughout the complete cell culture process.

During the whole cell culture workflow, precautions shall be taken to avoid cross contamination between different samples/specimens, e.g., by using disposable material whenever feasible or appropriate cleaning procedures between processing of different specimens/samples.

5.2 Legal and ethical issues

The general requirements on legal and ethical issues of ISO 20387:2018, 4.1.6 apply.

The biobank shall establish an ethics investigation committee, which is responsible for investigation and evaluation of any related ethical issues.

The biobank shall be aware of and able to demonstrate compliance with relevant national, regional and international approved ethics, laws and regulations relating to mesenchymal stromal cells derived from bone marrow.

5.3 Facilities and environmental conditions

ISO 20387:2018, 6.3 shall be followed.

Biobank should define a protocol to test the quality of the biobanking environment(s). It is recommended to consider test methods proposed in ISO 14644.

5.4 Reagents and equipment

ISO 20387:2018, 6.5 shall be followed.

Reagents and equipment used in biobanking should be selected under quality specific criteria defined by the biobank, evaluating risks that can affect or impact performed assays, tests or their validity.

The biobank should establish, document and implement a procedure for evaluating the reagent stability; this procedure should define criteria for internal or external quality control (i.e., antibodies, diluents, calibrators).

As changes in temperature can induce thermic stress in cultured cells, all incubators used, for working in accordance with this document, shall be qualified.

6 Collection of bone marrow and associated data

6.1 Informed consent

A procedure for obtaining the informed consent shall be established, documented and implemented according to ISO 14155:2011, 4.7.4 and 4.7.5.

A procurement protocol shall be written and reviewed. The review of the procurement protocol(s) shall ensure that the bone marrow donor or the donor's authorized relative is adequately informed about the specific aspects of his/her voluntary donation.

The donor or the donor's authorized relative shall understand the purpose of the bone marrow donation, and sign a written and valid informed consent form, including but not limited to:

a) potential research under appropriate conditions;

b) potential use in many laboratories in many countries;

c) research and treatment applications;

d) unexpected feedback (for example, the cells' future use);

e) potential commercial exploitation;

f) other issues applicable to the research;

g) statements regarding manipulation, retention and personal data protection; and

h) required information according to ISO 14155:2011, 4.7.4 and 4.7.5.

The informed consent form shall make the donor or the donor's authorized relative aware of and give him/her the ability to accept or reject the donation based on the information of:

1) mesenchymal stromal cells, as a new type of biological resource, being a set of biological material and its associated data;

2) that these cells will be stored, and may be sequenced, used in animal studies, or sent to other labs for analyses;

3) the potential extent of testing to be performed on the tissue and/or the cells derived;

4) that donations will be anonymized/pseudonymized/codified to maintain the donor's confidentiality.

The informed consent form and discussion should cover information that addresses:

— key aspects of human mesenchymal stromal cell research; including but not limited to the fact that an immortal mesenchymal stromal cell line can be established, which is a partial or full genetic match to the biomaterials donor; and

— that the mesenchymal stromal cell line can be shared with other researchers outside the institution for other research purposes that are not fully anticipated at this time.

Consent shall also be obtained from the attending physician or surgeon. Appropriate approval shall be obtained from the relevant ethical committee.

The agreement on the informed consent shall not be affected by coercion, inducements and or misconduct. A personal privacy protection mechanism should be established for the donor.

Informed consent should be available in paper or digital for each donor or the donor's/patient's authorized relative and approved by the biobank.

Bone marrow shall only be obtained after the informed consent form has been signed by the donor or the donor's authorized relative.

6.2 General characteristics of bone marrow donors for mesenchymal stromal cell isolation

For research studies bone marrow donors:

a) shall be of legal age.

b) shall not be older than 60 years of age [17], [18], [19], [20], [21], [22].

c) may be those undergoing prosthetic hip replacement or bone marrow aspiration, who voluntarily accepted informed consent for tissue donation [5], [23].

d) should be serologically negative for infectious agents: HBsAg, hepatitis C, HIV, VDRL [24], [25].

e) should have no hematological malignancies [26], [27] or primary immunodeficiencies [28].

NOTE Depending on the basic research interest, hematological malignancies can be considered.

The isolation of mesenchymal stromal cells should not be performed from donors:

1) with contraindications for extraction of bone marrow aspirate, sedation or anesthesia locally (i.e., thrombocytopenia); and

2) in gestation or lactation.

Any risk to donors should be considered and evaluated under clinical decision.

It has been shown that the characteristics of bone marrow donors such as age, viral infections, neoplastic and immunological disorders affect some of the mesenchymal stromal cells' characteristics. Thus, the final inclusion criteria for a donor of bone marrow for mesenchymal stromal cells to be used in research should be defined by the investigators.

6.3 Information about the bone marrow donor

The biobank shall establish, document and implement inclusion and exclusion criteria based on the purpose of research. The criteria shall be documented. A risk assessment shall be performed and documented.

The documentation of the donor information shall include, but is not limited to:

a) the ID of the donor, which can be in form of a code (e.g., pseudonymized);

b) the relevant health status of the bone marrow donor (e.g., healthy, disease type, concomitant disease, demographics (e.g., age and gender));

c) the information about medical treatment and special treatment prior to bone marrow collection (e.g., date, terms of treatment, medication, conclusion of medical specialist);

d) a statement about the absence of hepatitis C, HIV and VDRL;

e) the informed consent form (see 6.1) of the specimen donor or the donor's authorized relative;

f) the absence of congenital abnormalities in the neonates, when appropriate.

Donors shall not be considered suitable for donation, if they:

1) were unwell at the time of donation. Deferral periods for specific infections can exist and/or vary in accordance with local regulatory guidelines for other cell and tissue products;

2) have tested confirmed repeat positive for infectious disease (see 6.3, b) to d));

NOTE Inclusion and exclusion criteria can be strategically based on specific factors (e.g., ethnic background or immune haplotype).

6.4 Anatomical collection site

Following the recommendations of the International Council for Standardization in Hematology (ICSH) protocols [29], the puncture of the iliac crest should be performed to obtain bone marrow aspirates.

If the bone marrow will be collected by reaming the intramedullary canal, the collection should be made from the head of the femur [23].

6.5 Collection volume

It has been shown that the volume of bone marrow collection influences the number of isolated mononuclear cells, and therefore plays an important role in the efficient isolation of mesenchymal stromal cells from bone marrow [30]. Thus:

1) for samples of bone marrow aspirate, collect a bone marrow volume between 10 ml and 80 ml [31], [32], [33].

2) for rhymed samples of the intramedullary canal, collect a bone marrow volume between 30 ml and 35 ml [30].

6.6 Collection procedure

6.6.1 General

The biobank shall establish, document, validate and implement a mesenchymal stromal cell collection procedure for each collection method. ISO 20387:2018, 7.2 shall be followed.

The biobank shall establish, document and implement a collection protocol for each used collection method (see 6.6.2 and 6.6.3).

All reagents and materials used to collect the bone marrow shall be sterile.

Biorisk management should be considered.

6.6.2 Obtaining bone marrow by reaming the intramedullary canal

During the prosthetic hip replacement procedure, the orthopedic surgeon collects the bone marrow from the intramedullary canal with the help of the reamer [5]. The sample should be collected with a disposable sterile container, tagged according to ISO 20387:2018, 7.5.1, and filled with EDTA plus 2 mg/ml anticoagulant (ethylenediaminetetraacetic acid) or 15 IU/ml to 20 IU/ml heparin.

6.6.3 Obtaining bone marrow by aspirate

Bone marrow aspiration of the anterior iliac crest shall be performed according to the International Council for Standardization in Hematology (ICSH) protocols [30].

Bone marrow aspirate should be taken in heparinized syringes of 15 IU/ml to 20 IU/ml [31].

7 Transport of bone marrow and associated data to the biobank

7.1 General

The biobank shall establish, implement, validate and document procedures for the transport and handling, including safety aspects, of bone marrow and its associated data. The requirements of ISO 20387:2018, 7.4 and ISO/DIS 21973 apply. ISO 15189 can be used to consider transport and handling, and safety requirements on facilities.

Unnecessary exposure to radiation shall be avoided during shipment.

Biological material is in general categorized as dangerous goods, and therefore requires all staff involved in the shipping of these materials to be suitably trained. The staff training shall be according to ISO 20387:2018, 6.2.2, and 6.2.3.

Biorisk management should be considered.

7.2 Transport of bone marrow obtained by reaming the intramedullary canal

The sample shall be transported under appropriate biosafety conditions [31] and with a transportation temperature of 15 °C ± 2 °C. The transport shall be finalized within a duration < 2 h after the bone marrow has been obtained (see 6.6.2) and shall be in accordance with the requirements for the cell isolation start point (9.1) [24], [30].

7.3 Transport of bone marrow obtained by aspirate

The sample shall be transported under appropriate biosafety conditions [31] and with a transportation temperature of 15 °C ± 2 °C. The transport shall be finalized within a duration < 2 h after the bone marrow has been obtained (see 6.6.3) and shall be in accordance with the requirements for the cell isolation start point (9.2).

8 Reception and traceability of bone marrow and associated data

ISO 20378:2018, 7.3.1, 7.3.2 and 7.5 apply.

9 Isolation and expansion of mesenchymal stromal cells

9.1 Isolation from bone marrow obtained by intramedullary canal reaming [5], [18], [31], [32], [33], [34]

The mesenchymal stromal cell isolation protocol shall be performed within a duration ≤ 2 h after the bone marrow has been obtained (see 6.6.2) [24], [30].

1) Perform a sterile mechanical disruption of reaming the intramedullary canal with PBS1X (without Ca^{++} and Mg^{++}) with antibiotics (1:2 ratio) in a sterile mortar. Filter the disintegrated sample through a Buchner funnel.

2) Collect the filtered sample and centrifuge it for 20 min at 800 g at 15 °C to 20 °C.

3) Collecting cells (buffy coat) and dilute with PBS1X (without Ca^{++} and Mg^{++}) with antibiotics. Subsequently separating mononuclear cells by density gradient Ficoll ™ or Percoll ™ (1-part ficoll ratio: 3 parts blood), centrifuged for 30 min at 450 g at 15 °C to 20 °C.

4) Separate the mononuclear cells and perform three washes with PBS1X. Make a cell count and determine cell viability by exclusion techniques (trypan blue) (manually or automated).

5) It is suggested to start cultures, when the cell viability is higher than 90 % and the cell density has reached 100×10^3 cells/cm^2 to 160×10^3 cells/cm^2 with a ratio of 0,2 ml of culture medium per planting area. Incubate at approx. 37 °C, 5 % CO_2 and 95 % relative humidity. For culture under hypoxic conditions adjust the gas mixture and/or hypoxia chamber as required for research.

6) After 72 h of culture, remove non-adherent cells and add fresh culture medium. Do not remove the culture medium to observe adherent cells (approx. 5 days to 7 days).

7) When a confluency of adherent cells is observed between 70 % to 80 %, making the pass cells by trypsinization (trypsin 0,25 %, EDTA 1 mM). The cell population obtained from intramedullary reaming after the first passage should be grown at a density between 10×10^3 cells/cm^2 to 25×10^3 cells/cm^2. A sterility test to the culture media should be performed before use. Additionally, the culture media should be tested for endotoxins levels and mycoplasma spp. To assess the performance of the culture media, a characterized cultured mesenchymal stromal cell line should be used.

8) It is suggested to use the culture medium DMEM (Dulbecco's modified Eagle's medium), alpha-MEM (Minimum Essential Media) or IMDM (Iscove's Modified Dulbecco's Medium) supplemented with fetal bovine serum (10 % to 20 %), lysate platelet or human serum. The use of free culture media serum designed for expansion of the mesenchymal stromal cells is also suggested. The

selection of the culture medium depends on research purposes [35], [36], [37], [38]. Similarly, researchers shall decide, if the mesenchymal stromal cells will be cultured in 2D or 3D systems.

9.2 Isolation from bone marrow obtained by aspirate [39], [40]

The mesenchymal stromal cell isolation protocol shall be performed within a duration ≤ 2 h after the bone marrow has been obtained (see 6.6.3) [24], [30].

1) Transfer the bone marrow aspirate into sterile tubes and centrifuge them for 10 min at 400 g at 15 °C to 20 °C, then resuspend the cells in 50 ml of culture medium [35], [36], [37], [38]. Perform cell counting and determine the viability by exclusion techniques (trypan blue) (manual or automated).

2) Direct seeding; the culture should be started with a cell viability ≥ 90 % and a cell density of 1×10^6 cells/cm^2 at a ratio of 0,2 ml of culture medium per planting area (for separation of mononuclear cells, see 9.1, points 3) and 4)).

a) Incubate at approx. 37 °C, with 5 % CO_2 and 95 % relative humidity. Then, follow 9.1, points 6), 7) and 8).

3) It is suggested to grow mesenchymal stromal cells obtained from bone marrow aspirate, after the first passage, at a density of 7×10^3 cells/cm^2.

10 Biological and functional characterization of mesenchymal stromal cells

10.1 Determination of cell viability (supravital staining with trypan blue 0,5 %) [41], [42]

10.1.1 Dilute 1:2 of cell suspension with trypan blue 0,5 %, immediately transfer the indicated volume of dilution according hemocytometer (manual method Neubauer or automated equipment).

10.1.2 For manual a method, perform the counting of cells in the quadrants of the chamber (Neubauer chamber 4 quadrants of 0,1 mm^3) simultaneously discriminating between living cells (unstained) and dead cells (stained blue).

10.1.3 Calculate the cell count with Equation 1:

$$\text{cell count} = \text{amount of cells} \times \text{dilution factor} / 4 \times 0{,}1\ \text{mm}^3 \quad (1)$$

10.1.4 Calculate the percentage of cell viability with Equation 2:

$$\text{cell viability (\%)} = \text{amount of living cells} \times 100 / \text{amount of total cells} \quad (2)$$

10.1.5 Quality control for the cell viability test should be performed using live and death cells.

Depending on the biobank facilities and resources, an automated cell viability test should be performed. The instrument conditions and controls to perform the assays should be considered.

10.2 Morphologic features

Microscopically, seeded mesenchymal stromal cells are fibroblastoid like (spindle-shaped and fusiform) and grow by attaching to the bottom of the culture dish, with diameters ranging from 9 μm~15 μm. Colonies have spiral or radial patterns (Figure 1 (A) and (B)).

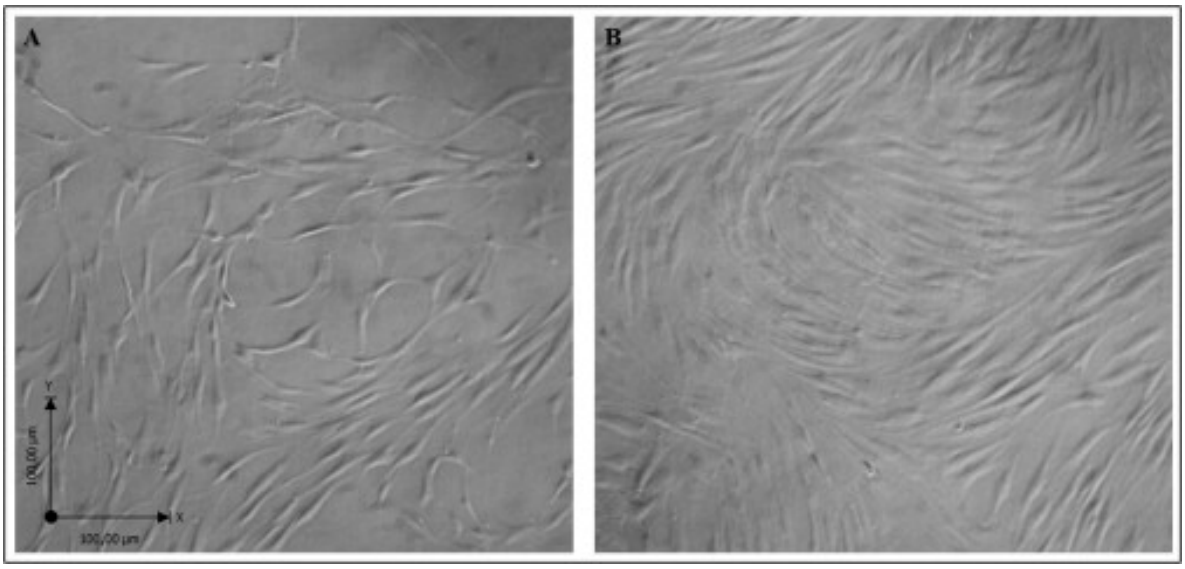

Figure 1. Morphology of Mesenchymal stromal cells isolated from bone marrow. Cells after 8 days (A) and 15 days of culture (B). (Olympus CK2, 10x magnification Scale bar: 100 microns). Photographs were taken with a Sony Cybershot DSC-W7 digital camera, and colours were corrected with Adobe Photoshop *(From Rodriguez-Pardo, Viviana M et al Mesenchymal stem cells leukaemic cells Promote aberrant B-cell phenotype from acute lymphoblastic leukemia Hematology / Oncology and stem cell therapy 2013; 6 (3-4):89-100)* [5].

To evaluate morphology characteristics of mesenchymal stromal cells under the influence of the culture media a comparison with a characterized mesenchymal stromal cell line should be performed.

10.3 Population doubling time (PDT) [34], [43], [44]

The population doubling time is the time (measured in hours) required for the replication of the population of mesenchymal stromal cells. The PDT is calculated with Equation 3 using the cell counts obtained before and after performing crops:

$$PDT = (T - T_0) \times \log_2 / (\log N - \log N_0) \quad (3)$$

Where:

$(T-T_0)$	is the incubation time in hours
N	is the count of cells harvested
N_0	is the count of cells seeded

The average PDT of mesenchymal stromal cells isolated from human bone marrow ranges between 40 h and 100 h, depending on the characteristics of the donor (e.g., age) and culture passage [34], [44].

10.4 Colony formation assay

1) Isolate and culture mesenchymal stromal cells as shown in Clause 9. Obtain the cells at a confluence of 70 % to 80 %, and trypsinized.

2) Perform cell counting of the mesenchymal stromal cells, and seed 100 cells per 100 mm tissue culture dish in complete culture medium.

Incubate for 10 days to 14 days at approx. 37 °C in humidified 5 % CO_2; then wash and stain with PBS 0,5 % Crystal Violet in methanol for 5 min to 10 min at 15 °C to 20 °C.

3) Wash the plates with PBS twice and visible colonies count (Figure 2).

Figure 2. Assay fibroblastoid forming unit (CFU-F assay) *(From Taghiyar Leila et al Isolation, Characterization and Osteogenic Potential of Mouse Cells in Digit Tip Blastema Comparison With Bone Marrow Derived Mesenchymal Stem Cells-In Vitro, Cell J. 2018; 19 (4):585-598)* [46].

10.5 Cell proliferation

10.5.1 Determination by incorporation of CFSE [5]

1) Suspend mesenchymal stromal cells (1 x 10^6 cells/ml) in 1 000 ul of 0,1 % albumin PBS1X.

2) Add 1 ul of 5uM CFSE (or CFSE dilution 1:1 000) to the cell suspension.

3) Incubate for 20 min at approx. 37 °C in the dark.

4) Add 1 000 µl of culture medium with 10 % FBS.

5) Incubate for 5 min at approx. 37 °C in the dark.

6) Centrifuge for 5 min at 450 g at 15 °C to 20 °C.

7) Resuspend the cells in fresh medium (1 000 µl) of pre-warmed to 37 °C.

8) Analyze by flow cytometry (488 nm laser, detector FL1 for fluorescein isothiocyanate). Adjust compensation according to the instrument and internal quality control conditions.

To evaluate the cell proliferation by CFSE incorporation serum-depleted cells should be used as a negative control for proliferation assay.

10.5.2 Method of 3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide (MTT) [46] [47], [48]

1) Suspend mesenchymal stromal cells (1× 10^3 cells/ml) in 96-well plates with 100 µl of MTT working solution (5 mg/ml in culture medium without serum).

2) Incubate 4 h at approx. 37 °C, remove supernatant and resuspend the pellet in 100 µl of DMSO.

3) Perform the evaluation by spectrometry at 490 nm.

10.6 Immunophenotyping by flow cytometry [5], [19], [50]

10.6.1 General guidelines:

1) Make the adjustment and calibration according to the type of cytometer available.

2) Use the compensation strategy according to the number of fluorophores to be detected.

3) The expression of antigens associated with mesenchymal stromal cells should be determined according to the consensus of the International Society for Cellular Therapy (ISCT) [12]. Consider evaluating the expression of other antigens associated with bone marrow mesenchymal stromal cells such as CD146 and CD271.

4) Prepare the buffer for the cytometry (PBS1X, 2 mM EDTA, 0,5 % FBS).

5) Add the suspension of mesenchymal stromal cells according to standardized biobank values. A 1×10^5 cells/ml to 5×10^5 cells/ml mesenchymal stromal cell suspension should be used.

6) The volume of each antibody to be added should be established by the biobank. Table 1 shows the clones of antibodies suggested for phenotyping of mesenchymal stromal cells [50], [51], [52], [53], [54].

Table 1 — Clones of antibodies suggested for immunophenotyping of bone marrow Mesenchymal stromal cells

Antigen	Antibody clones [5], [49], [50], [51], [52], [53], [54]
CD34	581, Q-BEN 10 8G12, AC136, 4H11
CD45	2D1, t29 / 33, HI30
CD105	SN6
CD73	AD2
CD90	Thy-1, F15-42-1, eBio5E10
CD44	MEM-85, G44-26
CD146	Insufficient information to suggest clone
CD271	Insufficient information to suggest clone

10.6.2 Remove the culture medium of the mesenchymal stromal cells by centrifugation (5 min of washing with cytometry buffer performed twice at 450 g) at 15 °C to 20 °C.

10.6.3 A 1×10^5 to 5×10^5 mesenchymal stromal cell suspension should be used.

10.6.4 Add concentration of antibodies defined by the biobank. Incubate 15 min at 15 °C to 20 °C in the dark.

10.6.5 Add 2 000 µl of cytometry buffer and centrifuge for 10 min at 300 g (two times) at 15 °C to 20 °C.

10.6.6 Resuspend cells in 300 µl of cytometry buffer and read out with the flow cytometer.

To evaluate immunophenotyping of mesenchymal stromal cells by flow cytometry it is suggested to be compared with a characterized mesenchymal stromal cell line.

10.7 Potency assay: multilineage differentiation (osteogenesis, chondrogenesis, adipogenesis)

10.7.1 WARNING — To evaluate multilineage differentiation of mesenchymal stromal cells, the biobank should define, document and implement protocols and requirements for the specific assays, (i.e., mesenchymal stromal cell line with ability to multilineage differentiation).

10.7.2 Adipogenesis:

1) Use mesenchymal stromal cell cultures with a confluence between 60 % and 80 % in the 3rd culture passage.

2) Remove the culture medium and wash the cells with PBS one time. Replace the culture medium by the adipogenic differentiation medium. Incubate at approx. 37 °C, with 5 % CO_2 and 95 % relative humidity for 14 days.

3) Change the culture medium every three days. Perform the negative control of differentiation with the culture medium for expansion.

4) After 14 days of incubation, determine the presence of lipid vacuoles by staining techniques, e.g., sudan black.

5) To confirm the adipogenic capacity of mesenchymal stromal cells, evaluate the expression of genes encoding proteins associated with these cells such as PPAR (peroxisome proliferator-activated receptor gamma), CEBPα (CCAAT / enhancer-binding protein alpha), AP2 (adipocyte protein-2) and LPL (lipoprotein lipase) using quantitative Real-time RT-PCR [55], [56].

10.7.3 Chondrogenesis:

1) Use mesenchymal stromal cell cultures with a confluence between 60 % and 80 % in the 3rd culture passage.

2) Remove the culture medium and wash with PBS one time. Replace the medium by the chondrogenic differentiation medium. Incubate at approx. 37 °C, with 5 % CO_2 and 95 % relative humidity for 14 days.

3) Change the culture medium every three days. Perform the negative control of differentiation with culture medium for expansion.

4) After 14 days incubation, stain the cells with alcian blue staining or safranin.

5) To confirm the chondrogenic capacity of mesenchymal stromal cells is suggested to evaluate the expression of genes encoding proteins associated with this cell line such as SOX9 (SRY-related HMG box 9), COL2A1 (Collagen type 2A1), col10 (Collagen type X) and Agg (aggrecan) using quantitative Real-time RT-PCR [56], [57].

10.7.4 Osteogenesis:

1) Use mesenchymal stromal cell cultures with a confluence between 60 % and 80 % in the 3rd culture passage.

2) Remove the culture medium and wash once with PBS. Replace the medium by chondrogenic differentiation medium. Incubate at approx. 37 °C, with 5 % CO_2 and 95 % relative humidity for 21 days.

3) Changing the culture medium every three days. Remember to negative control of differentiation with culture medium for expansion.

4) After 21 days of incubation, stain the cells with alizarin red staining, safranin or detect the expression of alkaline phosphatase.

5) To confirm the osteogenic potential of mesenchymal stromal cells is suggested to evaluate the expression of genes encoding proteins associated with this cell line such as ALP (alkaline phosphatase), RunX2 (runt-related transcription factor 2), OCN (Osteocalcin) and OPN (osteopondin) using quantitative Real-time RT-PCR [55], [56].

10.8 Potency assay: mesenchymal stromal cells stimulation assay with IFN-γ (Interferon-gamma)

WARNING — To evaluate immunomodulatory capacity of mesenchymal stromal cells, the biobank should define, document and implement protocols and requirements for the specific assays, (i.e., mesenchymal stromal cell cell line with immunomodulatory capacity).

Studies have shown that the functional heterogeneity of mesenchymal stromal cells depends on the culture conditions, number of passes and activation status, among others. Therefore, it is suggested to perform functional evaluations, in addition to multilineage differentiation, related to the immunomodulatory capacity of the mesenchymal stromal cells to confirm their functional identity. [58], [59], [60], [61].

To confirm the functional capacity, mesenchymal stromal cells (previously isolated and characterized by immunophenotype and multilineage assay) should be cultured for 12 h to 14 h under culture conditions at approx. 37 °C with 10 ng/ml of IFN-γ. Afterwards, the expression of HLA-DR, CD54 and CD274 shall be evaluated by flow cytometry.

Additionally, the expression of genes that encode proteins, e.g., IDO (indoleamine 2,3-dioxygenase), TRAIL (TNF-related apoptosis-inducing ligand) and COX-2 (Cyclooxygenase-2) by quantitative Real-time RT-PCR [61] should be tested. Other tests are suggested to evaluate the functional capacity of the mesenchymal stromal cells as their potency in inhibiting t-lymphocyte proliferation [58], [62] or angiogenic endothelial cell induction [63].

11 Evaluation senescence by detection of β-Galactosidase-SA

Establish mesenchymal stromal cell cultures with a density of $2{,}5 \times 10^4$ cells. After 48 h of culture under culture conditions at approx. 37 °C, evaluate the expression of SA-β-Galactosidase by histochemistry [64].

12 Cryopreservation

Collected cells for freezing should be in the growth phase, the cell count shall be between 500 000 cells/ml to 1 000 000 cells/ml. The morphologic features shall be tested and shall be coherent with features of mesenchymal stromal cells isolated from bone marrow.

The cell viability shall be higher than 90 % and free of contamination (adventitious agents).

Resuspend the cells to between 2×10^6 /ml to 2×10^7/ml.

The cooling rate shall be adjusted to decrease 1 °C per min, progressively in 4 h to 6 h.

Maintain the cells all night or for 12 h at -80 °C, afterwards quickly transfer them into liquid nitrogen to maintain the cell line at -196 °C.

13 The biobank shall maintain records of the cryopreservation process, including the cell density, viability and temperature control. Thawing

For thawing, the vial shall be removed from the LN tank and plunged into a water bath at 37 ° C with gentle swirling.

The contents of the vial shall be added slowly to 5 ml of fresh medium + 30 % FCS with gentle swirling. The cells shall be centrifuged with 20 ml of twice saline solution at 1 000 rpms to clean traces of the culture media.

The cells are pelleted and shall be resuspended in 1 ml of culture media defined by the biobank. Afterwards, a live/dead count shall be made.

14 Transport of cells under culture

The biobank shall establish, implement, validate and document procedures for the transport and handling of mesenchymal stromal cells and their associated data. The requirements of ISO 20387:2018, 7.4 and ISO/DIS 21973 apply. Unnecessary exposure to radiation shall be avoided during shipment.

According to the specific type of application, cells can be included in one or more syringes of different volumes. The recommended media of transport are DMEM without phenol red or Ringer Lactate.

As a primary container, each syringe is introduced into a self-adhesive seal package pre-sterilized with ethylene oxide. The packaging is labeled with:

1) the sample's data;

2) the production and expiration date;

3) the name of the entity; and

4) the address and telephone number.

Each syringe is included in a zip plastic bag to avoid the moisture, which is produced by the refrigerated transport gels.

The secondary container is a portable polyurethane or polystyrene (styrofoam) refrigerator, which was previously disinfected with alcohol, with gels and a refrigerated or data logger thermometer for temperature control (between 4 ° C and 8 ° C).

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ISO 14644, cleanrooms and associated controlled environments

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