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Biotechnology — Biobanking — Requirements for human neural stem cells derived from pluripotent stem cells

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by 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

Neural stem cells (NSCs) are the adult stem cells in the central nervous system (CNS). NSCs have self-renewal ability and multipotency. NSCs can differentiate into various neurons and glial cells including astrocytes and oligodendrocytes. NSCs play a major role in embryonic development and adult neurogenesis. According to the hypothesis by Alvarez-Buylla, there are several types of cells can be called NSCs, including neuroepithelium – epithelial cells of the ventricular zone (VZ) of the neural tube[1], radial glial cells (RGCs) and basal (intermediate) progenitor cell (IPC)[2-4]. In the adult, NSCs are restricted to specific brain regions, such as the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus[5].

NOTE The term "neural" refers to any type of nerve cell, including a mixture of brain cells, whereas "neuronal" is specifically related to neurons.

Despite these advances, substantial ambiguities persist regarding the nomenclature, nature, identity, function, mode of isolation and experimental handling of these cells. NSCs are not fully defined by the initial minimal criteria proposed out, and as such require careful characterization by a matrix of functional assays.

NSCs have been isolated from human fetal CNS (brain or spinal cord), cerebrospinal fluid, biopsy and autopsy material, or differentiated from pluripotent stem cells (PSCs), which are widely used for animal and clinical research[6]. NSCs generated from different sources or differentiation protocols have different properties. Different institutions use different practices for isolating, processing and biobanking these NSCs, making it difficult to compare data and results across institutions. Thus, there is a need for standardized approaches to isolate, process, expand and cryopreserve these NSCs.

The aim of this document is to provide general guidance for biobanking of human NSCs derived from pluripotent stem cells (hPSC-NSCs) for research purposes. This document is applicable for academic centers, public and private institutions performing a biobanking service of hPSC-NSCs for R&D (Research and Development) and preclinical studies, not for clinical use.

Importantly, this document is focused on hPSC-NSCs that have been isolated, manipulated and/or propagated from hPSCs in culture for research purposes.

Biotechnology — Biobanking — Requirements for human neural stem cells derived from pluripotent stem cells

1 Scope

This document specifies requirements for the biobanking of human neural stem cells (hPSC-NSCs) derived from human pluripotent stem cells (hPSCs), including the requirements for the differentiaton, culture, characterization, quality control (QC), storage, thawing and transport of hPSC-NSCs.

Requirements for the collection of biological source material, the transport to and reception of biological source material and hPSCs at the biobank, as well as the establishment, expansion and QC of hPSCs are covered in ISO 24603.

This document is applicable to all organizations performing biobanking of hPSC-NSCs used for research and development in the life sciences.

This document does not apply to hPSC-NSCs for the purpose of in vivo application in humans, clinical applications or therapeutic use.

NOTE International, national or regional regulations or requirements or multiple of them 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 8601-1, *Date and time — Representations for information interchange — Part 1: Basic rules*

ISO 20387:2018, *Biotechnology — Biobanking — General requirements for biobanking*

ISO 21709:2020, *Biotechnology — Biobanking — Process and quality requirements for establishment, maintenance and characterization of mammalian cell lines*

ISO 24603:2022, *Biotechnology — Biobanking — Requirements for human and mouse pluripotent stem cells*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 20387, ISO 21709 and the following apply.

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

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

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

3.1
authenticity
quality of being genuine or true

[SOURCE: ISO/TS 22859:2022, 3.1]

3.2
biobank
legal entity or part of a legal entity that performs *biobanking* (3.3)

[SOURCE: ISO 20387:2018, 3.5]

3.3
biobanking
process of acquisitioning and storing, together with some or all of the activities related to collection, preparation, preservation, testing, analyzing and distributing defined biological material as well as related information and data

[SOURCE: ISO 20387:2018, 3.6]

3.4
biorisk
effect of uncertainty expressed by the combination of the consequences of an event (including changes in circumstances) and the associated "likelihood" (as defined in ISO Guide 73) of occurrence, where biological material is the source of harm

Note 1 to entry: The harm can be the consequence of an unintentional exposure, accidental release or loss, theft, misuse, diversion, unauthorized access or intentional unauthorized release.

[SOURCE: ISO 35001:2019, 3.17]

3.5
cell culture
growth of cells dissociated from the parent tissue by spontaneous migration, mechanical or enzymatic dispersal for propagation under in vitro conditions

[SOURCE: ISO/TS 22859:2022, 3.5]

3.6
cell master file
complete dossier of all procedures and records used to generate cells

[SOURCE: ISO/TS 22859:2022, 3.6]

3.7
cell morphology
form and structure of the cell

Note 1 to entry: Morphology can be represented by a single parameter or a combination of two or more parameters.

[SOURCE: ISO 21709:2020, 3.3]

3.8
cell population purity
percentage of a particular cell type in a population, of which has the same specific biological characteristics, such as cell specific markers, genetic polymorphisms and biological activities

[SOURCE: ISO/TS 22859:2022, 3.8]

3.9
cryopreservation
process by which cells are maintained frozen at an ultra-low temperature in an inactive state so that they can be revived at a later time

[SOURCE: ISO 21709:2020/Amd.1, 3.6]

3.10
differentiation
process to bring the stem cells into a defined cell state/fate

[SOURCE: ISO/TS 22859:2022, 3.11]

3.11
differentiation potential
ability that refers to the concept that stem and progenitor cells can produce daughter cells which are able to further differentiate into other cell types

[SOURCE: ISO/TS 22859:2022, 3.12]

3.12
flow cytometry
methodologically oriented subdiscipline of analytical cytology that measures cells in suspension in a liquid vehicle as they pass, typically one cell at a time, by a measurement station

Note 1 to entry: The measurement represents transformations of changes in the output of a detector (or detectors) due to changes in scattered light, absorbed light, light emitted (fluorescence) by the cell, or changes in electrical impedance, as the cell passes through the measuring station.

Note 2 to entry: Flow cytometry allows simultaneous evaluation of morphological characteristics of cells (size and internal complexity) with membrane or intracellular antigens.

[SOURCE: ISO/TS 22859:2022, 3.13]

3.13
human neural stem cells derived from pluripotent stem cells
hPSC-NSCs
immature cellular population differentiated from pluripotent stem cells, which has the ability for self-renewal and differentiation to neurons and glia cells (astrocytes or oligodendrocyte) in vitro and in vivo.

Note 1 to entry: Without any manipulation, culture-adapted hNSCs (human neural stem cells) is an alternate term used to denote cells that are different from cells that are found in vivo. It is increasingly clear that these cell types have different properties in terms of gene expression, functionality and phenotype.

3.14
identity verification
part of the process of verifying authenticity of a cell line in which cell origin is genetically confirmed

[SOURCE: ISO 21709:2020, 3.10]

3.15
multipotent cells
cells that have the ability to differentiate into more than one, but a limited number of related cell types

3.16
passage
subculture
process of further culturing of cells in a new culture vessel to provide higher surface area/volume for the cells to grow

[SOURCE: ISO/TS 22859:2022, 3.18]

3.17
passage number
number of subculturings that occurred

Note 1 to entry: For this document P_0 is understood as the starting population of the cells.

[SOURCE: ISO 21709:2020, 3.13, modified — Note 1 to entry has been added.]

3.18
doubling time
PDT
population doubling time
time taken for cultured cell count to double

Note 1 to entry: The time is measured in hours.

[SOURCE: ISO 21709:2020, 3.8, modified — “population doubling time” and “PDT” have been added as the preferred term and Note 1 to entry has been added.]

3.19
primary cells
cells isolated directly from body fluid, tissue or organs taken directly from an organism, using enzymatic or mechanical methods

[SOURCE: ISO 21709:2020, 3.15, modified — "body fluid" added to definition.]

3.20
primary culture
initial in vitro cultivation of *primary cells* (3.19)

3.21
primary human neural stem cells derived from pluripotent stem cells
primary hPSC-NSCs
initial neural stem cells (NSCs) derived from in vitro human pluripotent stem cell (hPSC) differentiation

3.22
proliferation
cell number expansion by cell division

[SOURCE: ISO/TS 22859:2022, 3.22]

3.23
self-renewal
ability of *stem cells* (3.24) to divide symmetrically, forming two identical daughter stem cells

Note 1 to entry: Adult stem cells like neural stem cell, bone marrow stem cell etc. can also divide asymmetrically to form one daughter cell which can proceed irreversibly to a differentiated cell lineage and ultimately lead to focused functional differentiated cells, whilst the other daughter cell still retains the characteristics of the parental stem cell.

[SOURCE: ISO/TS 22859:2022, 3.23]

3.24
stem cell
non-specialized cells with the capacity for *self-renewal* (3.23) and *differentiation potential* (3.11), which can differentiate into one or more different types of specialized cells

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

[SOURCE: ISO/TS 22859:2022, 3.24]

3.25
viability
attribute of being alive (e.g., metabolically active, capable of reproducing, have intact cell membrane, or have the capacity to resume these functions) as defined based on the intended use

[SOURCE: ISO 21709:2020, 3.17]

3.26
viable cells
cells within a sample that have an attribute of being alive (e.g. metabolically active, capable of reproduction, possessed of intact cell membrane, or with the capacity to resume these functions) defined based on the intended use

[SOURCE: ISO 20391-1:2018, 3.29]

4 Abbreviations

Abbreviation	Term
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
B27	The most cited neural cell culture supplement, which is an optimized serum-free supplement used to support the neural cell culture
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
CFSE	carboxyfluorescein succinimidyl ester
CNS	central nervous system
CORIN	atrial natriuretic peptide-converting enzyme
DCX	doublecortin
DG	dentate gyrus
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
EB	embryoid body
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FL	fluorescence spectrophotometer
FOXA2	forkhead box protein A2
GABA	gamma-aminobutyric acid
GPCs	GABA-ergic progenitor cells
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCsAg	hepatitis C surface antigen
HCV	hepatitis C virus
HIV	human immunodeficiency virus
hPSC-NSCs	hPSC derived NSCs
hPSC	pluripotent stem cell
IPC	intermediate progenitor cell
KOSR	KnockOut™ [a] Serum Replacement
Lif	leukemia inhibitor factor
MNs	motor nuerons

[a] KnockOut™ Serum Replacement is a trademark of ThermoFisher Scientific. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

[b] Neurobasal™ media is a trademark of ThermoFisher Scientific. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

Abbreviation	Term
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N2	N-2 Supplement is a chemically defined, serum-free supplement, which is intended for use with Neurobasal™ [b] media
NESTIN	neuroepithelial stem cell protein
NeuN	neuronal Nuclei
NIM	neural induction medium
NPM	neural proliferation media
NSC	neural stem cell
PAX6	paired box 6
PBS	phosphate-buffered saline
qPCR	quantitative polymerase chain reaction
RGCs	radial glial cells
RT-PCR	real time polymerase chain reaction
SGZ	subgranular zone
SOX2	Sry-related HMG box 2
SOX1	Sry-related HMG box 1
SVZ	subventricular zone
TGFb	transforming growth factor-beta
TP	treponema pallidum
TPPS	transferrin putrescine, progesterone sodium selenite
TUBB3	neuron-specific class III β-tubulin
LGE	lateral ganglionic eminence

[a] KnockOut™ Serum Replacement is a trademark of ThermoFisher Scientific. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

[b] Neurobasal™ media is a trademark of ThermoFisher Scientific. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

5 General requirements

5.1 General

The biobank shall follow ISO 20387 and ISO 21709 in addition to this document. ISO/TR 22758 can be used as additional reference for the implementation of ISO 20387.

ISO 24603 shall be followed for human biological source material.

The biobank shall establish criteria and procedures for the establishment and expansion of PSCs, differentiation, culture, characterization, quality control, storage, thawing and transport of hPSC-NSCs.

A data analysis procedure shall be established, documented, implemented, regularly reviewed and updated.

The biobank shall use validated and/or verified methods and procedures for activities pertaining to hPSC-NSCs according to ISO 20387:2018, 7.9.2 and 7.9.3 at all stages of the biological material life cycle (ISO 20387:2018, 3.29).

According to the characteristics of hPSC-NSCs, procedures, quality control (QC) documents for collection, separation, expansion, storage, transportation and testing, and data analysis shall be established, documented, implemented, regularly reviewed and updated.

The initial cell state of PSCs as well as culture methods can influence the properties of hPSC-NSCs. Workflow steps cannot always be controlled. Thus, their impact on the hPSC-NSCs properties 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 biobank shall assess biorisks of hPSC-NSCs at the facilities and implement appropriate biosafety measures for the protection of personnel and environment.

The authenticity and properties of hPSC-NSCs shall be monitored throughout the complete biobanking process from generation to distribution.

5.2 Legal and ethical requirements

ISO 20387:2018, 4.1.6, 4.3, 7.2.3.4, 7.3.2.4, A.7 a), and ISO 21709:2020, 4.2 and ISO 24603:2022, 5.2 shall be followed.

The biobank shall collect relevant information on ethical requirements, implement and regularly update them, where relevant.

The biobank shall establish, document and implement policies on the procurement and supply of PSCs.

Experimental plans using or establishing human PSCs should be consulted in a specialized ethics review committee with particular expertise in topics relevant to the type and intended use of the PSC lines in the biobank.

The biobank shall establish a process to verify and document cell line provenance, to be able to provide evidence of ethical and regulatory compliance.

The biobank shall be aware whether reimbursement was made for the donation of human tissues.

5.3 Personnel, facilities and equipment

ISO 20387:2018, Clause 6, and ISO 21709:2020, 4.3, 4.4, 4.7 shall be followed.

The biobank personnel shall be appropriately and specifically trained in hPSC-NSCs generation, characterization, culture, cryopreservation, recovery and transport.

The biobank shall ensure that external operators providing hPSC-NSCs services demonstrate relevant knowledge, experience and corresponding skills.

The biobank shall ensure that facilities, equipment and environmental conditions do not adversely affect hPSC-NSCs quality or invalidate the test results.

Equipment management procedures should be established, including the use of equipment and maintenance plan.

The biobank shall control the operating environment and conditions (e.g., temperature, humidity, cleanliness) according to the relevant characteristics of hPSC-NSCs and the need for aseptic processing.

5.4 Reagents, consumables and other supplies

ISO 21709:2020, 4.5 and ISO 24603:2022, 5.4 shall be followed.

The biobank shall establish acceptance criteria for materials, including reagents and consumables, necessary for hPSC-NSCs differentiation, culture, establishment, expansion, preservation, storage, thawing and transport.

5.5 Management of information and data

ISO 20387:2018, 7.8.3 and 7.10 shall be followed.

The biobank shall manage and maintain associated data of hPSC-NSCs, including but not limited to the following:

a) the technical information: methods used in the generation of cells, culture conditions, passage data including passage number, characterization, microbiological test data;

b) the preservation and storage information;

c) the characterization and safety testing data;

d) the cell identity verification methods, e.g. by short tandem repeat (STR) analysis and/or HLA-typing or equivalent validated methods.

Certain data retention times, data integrity and security of data storage shall be ensured.

For hPSC-NSCs, a minimum period of retention of records shall be established. Special requirements for storage and retention times can apply for future applications. Personal data of each human donor shall be held in a protected location and shall be handled in accordance with ISO 20387:2018, 4.3.

The cell master file shall be kept to enable review of the data and records for specific applications.

6 Collection of biological source materials and associated data to the establishment, characterization and QC of hPSCs

ISO 24603 shall be followed.

7 Generation of hPSC-NSCs from hPSCs

7.1 Processes

For establishing hPSC-NSCs ISO 21709:2020, 5.1 shall be followed.

The biobank shall establish, implement, validate, document and maintain procedures for hPSC-NSCs generation from hPSCs.

Processes should be performed in a biosafety cabinet or under a laminar flow hood using appropriate aseptic techniques.

Each culture expansion is referred to as a ”subculture” or “passage”.

7.2 Unique identification

The unique identification of hPSC-NSCs shall be established in accordance with ISO 20387:2018, 7.5. This should include a unique cell name or sample number, a biobank batch number and biobank vial number. Cells should be anonymized or de-identified.

7.3 Testing for infectious agents

The starting hPSCs should be tested for relevant transmittable infectious agents, e.g., HIV, HBV, HCV, HTLV, HCMV, toxoplasmosis and TP.

The analytical data and results as well as the associated analyses shall be documented and available to authorized biobank personnel and researchers who process biological material and established cells.

7.4 Generation of hNSCs and culture

hPSC-NSCs can be generated from hPSCs by methods combining set of differentiation-inducing factors (e.g., growth factors, low-molecular weight compounds, oxygen concentration during cell culture) and cell culture condition (e.g., suspension, monolayer). The dual SMAD inhibition (inhibition of bone morphogenetic protein

(BMP) and transforming growth factor-beta (TGFb) signaling) is widely used for inducing differentiation of hPSCs[7]. Examples of suitable methods for the differentiation and culture of hPSC-NSCs are given in Annex B and references [8, 9].

The biobank shall establish, implement, validate, document and maintain procedures for differentiation and culture of relevant cell lines.

Processes should be performed in a biosafety cabinet or under a laminar flow hood using appropriate aseptic techniques.

For deriving neural stem cells from pluripotent stem cells, the differentiation strategies shall be clearly documented.

NOTE 1 The self-renewal of hPSC-NSCs depends on the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF).

NOTE 2 Long-term maintenance of hPSC-NSCs in vitro remains a challenge, because it can cause change in the characteristics of hPSC-NSCs, including but not limited to:

a) tendency to differentiate into specific region of brain, e.g., forebrain to mid/hindbrain[9];

b) population doubling time (both decreasing and increasing can happen);

c) ability to differentiate into neuron;

d) genetic instability (karyotype abnormality)[8,10].

7.5 Subculture and limited expansion

A culture can be further expanded for biobanking after successful establishment of the primary hPSC-NSCs; this is then known as a subculture. Each culture expansion is referred to as a "subculture" or "passage".

Cultures should be tested for microbiological contaminants (including bacteria, fungi, mycoplasma, endotoxins and adventitious viral agents) before any further expansion.

Cell passaging follows the relative protocols after establishment of primary culture. Expansion of hPSC-NSCs is recommended for up to 3 passages to ensure sufficient availability of material while preserving the biological features of the original culture, thus preventing culture-associated adaptations. The biobank shall monitor the expansion for changes in specific biological characteristics (e.g., undifferentiation status and immunophenotyping).

During hNSC culture and expansion it is important that the cells do not differentiate prematurely. This can have an unfavorable effect on product quality and yield[11].

8 Characterization of hPSC-NSCs

8.1 General

The biobank shall establish, document and implement procedures to characterize hPSC-NSCs and report the relevant data so that users can determine suitability for their intended use.

The biobank shall define a matrix of assays and a set of markers based at least on Clause 7.

The biobank shall perform ongoing characterization of hPSC-NSCs in culture. The characterization shall include, but is not limited to:

a) authentication;

b) cell morphology;

c) growth kinetics;

d) viability;

e) differentiation capability in vitro;

f) immunophenotype;

g) functional characterization in vitro;

h) being free of microbial contamination;

i) karyotyping.

Exemplary methods for hPSC-NSCs characterization tests can be found in Annex B.

8.2 Viability

The biobank shall define, implement and document a procedure to determine cell viability.

Quality control for cell viability test shall be performed using live and dead cells. Cell viability shall be determined and documented.

The biobank shall assess the amount of viable cells in the cell culture at regular intervals and especially after changes of cell culture conditions.

Viability shall be assessed following recovery from cryopreservation.

The biobank shall limit the acceptable percentage of nonviable cells in the population during the test procedure.

The amount of viable hPSC-NSCs should be ≥ 80 % prior to cryopreservation.

The amount of viable hPSC-NSCs should be ≥ 60 % immediately post-thaw and determined with a validated method.

NOTE A viability assay is usually performed following 24 h to 48 h in culture after recovery to avoid false overestimation of viability.

An automated cell viability test should be performed.

The biobank can define, implement and document a procedure to evaluate apoptosis in the cell culture.

EXAMPLE Assessment on any cryopreserved and thawed hPSC-NSCs for apoptosis is typically done using flow cytometry (see B.7).

8.3 Morphology

Cell morphology can be very different depending on growth conditions and different differentiation strategies. In addition, at different stages of culture cells have different morphologies reflecting changes in status, such as generation of EB (embryoid body) followed by differentiation into neural rosettes. EBs are 3D spherical aggregates that recapitulate several aspects of early embryogenesis.

A description of cell morphology should include the conditions of culture as well as the stages of culture.

NOTE hPSC-NSCs can be maintained, and possibly expanded, without adherence under specific culture conditions. But these cells, if maintained under more standardized conditions, would be expected to demonstrate adherence.

The biobank shall document significant cell morphology changes.

8.4 Population doubling time and subculture/passage

8.4.1 PDT

The PDT is the time (measured in hours) required for the replication of the population of hPSC-NSCs. The PDT is calculated with Formula (1) using the cell counts obtained before and after harvest:

$$D = (T - T_0) \times \log 2 / (\log N - \log N_0) \quad (1)$$

where

$(T\text{-}T_0)$ is the incubation time in hours;

N is the count of cells harvested;

N_0 is the count of cells seeded;

D PDT.

NOTE 1 Formula (1) is applicable in a linear range of cell expansion.

The average PDT of hPSC-NSCs isolated from hPSCs ranges between 20 h and 40 h.

NOTE 2 Depending on the culture conditions, culture passage, cell density and characteristics of the donor (e.g., age), the PDT can vary.

The PDT of hPSC-NSCs should be determined by the biobank after secondary culture.

PDT can reflect the growth kinetics of hPSC-NSCs in culture. The biobank can utilize the PDT of hNSC cultures at different passages to evaluate changes in culture cell growth kinetics.

The PDT shall be documented.

8.4.2 Subculture/passage

P_0, the passage number(s) together with the seeding and final cell density, and the culture vessel surface area shall be documented. For 2D culture, when the hPSC-NSCs cover the culture vessel at 80 % to 90 %, the cells can be passaged.

Passage numbers are frequently used by laboratories. However, passage number is correlated with the surface area/volume of a culture vessel and how the initial P_0 is defined. It is recommended that the biobank defines P_0 as the initial plating of hPSC-NSCs.

Documenting PDT along with passage numbers can facilitate a better understanding of growth dynamics of the hPSC-NSCs and the relationship between passages and PDT.

8.5 Cell population purity

The biobank shall evaluate the purity of hPSC-NSCs and unwanted cell populations such as astrocyte, neuron and other mature neural cells should be < 10 %. Immunophenotyping of hPSC-NSCs as described in 8.8 can be used for evaluating and verifying purity and identity. Unwanted microbial contaminants shall be defined and checked, as described in 8.9.

NOTE Since definitive markers for neural stem cells have not been established, purity can vary depending on the markers used for evaluation.

8.6 Proliferation

The biobank shall define, implement and document a cell proliferation assay.

The assay for cell proliferation shall be documented including internal QC criteria as described in Clause 8.

NOTE Cell proliferation can be evaluated by CFSE or by MTT (see B.5 and B.6).

8.7 Differentiation capability — in vitro multi-cell type differentiation

8.7.1 General

To evaluate in vitro multilineage differentiation of hPSC-NSCs, the biobank should establish, document and implement procedures and requirements for the specific assays, (i.g., hPSC-NSCs with ability to undergo in vitro multi-cell type differentiation). The following in vitro multi-cell type differentiation assays are part of the in vitro characterization of hPSC-NSCs, but do not always reflect the in vivo differentiation capacity of these cells.

In vitro multi-cell type differentiation into neuron and astrocyte under appropriate conditions is a valuable tool for characterization of hPSC-NSCs. This information can be useful, if assessed in a quantitative assay format that is validated and sufficiently sensitive to assess hPSC-NSCs grown for different lengths of time, and under different conditions.

8.7.2 In vitro neuronal and glial differentiation

hPSC-NSCs can differentiate into various types of neurons and astrocytes in different induction medium. hNSCs can differentiate into motor neurons, dopaminergic neurons and other types of neurons as well as various types of glial cells. For cells at these stages of development, there are several protocols for differentiation, see in Annex B.

NOTE Typical neural induction medium in general contains DMEM/F12, N2, B27, L-Glutamine and other cytokines.

8.8 Immunophenotyping by flow cytometry

NOTE Cell surface marker expression has been described for the identification and isolation of many neural cell types by fluorescence-activated cell sorting (FACS) from embryonic and adult tissue from multiple species. hPSC-NSCs have been isolated from human brain using genetic promoter-reporters of hPSC-NSCs markers[12,13]. hPSC-NSCs are characterized by the presence of specific cell markers and the absence of others, for example, hPSC-NSCs from neural induction cultures of hPSCs are SOX1+/SOX2+/NESTIN+/CD133+/OCT4-[14].

CD133 has proven useful for isolating hNSC in multiple studies[15]. However, due to its high expression in hPSCs and dim expression in EB-rosette(+) cultures, SOX2 or CD133 should be avoided as a selection marker for hPSC-NSCs, which reduces the risk of insufficient purification away from hPSCs[14][16].

hPSC-NSCs can be characterized by a panel that includes at least the markers (antigens) and expressions of antigens listed in Table 1. Additionally, other international guidelines should be followed, where appropriate.

Table 1 — Antigens and antibodies recommended for immunophenotyping of hPSC-NSCs

Antigen	Antibodies (clones)	Required detection rate in %
NESTIN	clone 10C2, 25/NESTIN	≥ 80,0
SOX1	Asn242-Gly379 (Leu276Ile), N23-844	≥ 80,0
SOX2	Poly6308	≥ 80,0
CD133	W6B3C1	≥ 80,0

The expression of NESTIN, CD133, SOX1 and SOX2 should be ≥ 80,0 % of the hPSC-NSC cell population.

Subclause B.7 includes an informative general guideline on immunophenotyping by flow cytometry.

8.9 Microbial contamination

Procedures for microbial contaminant testing of hPSC-NSCs shall be established, validated, implemented and documented throughout the whole process.

Throughout the whole process from donation and procurement, preparation of culture reagents and equipment, to maintenance and cryopreservation of cultures, it is important to take a holistic view and establish microbiological testing at all critical points of the process. In addition, procedures to minimize risks to other established cultures should be in place. It is good practice to maintain QC procedures for primary tissues or cells newly brought into the biobank. Such cultures should be maintained in a dedicated area and in segregated equipment until sufficient data is available to justify their relocation.

Test methods used for microbiological testing shall be validated. It is important to be sure that appropriate levels of sensitivity, specificity and robustness are being used in respect of testing cell cultures.

Microbial contamination shall be assessed by risk management throughout the process.

hPSC-NSCs used for research and development shall be free of contaminants. These contaminants include, but are not limited to bacteria, fungi, and mycoplasma:

a) Tests for the presence of bacteria, fungi, and mycoplasma shall be conducted routinely. The use of antibiotics should be eliminated as soon as possible. However, where they are used in the culture medium, these should be removed prior to sampling.

b) It is also necessary to be aware of the impact, which some contamination can have on the biological characteristics of the cell population being cultured. For example, a low level of viral infection will probably not have a significant impact on cell death, but can dramatically influence the biological activity. This type of contamination can impact any research data being generated.

EXAMPLE Mycoplasma is recognized as a common contaminant of cell cultures due to the risk of contamination from numerous sources. Mycoplasma can be very difficult to remove from cell culture as their small size limits filtration and they can be difficult to detect without establishing routine testing procedures.

The risks posed by transmissible spongiform encephalopathies (TSEs) should be considered irrespective of the origin or history of the cells. There are a number of TSE diseases across the globe showing their ability of transmitting to humans.

8.10 Neural replacement and functional recovery

The function of hPSC-NSCs can vary depending on the animal model, disease of interest and immune rejection features. hPSC-NSCs should be injected into wild-type immunodeficient mice or use immunosuppressants to measure the in vivo performance of NSCs.

NOTE 1 When transplanted into a diseased brain, hPSC-NSCs repair and replace injured tissues after migration toward and engraftment within lesions[17].

NOTE 2 In Amyotrophic lateral sclerosis (ALS), studies showed that hPSC-NSCs derived from iPSCs improved the ALS phenotype, the hPSC-NSCs comprise amelioration of neuromuscular function and survival, protection of host motor neurons (MNs) and positive host–environment alterations such as reduced micro/macrogliosis, as revealed by neuropathological analyses[18].

NOTE 3 In Alzheimer's disease (AD), implanted NSCs derived from human fetal cells extensively migrated and engrafted, and some differentiated into neuronal and glial cells, although most NSCs remained immature[17].

NOTE 4 In spinal cord injury, NSCs from human fetal brain (named as NSI-566) has shown the ability to provide neurotrophic support via paracrine factors, achieve synaptic integration with host neurons, and promote regeneration of host neuronal fibers[19].

Factors to be taken into account in this functional assay should include the injection site, observation time and animal background.

mRNA and protein expression levels for paracrine secretion/expression should be tested by qPCR or multiplex PCR methods on hPSC-NSCs. Additionally, protein levels should be measured by enzyme-linked immunosorbent assay (ELISA) or multiplex assays in medium.

9 Quality control

ISO 20387:2018, 7.8 and ISO 21709:2020, 5.5 shall be followed.

The biobank shall establish, implement and document a QC procedure which shall include the testing of biological characteristics related to the in vitro functionality of hPSC-NSCs as given in Clause 7.

QC of biological characteristics (see Clause 7) of hPSC-NSCs shall be performed for all critical procedures, from isolation to thawing. A recommended QC procedure for biobanking of hPSC-NSCs is given in Table A.1, Annex A.

The biobank shall establish, implement and document QC acceptance criteria for all the biological characteristics of hPSC-NSCs included in Clause 7.

The biobank shall establish, implement and document QC acceptance criteria for all critical control points, e.g. culture media, reagents and equipment.

Throughout the biobanking processes, the culture media shall be periodically tested for *Mycoplasma spp.*

QC shall be established with a risk-based approach related to laboratory safety.

10 Storage

10.1 ISO 20387:2018, 7.5, 7.7, A.6 and ISO 21709:2020, 5.3.4 shall be followed.

10.2 Optimization of the cryopreservation procedure and method(s) to minimize damage to cells during freezing and thawing is critical to ensure reliable availability of viable cells.

NOTE Controlling the freezing rate, using an appropriate cryoprotectant, and maintaining a stable storage temperature can minimize negative effects on the cell viability.

10.3 For cryopreserved hPSC-NSCs, the following information shall be documented:

a) the cell name;

b) the preserved hPSC-NSCs batch number;

c) the date of preservation according to ISO 8601-1;

d) the culture conditions;

e) the passage number;

f) the operator name.

Each stored vial derived from the same batch of cultured cells shall have a unique identification reference number (i.e., a biobank or batch number), which is traceable throughout the processes of collection, separation and expansion according to ISO 20387:2018, 7.5.

10.4 Prior to freezing, cell morphology (8.3), immunophenotyping (8.8), viability (8.2) and differentiation capability (8.7) shall be tested and the results shall be consistent with features of hPSC-NSCs isolated from human fetal brain tissue.

10.5 The amount of viable cells shall be ≥ 80 %. They should be free of contamination(s).

10.6 Cells should be in the growth phase, centrifuged and resuspended in cryopreservation medium. The cell count can range from 1 000 000 cells/ml to 10 000 000 cells/ml, but samples with a cell count in the range of 2 000 000 cells/ml to 5 000 000 cells/ml should be used.

10.7 Important factors for the cryopreservation procedure.

a) Cryovials shall be appropriate for cryopreservation.

b) For cryoprotectant reagent/s the following applies.

— Cryoprotectant formulas (containing DMSO or other constituents) should be optimized by the biobank to preserve cell viability and function. The most common cryopreservation medium to store hPSC-NSCs is a solution of 5 % to 10 % (v/v) DMSO and up to 90 % to 95 % (v/v) complete medium (e.g., KOSR). DMSO facilitates entry of organic molecules into tissues. Hazardous materials shall be handled by using appropriate safety practices[20].

— The time that cells are in contact with DMSO should be minimized until frozen. If using controlled-rate freezing, additional considerations can apply.

NOTE 1 Exposing cells to DMSO at high concentrations or for prolonged periods of time is damaging to the cells.

NOTE 2 Cryopreservation can lead to cell damage during loading/unloading of the cryoprotectant agents (CPAs), freezing and thawing steps.

For long-term biobanking of hPSC-NSCs (e.g. more than 12 months), the vapor phase nitrogen method should be used.

10.8 Controlled-rate cooling of hPSC-NSCs can be performed as follows:

a) document the date and time of the start of the cryopreservation process in accordance with ISO 8601-1;

b) begin fast cooling from room temperature down to –10 °C at -0,5 °C/min to -1 °C/min;

c) hold at –10 °C for 5 min. This step is critical to ensure the formation of the ice crystal seed.

d) cool down to –80 °C at -1 °C/min (target temperature).

e) transfer into liquid nitrogen for subsequent storage.

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

11 Thawing

In the cell recovery process, frozen cells should be thawed at 36,5 °C ± 0,5 °C, or processed for culture with drop-wise addition of medium, put into culture and then transferred into an incubator with appropriate gas atmosphere and humidity. To optimize the process, the incubator should be set to an appropriate culture temperature of 36,5 °C ± 0,5 °C.

The frozen cells should be quickly thawed by warming and transferred directly to pre-warmed culture medium at 36,5 °C ± 0,5 °C to ensure maximal hPSC-NSCs viability and biological activity.

For cells preserved by vitrification methods, this can be more critical and expert advice should be sought.

The following information should be clearly documented, including but not limited to:

a) the batch number for the set of frozen vials;

b) the cell name;

c) the passage number;

d) the culture condition;

e) the operator name;

f) the thawing date of thaw operation in accordance with ISO 8601-1;

g) the thawing time in accordance with ISO 8601-1 as the time point when frozen cells leave liquid nitrogen to the time point when the cells are put into culture;

h) the date in accordance with ISO 8601-1 at which the culture reaches sufficient colony density to be passaged.

Cell viability shall be tested after thawing.

12 Disposal

For managing waste disposal ISO 20387:2018, 4.1.8, 7.1.1, 7.5.3, 8.4.2, A.7 and ISO 21709:2020, 5.3.6 shall be followed.

Any disposal of hNSCs shall be conducted in accordance with applicable environmental, biosafety, ethical requirements.

13 Distribution of hPSC-NSCs — Information for users

ISO 20387:2018, 7.12 shall be followed.

“Instructions For Use” (IFU) and/or standard operational procedures for differentiation, culture, preservation, storage and transport of hPSC-NSCs should be provided to hPSC-NSCs users. The IFU should typically contain information prescribing general culture, preservation methods and procedures.

Batch numbers, traceable to the "batch" or biobank, and a statement or material safety data sheet on hazards for the cells shipped shall be provided to hPSC-NSCs users.

Terms and conditions or a warranty, which qualifies hPSC-NSCs potential and characteristics based on testing performed by the biobank, should be provided.

The characterization and microbiological test data from the depositor for hPSC-NSCs in the biobank shall be available for users.

The biobank should have a documented policy for the quality and sourcing of raw materials that can impact the quality of cell preparations, subject to national or international restrictions e.g. fetal bovine serum, trypsin, growth factors.

The biobank should provide information to facilitate efficient selection of suitable cells. Information should include, but is not limited to:

a) the date of collection and preservation of hPSC-NSCs in accordance with ISO 8601-1;

b) the date, in accordance with ISO 8601-1, of differentiation (for hPSC-NSCs, this is usually considered to be the date the cells were differentiated from the hPSCs in vitro);

c) the passage number of hPSC-NSCs;

d) whether fresh or frozen biological source material has been used;

e) where applicable, relevant information regarding informed consent obtained from the human donor for use of the original tissue for research;

f) any associated constraints on the use of the derived cells;

g) the data and interpretation resulting from characterization and QC.

14 Transport of hPSC-NSCs

14.1 General

ISO 20387:2018, 7.4 and ISO 21709:2020, 5.4.4 shall be followed.

The biobank shall establish, implement and document procedures for the transport and handling of hPSC-NSCs and their associated data.

Unnecessary exposure to radiation should be avoided during shipment.

hPSC-NSCs can be transported as frozen ampoules/vials or as living cultures; in either case:

a) advise the recipient as to when the cells are to be shipped;

b) provide written instructions on the following:

   1) instructions upon reception of hPSC-NSCs;

   2) instruction for thawing and reconstitution of hPSC-NSCs;

   3) instructions for secondary storage conditions;

   4) medium or serum required;

   5) any special supplements;

   6) subculture regimen;

c) tape the cells' data sheet and a copy of the instructions to the outside of the package so that the recipient knows what to do before opening it.

Each frozen ampoule/vial or living culture container (primary container) can be introduced into a pre-sterilized self-adhesive seal package. The packaging shall be labelled with:

— the sample's data;

— the production and expiration date;

— the name and contact information of the entity that performs biobanking.

14.2 hPSC-NSCs frozen in ampoules or cryovials

For long distance transport, hPSC-NSCs frozen in ampoules or cryovials should be shipped in but not limited to dry ice or a liquid nitrogen Dewar flask or in dry-shippers filled with liquid nitrogen vapor.

NOTE 1 The appropriate amount of dry ice, or liquid nitrogen in the Dewar flask, or liquid nitrogen vapor in the dry-shipper depends on the condition of the transport, e.g., duration.

An outer container with thermal insulation, e.g., a thick-walled polystyrene foam container, should be used for shipping the ampoules or cryovials or both. If an outer container is used, the outer container shall be disinfected (e.g. with 75 % alcohol), shall be filled with appropriate cold charge (e.g. dry ice), and should include a data logger thermometer for temperature control.

NOTE 2 If hPSC-NSCs thaw slowly, their viability will decline rapidly.

Ampoules/cryovials shall be hermetically closed to prevent leakage.

14.3 Living hNSC cultures

hPSC-NSCs can be transported as a living culture in culture media in appropriate culture flaskstypically over short durations, i.e. ≤ 2 h. In such case, hPSC-NSCs in the middle and late stages of the growth curve should be used for shipping. The temperature during handling and shipping shall be ≤ 37 °C and > 4 °C.

As much culture medium as possible should be used (e.g. fill the flask to the top with medium).

NOTE 1 Medium touching the lid of the flask can cause contamination.

NOTE 2 Confluent or post confluent cultures will exhaust the medium nutrients more rapidly and can detach cells in transit.

Annex A
(informative)

Exemplary quality control test procedure for biobanking of hPSC-NSCs

Table A.1 — Exemplary quality control test procedure for biobanking of hPSC-NSCs

Quality test	Isolation and primary culture	Subculture	Cryopreservation	After thawing
Cell morphology	+	+	—	+
Cell marker	+	+	—	+
Growth kinetics	—	+	—	+
Count and viability	+	+	+	+
Differentiation capability in vitro	—	+	—	+
Microbial contamination	+	—	+	+
Key "+" represents test in procedure "—" represents test not in procedure				

NOTE Quality control of hPSC-NSCs is described in Reference [10].