Standards for Human Stem Cell Use in Research

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Summary

This document identifies quality standards and outlines basic core principles for the laboratory use of both tissue and pluripotent human stem cells and the in vitro model systems that rely on them. Building upon previous recommendations for the characterization of cell lines (Crook et al., 2010; Crook and Stacey, 2014; Stacey et al., 2013; The International Stem Cell Banking Initiative, 2009; The International Stem Cell Initiative, 2007; The Steering Committee of the International Stem Cell Initiative, 2005), these standards are best practice recommendations designed to improve the reproducibility of stem cell research within and between laboratories, and from cell line to cell line. While some of the principles articulated also apply to other species, this document focuses on human stem cells exclusively, and does not seek to provide recommendations regarding non-human species. It addresses standard research practices shared by human pluripotent and tissue stem cells but does not include specific protocols or address individual differences in methodologies used to culture or characterize stem cells, which are extremely diverse. **Overall, the emphasis of this document is creating a set of recommendations that, when taken together, establish the minimum characterization and reporting criteria for scientists, students, and technicians in basic research laboratories working with human stem cells.** This document focuses on 1) basic characterization to describe cell identity, ensure culture integrity, and promote material safety; 2) the assessment of the undifferentiated state and pluripotency to appropriately evaluate cells and their developmental potential; 3) genomic characterization to assess genetic integrity and monitor the emergence of cellular changes that could interfere with the interpretation of results or manifest as potentially malignant traits; and 4) stem cell-based model systems to improve fidelity and utility of stem cell-derived model systems (organoids, microphysiological systems, engineered cells, etc.) in basic and preclinical research. The guidelines provided are intended to be both technically and financially feasible for the average research laboratory and, if undertaken, will promote rigor and reproducibility not only within the laboratory but broadly throughout the field.

Introduction

One of the primary goals of stem cell research is to identify and harness the mechanisms that control stem cell behavior, enabling the generation of cell types or tissues for basic and preclinical research. For the results and outputs from this research to be accurate and durable, high standards that ensure reproducibility and reliability should be applied to all stages of the research pipeline. For experiments involving in vitro stem cell cultures this is underpinned by the application of standardized characterization principles, that, when carefully and consistently implemented, increase confidence in experimental outputs and facilitate reproducibility across research laboratories.

All cultured cells are dynamic, living model systems that seek to capture a cellular state and serve as a surrogate that facilitates experimental interrogation of a biological system. In the case of stem cells, these cultures provide accessible experimental models for studying early development and disease and allow for the generation of functional cells and tissues with therapeutic potential. Characterization is an essential practice for ensuring culture integrity, establishing baseline phenotypic profiles, and providing insight into the fidelity with which the cells will accurately model the target biological system. For this reason, appropriate, systematic characterization has a direct and significant impact on the ability to obtain reproducible data, and the accuracy of the interpretation of that data.

Reproducibility issues in both basic and translational research can hinder progress and erode trust (Baker, 2016; Drucker, 2016). When systematically implemented, the deployment of appropriate characterization strategies, combined with good documentation practices, drive rigor and harmonization, which in turn facilitates the reproducibility and accuracy of experimental outputs. The benefits of adopting systematic characterization are substantial: Long-term efficiencies are realized, as waste and time lost to irreproducible experiments are reduced. Further, publications based on suitably characterized cell lines provide more accurate data that enables and accelerates research progress.

The need to implement standards and improve reproducibility of published scientific data has been a topic of high-level discussion in recent years and has been highlighted by major scientific journals ("Nature Editorial," 2013; Baker, 2016). The advancement of culture methods to include tissue and pluripotent stem cells, and, specifically, the technical demands of generating consistent cell cultures and reproducible data outputs with these cells, is an area requiring considerable diligence and expertise. Thus, universally accepted quality standards are needed to improve the rigor and reproducibility of all research utilizing stem cells. Standards set researchers up for success, ensure rigor in preclinical research, ultimately strengthening the pipeline of therapies for patients.

SECTION 1

Basic Characterization

Crucial to the reproducibility effort is the consistent generation and accurate characterization of research materials, particularly those used to initiate experiments. This section highlights key principles in the acquisition/generation, preservation, and early characterization of human stem cells essential to promote reproducibility in research, safety in the laboratory, and clarity in literature. Specific recommendations are offered in the following areas:

- acquisition of material
- initial biobanking
- establishing identity and authentication
- transgene clearance
- assuring basic cell hygiene

Undertaking these necessary practices lays the foundation for rigor and reproducibility within the laboratory, resulting in the reliability and validity of subsequent publications.

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Acquisition of Materials

Recommendation 1.1.1: Materials (e.g., donor samples, primary tissues, or cell lines) must be transferred between laboratories using appropriate transfer agreements that capture all associated donor and supplier/provider restrictions. Researchers and laboratory staff should read and understand these agreements prior to beginning any experiments, and be familiar with any additional local (e.g., institutional, regional, federal) restrictions and obligations relating to the use of materials and associated data.

A Material Transfer Agreement (MTA) or similar agreement (Memorandum of Understanding (MoU), etc.) is a legally binding agreement that dictates the terms of transfer and use of materials and data (Bubela et al., 2015). This agreement is essential in the appropriate transfer of materials to inform potential users of restrictions on use, and to protect the rights of the user, their institutions, and most critically in human research, the donors. The MTA should be designed to include any informed consent provisions and licensing restrictions necessary for the use of the materials and the allowable metadata associated with the material(s) . Anyone handling human derived materials or data should read and understand the MTA associated with the materials prior to use. Failure to do so could result in violation of donor consent obligations, or misuse of materials that may waste resources, result in invalid research, or prevent publication. These documents should be stored in a central but secure location accessible to authorized laboratory personnel.

It is important to note that regulations may differ from region to region on the use of materials and the associated data (e.g., allowable primary materials sources, sharing of genetic information, etc.), thus, knowledge of local, regional, and national regulations, such as GDPR (General Data Protection Regulation) regarding use is essential prior to initiating any use or transfer of materials or sharing of associated data. A data protection strategy should also be put in place (e.g., https://ria.princeton.edu/human-research-protection/data/what-kind-of-data-protect). Additionally, while laboratories must comply with domestic laws and regulations, ideally they should also verify compatibility with international principles put forth by relevant bodies such as the International Society for Stem Cell Research (ISSCR Guidelines for Stem Cell Research and Clinical Translation, 2021). Importantly, where local conditions and procedures prohibit any or all the requisite conditions and obligations for quality assured stem cell research, the laboratory in question should be able to justify alternate measures taken to assure quality (The International Stem Cell Banking Initiative, 2009).

Principles of Cell Line Biobanking for Preservation

Recommendation 1.2.1: Following derivation or acquisition of stem cell lines, a Master Cell Bank (MCB) should be generated prior to any experimental use or distribution. The MCB should be created from the earliest possible passage of the established cell line or development of non-adherent culture and should be characterized post-thaw prior to any experimental use.

The biobanking of cell lines is critical for assuring the preservation and availability of quality-controlled cells for scientific research (Coecke et al., 2005; Crook and Ludwig, 2017; International Society for Biological and Environmental Repositories (ISBER), 2005; "OECD Best Practice Guidelines for Biological Resource Centres," 2007; Pamies et al., 2022). Frozen stocks of cells should be prepared from traceable and reliable source materials (e.g., cell lines, donor samples, or primary tissues) and quality reagents (media, matrices, and supplements) using standardized protocols and procedures that are well documented and do not negatively impact the quality of the cells and future research. Any major selection or manipulation event (subcloning, gene editing, etc.) creates a new line and would necessitate the creation of a new MCB and all associated testing.

Upon acquisition or derivation of a cell line, prior to any experimental work, a MCB should be established, and the generation of Working Cell Banks (WCB) is strongly recommended. For the purposes of these recommendations, a cell line is defined as any material capable of being serially passaged and biobanked prior to senescence. Creating even small MCBs is necessary even for the average academic laboratory as working from established, well characterized stocks is the cornerstone of rigor and reproducibility within the laboratory. These materials, whether immortal or not, serve as the basis for continuing research and can be distributed to other research groups, and therefore should undergo similar preservation and characterization. The preservation of early passage materials (seed vials) protects against early loss due to contamination or inadvertent mishandling. A two-tier biobanking system is recommended to allow ongoing production of cell stocks for experimental use (Figure 1). To ensure the MCB is a single homogenous lot, expanded cells should be pooled prior to cryopreservation. This creates a consistency of materials that will promote reproducibility and would apply equally to the generation of any WCBs. Because the MCB will be the basis of all future work with the cell line, it should be well characterized (see Appendix 1, Table A1.1). The two-tier system allows strategic characterization of the MCB and WCBs to promote cost effectiveness and ensure high quality materials for ongoing experimental use. Securing a portion of the characterized MCB off-site, preferably out of region, is highly recommended to guard against loss due to local catastrophic events (i.e., freezer failure, natural disaster, etc.).

[1] For more information on informed consent documents and templates, please see sections 2.3, 2.4, 3.2.1 and Appendix 2-4 of the ISSCR Guidelines for Stem Cell Research and Clinical Translation.

[2] For examples of step-by-step protocols for cryopreserving human pluripotent stem cells (hPSCs), which are easily adaptable for use with tissue stem cells – subject to careful validation of recovery of representative functional populations [e.g., 'Vitrification' method, 'Slow Freezing' method, or 'Control Rate Freezing' method], refer to Crook et al., 2017).

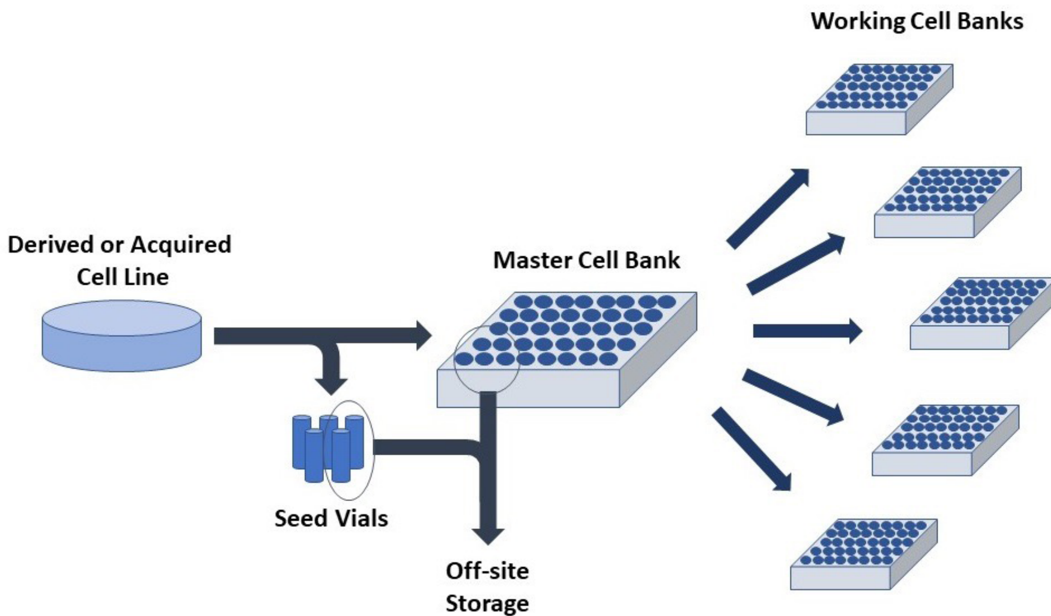

Figure 1. *Two-Tiered Cell Biobanking Strategy. The initial culture (derived or acquired cell line) is expanded, and seed vials are preserved at the earliest possible passage of stable and consistent cell cultures. The Master Cell Bank is then cryopreserved, from which Working Cell Banks (WCBs) can be produced for ensuing research. Seed stocks (intended only for recovery following loss of initial culture or for production of future MCBs) and a portion of the initial MCB should be stored off-site to guard against catastrophic loss.*

Stem cell laboratories should consistently conduct their research using authenticated, stringently tested, well-characterized cells that are cryopreserved and cultured within a quality framework for quality assurance (Crook et al., 2010; Crook and Stacey, 2014; Stacey et al., 2013; The International Stem Cell Banking Initiative, 2009). At a minimum, all routine laboratory protocols should be well documented, all work traceable, and all critical equipment regularly monitored and maintained. While it may not be achievable for all academic laboratories, researchers should strive to adhere as closely as possible to Good Laboratory Practice Standards (https://www.ecfr.gov/current/title-21/chapter-I/subchapter-A/part-58). These practices and processes ensure that the quality and integrity of cell lines preserved are assured, affirmed by generations of reliable data regarding cell safety and performance.

Cell Line Authentication

Recommendation 1.3.1: Cells for experimental use should be authenticated. Short Tandem Repeat (STR) analysis is recommended for authentication.

Authentication of research materials is important to confirm that investigators are working with the expected material and to demonstrate it is free from cross contamination with cells from another source. Unfortunately, by its very nature, in vitro culture allows opportunity for errors that can lead to the misidentification and/or cross-contamination of cell lines within the laboratory, a long standing and well documented issue and a major contributor to erroneous experimental conclusions and publication retractions (Casadevall et al., 2014; Freedman et al., 2015; Horbach and Halffman, 2017; Souren et al., 2022). This highlights the need for authenticating the identity of cell lines in particular and all cell cultures in general, a practice that will instil confidence in the interpretation and reliability of research data obtained using them (American Type Culture Collection Standards Development Organization Workgroup ASN-0002, 2010; Nelson-Rees and Flandermeyer, 1977).

In principle the need to authenticate research materials is fundamental to good science and this can be achieved by direct testing of the material and rigorous traceability. In the case of cell lines that can be passaged indefinitely, there is a significant risk that misidentified lines could be transmitted widely, potentially leading to corruption of research data on an international basis. Thus, in the case of cell lines, identity testing is strongly recommended. For tissues and low passage materials, such as tissue stem cell (TSC) cultures, that will not be distributed, the impact of switched or cross-contaminated cells is lower. Thus, for such materials it may be argued that genetic testing of all cultures is not necessary. However, in these situations the investigators should provide assurance of the provenance of the material they are using, and this may involve enhanced levels of control and traceability.

Funding agencies and journals are increasingly requiring evidence of cell line authentication to receive funds or publish (“Nature Editorial,” 2013; “Guidance: Rigor and Reproducibility in Grant Applications,” 2019). While rigorous documentation and testing by centralized cell banks can reduce the potential for misidentification at the point of sourcing stem cells, the onus still lies with the end researcher to authenticate materials used within the laboratory.

Several methods can be used to authenticate cell lines including short tandem repeat (STR) analysis, single nucleotide polymorphisms (SNP) profiling, whole genome sequencing (WGS), and other DNA profiling technologies. All of these are acceptable authentication strategies which can be used in the laboratory to properly identify cell lines. However, only STR analysis has been formally developed into an internationally recognized and accepted consensus standard for human cell line authentication (Almeida et al., 2016). The STR standards document "Authentication of Human Cell Lines: Standardization of Short Tandem Repeat (STR) Profiling. ANSI/ATCC ASN-0002-2021" provides information regarding the reasoning behind authentication, and detailed protocols for STR analysis. The advantages of STR are numerous, including cost efficiency, reproducibility, comparability across platforms, and ability to detect multiple cell sources within a culture. For these reasons it is recommended for use in authentication. Regardless of authentication strategy chosen, in order to protect donor privacy, genetic profiles used for authentication should not be made public.

Recommendation 1.3.1.a: When authenticating cells or a cell line, a reference sample from the original donor should be used for confirmation of origin. Where donor material is not available, a profile obtained from the earliest passage stocks available may be used for reference.

It is recommended to use cell materials directly from donors to generate a reference profile to authenticate materials derived from that donor. This allows unambiguous confirmation of identity and clear traceability of consent. Occasionally it is necessary to use materials for derivation or experimental use where no donor profile information exists. When no donor sample is available for existing research materials, the material provider should have available a profile from early stocks that can be used to authenticate laboratory materials.

Recommendation 1.3.1.b: At a minimum, authentication of cell lines should be performed at the establishment of the MCB.

Once the MCB is established (section 1.2), it should be assessed and ensured to match the original donor or early reference sample. For materials that are manipulated or passaged, it is also strongly recommended to assess at significant manipulation points (e.g., gene editing, clonal isolation, etc.) and/or at the end of studies to assure continuity of materials throughout experimental processes. Ideally, materials being provided externally (shared with collaborators or otherwise distributed) should come only from tested, authenticated stocks (MCB or WCBs) and the receiving laboratory should authenticate the cell line upon receipt. Any live cultures transferred between laboratories should be considered to have unknown identity until they have been properly authenticated (for more on the timing of authentication during the experimental process please see Appendix 1, Table A1.1).

Nomenclature/Assigning a Unique Identifier

Recommendation 1.4.1: Cell lines should be assigned an unambiguous identifier to safeguard provenance of data associated with that line in the public domain. The identifier should be generated by an international open-sourced registry to ensure it is persistent between laboratories, interoperable, and unique. Published reports should reference this unique identifier.

Cell lines have a physical entity, as well as a digital identity, a unique and persistent online record of the line. Ensuring that the digital identity of the line is unambiguous is an essential part of building confidence regarding the provenance and reproducibility of the physical entity. The recommendation for a unique, persistent, and unambiguous digital reference assigned by a trusted third-party registry assists interoperability of that identifier, as registries maintain a list of existing identifiers and cell lines. Registration of lines is an essential component of good stewardship of the line, as it allows it to be globally findable, and further ensures provenance of derivatives is linked to the originating line (such as genetically modified transgenic reporter lines, gene edited isogenic lines, or subclones that have distinct properties). Examples of such registries are Cellosaurus (https://web.expasy.org/cellosaurus/) for cells used in biomedical research (Bairoch, 2018), and the human pluripotent stem cell registry (hPSCreg; https://hpscreg.eu) for hPSCs specifically (Kurtz et al., 2018). The hPSCreg links cell lines to established data compliant with these recommendations and to the RRID of the ExPasy-resourced cell line data base 'Cellosaurus' (see Appendix 2 for general principles of a registry).

Registration is an important step in adhering to FAIR (FAIR principles of Findable, Accessible, Interoperable and Reusable) principles, even if the lines themselves have restricted availability. It simplifies the process of collating minimal information about the generation, provenance and availability of a line. Line registration assists stem cell researchers seeking to meet local governance requirements and is mandated by some funding bodies and in some national jurisdictions. Note that biobanking a line (Recommendation 1.2) does not preclude the need to register the line. While registration and biobanking are different activities, the biobanking of the physical entity is underpinned by assignment of a unique and persistent identifier for each cell line.

There is currently no international registry designed for primary tissues, or cells that can only be propagated for a finite time from those tissues. Nevertheless, where data is generated and placed in the public domain, there is a need for an unambiguous digital identity to ensure that data generated from the same donor line can be reconciled together, and data from different donor lines can be distinguished from one another. In these instances, we recommend that laboratories working with tissue or primary cells adopt nomenclature rules that allow digital traceability (Kurtz et al., 2018). We encourage the use of 'common-use' names associated with primary cells that identify the institute/originating laboratory, cell type, and a unique donor ID that is 3 alpha-numeric digits or more, to reduce the chance of duplicated identifiers. We also encourage the use of tissue, cell and cell line ontologies to reduce ambiguity about the origin or propagation of the material (Sarntivijai et al., 2014). Best practice on publication would include generation of a Research Resource Identifier using webtools at the Research Resource Identification site (https://www.rrids.org).

Reprogramming Transgene Elimination

Recommendation 1.5.1: Verification of the elimination of the transgene expression in newly derived human induced pluripotent stem cell (hiPSC) lines should be performed prior to biobanking, distribution, and experimental use.

Viral systems are commonly used to overexpress the reprogramming factors in somatic cells to generate hiPSCs. Non-integrative reprogramming methods include viruses (Sendai virus (SeV), adenovirus), self-replicative RNA from Equine encephalosis virus (EEV) and episomal vectors which contain sequences from the Epstein-Barr virus (EBV) (Haridhasapavalan et al., 2019). Cells transduced with these engineered viruses or vectors do not produce infectious viral particles, but they transiently express viral sequences containing the reprogramming factors. Clearance of these factors from the cell line is critical, as the persistent expression of the reprogramming factors in hiPSC cells can affect their proliferation and differentiation potential and increase the risk of tumor formation in a mouse model (Okita et al., 2007). Thus, clearance of the reprogramming vectors should be confirmed in newly derived hiPSC lines prior to biobanking or any experimental use.

The transgene expression is retained in newly derived hiPSC lines but can usually be eliminated through multiple cell passages. Viral clearance timing is dependent on the system used. For Sendai virus derived hiPSC lines, the vector clearance should be confirmed by immunostaining with antibodies against the virus or by quantitative PCR (qPCR) for Sendai virus specific sequences. Most of the SeV derived hiPSC lines are transgene free by passage 10. Newly derived hiPSC lines generated with the episomal vector method show faster clearance of the vectors, generally by passage 3-5, however, it was reported that in as many as 30% of the hiPSC lines, the vector was not eliminated, most likely due to genome integration. The clearance episomal vectors can be assessed using qPCR (Schlaeger et al., 2015).

Cell Hygiene

Recommendation 1.6.1: Cell cultures should undergo microbiological and viral testing including mycoplasma, sterility, and adventitious agent screening to promote cell competence and technical staff safety.

Recommendation 1.6.1.a: Cell cultures (both primary and stem cell cultures) should be assessed to confirm the absence of mycoplasma upon entering the laboratory and regularly monitored (quarterly at a minimum) to ensure the absence of mycoplasma infection during routine culture. Cultures intended for experimental research should be monitored at the initiation and completion of studies. Any lines shared outside the laboratory should be confirmed mycoplasma negative prior to distribution. If culture lines are found to be contaminated, they should be discarded.

Mycoplasma contamination of cell cultures is well known to be a significant issue in cell repositories, with contamination rates worldwide ranging from 15% to >80% depending on the location and level of monitoring (Chernov et al., 2014; Corral-Vázquez et al., 2017; Drexler and Uphoff, 2002; Hay et al., 1989). The impact a mycoplasma infection can have on a cell culture is significant, compromising both structure and function of the host cells (Cimolai, 2001; Drexler and Uphoff, 2002; McGarrity et al., 1984; Tsai et al., 1995; Zhang et al., 2006). This can affect all measurable parameters of cell morphology and physiology, rendering results obtained using an infected culture unreliable. Mycoplasma is undetectable with standard laboratory equipment, lacks a cell wall making it resistant to most antibiotics, is ubiquitous within the environment, and its size and flexibility permit it to evade filtration devices. These aspects, combined with a rapid expansion rate, allow it to overtake a culture quickly and easily, making screening and exclusion of infected cultures critical to improve rigor.

Maintaining a mycoplasma free culture environment requires both initial vigilance and routine monitoring. All incoming cultures, regardless of origin and testing certification, should be quarantined and tested before being maintained with existing cultures. Once confirmed as mycoplasma negative, the culture can be placed in standard culture areas, and routinely screened as part of an ongoing testing program. When banking cells for future use, each MCB and WCB should be screened and confirmed negative prior to use for experimental purposes or sharing of the culture. If found to be positive at any point, unless the culture is absolutely irreplaceable, it should be discarded (for more on the timing of testing during the experimental process, please see Appendix 1, Table A1.1).

Recommendation 1.6.1.b: Cultures should be screened to ensure that they are free of microbial and viral contamination.

Microbial or viral contamination can alter cellular behavior and integrity, pose an immediate health risk to researchers, and preclude the future therapeutic use of cell products (Barone et al., 2020). Concerns regarding the potential effects, processes for identifying contamination, and potential resolution are common to all culture systems and have been well reported. See Appendix 3 for more detail on identifying and mitigating this risk in culture.

SECTION 2

Pluripotency and the Undifferentiated State

An aspect unique to human pluripotent stem cells (hPSCs) is their undifferentiated developmental state and the potential to give rise to all somatic lineages. In working with hPSCs, these defining features should be rigorously demonstrated not only for newly derived lines, new culture systems and genetically modified lines, but also for experimentation with more established lines to ensure the cells are behaving as expected.

With mouse ES cells, pluripotency has ultimately been demonstrable by the ability of the cells to participate in development and form germ line chimeras when transferred to a blastocyst that is allowed to develop to term. For obvious ethical reasons such a test is not possible for hPSCs, so pluripotency has to be demonstrated by surrogate assays – either the ability to form teratomas containing tissues of all three germ layers when allowed to form xenograft tumors, or by differentiation in vitro. Human PSCs typically express a number of particular genes and cell surface antigens that can be used to monitor the differentiation of these cells. However, none of these markers are uniquely associated with pluripotent differentiation capacity, and many are also expressed by stem cells that have lost the capacity to differentiate, (referred to as “nullipotent” stem cells). Thus, these markers cannot be used to identify pluripotent cells in the absence of functional evidence of pluripotency and further, they should not be referred to as ‘pluripotency markers,’ but as markers of the undifferentiated state.

As the field has grown, especially following the development of hiPSCs, the rigor and reproducibility of research has been hampered by imprecise reporting of experiments and confusion in terminology. In this section we provide clear guidance on how human pluripotent stem cells should be defined and characterized, to ensure accurate and unambiguous reporting of results obtained with these cells.

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Assessing Pluripotency

Recommendation 2.1.1: Pluripotency in human cells should be demonstrated experimentally by assays that assess differentiation capacity. Differentiation should be shown by quantitative measurements of the induction of marker combinations representative of ectoderm, endoderm, and mesoderm lineages, alongside loss of markers of the undifferentiated state.

Pluripotency is a functional property implying the capacity of a single cell to differentiate into all the somatic cell types of an organism. A cell line should be designated as pluripotent only if it has been experimentally shown to be capable of differentiating into cells representing all three embryonic germ layers that give rise to the somatic lineages of a developing organism. Human PSCs may exhibit a bias in the lineage(s) generated but should be able to make ectoderm, definitive endoderm, and mesoderm without genetic or epigenetic manipulation. Typically, hPSCs also have potential to produce primordial germ cells, but this feature may be absent in later stages of pluripotency. They also may or may not be capable of contributing to chimeras, but such capacity is not an essential part of the definition.

To claim differentiation into specific lineages or cell types, in vitro assays are recommended. For pluripotency designation, unequivocal evidence should be presented of differentiation into progenitors of definitive endoderm, mesoderm and neuroectoderm. Preferably, evidence of further differentiation into lineage-specific cell types should also be provided. The efficiency and extent of differentiation into cells of each germ layer should be reported (see Section 5).

Evidence of differentiation should be based upon multiple criteria, which may include morphology, expression of appropriate combinations of lineage or cell type specific mRNAs or proteins, cell surface markers, and assessment of functional properties. Additionally, ultrastructural features, multi-'omics profiling and transgenic reporters may be used where feasible. There should also be downregulation of markers of undifferentiated cells. Examples of markers that have been widely used to monitor differentiation are provided in Appendix 4 (Tables A4.1, A4.2, and A4.3). Where possible, marker expression should be quantified by techniques such as flow cytometry or quantitative imaging.

The degree of stringency required for determination of undifferentiated stem cell status and differentiation potential depends on the context of the experiments reported and the conclusions drawn from them (see Figure 2, below).

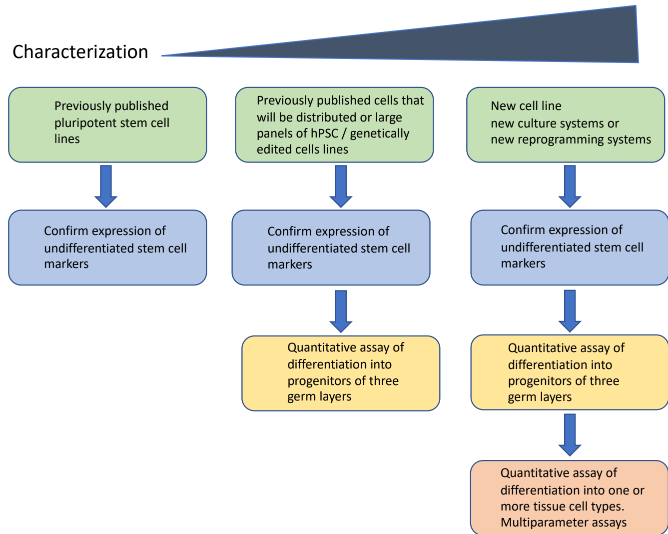

Figure 2. *The status and purpose of hPSC lines influence the characterization of the undifferentiated state and pluripotency potential. hPSC lines that have been previously published (left column) require less characterization than new lines or new culture or reprogramming systems (right column).*

Recommendation 2.1.1.a: For studies using cell lines where pluripotency has been established as described above and reported in peer-reviewed publications, it may not be necessary to repeat multi-lineage differentiation assays. Minimally, however, the undifferentiated status of the cells should be monitored by quantitative marker analysis (see Table A4.1).

Recommendation 2.1.1.b: For large scale studies describing the derivation of extensive panels of new pluripotent cell lines by well-established techniques, where in depth characterization of all lines may not be possible, a subset of the lines should be confirmed to be pluripotent by differentiation assays. For the remaining lines, the undifferentiated status of the cells should be monitored by quantitative marker analysis; these lines should then be designated putative pluripotent lines.

Recommendation 2.1.1.c: Where novel reprogramming techniques, cell culture methodologies, or other non-established protocols are used, confirmation of the undifferentiated status and developmental potential should be comprehensive. These include evaluation of larger panels of markers of the undifferentiated state and the capacity for differentiation into progenitors of the three embryonic germ layers, and of more differentiated cells, by multiparametric analysis.

Recommendation 2.1.2: Xenograft (teratoma) assays are not required to indicate pluripotency.

Although xenografting of pluripotent stem cells into immunocompromised animals provides a strong test of pluripotency and has been widely used in the past, concerns for animal welfare and increasing regulation in different jurisdictions make this assay undesirable if equivalent information can be derived from in vitro assays. The xenograft assay can provide evidence of the ability of differentiated cells to undergo histogenesis to yield complex tissues. However, several studies have confirmed that adequate evidence for pluripotency can be obtained from in vitro differentiation (Allison et al., 2018; Bock et al., 2011). Organoid or 3D assays in vitro may yield information on capacity for morphogenesis and histogenesis. Thus, xenograft assays are not recommended as a routine method for assessing pluripotency, although they may be a useful adjunct for the assessment of potential malignancy. If xenograft assays are used, criteria for assessment of teratomas and teratocarcinomas are described in Appendix 4.

The Undifferentiated State

Recommendation 2.2.1: The expression of recognized cell surface markers and transcripts can be used to assess and monitor the undifferentiated status of a cell line. However, the expression of such markers does not demonstrate pluripotency.

None of the markers present on undifferentiated cells are uniquely expressed in these cells, although they are often incorrectly used to identify pluripotent stem cells. Equally, there are many examples of cells that have little or no capacity for differentiation, notably nullipotent embryonal carcinoma cells from germ cell tumors, but that express many of these markers of undifferentiated cells, including OCT4 (POU5F1) and NANOG (see Table A4.1, for a list of markers). Therefore, these markers should not be called pluripotency markers as pluripotency cannot be defined by marker expression from undifferentiated cells. Nevertheless, such markers and gene expression profiles are useful to indicate undifferentiated status and as surrogate measures for retention of identity in cell types whose pluripotency has been well-established by prior differentiation assays, e.g., conventional hESCs or hiPSCs. They may also be used to screen putative pluripotent cells created by well-established methods for producing pluripotent cells. The absence of certain key markers, e.g., OCT4, can be strong indicators of loss of pluripotency, but these data cannot be considered definitive.

Developmental State

Recommendation 2.3.1: Evidence that a stem cell culture represents a particular developmental state should be based on relatedness to a stage and region of embryo development as assessed by specific gene expression and as far as possible by global profiling. Developmental state should be corroborated by demonstration of appropriate lineage-specific differentiation, including potential lineage biases. Profiling comparisons need to clarify the relationship to other reported stem cell states, and information should be provided on differentiation or interconversion between states. The culture conditions for generating and propagating the specific stem cell state should be reported in full, together with information on stability (or transience), homogeneity/heterogeneity and clonogenicity.

Stem cells in culture are expected to correspond to staging points along the developmental trajectory in the embryo between zygote and late gastrulation. Such correspondence means high global similarity in transcriptome and epigenome together with relatedness in features such as cell morphology, metabolic parameters, and differentiation competence.

Epiblast cells in the preimplantation human embryo and corresponding pluripotent stem cell lines are designated naïve. Naïve pluripotency is considered a discrete state. It is succeeded by an intermediate or formative stage of pluripotency that has shed naïve characteristics but not gained lineage specification. The formative phase appears to last several days in humans, and it is probable that a series of formative sub-states may be captured in culture. Formative pluripotency is a continuum with regionally specified and fated epiblast at the time of gastrulation, termed primed. Studies with mouse epiblast stem cells (EpiSCs) demonstrate that primed pluripotent stem cells can reside in different sub-states, related to different regions of gastrulation stage epiblast.

While cells in culture cannot be completely identical to cells in vivo, it should be considered that adaptations to the culture environment may induce features and identities that do not exist in the embryo. Stem cell properties that are discordant with those known for cells in the embryo should be declared. These aberrant pluripotent states may be of interest for certain applications, but caution is required in interpretations related to normal development.

Recommendation 2.3.1.a: Heterogeneity within the culture should be quantitatively addressed at the single cell level, by flow cytometry, high content screening, live cell imaging, and/or single cell -omics. Composition of the cultures should be assessed at multiple time points. Ideally, sub-populations should be characterized for inter-conversion and for clonogenic potential.

Pluripotent stem cell cultures are often mixed in composition. Such heterogeneity may arise in several ways including: continuous differentiation of a fraction of cells; maintenance of different pluripotent states in the same culture conditions; hierarchical pluripotency progression with more primitive naïve or formative stage cells giving rise to later stages; interconversions between pluripotent sub-states reflecting inherent plasticity but not necessarily relevant to developmental events; and spread of genetic or epigenetic changes that influence cell identity.

SECTION 3

Genomic Characterization

Stem cells are subject to the acquisition of genetic changes in culture (Draper et al., 2004; Mayshar et al., 2010; The International Stem Cell Initiative, 2011; Weissbein et al., 2014). Although variants initially appear as a single abnormal cell, their level of mosaicism can change over time, depending on the properties they confer upon cells. For example, variant cells that possess a selective growth advantage can rapidly expand in culture and outcompete wild-type cells to dominate cultures (Draper et al., 2004; Olariu et al., 2010; Price et al., 2021). Apart from a growth advantage, genetic changes can alter many different aspects of stem cell phenotype and behavior (Barbaric et al., 2014; Ben-David et al., 2014; Markouli et al., 2019). Moreover, culture-acquired genetic changes may also impact the behavior of differentiated cells derived from variant stem cells. By changing properties of cells, genetic alterations can affect reproducibility and repeatability of results obtained from stem cells and their differentiated derivatives. Hence, this section advocates monitoring of stocks and cultures of stem cells for the presence of culture-acquired genetic changes.

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Assessing Genetic Status

Recommendation 3.1.1: Cultures should be monitored for the presence of culture-acquired genetic changes as these can have myriad irreversible effects on stem cells and their differentiated progeny, such as altered growth rate, tumorigenicity, differentiation potential, and functionality, that may significantly impact the reproducibility and reliability of data collected.

All cell cultures are vulnerable to cultured-acquired genetic changes and should be monitored to ensure the consistency of the genetic composition over time. Human PSCs in particular are prone to culture-acquired genetic changes at the chromosomal, sub-chromosomal and nucleotide levels (reviewed in Halliwell et al., 2020). Some of the commonly acquired genetic changes in hPSCs include, but are not limited to, gains of chromosomes or parts of chromosomes 1, 12, 17, 20 and X (Draper et al., 2004; Baker et al., 2016) and single nucleotide variants in TP53 (Merkle, et al., 2017; Lezmi and Benvenisty, 2021). Thus, routine monitoring of hPSCs should entail analysis of the chromosomal complement to detect numerical and structural aberrations. Since many recurrent culture-acquired changes in hPSCs known to date are numerical and structural abnormalities, this analysis is expected to be sufficient to detect most large aberrations likely to be encountered. However, researchers should risk-assess the need for higher resolution detection methods to identify any recurrent changes in hPSCs that are not detectable by analysis of the chromosomal complement, such as small copy number variants (e.g., 20q11.21 CNV) and single nucleotide variants, including those in TP53 and additional cancer-related genes. For example, additional monitoring may be warranted if any alterations are observed in the traits of hPSCs during culture, such as changes in growth patterns or differentiation ability. Moreover, specific applications (i.e., establishment of new culture conditions, reprogramming methods or preclinical work) may also require more extensive monitoring for the presence of genetic changes.

Although a range of methods for assessment of genetic changes is available, currently no single method detects all types of genetic changes with equally high sensitivity and specificity. Thus, the specificity and detection limits of the assays should be considered when choosing specific methods for assessment of cultures and interpreting negative results, i.e., variants may be present at a mosaicism or resolution below levels of detection for a given assay (Appendix 5, Table A5.1). Moreover, this area is constantly evolving and therefore the particular changes to focus on and the most appropriate methods to use should be subject to regular review. Some examples of presently relevant recurrent genetic changes in hPSCs are listed in Table A5.2 (Appendix 5) including some of their known phenotypic effects and suitable methods for detecting them.

Timing of Assessment

Recommendation 3.2.1: Master and working cell banks should be evaluated to determine their genetic status.

A crucial timepoint for genetic assessment is during the preparation of the master and working cell banks. Cell lines are more likely to acquire genetic changes after a culture bottleneck, such as cloning events or gene editing. Ideally, master and working cell banks are created after such interventions, following genetic assessment. No method for detection of genetic changes can detect low levels of variant cells. Since the common recurrent variants provide the cells with a growth advantage, variants present below the level of detection in an initial screen may become detectable several passages later (e.g., see Olariu et al., 2010).

Recommendation 3.2.2: Monitoring for genetic changes should ideally cover the timespan of experiments.

Cells carrying variants providing a selective advantage can overtake a culture rapidly, often within 5-10 passages (Avery et al., 2013; Olariu et al., 2010). Therefore, not using cells that were drawn from the tested bank beyond passage 10 after thawing significantly decreases the risks of genetic drift. To avoid unnoticed genetic drift of the cell cultures used in experiments, there are several timepoints when genetic monitoring should ideally be carried out:

1) before starting the experiments (e.g., on the master or working cell banks).

2) during experiments (e.g., approximately every 10 passages*) to aid timely detection of genetic changes and/or on completion of experiments to assess whether the cell cultures used in experiments had retained their genetic integrity throughout the duration of the experiments.

3) after major culture bottlenecks, as these increase the chance of clonal expansion of genetically abnormal cells.

4) If any alterations are observed in the growth characteristics of stem cells during culture or in their patterns of differentiation, assessing the presence of genetic changes is recommended to check whether these alterations are caused by culture-acquired genetic changes. If no karyotypic abnormalities are apparent, but cells show altered properties, assessing the cells for presence of recurrent copy number variants not readily detected by karyotype analysis (e.g., 20q11.21 for hPSCs) and point mutations (e.g., in TP53 for hPSCs) is recommended.

*Passage is used here as it is a widely used and long established, convenient measure of population growth per passage, but the number of population doublings is affected by the split ratio and the cell cycle time, so it is advisable to consider these factors when choosing the interval between monitoring).

Recommendation 3.2.3: When a new cell line or derivative is generated by modifying culture conditions, implementing new reprogramming techniques, or after performing complex genome or epigenome manipulation, the cells should be evaluated for genetic changes after the intervention.

Analyzing cell material before and after generation of a new cell line or derivative enables comparison of genomic profiles and identification of newly introduced variants versus variants which were already present in the original cell material (Steeg et al., 2021).

For an overview of when and how to assess the genomic status of stem cells, see Figure 3 (below).

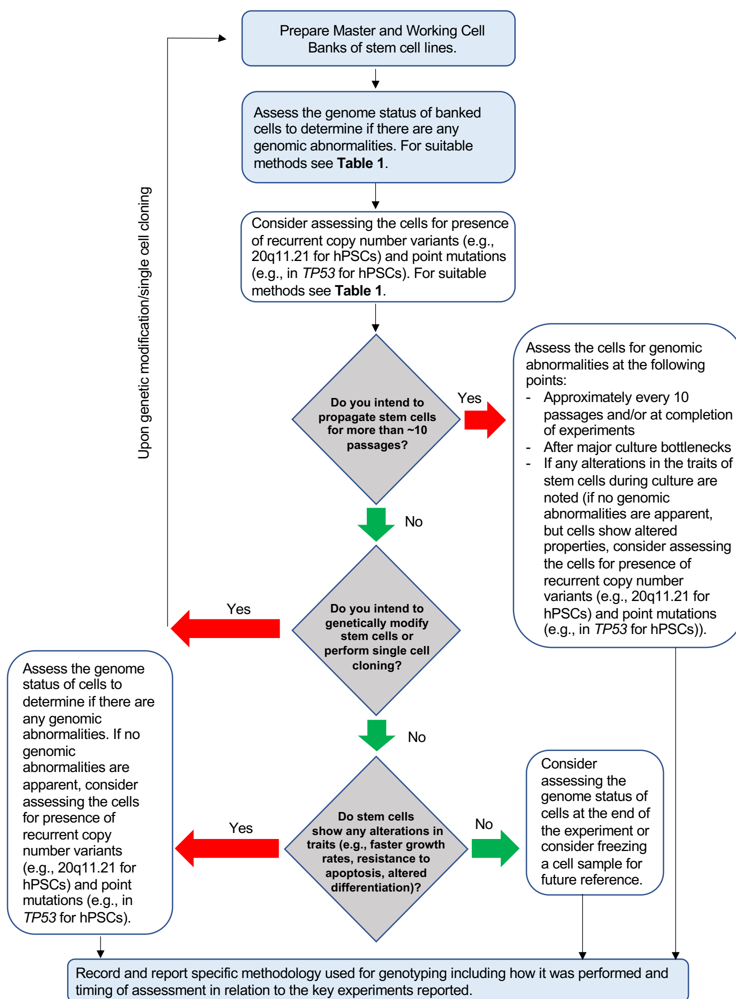

Figure 3. *Decision process for when and how to assess the genomic status of stem cells.*

SECTION 4

Stem Cell-Based Model Systems

Stem cell derivatives, organoids, and microphysiological systems to model normal and abnormal tissue physiology.

Stem cells and their differentiated progeny can be used to model tissue physiology, but more complex in vitro models are needed to recapitulate higher-level anatomical and physiological or pathological aspects of human biology. Organoid and organ-on-a-chip technologies (also referred to as microphysiological systems) are rapidly advancing platforms for such complex in vitro models. These models, from 2D to printed technologies, represent different aspects of human organs and tissues, and promise to reproduce human physiology that resembles the human situation well enough for predictive testing of interventions. Guidelines for best practice are needed to realize opportunities and address limitations of the models. These technologies aim towards the same overall goal of being physiologically reliable, albeit simplified, tissue representations.

Crucial to ensuring that these human model systems are widely adopted by academia and industry is confirming their reproducibility between developers and end-users, and individual laboratories and operators. The goal of this section is to improve the utility of model systems in fundamental research by:

- Improving the rigor and interpretability of model systems.
- Improving their reproducibility by reducing variability in their derivation, composition and use.
- Assessing the quality and validity of model systems, including their ability to recapitulate human (patho)physiology.

We provide comprehensive indications of which aspects of the models need particular attention to achieve these goals and ensure that they can be used optimally to understand and advance human health.

Understanding Your Starting Material

Recommendation 4.1.1: Consider the cell line or tissue of origin and, if known, identify the cell type of the starting material for the model.

There are several important points to consider when sourcing material for the generation of stem cell-based models as in vitro platforms to study normal physiology or disease pathology.

The starting material can influence variability and reproducibility. Firstly, the starting cell type(s) (e.g. fibroblast, epithelial cell), should be considered and documented. Secondly, whether the cells are obtained from fetal or adult tissues or banked material should be noted. Thirdly, consider the site that the sample was derived from, describing the anatomical location as well as possible. Fourthly, the isolation procedure should be carefully considered as this can lead to enrichment of specific cell and different types. Given the inherent nature of clinically derived material, it is essential that the origin is consistently described. The tissue or cell of origin of the starting material should be characterized as early as possible. Lastly, researchers should carefully consider the culture conditions since differences, even if small, can change the phenotype of the cell of origin or lead to selective growth of different cell populations.

Recommendation 4.1.2: Consider the sex, age, ethnic and genetic background, health status, risk factors and any additional clinical signs or symptoms of the donor, where available and as permitted by local regulations.

The background of the donor cells for derivation of model systems can influence the outcome of these models and may affect the generalizability of findings. Thus, details of the donor background should be documented even in the case of donors with no known relevant diseases. Laboratories should aspire to collect as much metadata as possible to assess how broadly applicable the findings from these model systems are. Include sex, age, health status and histopathological analysis of the starting material where available. Whenever possible, the donor's information should also be expanded to include self-reported lifestyle risk factors (e.g., smoking, diet, and exercise), known infections, prior treatments and/or disorders, and family history/genetic predisposition. For disease modeling, disease status and health of the donor should be provided, including genetic mutation and associated clinical data. Consider best practices in data management and donor privacy in your jurisdiction, noting that this may mean that not all information collected will be publicly available at the end of the study.

Generating the Model System

Recommendation 4.2.1: Quality control metrics of the method and the intended model should be established, fully documented, and validated across different stem cells and donors.

Researchers should use and establish, where not already available, metrics to assess the quality of components used to generate the model, and basic quality control metrics of the model system itself. Components and reagents for the development and maintenance of the model system should be tested, either by the manufacturer or the experimenter, for key metrics relevant to the model system.

A model system based on an engineered device should ideally be produced from ready-to-use devices and components. If this is not possible, the devices should be manufactured using processes that are widely available at academic institutions or via companies. Detailed protocols should include methods of device manufacture, companion reagents and their source. Descriptions of the fabrication of the devices should indicate potential problems and provide troubleshooting advice. Each step should be specified so that the fabrication processes can be reproduced. The success rate in producing the final model system should be stated so as not to create false expectations.

Recommendation 4.2.2: Ensure reproducibility within and between laboratories by describing the operational microenvironment and identifying conditions that affect variability for the given model system.

Methods of measurement need to be described and repeated to document any technical variability. The conditions that can affect variability, such as cell seeding density, culture reagents, fluid flow rate in microfluidic devices, oxygen tension, exogenous extracellular matrix components, frequency of media changes, and media batches, should be elaborated, and details of quality control metrics should be recorded. Sufficient replicates should be performed to assess technical and biological variability. For example, independent experiments are needed to assess intra-batch, batch-to-batch and line-to-line variability.

Validating Stem Cell-Derived Models

Recommendation 4.3.1: Demonstrate that the cellular model is functionally and phenotypically representative of the native cell/tissue by multiple, appropriate criteria.

The successful application of stem cells for modelling requires verification of cell specialization to target cells that recapitulate native cellular phenotypes. This is achieved by systematically evaluating differentiation to lineage-specific cell and tissue morphology, function, and expression of cellular markers (such as cell surface antigens and RNA transcripts). Importantly, in addition to being dependent on the genetic makeup of the donor organism, cellular phenotype is influenced by the surroundings to which the cells are subjected, including various epigenetic processes. As such, recurrent assessments during the development (i.e., at different developmental timepoints) and maintenance of cellular models are necessary.

Different levels of complexity in the model system may be needed to represent different aspects of the physiological system. There are four basic tissue- and component cell-types derived from stem cells for modelling, which are defined by their morphology, function, and cellular markers: epithelium, connective tissue, muscle and nervous tissue/cells. Epithelial cells and tissues form organ boundaries and are involved in protection, secretion, absorption, excretion, filtration, diffusion, and sensory reception. Connective tissues and cells (including cartilage, adipose, bone, and blood/lymph vessels) support and provide structure to other tissue types and help transfer nutrients and other substances between tissues and organs, repair damaged tissue, and defend the body against infection and disease. Muscle tissue (skeletal, cardiac, or smooth) is composed of cells that contract to produce movement of body parts, while nervous tissue, including neurons and glial cells, transmits and integrates information through the central and peripheral nervous systems. Models of these tissues and constituent cells should exhibit established native cellular morphological and functional traits. Morphological assessment should, therefore, confirm the shape, structure, form, and/or size of target cells, with alterations in the morphology of cells, potentially indicative of changed cellular function, such as during stem cell differentiation, tumor formation, and cell-pathogen interactions. Cell functionality should similarly recapitulate in vivo cellular processes underpinning the fundamental activities (intra- and intercellular) and the role of a target cell or tissue, such as metabolism, proliferation, respiration, diffusion, osmosis, active transport, ion flux, motility, and electrophysiology.

It is important that phenotypes identified in stem cell disease modelling are relevant to the human disease. Researchers should make necessary efforts to corroborate cell and molecular features that emerge from disease stem cell studies in a patient with the disease, through comparison with post-mortem tissue, relevant patient cells or tissues, or published data.

Assessment of cell specific markers can be performed by common immunophenotyping methods, including flow cytometry for cell antigen analysis of cell suspensions, or immunocytochemistry to evaluate single cell layers of fixed or unfixed cells, or immunohistochemistry, which is performed on fixed-, whole- or sectioned tissue specimens. Common methods for transcript analysis include qPCR, single-cell RNA sequencing (scRNAseq) and gene expression microarrays.

Recommendation 4.3.2: Where the development of new benchmarking tools is required to assess a stem cell model, the readout should be extensively validated against reference tissue panels and reproduced in multiple stem cell derivatives.

The highly coordinated networks of gene products that underpin cell states and functions present opportunities to use high-dimensional data such as RNA-sequencing to phenotype cultured cells. However, single markers should not be used to indicate cell lineage, stage, or specific cell identity. Molecular phenotypes should consist of panels of gene products whose correlated behavior is reproducibly associated with functional behaviors. New marker panels should be generally applicable across multiple experiments, or between laboratories; these should be derived using appropriate statistical approaches to benchmark their sensitivity and specificity.

Recommendation 4.3.3: Phenotypes that are associated with perturbation assays should ensure phenotypic measurements can distinguish general stress responses from targeted changes. Measurements should control for density-dependent phenotypes if cell cycle, cell growth or cell death are altered.

Generalized stress responses should be expected any time the environment of a cell is altered, and the resulting phenotypes may overwhelm the impact of the planned perturbation. In any screen, controls should be included to monitor for phenotypes that are a consequence of perturbation, or measurement. For example, anti-viral responses should be expected in samples exposed to nucleic acids, including guide-RNAs for genome editing, and may activate cellular shutdown or cell death pathways. If evaluating the perturbation of a specific gene, then controls should include guide RNAs of similar purine/pyrimidine composition. This requires careful evaluation of expected phenotypes (e.g., changes in drug sensitivity, response to a differentiation agent), proliferative responses, morphology changes, or cell death. If any of these form part of the target phenotyping panel, then understanding and reporting on specific types of proliferative signal, or form of cell death (e.g., apoptosis vs pyroptosis) is essential to identify target activities over background phenotypes.

Recommendation 4.3.4: Where the model is assessing the impact of a known genotype on the phenotype, it is essential to confirm the stem cell-derived disease model carries the expected genotype.

Genetic validation of patient-derived stem cells is important to confirm the known mutation(s) and may also be useful in authentication of the sample and its origin (see Recommendation 1.3.1). Genetic instability, as well as genetic mosaicism of donor tissue, may contribute to stem cell pools of mixed genotypes.

Proper Controls

Recommendation 4.4.1: Consider variability when determining the necessary number of disease and control stem cell derivatives to be included in a study.

Power analysis should be used to determine sample size. This will be impacted by the effect size and penetrance of the phenotype. If effect sizes of the biological readouts are unknown, then aim for the largest sample size available. A strong rationale should be provided for the chosen sample size. If replication is not possible, then variability should be reduced by using isogenic controls. For disease modelling, it is important to select appropriate non-diseased samples to establish base lines for controls (see Recommendations 4.4.2 and 4.4.3).

Recommendation 4.4.2: In comparing disease models with healthy controls, the meaning of “healthy” should be clearly defined.

Many models require comparisons of diseased states with non-diseased states. Therefore, it is important to define the parameters of the disease that are being assessed to choose an appropriate control, so that conclusions of any study can be properly placed in context. Given the penetrance of most diseases is age-related, and that many control samples may carry additional risk factors towards the given disorder or be chosen from well-characterized stem cells derived from an unrelated disease cohort, the term healthy is subjective. Therefore, the choice of controls requires consideration of age-matched, ethnicity, sex, familial associations, genotype, and clinical history.

Recommendation 4.4.3: The genetic background should be considered when selecting cells to introduce or correct disease-associated mutations.

Stem cells may manifest disease-associated traits because of polymorphisms in the donor or because of culture acquired genetic changes. Beyond considerations of controls outlined in Recommendation 4.4.1, these genetic aspects can confound the phenotypic readout. (See also Recommendation 3.1.1).

Recommendation 4.4.4: When comparing isogenic cells derived from genome manipulation, multiple independent clones should be assessed. Where bulk cultures but not independent clones are used, this should be documented.

All current methods for gene editing/gene correction risk introducing unintended genetic changes. These include CRISPR-based approaches, prime-, or base editing, multiple conventional methods of homologous recombination, etc. Researchers should take necessary measures to identify genetic changes and to select lines for phenotypic characterization (of differentiated derivatives) in which these common or rare mutations have not been introduced. Further, it should be noted that healthy individuals and patients can be mosaic in cell composition and the relative proportions of mutant and healthy cells can vary depending on the tissue sample available for stem cell generation. Ideally, the products of independent genetic modification experiments would be compared. In the case of time consuming- or costly experiments, or long differentiation protocols, it may be sufficient to carry out only key experiments relevant to the goals of the study. 'Independent lines' are considered to mean derivatives of a single cell selected during reprogramming or gene modification (correction or mutation) arising in an independent well.

SECTION 5

Reporting

It is essential that any published paper includes detailed information on the following parameters to ensure that the published results are reproducible. The following section highlights the requisite details that should be reported in manuscripts using pluripotent or tissue stem cells. For a complete list of reporting recommendations, see Appendix 6.

Basic Characterization

Recommendation 5.1.1: Published reports should include the source of the cell line or the details of its derivation, complete descriptions of the methods used for stem cell maintenance and preservation (including culturing, passaging, freezing and thawing methods), the passage number (or ideally population doublings) of cryopreserved MCB or WCB stocks, and number of subsequent passages prior to and during experimentation.

Understanding the nature of cell materials used in experimentation is essential in the evaluation of research techniques and results, and in their comparison between laboratories. All published reports should include the unique cell line identifier, the specific source of the initial cell materials (i.e., commercial group, repository, collaborator), and detailed protocols for propagation and preservation during experimental use should be provided or referenced. Overall passage number of the cell line from derivation should be noted, and the passage duration of experimental use and its relation to characterized stocks.

Recommendation 5.1.2: Published reports should include the registry number of the originating cell line (hiPSC, hESC, somatic cells) and a unique number for any modification(s) made to a line, such as reprogramming to an hiPSC (see Recommendation 1.4.1).

Pluripotency

Recommendation 5.2.1: Tests of pluripotency and the undifferentiated status should be thoroughly described, including assay methodology, source of reagents, readouts, and quantitation and statistical analysis, and should indicate the point in the culture history of the cell line relative to experimental studies at which assays were performed. The term 'pluripotency marker' should not be used to describe markers used to characterize the undifferentiated state.

Tests for pluripotency and the undifferentiated state should span the period in culture during which experiments were carried out. For example, if experiments were conducted within 10 passages of recovery of the stocks from a working cell bank, these tests should be performed early after recovery from cryopreservation and after 10 passages.

Appendix 4 provides guidelines on minimal criteria for assessment of pluripotency and undifferentiated status. However, the necessity and degree of stringency required for such an assessment will be dependent upon the context of the experiments reported and the conclusions that are drawn from them (see Figure 2, above). These are matters for assessment by reviewers, and it is important to clearly document what has been done.

Genomic Characterization

Recommendation 5.3.1: The specific methodology used for genotyping should be reported, including how it was performed (e.g., number of cells analyzed) and timing (passage/population doublings) in relation to the key experiments reported.

While we do not recommend the specific methods for genotyping that should be used, the specific methodology should be described in sufficient detail so that the scope of the assays (i.e., the range of genetic variants that could have been detected) and their sensitivity (i.e., the lower limit for detecting variant cells in a mosaic culture) are clear to the reader (see Appendix 5). The author should provide a clear indication of when genotyping was carried out in relation to specific sets of experiments that generated key data for the study. In particular, the relationship between cultures of cells used for genotyping and those used for key experiments should be explicit.

Recommendation 5.3.2: The appearance of genetic variants during experimental procedures does not preclude publication provided that their potential effects are appropriately considered.

There is currently no general approach to predict the effect of a particular culture-acquired genomic change on traits of hPSCs or hPSC-derived differentiated cells, and on those of human somatic cells. This is because the traits may reflect the complex effects of multiple mutations, and the effects of mutations on the traits may depend on the cell type of interest and their surrounding environment. Therefore, the accumulation of knowledge on the relationship between culture-associated cellular trait changes and culture-acquired genomic mutations is important as a common resource for the stem cell research community to inform the scientifically valid interpretation of experimental results.

Stem Cell-Based Model Systems

Recommendation 5.4.1: Information should be reported so others can understand the work and readily compare across studies. At a minimum, this should include the source of cells or tissues, relevant disease information, if applicable, and any genetic mutations or abnormalities.

Methodological details of model systems should include enough information so that others may accurately judge the results and reproduce findings. In particular, the source of cells and/or tissue used to generate models should be provided, such as species, tissue of origin, and cell type. This includes not only donor information relevant to the model system (patient status, genotype, species) but also details of how the cells or tissues were isolated. Cells seeded from tissues should include details on biopsy site, dissociation method, a detailed characterization of starting population using recognized markers and methods, media composition, doubling rates, and phenotype of expanded cells including morphological observations and relevant molecular markers. While anonymized information relevant to the model should be included, care should be taken to avoid any potential reidentification of donors, i.e., genetic information shared through databases with restricted access.

How cells were prepared or treated before establishing the model system should also be carefully described. If cells were purified for example through FACS or other sorting strategies, details of markers used should be reported. If pluripotent stem cell lines were used as a starting point for differentiation, details of their origin should be included, such as whether they were genotyped, exhibit relevant disease mutations, and how they were cultured (media, coating material, passage number) prior to differentiation. Details of how long the cells or tissues were kept in culture (including the passaging method) before being used as a model system should be provided if known.

Recommendation 5.4.2: Information regarding the experimental unit, or sample type, should be reported for each experiment performed. Whether samples are individuals, cell lines, clones, tissues, organoids, batches, cells, etc., should be reported.

Quantitative analysis of model systems may include measurements across different scales. For example, when measuring a particular cellular feature (i.e., division angle) the measurement is performed on individual cells, whereas when measuring a cell population effect (i.e., number of cells of a particular identity) the measurement is performed on a tissue level. Individual experimental units (data points) should be clearly defined, whether they represent each individual cell, each organoid, each cell line, etc. For data presented as a distribution (violin plot, box plot, etc.) the individual units used to generate the data should be defined. This information will often be hierarchical for in vitro model systems (i.e., organoids derived from multiple clones, across multiple individuals) and this hierarchy should be reported.

If statistics are performed (for example, significance testing) the experimental unit should be defined (what is n?). This may include a description of technical and biological replicates, and if so, what these refer to should be explained. As a general rule, technical replicates refer to replicates of the same biological material (i.e., extracted genetic material from a single experiment) run again on the same machine, while biological replicates would represent independent biological samples. As such, technical replicates capture the variability in the assay or readout, while biological replicates reflect true biological variability.

Biological replicates may refer to many different types of replicates. For example, if protein localization was measured in 100 cells of 3 organ-on-chip models each made from 3 batches of 3 different cell lines, which of these was used for statistical comparison should be defined and this hierarchical information included, ideally even displayed in the data representation (Lord et al., 2020). The rationale for choosing the experimental unit (i.e., single organoids versus batches) should be explained. Generally, the experimental unit should be chosen based on known sources of variability in the model itself, keeping the aim of the experiment in mind. For example, if the aim is to compare a disease state to healthy control, then several different disease cell lines and several healthy control cell lines should be used to generate the model, and the experimental unit, or n, would be the number of healthy and diseased cell lines used to generate the model, rather than the model itself (i.e., the organoid or the batch).

The number of each experimental unit should be clearly defined for each experiment and statistical comparison. Ideally, pilot studies and consideration of the relevant variables should be conducted to enable adequately powered experiments (Shin et al., 2022) using appropriate power calculations (see Recommendation 4.4.3).

Recommendation 5.4.3: When reporting information on sample types, heterogeneity and unknown issues may arise and these should be documented as thoroughly as possible, including both known sources of heterogeneity and unknown, in which case the lack of relevant information that may influence heterogeneity should be reported.

Depending on the model system, the source of cells or tissue may contribute to phenotypic heterogeneity. This is especially relevant to tissue stem cell-derived model systems, such as tissue stem cell-derived organoids. In particular, samples taken from different parts of the same tissue may exhibit heterogeneous phenotypes. Tumor samples and other tissues with genetic abnormalities may especially exhibit such heterogeneity and so whether these sources of heterogeneity are present, and details of where cells originate from within the tissue, should be provided. However, this information may not be present, so for patient samples with missing information, those unknowns should also be outlined. Thus, if the source of heterogeneity is known, for example related to tissue of origin (i.e., tumor samples, genetic abnormality, etc.) this should be reported. If heterogeneity is likely present but relevant information is missing, that lack of information should be stated.

Recommendation 5.4.4: Publication of phenotypes that use computationally derived classifiers should include the data and annotated code used for phenotype classification. Researchers must follow FAIR (https://www.go-fair.org/fair-principles/) and CARE (https://www.gida-global.org/care) data management principles. Computational phenotyping can be very effective for benchmarking cell models, or discriminate confounding generic cell responses from cell or tissue-specific effects of an assay.

Computational phenotypes are derived from analysis of high-dimensional molecular data, which may include a combination of image-analysis, antibody panels, (phospho)proteomics, metabolomics, transcriptomics, or epigenome profiling. While several computational approaches may be gainfully employed to benchmark or evaluate cellular responses, all require reporting of experimental variables using FAIR data principles. This reporting should include the use of structured ontologies of metadata, cell line accession numbers, and standardized data formats that allow for parsing of experimental series into standardized computational workflows. Best practice includes using markdown to explain key steps in the code and publication of workflows in code notebooks or git-libraries. Documentation of phenotyping markers derived from these workflows should include evaluation of marker sensitivity and specificity when applied to independent datasets and marker recall when code is applied to new data.

Acknowledgements

Steering Committee

Tenneille Ludwig, Co-Chair
WiCell Research Institute (USA)

Peter Andrews, Co-Chair
The University of Sheffield (UK)

Ivana Barbaric
The University of Sheffield (UK)

Nissim Benvenisty
Hebrew University (Israel)

Madeline Lancaster
MRC Laboratory of Molecular Biology (UK)

Christine Mummery
Leiden University Medical Center (Netherlands)

Martin Pera
The Jackson Laboratory (USA)

Yoji Sato
National Institute of Health Sciences (Japan)

Glyn Stacey
International Stem Cell Banking Initiative (UK)

Christine A. Wells
University of Melbourne (Australia)

Tongbiao Zhao
Institute of Zoology Chinese Academy of Sciences, Beijing Institute for Stem Cell and Regenerative Medicine, Beijing (China)

Working Group Members

Basic Characterization Standards

Glyn Stacey, Co-Chair
International Stem Cell Banking Initiative (UK)

Tenneille Ludwig, Co-Chair
WiCell Research Institute (USA)

Jeremy Crook
The University of Sydney, Chris O'Brien Lifehouse, The University of Wollongong (Australia)

Laurence Daheron
Harvard Stem Cell Institute (USA)

Jon Draper
Stem Cell Network (Canada)

Lyn Healy
Francis Crick Institute (UK)

Andreas Kurtz
Fraunhofer Institute for Biomedical Engineering, Berlin Institute of Health at Charité, (Germany)

Undifferentiated Stem Cells and Pluripotency

Peter Andrews, Co-Chair
The University of Sheffield (UK)

Martin Pera, Co-Chair
The Jackson Laboratory (USA)

Lyn Healy
Francis Crick Institute (UK)

Maneesha Inamdar
Jawaharlal Nehru Centre for Advanced Scientific Research, Institute for Stem Cell Science and Regenerative Medicine (India)

Tenneille Ludwig
WiCell Research Institute (USA)

Noriko Shimasaki
Center for iPS Research and Application, Kyoto, Kyoto Prefectural University of Medicine, Nagoya University, Japan, National University of Singapore, Singapore (Japan)

Austin Smith
University of Exeter (UK)

Genomic Characterization

Ivana Barbaric, Co-Chair
The University of Sheffield (UK)

Tenneille Ludwig, Co-Chair
WiCell Research Institute (USA)

Peter Andrews
The University of Sheffield (UK)

Nissim Benvenisty
Hebrew University (Israel)

Martin Pera
The Jackson Laboratory (USA)

Yoji Sato
National Institute of Health Sciences (Japan)

Claudia Spits
Vrije Universiteit Brussel (Belgium)

Tongbiao Zhao
Institute of Zoology Chinese Academy of Sciences, Beijing Institute for Stem Cell and Regenerative Medicine, Beijing (China)

Stem Cell-derived Model Systems (Organoids, Microphysiological Systems, Engineered Cells, Disease Modelling)

Madeline Lancaster, Co-Chair
MRC Laboratory of Molecular Biology (UK)

Christine Mummery, Co-Chair
Leiden University Medical Center (Netherlands)

Anita Bhattacharyya
University of Wisconsin-Madison (USA)

Jeremy Crook
The University of Sydney, Chris O'Brien Lifehouse, The University of Wollongong, NSW, Australia (Australia)

Meritxell Huch
Max Planck Institute of Molecular Cell Biology and Genetics, and Center for Systems Biology, Dresden (Germany)

Kim Jensen
Novo Nordisk Foundation Center for Stem Cell Medicine, University of Copenhagen (Denmark)

Prisca Liberali
Friedrich Miescher Institute for Biomedical Research (Switzerland)

Matthias Lutolf
Swiss Federal Institute of Technology (EPFL) (Switzerland)

Jihwan Song
CHA University, and iPS Bio, Inc. (Korea)

Christine A. Wells
University of Melbourne (Australia)

Tongbiao Zhao
Institute of Zoology Chinese Academy of Sciences, Beijing Institute for Stem Cell and Regenerative Medicine (China)

Supported by ISSCR Staff

Tyler Lamb, Director of Policy
Jack Mosher, Scientific Advisor

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APPENDIX 1

Recommended Standard Characterization of Stem Cells

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Table A1.1. Characterization of pluripotent stem cells (PSCs) tissue stem cells (TSC) and stem cell-derived models (SCM)

This table summarizes the recommendations for the basic characterization of human pluripotent and tissue stem cells, including the timing of any characterization.

Assay	Initial Characterization						Interval			End of Study			Section Reference		
	PSC		TSC		SCM		PSC	TSC	SCM	PSC	TSC	SCM	PSC	TSC	SCM
Authentication (match to donor)	MCB	WCB	MCB	WCB	MCB	WCB									
	R	D*	R	D*	R	D*	repeat at bottlenecks			R			1.3	1.3	1.3
Sterility	R	D*	R	D*	R	D*	observe daily	observe daily	observe daily				1.6.1; 1.6.1b; Appendix 3	1.6.1; 1.6.1b; Appendix 3	1.6.1; 1.6.1b; Appendix 3
Mycoplasma	R	D*	R	D*	R	D*	quarterly	quarterly	quarterly	R			1.6.1; 1.6.1a; Appendix 3	1.6.1; 1.6.1a; Appendix 3	1.6.1; 1.6.1a; Appendix 3
Genomic evaluation	R	D*	R	D*	R	D*	every ~10 passages, or at bottle-necks and/or EoS			D[2]	D[2]	D[2]	3.1; 3.2; Appendix 5	3.1; 3.2; Appendix 5	3.1; 3.2; Appendix 5
Adventitious agents	D[4]		D		D								Appendix 3.2	Appendix 3.2	Appendix 3.2
Confirmation of Cell Type/ Molecular markers	R		R		R								2.2; 2.3, Appendix 4 Tables A4.1; A4.2; A4.3	4.1.1; 4.3.1; 4.3.2	4.1.1; 4.3.1; 4.3.2
Pluripotency (PSCs)	R[3]												2.1; 2.2; 2.3		
Confirmation of genetic modification	R[1]		R		R								3.2.3; 4.4.4	3.2.3; 4.4.4	3.2.3; 4.4.4
Confirmation of disease mutation	R[1]		R		R								4.3.4	4.3.4	4.3.4
Differentiation Potential (TSCs)			R											4.1.1	

R = Recommended
D = Desirable
1: as applicable,
2: recommended at end of study if not performed at intervals
3: for derivation of new cell lines or new culture systems
4: preferred at level of donor
* desirable for all; strongly recommended for core facilities or when distributing/transferring lines externally

APPENDIX 2

Nomenclature Criteria

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Acceptance Criteria

Appropriate nomenclature is critical to the unambiguous identification of cell materials used in stem cell research. Adherence to general principles for naming cell lines and attention to issues that impact robustness is essential in generating consistent, easily employed nomenclature to identify materials. Ideally, assigned nomenclature should adhere to the following standards:

GENERAL PRINCIPLES

- Nomenclature should provide informative code to generate persistent and unique identifier
- Identifier should be linked to donor and STR profile (authentication)
- Identifier should allow to trace clones and derivatives to source
- Free to generate (for example, by API)
- Free to access and referenced in a central registry
- Consistent across publishers and journals
- Machine readable and if possible human readable
- Fit on standard tube labels (14-18 symbols)
- Linked to authentication profiles
- Allow FAIR data principle application (findable, accessible, interoperable and reusable)

ROBUSTNESS

- Automation of nomenclature generation (hPSCreg)
- Automated authentication test and match/mismatch recognition (donor/stem cell line/clones and derivatives)
- Link to and harmonizing with related codes (RRID)
- Sufficient scalability

APPENDIX 3

Cell Culture Hygiene Practices

The following recommendations apply broadly to cell culture. While they are not exclusive to stem cell culture, their relevance to the field and importance to best practice standards makes their inclusion essential for completeness.

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Sterility

Recommendation A3.1: Researchers should demonstrate and document that cell lines are free of microbial contamination. Cell lines should be monitored daily for evidence of visible contamination. In addition, MCBs should undergo appropriate robust microbiological testing to detect microbial (bacteria, fungi, and yeast) contamination. If cell lines are found to be contaminated, barring exceptional cases, they should be discarded.

Throughout processing, from derivation to biobanking, stem cell cultures should be handled aseptically (Bykowski and Stevenson, 2020; Sanders, 2012) to prevent inadvertent contamination and ideally processed in the absence of antibiotics which can affect the biochemistry of cultured cells (Farzaneh, 2021; Llobet et al., 2015; Romorini et al., 2013; Ryu et al., 2017; Skubis et al., 2017; Varghese et al., 2017).

Culture contaminants can adversely affect cell culture, causing cell death, altering cell function, genetic stability, and growth rate (Langdon, 2003; Stacey, 2011) and daily observation of cultures under the microscope is advised to monitor infection. In general, due to their rapid growth, bacteria, yeast and fungi can easily be detected within a few days following contamination. Signs of infection can include cell death, turbidity and color changes in culture media containing a phenol red pH indicator. If cell cultures are found to be infected, unless irreplaceable, they should be discarded.

Good cell culture practice (Pamies et al., 2022) and appropriately timed screening should be applied to ensure the sterility of the cell culture. Robust microbiological testing is recommended to assure the sterility of the MCB, the foundation of all future work with a cell line. Testing could include the use of microbial culture media to reveal the presence of hidden microbial contaminants ("European Pharmacopoeia Method 2.6.1 Sterility," 2022), or growing the cell line for 14 days in glucose rich media (antibiotic-free) and evaluating signs of infection.

Adventitious Agents

Recommendation A3.2: Ideally, donors should be pre-screened for human virus pathogens. If this is not possible, donor cells or cell lines should be tested for human virus pathogens at the earliest timepoint possible. At a minimum, human immunodeficiency virus 1 and 2 (HIV1), (HIV2), Hepatitis B and Hepatitis C should be screened. Cell lines should be confirmed negative for these viruses before biobanking or distribution. All human materials, tested or not, should be treated as potentially infectious, and handled appropriately using BSL2 or Category 2 standards.

Viruses are typically the most difficult contaminants to detect in cell culture. Most of the viruses have a diameter varying from 20nm to 400nm and therefore cannot be seen under light microscope, nor can they be removed by filtration.

Viral contamination can result in loss of cell cultures, invalid scientific data, potential hazards to operators and risk of viral disease outbreak of animal facilities (when cells are used for in-vivo animal study). Overlooked viral contaminations may alter the function of the cells and lead to flawed results, wasting of technical and financial resources, and potentially necessitate retraction of publications (Merten, 2002).

The major risk of contamination by human viruses is from the source material used for the generation of stem cells. If the donor cannot be tested before tissue collection, the material should be assumed to be potentially infectious and handled appropriately, using BSL2 or Category 2 standards (Artika and Ma'roef, 2017). While consistent application of this level of containment and personal protection equipment will protect operators, there are certain research areas where containment is challenging (e.g., flow cytometry and cell sorting), making the need for testing more critical (Pamies et al., 2016).

Viruses can be tested by direct methods (assays detecting the presence of the virus) or indirect method (assays detecting the effects of the virus). Direct methods include detection of viral sequences by qPCR or detection of viral antigens by immunofluorescence or ELISA. The indirect method is based on the observation of cytopathic effects triggered by the viruses. Viral testing by qPCR is very sensitive and relatively easy to establish in the lab, thus, should be the method of choice (Uphoff, 2010). Alternatively, viral testing can be done by external certified laboratories.

Overall, testing for the most common human viruses is strongly recommended to protect operators and reduce potential impact on cultures. Finally, when considering biological reagents, whether human or animal sourced, the importance of investment in high quality reagents with good traceability cannot be overstated.

APPENDIX 4

Markers for the Identification of Undifferentiated hPSCs and Monitoring Multi-Lineage Differentiation

Few, if any, gene transcripts, proteins, or oligosaccharides are uniquely expressed by a single cell type so that identification and monitoring of undifferentiated hPSCs, or of cells corresponding to ectoderm, mesoderm or endoderm require the use of multiple markers. Building on a set of cell surface antigens previously defined and characterized in human embryonal carcinoma cells (Andrews et al., 1996), the International Stem Cell Initiative identified a set of cell surface markers (Table A4.1) that are typically expressed by undifferentiated human ES cells (The International Stem Cell Initiative, 2007). The International Stem Cell Initiative also used transcriptome analysis of a panel of genes (Table A4.2, Table A4.3) to monitor undifferentiated human hPSCs and their differentiation into derivatives corresponding to ectoderm, mesoderm and endoderm. A similar panel of genes was used in the study by Bock et al (2011), which was then used by Tsankov et al (2015) to quantify differentiation to the three germ layers.

Table A4.1. Cell surface marker antigens for undifferentiated human ES cells

Antigen	Antibody
SSEA3	MC631
SSEA4	MC813-70
TRA-1-60	TRA-1-60
TRA-1-81	TRA-1-81
GCTM2	GCTM2
L-ALP	TRA-2-54
L-ALP	TRA-2-49
CD90(Thy-1)	F15-14-1
CD9	TG30

Source: (The International Stem Cell Initiative, 2007)

Table A4.2: Marker genes used in transcriptome panels to monitor undifferentiated hPSCs and their multi-lineage differentiation

This table lists genes the expression of which has been used in published studies to provide evidence of undifferentiated cells or differentiation into the different germ layers. It is important to note that no single gene is expressed uniquely by a single cell type or lineage. Thus, conclusions should be based upon the patterns of gene expression observed compared with proper controls. Minimally, conclusions can be based on the expression of two or three genes indicative of each germ layer, but the strength of any conclusions is enhanced further as additional markers are included in the panel.

Gene	Undiff	Ectoderm	Mesoderm	Endoderm	Trophoblast
ABCB1		3	3		
ABCG2	3	2, 3	2, 3		
ACTA2			3		
ACTB			3		
ACTC			1		
ACTN1			3		
ADIPOQ			2, 3		
AFP				1, (3?)	
ALB				3	
ALPL	3				
ANPEP			2, 3		
APOE		2, 3		2, 3	
BMP2			3		
BRIX	1				
CAMK2A	3				
CAPN1			3		
CD14			3		
CD151			3		
CD19			3		
CD24	3	3	3		

TABLE A4.2 CONTINUED

CD34			2, 3	1	
CD36			2, 3		
CD4			2, 3		
CD44		2, 3	2, 3	2, 3	
CD59			3		
CD9	1, 3		3		
CDCP1			3	3	
CDH1	3		2, 3		
CDH15			3		
CDH2		2, 3	2, 3	2, 3	
CDH5			2, 3	1	
CDKN2A	3				
CDX2	3			2, 3	
CEACAM1			2, 3		
CGB					1
COL1A1			1		
COL2A1			1		
COMMD3	1				
CRABP2	1	2, 3			
CREBBP	3				
CSF1R			3		
CTNNB1	3			2, 3	
CXCR4		3	3	3	
DDX3X	3				
DES			1, 3		
DIAPH2	1				
DLL1	3		2, 3		
DLX5				3	
DNMT3B	1				
DPPA3	3				
E2F1	3				
EBAF	1				
EDNRB	1				
EN1		2, 3			
ENG			3		
ENO3			3		
EOMES					1
EP300	3				
EPCAM	3				

EPHB2	3				
ERAS	3				
FABP1				3	
FABP2				3	
FABP4			3		
FAS		2, 3			
FCGR3A			3		
FCGR3B			3		
FGF10	3				
FGF2	3				
FGF4	1				
FGF5	1				
FGFR2		2, 3			
FLT1				1	
FN1		3	3	1	
FOXA1				3	
FOXA2			3	1, 2, 3	
FOXD3	1, 3				
FUT4	3	2, 3	2, 3		
GABRB3	1				
GAL	1				
GATA2		2, 3			
GATA3		2, 3	2, 3	3	
GATA4			2, 3	1, 2, 3	
GATA6	1			2, 3	
GBX2	1				
GCG				1, 2, 3	
GCM1					1
GDF3	1				
GFAP		1, 3			
GNL3	3				2, 3
GRB7	1				
GREM1	3				
GSC			3		
GSK3B	3				
HAND1		2, 3	3		
HBB			1		
HBZ			1		
HHEX			2, 3		

HLA-DRA			3		
HLXB9		1			
HNF1A				2, 3	
HNF1B				2, 3	
HTATSF1				3	
IAPP				1	
ICAM1		2, 3	2		
IFITM1	1				
IFITM2	1				
IFNGR1	3				
IGFBP3			3		
IL6ST	1				
IMP2	1				
INHBA			2, 3		
INS				1	
IPF1				1	
IRF6	3				
ISL1		1		2, 3	
ITGA2B			3		
ITGA4		2, 3	2, 3		
ITGA6	3	2, 3	2, 3	2, 3	
ITGAL			2, 3		
ITGAM			2, 3		
ITGAV			2, 3		
ITGAX			2, 3		
ITGB1	3	2, 3	2, 3	2, 3	
ITGB2			3		
ITGB3			2, 3		
JAG1	3	3			
JMJD6	3				
KDR			2, 3		
KIT	1, 3		2, 3		
KLF4	3				
KRT1					1
LAMA1				1	
LAMB1				1	
LAMC1				1	
LEF1			2, 3		
LEFTB	1				

LIFR	1				
LIN28	1, 3				
LRP2				3	
MAP2		2, 3			
MAP2K1	3				
MAPK1	3				
MAPK3	3				
MAPT		2, 3			
MCAM		2, 3	2, 3		
MIXL1			3	3	
MME			2, 3		
MNX1		2, 3			
MYC	3				
MYF5			1		
MYH3			3		
MYOD1			1, 2, 3		
MYOG			2, 3		
NANOG	1, 3				
NCAM1		2, 3	2, 3		
NEFL		2, 3			
NES		1, 2, 3	2, 3		
NEUROD		1			
NEUROG3	3	2, 3		2, 3	
NGFR		2, 3	2, 3		
NKX2-5				2, 3	
NODAL	1, 3		3		
NOG	1				
NOG		2, 3			
NOTCH1	3	2, 3	2, 3		
NPPA			1		
NR5A2	1, 3				
NR6A1	1				
NUMB	1				
OLIG2		1			
OTX2		2, 3			
PAX3		2, 3			
PAX4				1	
PAX5		3			
PAX6		1, 2, 3		2, 3	

PAX7		2, 3			
PDGFRA		2, 3			
PDX1				3	
PDX1				2	
PECAM1			2, 3	1	
PODXL	1, 3				
POU5F1	1, 3				
PROM1	3				
PTEN	1				
PTF1A				1	
PTPRC			3		
RAF1	3				
REST	1				
ROCK1	3				
RUNX2			1		
S100B		3			
SDC1			2, 3		
SEMA3A	1				
SERPIN				1	
SFRP2	1				
SHH	3				
SLC2A2				2, 3	
SNAI2		2, 3			
SOX10		2, 3			
SOX17	3			3	
SOX17				1	
SOX2	1, 3	2, 3			
SOX7				3	
SOX9		2, 3			
SP1	3				
SPARC				3	
SPI1			2, 3		
SRF			2, 3		
SST				1, 2, 3	
STAT1	3				
STAT3	3		2, 3		
STAT5A	3				
SYP		1, 2, 3		2, 3	
SYT1		3			

TBXT			1, 2, 3		
TAT				1	
TAZ			3		
TDGF1	1, 3	2, 3			
TERT	1, 3				
TFCP2L	1				
TFRC			3		
TGFB1			3		
TGIF1	3				
TH		2, 3			
TH0		1			
THBD			3		
THY1		2, 3	2, 3	2,3	
TNFRSF1A			2, 3		
TNFRSF1B			3		
TNFRSF8	3				
TNNI3			3		
TP63		3			
TWIST1			2, 3		
UTF1	1, 3				
VCAM1			3		
VIM		3	3	3	
WT1			1		
XIST	1				
ZFP42	1, 3				
ZFX	3				

1) Characterization of human embryonic stem cell lines by the International Stem Cell Initiative (The International Stem Cell Initiative, 2007).
2) Reference maps of hESC and hiPSC variation enable high-throughput characterization of pluripotent cell lines (Bock et al., 2011).
3) Assessment of established techniques to determine developmental and malignant potential of human pluripotent stem cells by the International Stem Cell Initiative (Allison et al., 2018).

Table A4.3: Reduced marker gene set used for embryoid body analysis

Undifferentiated	Ectoderm	Mesoderm	Endoderm
TNFRSF8	TH	PECAM1	FABP1
TERT	PAX3	CSF1R	ALB
FOXD3	MAP2	ITGB3	FOXA1
MYC	OTX2	VCAM1	HNF1A
ALPL	MAPT2	ANPEP	LRP2
UTF1	PAX7	IGFBP3	HNF1B
POU5F1	EN1	CDH5	SST
NANOG	NOG	SPI1	ISL1
E2F1	MNX1	THBD	DLX5
PODXL	CRABP2	ITGB2	SPARC
GREM1	PDGFRA	PTPRC	GATA6
FGF2	SNAI2	CD36	FABP2
SHH	SOX9	BMP2	HTATSF1
DPPA3	S100B	CD34	SLC2A2
LIN28	NEFL	CD14	GCG

Source: International Stem Cell Initiative (Allison et al., 2018).

Xenograft Tumor Assays

We have not recommended the routine use of xenograft tumor (teratoma) assays to assess pluripotency since in vitro assays can adequately confirm the ability of hPSC to differentiate into cells of the ectoderm, mesoderm, and endoderm lineages. However, xenograft tumor assays may be useful to assess the degree to which the differentiated derivatives of hPSC can undergo maturation and histogenesis. Further, xenograft assays can indicate malignant potential of a cell line, revealed by the presence of undifferentiated pluripotent stem cells, or yolk sac elements and immature neurectoderm, within the tumor (Allison et al., 2018). In addition, xenografts can reveal if patient-derived cultures considered “healthy” contain tumorigenic cells (Georgakapoulos et al., 2020). If xenograft assays are used, full details of the strain of mouse used, the site and method of inoculation and the method of cell preparation should be provided. The histology of tumors should be assessed by a qualified histopathologist using WHO criteria and/or with reference to suitable histology atlases (Damjanov and Andrews, 2016). The term teratoma’ should be reserved for tumors containing tissues corresponding to all three germ layers, but without evidence of undifferentiated stem cells; the term teratocarcinoma should be used for tumors containing tissues corresponding to all three germ layers and undifferentiated stem cells (Damjanov and Andrews, 2007). Traditional histological morphological approaches can be combined with computational approaches, such as the Teratoscore assay (Avior et al., 2015), in which RNA-seq data from teratomas is used to obtain detailed information on their cellular composition, including the presence of undifferentiated stem cells (Allison et al., 2018).

Appropriate antigens for the detection of undifferentiated stem cells and yolk sac elements are listed below.

Undifferentiated stem cells: OCT4, TNFRSF8, TRA-1-60/GCTM2
Yolk sac carcinoma: CDX2, GPC3, AFP, SALL4
Primitive neuroectoderm: PAX6, CD99, CD56, POU5F1, TNFRSF19

APPENDIX 5

Assessment of Methods for Genetic Analysis

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Table A5.1. Commonly used methods for detection of genetically variant cells

Method	Types of Abberations Detected	Resolution	Sensitivity	Cost	Speed	Advantages	Disadvantages
Karyotyping	Aneuploidy Duplications/ deletions Balanced and unbalanced translocations	5-10Mb (depending on the level of banding and chromosomal region)	Depends on the number of metaphases analyzed, e.g., 20 metaphases analyzed can exclude 15% mosaicism	Moderate	Slow	Provides an overview of the entire chromosome complement in a cell in a single assay. Makes a distinction of individual clones. Can detect balanced translocations.	Requires testing proliferative cells. Has limited resolution. Requires specialist skills.
SNP/CGH arrays	Aneuploidy Duplications/ deletions Unbalanced translocations	10-100kb	Typically, 10-20%	Moderate	Moderate	Can be performed on non-proliferative cells. Provide higher resolution than karyotyping, allowing the detection of small copy number variants.	Cannot detect balanced translocations or inversions. Cannot detect very small copy number changes.

eSNP karyotyping	Aneuploidy Duplications/ deletions	5-10Mb	Depends on the sequencing depth	Moderate	Slow	Allows using existing RNAseq data to assess for presence of genetic changes and thereby both genetic integrity and expression analyses can be done on the same sample.	Limited resolution and sensitivity.
qPCR/ ddPCR	Single nucleotide variants Copy number variants Aneuploidy	>1bp	Depends on the type of abnormality analyzed; for SNVs the sensitivity can be <0.1%; for copy number changes it is typically around 10%	Low	Rapid	Accessible to any standard lab. Allows rapid turnover. Relatively cheap.	Targeted approach, i.e., only detects aberrations at predetermined loci.
FISH	Aneuploidy Copy number variations Gene fusions	100-1Mb	Depends on the number of cells examined and the type of an aberration assessed; e.g., for whole chromosome gains, screening of 100 cells typically yields 5% sensitivity	Moderate	Moderate	Can be performed on proliferative or non-proliferative cells. Can afford higher resolution and sensitivity compared to karyotyping. Makes a distinction of individual clones. Limited resolution and sensitivity.	Targeted approach, i.e., only detects aberrations at predetermined loci. Tandem duplications can be difficult to detect, because of the overlap in the signal.

Next generation low-pass sequencing	Aneuploidy Copy number variations	0.5-5Mb (depending on the sequencing depth)	Depends on the sequencing depth	Moderate	Moderate	Can be performed on proliferative or non-proliferative cells. High throughput.	
Next generation deep sequencing (whole genome (WGS) or whole exome (WES))	Sequence changes Indels Copy number variations	1bp	Depends on the sequencing depth	High	Slow	Nucleotide-level resolution allowing detection of single nucleotide variants. High throughput. May require ethics approval.	High demands for data storage and relatively long time for bioinformatics processing (both are reduced in WES, but WES only interrogates around 1% of the genome).

This table was adapted from McIntire et al., 2020.

Table A5.2. Common recurrent genetic changes in hPSCs, some of their phenotypic consequences and suitable methods for their detection

Recurrent Genetic Change	Acquired as...	Some of the Reported Phenotypes of Variant Cells	Suitable Methods for Testing	Comments
Chromosome 1q gain	Gain of the whole chromosome (trisomy 1), whole q arm or an interstitial duplication.		Karyotyping (e.g., G-banding or low pass sequencing)*; qPCR or ddPCR; SNParrays or aCGH arrays; eSNP-karyotyping; FISH	*In some instances, the gain of 1q is acquired as an interstitial duplication below the resolution of karyotyping
Chromosome 12 gain	Gain of the whole chromosome 12 (trisomy 12), isochromosome 12p or a gain of the whole or parts of the p arm.	In undifferentiated state, trisomy 12 cells exhibit increased proliferation and teratocarcinoma formation (Ben-David et al., 2014); Variants with a 12p gain also show reduced ability for differentiation (Keller et al., bioRxiv)	Karyotyping (e.g., G-banding or low pass sequencing)*; qPCR or ddPCR; SNParrays or aCGH arrays; eSNP-karyotyping; FISH	*In some instances, the gain of 12p is acquired as an interstitial duplication below the resolution of karyotyping.
Chromosome 17q gain	Gain of the whole chromosome 17 or a gain of the whole or parts of the q arm	Variants with a chromosome 17q gain exhibit selective growth advantage, supercompetitive phenotype in high cell density cultures (Price et al., 2021) and altered differentiation patterns (Lee et al., 2015)	Karyotyping (e.g. G-banding or low pass sequencing); qPCR or ddPCR; SNParrays or aCGH arrays; eSNP-karyotyping; FISH	

Chromosome 18q loss	An interstitial deletion of q arm		Karyotyping (e.g., G-banding or low pass sequencing)*; qPCR or ddPCR; SNParrays or aCGH arrays; FISH	*Deletion may be below the resolution of karyotyping
Chromosome 20q gain	Gain of the whole chromosome 20 (trisomy 20), gain of isochromosome 20q or an interstitial duplication	Selective growth advantage in undifferentiated state (Avery et al., 2013; Nguyen et al., 2014) reduced genetic stability (Zhang et al., 2019) and altered differentiation patterns (Markouli et al., 2019; Werbowetski-Ogilvie et al., 2009)	Karyotyping (e.g., G-banding or low pass sequencing)*; qPCR or ddPCR; SNParrays or aCGH arrays; FISH	Karyotyping can detect trisomy 20 and isochromosome 20q, but interstitial duplications of 20q are often below the resolution of karyotyping
Single nucleotide variants in TP53	Typically acquired in DNA-binding domain of TP53 as dominant negative mutations	Selective growth advantage in undifferentiated state; also selected for during some differentiation protocols (Merkle et al., 2017)	Next-generation sequencing (DNA or RNA) Sanger sequencing	

APPENDIX 6

Reporting Practices for Publishing Results with Human Pluripotent and Tissue Stem Cells

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MANUSCRIPT NAME:

MANUSCRIPT NUMBER:

Reporting Practices for Publishing Results with Human Pluripotent and Tissue Stem Cells

This checklist is intended to help scientists, reviewers, and editors prepare and assess manuscripts for inclusion of critical details relevant to work with pluripotent stem cells (PSCs) and tissue stem cells (TSCs) with the goal of increasing the rigor and reproducibility of research through reporting. It is essential that any published paper includes detailed information on the following parameters to increase the transparency of the experimental details and ensure that the published results are reproducible. For additional details on the recommendations, please see the specific sections of the ISSCR's Standards for Human Stem Cell Use in Research referenced in the checklist (www.isscr.org/standards-document). All sections apply to PSCs and TSCs unless otherwise noted.

	Reference Section	Page Reported in Manuscript
Metadata		
Describe the source of the cells / cell line including:		
Name (or names) / alias of line	1.4; 5.1.2	
Unique ID / Registry # (name of registry)	1.4	
Source (vendor and catalogue number if obtained commercially); biopsy site and derivation details (if derived)	4.1.1; 5.1	
Additional metadata as applicable (e.g., sex, ethnicity, disease information, known mutations, etc.)	4.1.2; 5.4.1	

Culture Details		
Describe methods used for isolation, maintenance, and preservation of the cells including:		
Passaging / dissociation / split ratio	3.2; 4.2.2; 5.1.1	
Freezing and thawing	5.1.1	
Culture reagents used (e.g., media, matrices, growth factors, etc.) with vendor and catalogue number	4.2.2; 5.1.1	
The passage number of the cryopreserved / characterized Master Cell Bank or Working Cell Bank stocks used, and the number of subsequent passages prior to and during experimentation	1.2; 3.2.2; 5.1.1	

Basic Characterization		
Describe the assessment of the following including when they were performed relative to the experiments:		
Authentication	1.3; Appendix 1	
Mycoplasma	1.6; Appendix 1	
Sterility (bacteriostasis / fungistasis)	1.6; Appendix 3	

	Reference Section	Page Reported in Manuscript
Genomic Characterization		
Describe the genomic characterization including:		
Methodology used including sufficient detail to allow an assessment of sensitivity (e.g. the number of cells analyzed / resolution / depth of analysis)	3.1; 5.3; Appendix 5	
Timing of analysis in relation to key experiments reported	3.2	
Characterization of Pluripotency and the Undifferentiated State (PSCs only)		
Describe the following:		
Assay methodology	2.1; 2.2; 5.2; Appendix 4	
Quantitative results along with statistical analysis	2.1; 2.2; 5.2; Appendix 4	
Timing of analysis in relation to key experiments reported	2.1; 2.2; 5.2	
Confirmation of cell type (TSCs only)		
Describe the characterization of the following:		
The starting population(s) with recognized markers and methods	4.1; 4.3.1; 5.4.1	
Phenotype of expanded cells	4.1; 4.3.1; 5.4.1	
Demonstration of lineage potential	4.1; 4.3.1	
Molecular Characterization		
Describe the following:		
Confirmation of disease mutation (if applicable)	4.3.4	
Confirmation of genetic modification (if applicable)	4.4.3; 4.4.4	
Experimental Details		
Describe the following:		
Information regarding the experimental unit or sample type for each experiment (e.g. individuals, cell lines, clones, tissues, organoids, devices, batches, cells, etc.)	4.4.4; 5.4.2	
Number of replicates (biological / technical)	4.2.2; 5.4.2	
Data Practices		
Information on:		
Statistical methods used	4.4.1; 5.4.2	
Inclusion of the data and annotation code / software used for phenotype classification for computationally derived classifiers (if applicable)	5.4.4	
Verification that FAIR (https://www.go-fair.org/fair-principles/) and CARE (https://www.gida-global.org/care) data management principles were followed	5.4.4	

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