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t4 workshop report*

Good Cell Culture Practice for Stem Cells and Stem-Cell-Derived Models

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Summary

The first guidance on Good Cell Culture Practice (GCCP) dates back to 2005. This document expands this to include aspects of quality assurance for *in vitro* cell culture focusing on the increasingly diverse cell types and culture formats used in research, product development, testing and manufacture of biotechnology products and cell-based medicines. It provides a set of basic principles of best practice that can be used in training new personnel, reviewing and improving local procedures, and helping to assure standard practices and conditions for the comparison of data between laboratories and experimentation performed at different times. This includes recommendations for the documentation and reporting of culture conditions. It is intended as guidance to facilitate the generation of reliable data from cell culture systems, and is not intended to conflict with local or higher level legislation or regulatory requirements. It may not be possible to meet all recommendations in this guidance for practical, legal or other reasons. However, when it is necessary to divert from the principles of GCCP, the risk of decreasing the quality of work and the safety of laboratory staff should be addressed and any conclusions or alternative approaches justified. This workshop report is considered a first step toward a revised GCCP 2.0.

Keywords: Good Cell Culture Practices, *in vitro* methods, alternatives to animals, induced pluripotent stem cells

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1 Introduction to GCCP

The techniques available for *in vitro* cell culture have undergone massive developments in the last decade. The need to find cheaper, faster, humanized and more mechanistic approaches have been incentives for employing these methods in many areas such as toxicology (Suter-Dick et al., 2015), drug development and disease studies. A key problem when using these methods is that quality control is too often lacking. A number of concerns have been increasingly discussed in recent years (Marx, 2014; Freedman et al., 2015). These have raised awareness of quality problems in cell culture experiments of which the most frequent with serious impact on the quality of research and products are cross-contamination and microbial infection. Failure to adopt Good Cell Culture Practice (GCCP) in laboratories significantly increases the risk of generating erroneous data as well as risking worker health issues and legal liabilities.

The original GCCP document (Coecke et al., 2005) identified six principles of GCCP. The first of these emphasized the importance of cell line authentication. Investigations going back to the 1960s have revealed cases where cell lines were mislabeled or cross-contaminated and overgrown by other cells owing to poor cell culture practices and then circulated to other scientists (Yu et al., 2015; Gao and Sun, 2013; Nelson-Rees et al., 1981). In a recent report, 18-36% of all cell lines were shown to be wrongly identified (Hughes et al., 2007). A very useful list of such mistaken cell lines is available[1]. This problem has been raised numerous times (MacLeod et al., 1999; Stacey, 2000; Buehring et al., 2004; Rojas et al., 2008; Dirks et al., 2010).

The most commonly identified contaminating cell line so far is the HeLa cell line, the first human tumor cell line to be established (Gey et al., 1952). HeLa cells have contributed to more than 60,000 research papers. A study from 2004 showed that HeLa contaminants were used unknowingly by 9% of survey respondents (Buehring et al., 2004), but likely even underestimated the problem: only about a third of respondents were testing their lines for cell identity. Recently, the sequencing of the HeLa genome revealed dramatic genetic instability and changes compared to a normal genome (Landry et al., 2013). The cell line was found to be remarkably durable and prolific, as illustrated by its ability to contaminate many other cell lines. It is highly probable that today 10-20% of cell lines in use are actually HeLa cells (Hughes et al., 2007).

More recently, a new technical solution for cell line identification has been introduced by the leading cell banks (ATCC, CellBank Australia, sDSMZ, ECACC, JCRB, and RIKEN), i.e., STR profiling (typing). Short tandem repeat (STR) microsatellite sequence alleles that are highly polymorphic in human populations are selected to control the identity of human cell lines and their stability in cell cultures. When sufficient alleles are analyzed (typically 16 in current commercially available kits), their pattern should only result in the same profile when cell lines are derived from the same original donor (or donors who are identical twins). Still, recently severe genetic and functional differences in two samples from the same cell batch (not detectable with STR) were found in cultures from a major cell bank (Kleensang et al., 2016). Also, these systems do not generally work well in non-human species although STR panels have been developed for non-human species. Commercial kits typically comprise primers for a common subset of STR alleles, which permit comparison of profiles obtained with different kits (Andrews et al., 2015). However, this may not be feasible with array SNP systems, which are sometimes used for cell identification.

Another type of contamination that is astonishingly frequent and has a serious impact on *in vitro* results is microbial infection, especially with mycoplasma (Langdon, 2004; Callaway, 2014). Mycoplasma contamination within cell culture systems was first identified by Robinson and Wichelhausen in 1956 (Robinson and Wichelhausen, 1956) and numerous subsequent publications recognized the serious impact of such infection on *in vitro* cell cultures, including genetic instability, transformation, changes in physiological function and virus susceptibility. Mycoplasma infection is especially serious as these organisms tend to be resistant to certain antibiotics (having little cell wall material), may pass through some microbiological filters and may grow prolifically without being visibly evident (e.g., no effect on cell growth, no turbidity or obvious pH change in growth medium). Table 1 shows some reported frequencies of mycoplasma infection and the observed effects in culture. Such reports are likely to underestimate the problem because they arise from laboratories aware of and concerned about the consequences of mycoplasma contamination.

Non-sterilizable cell culture reagents, new cell lines brought into the laboratory and laboratory personnel are the main sources of *M. orale*, *M. fermentans*, and *M. hominis* contaminations. These species of mycoplasma account for more than half of all mycoplasma infections in cell cultures and are found in the healthy human oropharyngeal tract (Nikfarjam and Farzaneh, 2012). *M. arginini* and *A. laidlawii* are two other mycoplasma species that may contaminate fetal bovine serum (FBS) or newborn bovine serum (NBS). Trypsin solutions prepared from swine have been a major source of *M. hyorhinis*, though modern manufacturing practices have reduced this problem considerably. It is important to understand that mycoplasma implies resistance against penicillin (Bruchmuller et al., 2006), and can pass 0.2 μm sterility filters, especially at higher pressure rates (Hay et al., 1989), therefore it is extremely difficult to eradicate this intracellular infection.

There is a good understanding of this problem in the field of biotechnology where routine screening for mycoplasma contamination and disposal of positive cultures has reduced the incidence of such infection; however, this is not the case in basic research. Whilst mycoplasma testing by broth culture is internationally harmonized with validated methods (e.g., US and European Pharmacopeia), there is still no standardized PCR-based method, and numerous research laboratories do not test on a regular basis. The recent production of reference materials (Dabrazhynetskaya et al., 2011) offers hope for respective validation attempts. The problem lies in the fact that

[1] http://www.hpacultures.org.uk/services/celllineidentityverification/misidentifiedcelllines.jsp

at least twenty different species of mycoplasma are found in cell culture, though five of these appear to be responsible for 95% of contamination cases (Bruchmuller et al., 2006). For a comparison of the different mycoplasma detection platforms see Lawrence et al. (2010) and Young et al. (2010).

Current estimates indicate that probably only 60% of cell line studies use cell lines that have tested negative for mycoplasma infection and in fact are the cell lines they are thought to be (Hartung, 2013). These examples illustrate common deficits in the quality control of research laboratories which represents a significant risk to the quality of today's research using cell cultures.

Further important aspects of GCCP are appropriate documentation and reporting practices within laboratory work and in publications, the quality of which can vary significantly between laboratories. Failure to apply GCCP can have serious consequences for individual researchers and also for their employers. Such consequences have been known to include:

– Generation of erroneous data, leading to withdrawal of publications, loss of scientific reputation and wasted precious research time and resources
– Loss of crucial cell lines owing to microbiological contamination
– Failed patent applications when patent deposits are rejected due to contamination or lack of viability
– Laboratory worker exposure to infectious and other hazards as a result of working with cells, which have resulted in infection and in rare instances serious injury and death (e.g., frost-bite and asphyxia due to misuse of liquid nitrogen, cross-contamination of clinical samples in liquid nitrogen storage, poor aseptic technique or failure to adhere to appropriate laboratory procedures)
– Public exposure of work performed without appropriate ethical consent for use of cell lines leading to loss of laboratory reputation and potentially loss of funding from research sponsors

Thus, as a direct consequence of a failure to adopt GCCP a researcher and their employer/institution can be subject to risk of loss of scientific reputation, wasted time, wasted resources, lab worker infection and risk of legal prosecution. There is some guidance (under development for GLP and revision of GCCP see below) but to date it is only consistently applied in indus-

Tab. 1: Frequency and effect of mycoplasma infection of cell cultures

Reported frequencies of mycoplasma infection	**Effects of mycoplasma infection in cell culture** (compiled by Hartung, 2013)
Mycoplasma contamination of cell cultures is widespread, ranging from 5 to 35% in published reports (Hay et al., 1989).	Cell death and total culture degeneration and loss; increased sensitivity to apoptosis.
U.S. Food and Drug Administration (FDA) for more than three decades: 20,000 cell cultures examined, more than 3000 (15%) were contaminated with mycoplasma (Rottem and Barile, 1993)	Alteration of cellular morphology.
Studies in Japan and Argentina reported mycoplasma contamination rates of 80% and 65%, respectively (Rottem and Barile, 1993).	Alteration of proliferation characteristics (growth, viability).
An analysis by the German Collection of Microorganisms and Cell Cultures (DSMZ) of 440 leukemia-lymphoma cell lines showed that 28% were mycoplasma positive (Drexler and Uphoff, 2002).	Chromosomal aberrations (numerical and structural alterations); DNA fragmentation due to mycoplasma nucleases.
The Bionique Testing Laboratories in the US reported 11 and 7%, respectively, of infections in 10,000 samples each in 1994 and 2009 (Armstrong et al., 2010).	Alteration of cellular metabolism: Inhibition of cell metabolism; altered levels of protein, RNA and DNA synthesis with change of gene expression patterns;
A total of 301 cell cultures from 15 laboratories were monitored for mycoplasma using PCR and culture and found in 93 (31%) samples from 12 of the labs (Timenetsky et al., 2006).	Changes in cell membrane antigenicity (surface antigen and receptor expression).
Estimated that as much as 35 percent of the cell cultures currently used in research may be infected (Chi, 2013).	Interference with various biochemical and biological assays: Increase (or decrease) of virus propagation; reduction of transfection efficiencies; induction (or inhibition) of lymphocyte activation; induction (or suppression) of cytokine expression; influence on signal transduction; promotion of cellular transformation.
	Specific effects on hybridomas: Inhibition of cell fusion; influence on selection of fusion products; interference in screening of monoclonal antibody reactivity; monoclonal antibody against mycoplasma instead of target antigen; reduced yield of monoclonal antibody; conservation of hybridoma.

Tab. 2: Examples for Cell Culture Protocol Resources

General cell culture methods	
Nature Protocols	http://www.nature.com/nprot/index.html
Abcam – Cell Culture Guidelines	http://www.abcam.com/ps/pdf/protocols/cell_culture.pdf
Protocol online	http://www.protocol-online.org/prot/Cell_Biology/Cell_Culture/
Thermo Fisher Scientific – Cell Culture Protocols	https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols.html
Sigma-Aldrich: Basic Techniques – The "Do's and Don'ts" of Cell Culture	http://www.sigmaaldrich.com/technical-documents/protocols/biology/basic-techniques.html
Invitrogen – Cell Culture Basics	http://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics.html
Alternative methods	
EURL ECVAM DataBase Service on Alternative Methods to Animal Experimentation (DB-ALM)	http://ecvam-dbalm.jrc.ec.europa.eu/beta/
ZEBET database on alternatives to animal experiments on the Internet (AnimAlt-ZEBET)	http://www.bfr.bund.de/en/zebet_database_on_alternatives_to_animal_experiments_on_the_internet__animalt_zebet_-1508.html
CAAT repository of databases	http://altweb.jhsph.edu/resources/searchalt/searchaltdata.html
OECD test guidelines	http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm

try. The more recent growth in cell culture protocol resources is an important step (see Tab. 2), but it is still not common for researchers to stick closely to prescribed protocols, as they often adapt them to their own needs but fail to publish the details of their modifications.

2 The genesis of GCCP 1.0

Good Laboratory Practice (GLP) (at least originally) addressed only regulatory *in vivo* studies and the International Organization for Standardization (ISO) guidance is not really specific for life science tools and also does not address the relevance of a test. The relevance criterion is the truly unique contribution of validation, which, according to Organisation for Economic Co-operation and Development (OECD) consensus, is "the process by which the reliability and relevance of a particular approach, method, process, or assessment is established for a defined purpose" (OECD, 2005; Ferrario et al., 2014). This criterion is far too rarely applied in other settings (Hartung, 2007b). The limited applicability of GLP to *in vitro* studies was first addressed in a European Center for the Validation of Alternatives Methods (ECVAM) workshop in 1998 (Cooper-Hannan et al., 1999). Parallel initiatives (1996 in Berlin under the auspices of the German Society for Cell and Tissue Culture and 1999 in Bologna at the Third World Congress on Alternatives and Animal Use in the Life Sciences) led to a declaration toward Good Cell Culture Practice – GCCP (Gstraunthaler, 1999):

> *"The participants ... call on the scientific community to develop guidelines defining minimum standards in cell and tissue culture, to be called Good Cell Culture Practice ... should facilitate the interlaboratory comparability of* in vitro *results ... encourage journals in the life sciences to adopt these guidelines..."*

A GCCP task force was then established, which produced two reports (Hartung et al., 2002; Coecke et al., 2005).

The maintenance of high standards is fundamental to all good scientific practice, and it is essential for ensuring the reproducibility, reliability, credibility, acceptance, and proper application of any results produced. The aim of GCCP is to reduce uncertainty in the development and application of *in vitro* procedures by encouraging the establishment of principles for the greater international harmonization, standardization, and rational implementation of laboratory practices, nomenclature, quality control systems, safety procedures, and reporting, linked, where appropriate, to the application of the principles of Good Laboratory Practice (GLP). GCCP addresses issues related to:

- Characterization & maintenance of essential characteristics
- Quality assurance
- Recording
- Reporting
- Safety
- Education and training
- Ethics.

The GCCP documents formed a major basis for a GLP advisory document by the OECD for *in vitro* studies (OECD, 2005), which addresses:

- Test Facility Organization and Personnel
- Quality Assurance Program
- Facilities
- Apparatus, Materials, and Reagents
- Test Systems
- Test and Reference Items
- Standard Operating Procedures

– Performance of the Study
– Reporting of Study Results
– Storage and Retention of Records and Materials.

Therefore, the guidance documents have much in common: Inherent variation of *in vitro* test systems calls for standardization, and both the GLP advisory document and the GCCP guidance are intended to support best practice in all aspects of the use of *in vitro* systems, including the use of cells and tissues.

Notably, there are other activities in progress such as the Good *In vitro* Method Practice (GIVIMP) by ECVAM and the OECD that has been recently published[2]. The draft guidance supports the implementation of *in vitro* methods within a GLP environment to support regulatory human safety assessment of chemicals. GIVIMP will contribute to increased standardization and harmonization in the generation of *in vitro* information on test item safety. The guidance further facilitates the application of the OECD Mutual Acceptance of Data (MAD) agreement for data generated by *in vitro* methods and as such contributes to avoidance of unnecessary additional testing. GIVIMP takes into account the requirements of the existing OECD guidelines and advisory documents to ensure that the guidance is complementary and in line with these issued documents.

When comparing GLP and GCCP, there are some major differences: GLP still gives only limited guidance for *in vitro* work and cannot normally be implemented in academia on the grounds of costs and lack of flexibility. For example, GLP requires documented completed training of the personnel involved, while academic research often relies on people training on the job. GCCP, on the other hand, is intended for broad ranging applications, including research, and also aims to give guidance to journals and funding bodies.

All quality assurance of an *in vitro* system starts with its definition and standardization, which include:

– A definition of the scientific purpose of the method
– A description of its mechanistic basis
– The case for its relevance
– The availability of an optimized protocol, including:
  • standard operation procedures
  • specification of endpoints and endpoint measurements
  • derivation, expression, and interpretation of results (preliminary prediction model)
  • inclusion of adequate controls
– An indication of limitations (preliminary applicability domain)
– Quality assurance measures

This standardization forms the basis for formal validation, as developed by ECVAM, adapted and expanded by ICCVAM and other validation bodies, and, finally, internationally harmonized by OECD (2005). Validation is the independent assessment of the scientific basis, the reproducibility, and the predictive capacity of a test for a specific purpose. It was redefined in 2004 in the modular approach (Hartung et al., 2004) but needs to be seen as a continuous adaptation of the process to practical needs requiring a case-by-case assessment of what is feasible (Hartung, 2007b; Leist et al., 2012).

3 The need for GCCP 2.0

The advent of human pluripotent stem cells, first embryonic (1998) and then induced pluripotent (2006) stem cells, has greatly broadened the potential applications of human cell culture models. They promise to overcome the problem of limited availability of human primary cells. A variety of commercial providers nowadays make almost all relevant human primary cells available in reasonable quality but at costs that are challenging, at least for academia. Furthermore, human pluripotent stem cell (hPSC) lines promise to generate a broad variety of tissues, however, we do not yet have optimal protocols to achieve fully functional differentiation of any cell type. This will probably be achieved given time and effort, but many of the non-physiologic conditions taken over from traditional cell culture techniques contribute to the problems. Originally hPSC cultures were thought to be genetically stable, but we have lately learnt about their limitations in this respect (Mitalipova et al., 2005; Lund et al., 2012; Steinemann et al., 2013). Other limitations are costs of culture and complex differentiation protocols, which may require months of labor, media, and supplements. The risk of infection also increases with the duration and complexity of the procedures. Despite all the time and effort invested one may still not obtain pure cell types, and may need to sort them, which involves detachment of cells, disrupting the culture conditions and physiology.

GCCP guidance was developed before human stem cells became broadly used. We attempted a respective update in a workshop in 2007: "Human embryonic stem cells (hESC) technology for toxicology and drug development: summary of current status and recommendations for best practice and standardization. The Report and Recommendations of an ECVAM Workshop"[3].

Very much fueled by the availability of stem cells, but not restricted to these, a number of initiatives have started to develop organotypic cultures (also known as organoids, spheroids, microphysiological systems, 3D cultures, organ-on-chip, perfusion cultures, etc.) (Marx et al., 2016). These novel test types (Hartung and Zurlo, 2012) represent additional challenges regarding standardization of design and generation of optimized culture systems and devices. The systems are considerably more complex than traditional *in vitro* approaches, involving 3D constructs (Alépée et al., 2014), various cell types and other engineering methods (Andersen et al., 2014; Hartung, 2014). This must also be considered in the revision of the GCCP guidance.

A key element of this guidance is the advice on documentation and publication. Note that guidance also has been developed for the publication of journal articles on *in vitro* experiments (Leist et al., 2010). A CAAT workshop was held in March 2012 in San Francisco, and a taskforce was formed to further this work.

[2] http://www.oecd.org/env/ehs/testing/OECD_Draft_GIVIMP_in_Human_Safety_Assessment.pdf (last accessed 14 Dec 2016)

[3] https://eurl-ecvam.jrc.ec.europa.eu/about-ecvam/archive-publications/publication/hESC_%20010711.pdf (last accessed 23 Nov 2015)

These activities are currently united under the GCCP initiative (see below).

Taken together, GCCP 1.0 was a major step toward best practices for *in vitro* testing. A decade later, it requires updating, especially to incorporate stem cell technologies and organ-on-chip approaches and to include best practice for documentation and publication. We hope that GCCP 2.0 will improve cell culture work around the world and also will be guidance for journals and funding bodies, thereby enforcing the use of these quality measures.

4 Principle 1: Establishment and maintenance of sufficient understanding of the *in vitro* system and of the relevant factors which could affect it

All cell and tissue-based systems require establishing essential elements to ensure reliable and accurate work. These elements include among others authenticity, purity and stability of the cell line or tissue.

Special attention is required for pluripotent stem cell cultures. PSC are dynamic cells that can change their phenotype by differentiating into different cell types. All cells are *per se* prone to change in culture, but controlling the differentiation stages of pluripotent cells can be even more of a challenge. Moreover, reliable maintenance of cells in their undifferentiated state is critical for the propagation of these cells. Further, the method used to generate these cell lines (such as induction in the case of induced pluripotent stem cells (iPSC)) has direct repercussions on the identity of the cells and their properties. Incorrect characterization, accumulation of genetic aberrations and cell line misidentification are possible pitfalls with the consequences discussed above. It is also useful to consider different requirements for GCCP for stem cells used for different applications, e.g., in "organ-on-a-chip" applications, including disease models, versus for therapeutic use.

4.1 Pluripotent stem cells

Currently, iPSC are the most popular pluripotent stem cells used. Human somatic cells are reprogrammed to become embryonic stem cell-like iPSCs by a variety of mechanisms (see Section 4.6). Like embryonic stem cell (ESC) culture, maintenance of iPSCs in an undifferentiated state for propagation purposes is essential. Human iPSCs can be cultured on a supporting layer of feeder cells, such as mouse embryonic fibroblasts (MEF) or human foreskin fibroblasts (HFF), or on an extracellular matrix.

The respective technologies, such as reprogramming techniques, culture media and characterization methods (explained in section 4.6), are being refined constantly. Multiple methods of reprogramming have been developed to improve pluripotency and efficiency of iPSC derivation by minimizing genomic instability from unwanted integrations. Others create more defined methods to increase consistency, improve standardization in research and to bring us closer to clinical application.

Differences between iPSC and ESC

Whether iPSC recapitulate ESC characteristics exactly is still not clear (Feng et al., 2010; Hu et al., 2010). Although some studies have shown no significant differences between ESC and iPSC (Guenther et al., 2010; Mallon et al., 2014), other evidence suggests genetic (Chin et al., 2009; Muller et al., 2011), miRNA profile (Zhao et al., 2014), chromatin structure and methylation (Lister et al., 2011) differences.

It has been proposed that some of the differences between iPSC and ESC are effects of reprogramming or reflect persistence of epigenetic marks from the original tissue cells (Kim et al., 2010; Lister et al., 2011). During reprogramming, DNA methylation and other epigenetic marks are stripped and renewed to approximately resemble the naïve epigenetic state of ESCs. A few epigenetic markers from the somatic cell of origin, however, appear to be retained, see "Characterization of PSC" below. The differences may affect behavior in terms of tumorigenicity and spontaneous differentiation (Polouliakh, 2013), therefore understanding them will help to further improve technologies used to generate iPSC.

Authentication

The increasing use of different cell lines together with the lack of good practices have led to an increase in the number of cross-contaminations and lack of authenticity of cells. Experiments performed with cells that are not authenticated could produce erroneous data with the respective consequences. See "Cell Identification" in Section 5.1

Characterization of PSC

Phenotypic characteristics should be studied in both pluripotent cells (morphology, colony evaluation, markers of pluripotency potential) and in differentiated cells (morphology, differentiation markers, functionality). A set of markers, including a number of canonical cell surface markers (e.g., SSEA-3, SSEA-4, TRA-1-180, TRA-1-60) and expression of self-renewal genes (e.g., Oct-4, Nanog, Sox-2) is commonly used to confirm the typical undifferentiated PSC phenotype (see Tab. 3). Although commonly used, a standardized set of markers has yet to be established.

Phenotypic studies can help to isolate colonies of interest or specific cell types. For iPSCs, colony selection and removal of differentiated cells are typical methodologies employed to maintain undifferentiated iPSC cultures. During the iPSC selection process, the use of cloning techniques is required (this procedure is not necessary for miRNA/mRNA transfection reprograming). It is important to recognize that hPSCs are mosaic and can result in expansion of abnormal clones with enhanced growth rates, which may take over the culture. In addition, iPSCs can spontaneously differentiate (partially or completely) during their propagation, favoring such heterogeneity. Regular assessment of colony morphology is therefore a very important measure during maintenance of undifferentiated iPSC colonies. Proper technique to balance culture confluency, select the appropriate split ratio, and minimize differentiation is critical to high-quality cultures.

Genetic variation between donors may result in functional differences between iPSC lines. The most straightforward manner to confirm phenotypic differences between iPSCs (e.g., genetic disease-carrying and healthy patients) is to independently derive three or more cell lines from each patient to confirm differences can be replicated between these cell lines. Some

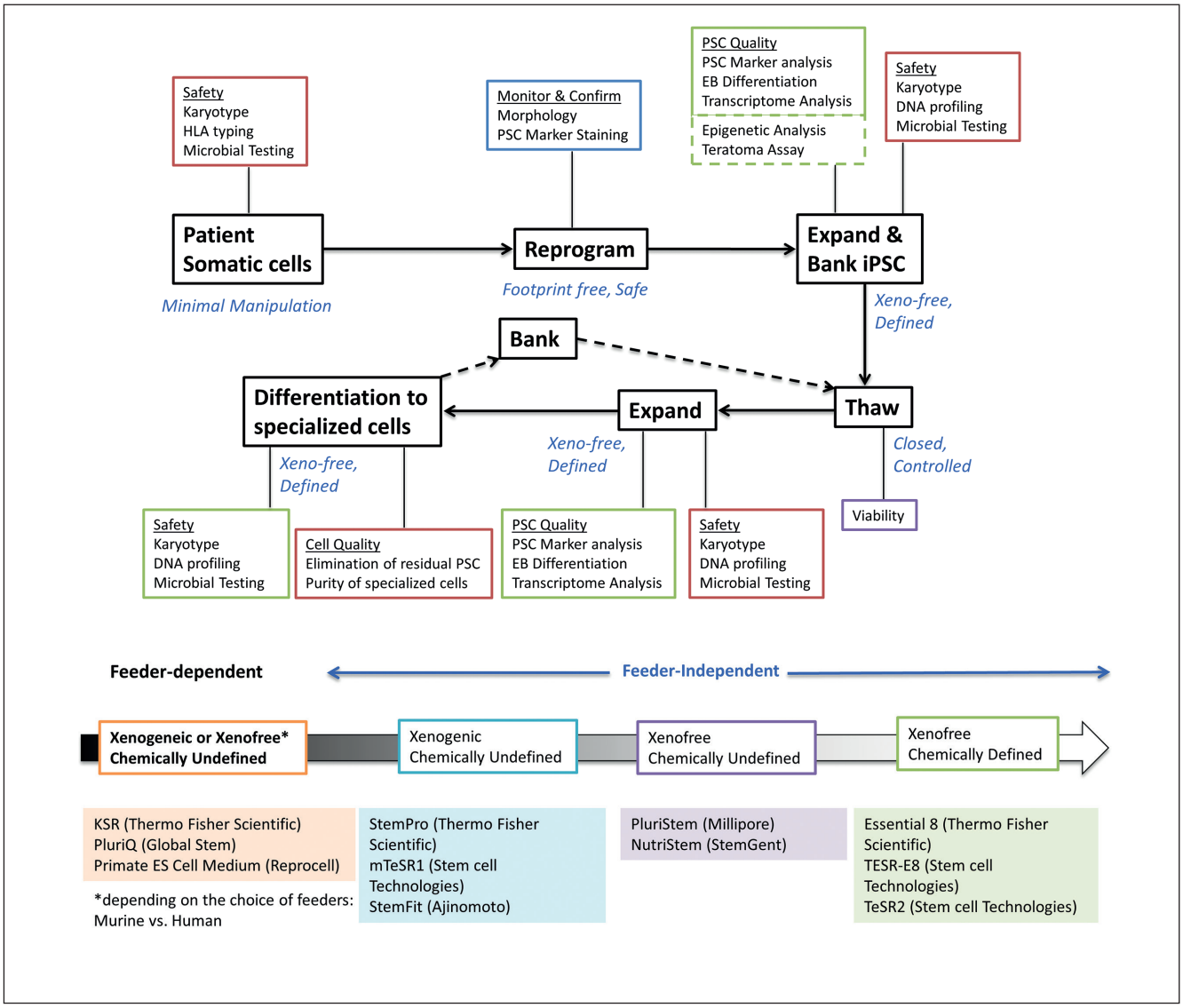

Fig. 1: Workflow of derivation and differentiation of patient PSC and stage-specific characterization requirements

genetic and epigenetic variations may change the PSC's properties while others may not. A donor may carry chromosomal abnormalities that do not have phenotypic effects. Moreover, acquired genomic abnormalities may not affect the purpose of the experiment. It is important to understand the aim of the experiment and possible effects of abnormal genotypes.

Genotypic or epigenetic variations in PSC can stem from variations inherent to the donor cells, changes induced in the reprogramming process, or accumulation during culture passaging (ISCBI, 2012; Liang and Zhang, 2013). These variations may change the differentiation potential of the cells, which can have significant impact on the suitability of a cell line for use in disease modeling and cell replacement therapy. Identifying changes which may adversely impact the characteristics of differentiated or undifferentiated cultures may not be straightforward. Low levels of aneuploidy in a diploid culture may be quite normal as this appears commonly in hESC and iPSC.

The stability of features of known significance such as the karyotype (via G-banding, where trisomy and gross chromosomal duplication/deletions and translocations can be detected (see additional text in Section 5.1)) or the faithful representation of the genetic profile of the disease under study in "disease-in-a-dish" applications should be monitored.

Clinically relevant specialized cells derived from PSCs undergo a series of workflow steps with varying requirements and complexity (Fig. 1). The various workflow stages and by-products (such as media and growth factors) are closely monitored according to stringent guidelines (Tab. 4). PSC cultured for reintroduction into patients should be examined carefully for mutations or large karyotype changes that might predispose them to tumorigenesis.

The International Stem Cell Banking Initiative (ISCBI) has developed guidelines for banking, characterization and distribution of research-grade ESCs (ISCBI, 2009). The active di-

Tab. 3: Commonly used characterization methods used to indicate pluripotent capability based on marker expression and differentiation potential

	Assay	Markers	Metric (Pros/Cons)	References
Marker analysis	Differential dye	Alkaline phosphatase	Robust positive staining by visual or microscopic observation (Fast & easy-to-use / Not highly specific)	Marti et al., 2013; Singh et al., 2012; Brivanlou et al., 2003; Gonzalez et al., 2011
	Surface marker expression	SSEA3, SSEA4, TRA-1-60, TRA-1-81	>70% positive for marker by flow cytometry (live staining / cost and potential for contamination)	Andrews, 2002; Draper et al., 2002; Henderson et al., 2002; Pera et al., 2000
	Marker expression	NANOG, POUF1, GDF3, DNMT3B	Uniform staining pattern by fluorescence microscopy (Highest specificity / terminal staining - not for live cells)	Sperger et al., 2003; Richards et al., 2004
	Lineage commitment assay	OCT4, SOX2	Differential expression profiling in response to signaling factors in 96CP platform-patterned 96-well plates (expression based on functional response / high technical expertise required)	Nazareth et al., 2013
In vitro differentiation	EB formation	SMA (mesoderm), TUBB3 (ectoderm), AFP (endoderm)	Positive detection of trilineage specific markers in spontaneously differentiating embryoid bodies (accepted method / duration of assay)	Itskovitz-Eldor et al., 2000
	Directed differentiation	SOX10 (ectoderm), SOX17 (endoderm), KDR, PDGFRA (mesoderm)	Positive detection of lineage specific markers with directed differentiation (newer methods / clonal bias may not be detected)	Chambers et al., 2009; Borowiak et al., 2009; Burridge et al., 2012; Kattman et al., 2011
***In vitro* differentiation**	Teratoma assay	Haematoxylin / eosin (H&E) stained histological sections	Identification of cells types that are derivatives of ectoderm, mesoderm and endoderm (gold standard / burden of animal testing)	Gertow et al., 2007; Gropp et al., 2012
	TeratoScore	Gene expression in teratoma	Algorithm using *in vivo* expression profiles to assess teratoma tissue and lineage composition (enables quantification of teratoma assay / burden of animal testing)	Avior et al., 2015
Transcriptome analysis	PluriTest	High density microarray	Pluripotency scores and novelty scores (easy analysis of global gene expression analysis / cell population assay, restricted to measurement of self-renewal patterns)	Muller et al., 2011
	ScoreCard	Medium/low density focused array	Scores measured by comparing l ineage expression levels to a reference standard (Confirms self-renewal signature and trilineage differentiation potential / cell population assay can diminish sensitivity)	Bock et al., 2011
	Cell Net	High density microarray	Computational platform to determine gene regulatory networks that govern cell identity (based on global gene expression / cost and complexity)	Cahan et al., 2014a, b

Tab. 4: Quality control in different stages of stem cell-derived therapeutic product

Stage*	Guidance/ Regulation	Research	Toxicology/product safety testing	Manufacture of cell-derived medical products	Manufacture of cell-based medicines
Cell bank or cell stock	Guidance	Coecke et al., 2005; ISCBI, 2009	Coecke et al., 2005; Pistollato et al., 2012; Stacey et al., 2016; ISCBI, 2009; Stacey et al., 2017	Coecke et al., 2005	Coecke et al., 2005; Andrews et al., 2015; EDQM, 2015
	Regulation	Ethics review for use of human tissue according to national regulation Laboratory health and safety regulations under national laws e.g., microbiological hazards, genetically manipulated organisms	Ethics review for use of human tissue according to national regulation Laboratory health and safety regulations under national laws e.g., microbiological hazards, genetically manipulated organisms	As for research and toxicology plus: WHO, 2010; EMEA, 1998; FDA, 2010b	FDA, 2001

*For regulation of manufacturing processes and end product in the EU under EC 1394/2007 (EU, 2007) & 2001 (EU, 2001b) and in the USA regulation and guidance can be found at the USFDA website (http://www.fda.gov/BiologicsBloodVaccines/default.htm) .

alog and collaboration under this umbrella between stem cell scientists, national cell banking groups, commercial suppliers of reagents and regulators has also helped to reach an understanding of the required quality control and on regulatory issues surrounding clinical-grade pluripotent stem cells (Andrews et al., 2015). Cells intended for therapeutic use fall under the guidance of US Food and Drug Administration (US FDA 21 CFR part 1271; FDA, 2001), the EU (European Union Tissues and Cells Directive, EU, 2012), and their equivalents in other parts of the world to ensure product safety by requiring manufacturers to confirm the absence of harmful agents and evidence of abnormalities (FDA, 1998). There is specific emphasis on the maintenance of high-quality cell stocks and end products characterized for sterility, purity, and tumorigenicity (FDA, 1998; EMEA, 1998; FDA, 2013; Adewumi et al., 2007; WHO, 2013). Manufacturing of cell products is also subject to thorough characterization, which includes monitoring of cell morphology, growth and functional activity, marker expression, HLA-type and contamination with microbial or endotoxin elements (FDA, 2003; Weber, 2006; EU, 2007). Specific regulation also applies to cell-based therapeutic products, e.g., Advanced Therapies Medicinal Products regulation (EU, 2007), whereby some principles are applicable to general research.

Cell purity, stability and functional integrity

Long-term cultures, especially PSCs, tend to acquire chromosomal changes. There is evidence suggesting that changes in the genetic machinery may confer a growth advantage of the aberrant population, producing a selective advantage to those cells (Baker et al., 2007) and affecting cell population homogeneity. Therefore, it is recommended not to maintain cultures for long periods of time and to avoid high (normally not higher than 40) passage numbers. Noteworthy, passage numbers are not precisely defined as it depends on the nature of the culture, the quality of the passage method, and the split ratio. It is important to minimize the passage level of cells in routine use and to replace the in-use stock from a frozen cell bank on a regular basis. This has been recommended in previous best practice documents to passage cells for no more than fifteen passages or for a maximum of three months by WHO guidance (WHO, 2013).

In order to avoid the risk of losing a new iPSC line due to contamination or differentiation, it is important to create a small cryopreserved stock of cells (seed bank) as soon as a stable iPSC culture has been established. This seed bank can then be used to establish a larger yet low passage, cryopreserved "master" stock, which provides the source of all cells for future work. The master stock or "bank" can then be used to generate a "working" stock which can then be used for all experimental routine work. In this way, the reliable supplies of low passage cultures can be made available over many years without the need to replace the master stock.

Viability (e.g., MTT, Alamar Blue, intracellular ATP assay, Phenol Red) and growth rate measurements (e.g., proliferation rate by cell counting) can be important tools to control the culture. Each cell viability assay has certain advantages and disadvantages depending on the cell line and culture model, so it is important to decide on the most appropriate technique for the cell type and the purpose of the experiment. Different factors, such as pH, medium type, temperature, incubation time, and evaporation, can influence these assays. Thus, it is important to select a viability assay adequate for the culture and methods studied (Stacey and Hartung, 2007).

Although such tests typically are used to detect toxicity in the cells, they also can be used to control the effects of different aspects of cell maintenance. Cells can be perturbed by different handling and maintenance processes such as cryopreservation, switching to different growth media, passaging, reprogram-

ming, cloning and gene editing. Viability can be used to study which processes are least harmful to the cells by comparing different protocols. However, other, more subtle processes such as micro-autophagocytosis may also be activated in suboptimal conditions without loss of viability.

Technologies such as metabolomics can be used to characterize PSC. Metabolism is involved directly or indirectly with cell function. Metabolomics technologies can be used to examine and identify metabolite changes in endogenous biochemical reactions and identify metabolic pathways and processes occurring within a living cell (Panopoulos et al., 2012; Bouhifd et al., 2013; Ramirez et al., 2013).

Genetic manipulation and differentiation

Genetic manipulation is the direct manipulation of an organism's genome or epigenome. This process may include the incorporation of new DNA, removal or silencing of a gene or group of genes, introduction of mutations or modification of the epigenome. Thereby, we can study the role of genes in diseases and other genetic pathways. Some of the most common tools used to perform genetic manipulation are zinc finger nucleases (ZFNs), meganucleases, transcription activator-like effector nucleases (TALENs), CRISPR and siRNA (see Section 4.9). All genetic manipulation requires quality controls through to the final stage of cell manipulation. Characterization of the cells must be done before and after genetic manipulation in order to identify any deleterious changes that may have occurred. Characterization and quality control of clonality is an important feature, see Section 4.9.

The most prominent characteristic of pluripotent cells is their capacity to differentiate to different mature phenotypes representing each of the three germ layers. This characteristic may be assessed by a number of techniques but has yet to be standardized (see Section 5.1). However, the generation of specific terminally differentiated cell types for experimental work requires directed differentiation protocols. iPSCs and other multipotent or unipotent cells possess a genetic "memory" (see "Differences between iPSCs and ESCs" in Section 4.1). As mentioned before, cells may retain some epigenetic "memory" after reprogramming that may affect resulting cells after the differentiation process (Vaskova et al., 2013). Generally, banked cells are at P8-P10 and iPSCs derived from most of the commonly used foot-print-free methods (Sendai, episomal and modified mRNA) do show elimination of reprogramming factors. It is harder to prove complete absence of epigenetic memory; however, a cell line may be considered a good quality iPSC as long as transcriptome analysis and tri-lineage differentiation does not show bias.

4.2 *In vitro* culture conditions

Cell and tissue culture environments differ in many respects from *in vivo* conditions (Hartung, 2007a). Different key elements such as culture medium, supplements, culture-ware, incubator conditions, are controlled *in vitro* in order to simulate the *in vivo* situation as well as possible and feasible. *In vitro* differentiation does not completely "phenocopy" the *in vivo* cell phenotype, however, it is unclear whether culture conditions are the sole limitation or whether epigenetic memory also contributes significantly.

Culture medium

In vitro work is generally performed in complex nutritive medium. Depending on the circumstances, the basal culture medium can be serum-supplemented (as in traditional cell culture methods) or serum-free but supplemented with the additives necessary for obtaining satisfactory cell proliferation and production, or for maintaining a desired differentiation status. Many, slightly different formulations exist under the same general medium names, such as Minimum Essential Medium (MEM), but even subtle changes to the medium formulation can substantially alter the characteristics of certain cells and tissues. In many cases, these variations are deliberate to achieve desired cellular characteristics for specific applications.

In order to maintain cultures of mammalian cells *in vitro*, it is necessary to provide an environment that closely mimics conditions present *in vivo* to provide the cell with the basic building blocks for nutrient metabolism and biochemical processes while maintaining the cell's phenotypes and characteristics. Notably, cell proliferation and differentiation counteract each other and thus most cultures have to compromise here, being less proliferative to obtain differentiated cells.

Early work in the area of mammalian cell culture design was based on the use of biological fluids such as blood plasma and serum matched with a basal medium consisting of minimally required components such as water, glucose, amino acids, vitamins, and a physiologically balanced pH-buffered salt solution (Amit et al., 2003; Crook et al., 2010). This combination is still widely used for many applications in cell culture research, but the many disadvantages mentioned above associated with serum use have precipitated a shift away from its use as a supplement to serum-free medium (SFM), protein-free medium (PFM) and chemically-defined medium (CDM).

Medium conditions

Supplements used in these media or added to basal medium supplemented with serum in stem cell culture may include proteins, hormones and growth factors and hydrolysates. Purified proteins are added to improve performance of the cells (such as growth, differentiation and maintenance). Proteins such as insulin, transferrin, and serum albumin are purified from animal sources or produced recombinantly in bacteria, yeast, or plants.

Similarly, purified growth factors such as activin, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and others are often added to stem cell culture media. Purified proteins and growth factors are more favorable than serum to ensure consistency of biological effects, but costs may be prohibitive, they may not support the growth of cells in the same way or as effectively as serum-containing growth media and they may, like serum, need to be eliminated at a later stage by complicated purification processes.

Serum is still used in some stem cell differentiation protocols. It is a complex mixture of a large number of constituents, includ-

Tab. 5: Critical components driving pluripotency in different PSC culture media

Medium	Factors
Essential 8 (E8)	TGF-β, FGF2
TeSR	TGF-β, FGF2
L7 hPSC medium	Not available
StemPro hESC medium	Not available
PluriSTEM™ Human ES/iPS medium	Activin-A, TGFβ1, and b-FGF
Nutristem	Proprietary growth factors and low FGF2

ing low and high molecular weight biomolecules with a variety of physiologically balanced growth-promoting and inhibiting activities. However, due to its complexity and to batch-to-batch variations, serum introduces unknown variables into a culture system and can interfere with its performance. Animal serum may be derived from adult, newborn or fetal sources. Bovine sera are most commonly used (Festen, 2007), and during the last few decades, fetal bovine serum (FBS) has become the standard universal growth supplement for cell culture media.

As the composition of serum is highly variable, it is important that each new serum batch should be evaluated in parallel with the in-use batch. A range of growth promotion tests can be used for this purpose, one of the most convenient and most widely used of which is the plating efficiency test (Freshney, 2000).

It may also be useful for individual users to define serum specifications that meet their particular needs, including the maximum acceptable levels of serum components, such as immunoglobulins (which may have inhibitory effects), endotoxins (indicative of bacterial contamination, but which may also be powerful cell mitogens), and hemoglobin (a toxic contaminant indicative of hemolysis during clotting in the production of the serum).

Animal sera are a potential source of microbiological contaminants, notably mycoplasma, bovine viruses, and possibly the prion agent, which causes bovine spongiform encephalopathy (BSE). Suppliers use a variety of techniques, including filtration, irradiation and heat-inactivation, to reduce microbial contamination. Nevertheless, it is wise, and for some applications obligatory, to specify sourcing of serum from countries where there is a low risk of infection, and, in the case of bovine sera, from animals of less than 30 months of age. There is recognized guidance on risk assessment of potential sources of BSE (WHO, 2013), which may cause variant Creutzfeldt-Jakob disease (vCJD) in humans[4].

The use of human serum is restricted to specialized applications, as it carries additional risks, such as the potential presence of human-pathogenic viruses, e.g., human immunodeficiency virus (HIV) or hepatitis C. Its use must be subject to the strictest quality controls, including documentation to demonstrate origin and viral safety.

Because of the disadvantages inherent in the use of animal and human sera, as well as animal welfare issues (Gstraunthaler, 1999) and serum-induced spontaneous differentiation of iPSCs, there have been many attempts to find alternatives. In some cases, it is possible to use fully chemically defined media with appropriate hormones and growth factors (van der Valk et al., 2004). A compilation of commercially available serum-free media was published recently[5] (Brunner et al., 2010). A number of defined serum-free media are now manufactured specifically for PSC culture, e.g., E8, KODMEM, L7, Nutristem, PluriSTEM, StemPro, TeSR Tab. 5).

Medium replenishment

The exhaustion or inactivation of essential nutrients in cell culture media and rising levels of acidic metabolites will inhibit cell growth and cell function and will ultimately cause cell death. Planning an appropriate procedure for medium replenishment (e.g., frequency and volume of medium) and timely passaging (e.g., split ratio) is therefore essential. This should also be considered when using conditioned medium from one culture in an attempt to promote the growth of another.

Nutritional status of pluripotent stem cell cultures can be handled by two basic modes of operation, i.e., batch cultures and perfusion cultures. Both processes are used mainly to scale-up 2D planar cultures, where cell densities are a critical issue and sufficient numbers of cells cannot be generated by conventional 2D planar cultures.

Batch culture refers to a partially closed system, in which most of the materials required are loaded into the bioreactor vessels, such as spinner flasks or single use bioreactor systems that are closed systems. Usually the only material added and removed during the course of batch culture is the gas exchange and pH control solutions. In a quality reactor, these conditions are supposed to be controlled and uniform throughout the reactor at any moment, but many factors such as cell mass, nutrients, waste and accumulation of secreted factors change. Most critically, this kind of culture requires optimization of seeding and terminal cell densities, aggregate size of the cultures, shear force, duration of cultivation process, and if microcarriers are used different versions should be compared to optimize cell density/volume ratios with the media used. Specific disadvantages of batch-processing are down-time between batches, cleaning and sterilization processes associated with each bioreactor vessel that is to be used again.

In comparison to batch cultures, perfusion bioreactors allow culture of cells over much longer periods, by continuously perfusing the cells with fresh medium and removing spent medium (Whitesides, 2006). Ways to remove spent medium include per-

[4] SaBTO - Advisory Committee on the Safety of Blood Tissues and Organs (2014). Donation of Starting Material for Cell-Based Advanced Therapies. London, UK: Department of Health. http://bit.ly/2gXXY3O

[5] http://www.drhadwentrust.org/science-and-education/serum-free-media

fusion through the bioreactor via capillary fibers, membranes or carriers ("fixed bed" systems) or filtration systems that prevent cells being removed from the bioreactor with the medium or separating the cells from the medium by centrifugation. New perfusion technologies called high-density (HD) cell banking have been used in cell banks to produce large batches more quickly and cost-effectively while reducing the risk of contamination (Tao et al., 2011) and allowing a higher level of automation of the process.

Conclusion

Any significant change in cell culture conditions can alter cell differentiation state and functionality; thus, exact definition and documentation of culture conditions is essential. Comparability studies, a concept used for human biological product regulation by FDA[6], may be necessary when major process changes are to be implemented.

4.3 Handling and maintenance

Cell culture conditions have to be controlled in order to maintain cell viability. Cells should not be left outside incubators over prolonged periods of time. All the equipment used in the culture (such as incubators, microbiological safety cabinets, cryostorage systems) must be set up and used appropriately and maintenance protocols for cells should be established as Standard Operation Procedures (SOPs). Factors such as temperature, atmosphere and pH need to be controlled in order to obtain reproducible and quality cultures (Coecke et al., 2005). In the case of PSC, and specifically iPSC and ESC, some techniques differ from other culture methods.

Cell detachment methods

For routine culture of iPSCs, passaging can be achieved using chemicals, enzymes or mechanical means to facilitate cell detachment (Beers et al., 2012). The approach selected depends on the cell grade (e.g., research, manufacture, therapy), culture conditions (e.g., growth medium, surface matrix), and current state of the culture (high/low passage, extent of differentiation, etc.).

Mechanical passaging, often referred to as "cut-and-paste", is used when throughput is not of high concern or differentiation is notable. This involves selecting and propagating pluripotent cells by manually dissecting out areas of undifferentiated cells as colony fragments and transferring them to fresh culture plates, thus positively selecting stem cells. The opposite approach, known as negative selection, may be used where colonies are relatively small but are showing areas of excessive differentiation. These differentiated areas are selected and removed by aspiration, enabling the undifferentiated cells to continue to proliferate. Either method is an effective way to "clean up" cultures to leave predominantly undifferentiated stem cells. These alternatives are dependent on the proportion of differentiated cells and size of the colonies.

Chemical or enzymatic passaging, in which the type of reagent selected often depends on what grade of manufacture is desired as well as the matrix used to support the cells. For example, stem cells cultivated on mouse embryonic fibroblasts (MEFS) are typically dissociated with collagenase or trypsin. Stem cells cultured on other matrix coatings like Matrigel™ and Vitronectin™ may be more compatible with reagents like dispase or EDTA (ethylenediaminetetraacetic acid), respectively. Dispase may require direct contact with cells to scrape them from the culture surface, while EDTA usually does not require scraping. If therapeutic-grade iPSCs are desired, it is important to consider the dissociation reagent more carefully to ensure it is synthesized in a way that is defined and ideally free of animal-derived components. The aforementioned reagents are best when splitting colonies as clumps, whereas trypsin is often selected to create single cell suspensions. In some conditions cell detachment may result in significant loss of cell vigor and viability, which may be reduced by the addition of Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitors (Beers et al., 2012). However, the impact of routine use of ROCK inhibitors in culture media is yet to be determined in longer term passaging.

Passage characteristics

At each culture passage the majority of cells will have undergone at least one cell division. The number of times a culture is passaged should be recorded together with the split ratio (i.e., ratio of culture size before to after passage) and an estimate of the number of cell doublings to track the relative age of the cells in culture. High-passage cells typically double robustly and have minimal differentiation but are more likely to acquire genetic abnormalities as they adapt to *ex vivo* conditions. Early passage cells may exhibit less predictable growth rates and increased potential for spontaneous differentiation. When newly derived after reprogramming, iPSCs at their earliest passages may demonstrate some residual carryover of the parental cells and/or differentiation. There may also be a range of different growth propensities between clones, especially if the original colony selected following reprogramming varied in size and/or quality. Successive passaging and attention to lower split ratios typically brings cultures to a more predictable standard of maintenance.

The ability of cells to efficiently adhere and divide successfully (plating efficiency) is affected by a range of parameters including clump size after passaging, split ratio, intensity of the mechanical force used to break up cell clumps and growth conditions.

Determining the appropriate split ratio is of significant importance, especially for low passage cells. Inappropriately high ratios can impose additional stress on cultures resulting in poor recovery, low attachment, and can potentially affect genomic stability. If stem cell clumps are not handled consistently, they cannot be counted and split properly. Standardized stem cell passaging can be achieved by defining the number of triturations (cell dispersion through mild pumping action with a pipette), the rate of liquid dispensing from the pipette and the proper evaluation of clump size among other factors. If cells become too diluted and/or are reduced to single cell suspensions, then

[6] http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122879.htm

medium supplemented with reagents such as ROCK inhibitors may be required to facilitate attachment and recovery.

An increasing number of facilities now successfully employs automated platforms capable of feeding, passaging, cryopreserving, and/or selecting colonies (Paull et al., 2015). Automation removes some of the variability inherent to manual culture and enables the maintenance of hundreds of clones in parallel.

Adaptation to new culture conditions

Transitioning PSCs from MEF co-culture to more defined conditions depends on the given stem cell line to be transferred, being more difficult to change from MEF to feeder-free cultures. For example, an established line that has undergone multiple passages on MEFs can be successfully transitioned to Matrigel™ and defined medium within approximately 3-5 passages. Similar to newly derived iPSCs, successive passaging at the appropriate ratios can minimize contaminating MEFs and support viable cell cultures.

Clone-to-clone variation is a topic that remains of interest and while there may be some inherent property differences between cell lines, the nature of culture conditions may also influence the ease of adaptation to new culture conditions. Post-adaptation growth rates may vary and may be influenced by the media and matrix selection and other factors such as splitting densities after passing from MEF co-culture to defined feeder-free conditions.

Use of antibiotics

As long as the appropriate facilities, equipment, sterile reagents and aseptic technique are employed, cell culture can be done successfully in the absence of antibiotics. It is recommended to reserve the use of antibiotics for special cases (e.g., culture of primary cells where contamination is highly likely, positive selection of recombinant cells by an antibiotic) and to routinely screen for contaminants like mycoplasma. Antibiotics like penicillin and streptomycin are often used across laboratories to minimize risk but may simply mask more significant forms of contamination such as mycoplasma. Cell banks should be subject to some form of "sterility test" for bacteria and fungi as some such contaminants may not be evident during routine passaging simply by observation of antibiotic-free cultures.

4.4 Cryopreservation

Vials representative of a reasonable percentage of the overall cryopreserved material should be thawed in order to evaluate the quality of the cryopreservation of a bank of cells (see Section 5.4). For example, cells can be harvested and pooled from multiple vessels or pooled and banked from one culture vessel at a time. In addition, it is important to consider the culture conditions used prior to cryopreservation when obtaining stocks of iPSCs from a laboratory because it can impact judgment on the quality of the clones. A number of advisory websites for cryopreservation are available[7,8,9,10]. Thawing into different medium and/or matrix may impact growth kinetics and stability. For example, some iPSC or ESC lines are still maintained in the presence of feeder layers and thawing them into feeder-free conditions may lead to unexpected results. An extended time period post-thaw should be considered when evaluating the recovery of cells post thaw. An immediate assessment at thaw may lead to false conclusions about the integrity of the cells, which is not readily apparent until further culture. For example, cells may attach and appear completely viable but deteriorate after extended time in culture. Alternatively, a culture may appear sparse with low plating efficiency but expand with time while maintaining an undifferentiated state.

4.5 Microbial, viral and cellular cross-contamination

Cell culture contamination (e.g., bacteria, viruses, yeast and other fungi) can result in a loss of cell cultures, erroneous scientific data and possible hazards to laboratory workers. Next to overt contaminations, micro-organisms with slow growth rates or that are resistant to antibiotics can go unnoticed and interfere in later studies. Immediate disposal of contaminated cultures is recommended to avoid contamination of other cultures. Attempts to eliminate contamination should only be performed if the culture is irreplaceable and, in that case, it should be handled under strict quarantine.

GCCP requires minimizing the risk of microbial infection (Coecke et al., 2005). The stem cell-derived systems and complex models discussed here are prone to the same risks as any cell culture; however, the typically longer culture periods and extensive manual manipulations pose even larger risks of infection. Therefore, some general aspects are reiterated here. Cells intended for banking and processing must be tested for mycoplasma, bacteria, fungi, bovine viruses, porcine viruses, and human viral pathogens (FDA, 2007; Bickmore, 2001).

Viral contamination

Viruses are the most difficult cell culture contaminants to detect due to their small size. A virus can be lytic (destroy host cells) or persistent (sub-lethal infection). Sometimes cell lines carry and express viral sequences without producing infectious virus particles. Mammalian genomes contain many retrovirus-like sequences, which are not overtly infectious. Such virus-like sequences are also observed at the RNA level in human and other cell lines (Coecke et al. 2005).

The main sources of viral contamination are primary cultures (such a feeder cells), cell lines, animal-derived culture reagents that cannot be sterilized (especially trypsin and serum) and the operator. Bovine serum is for example a potential source of Bovine Virus Diarrhea Virus (BVDV) contamination and serum generally is sold as BVDV-tested. Contamination of cell lines

7 https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cryopreservation-of-mammalian-cells.html

8 http://www.sigmaaldrich.com/technical-documents/protocols/biology/cryopreservation-of.html

9 https://www.atcc.org/~/media/PDFs/Cryopreservation_Technical_Manual.ashx

10 https://unclineberger.org/research/core-facilities/tissueculture/general-protocol-for-the-cryopreservation-of-mammalian-cells

with BVDV may cause slight changes in growth rate but this virus is non-cytopathic and microscopic changes in the culture will not be detected.

The common viral pathogens tested in cells intended for banking, as per ISCBI guidance, are: hepatitis C, human immunodeficiency virus (HIV), human T-lymphotropic virus (HTLV) I/II, Epstein-Barr virus (EBV), human cytomegalovirus (hCMV), human papillomavirus (HPV), herpes simplex virus (HSV), and human herpesviruses (HHVs). This is done to avoid risk of exposing laboratory workers and to exclude the possible impact that viruses can have in the cultures. The risk of more likely but less serious viral contaminants should also be considered[4], although comprehensive screening for all possible viruses would be impracticable from a cost perspective. Next generation sequencing offers a potential means of comprehensive virus screening but has yet to be adequately standardized for wide use.

Mycoplasma contamination

Mycoplasma is a prokaryotic organism that is a frequent contaminant of cell cultures. This organism can modify many aspects of cell genetics and physiology, including cell growth, metabolism, morphology, attachment, membranes and it can induce cytopathic effects such as plaque formation (Lincoln and Gabridge, 1998). Its small size and lack of a cell wall allows mycoplasma to grow to very high densities in cell cultures often without visible signs of contamination, pH change or even cytopathic effects. Mycoplasma has the ability to alter virtually every cellular function and parameter, making such contamination devastating for cultured cells (Lincoln and Gabridge, 1998). Mycoplasma are resistant to common antibiotics because they lack cell walls and may pass through standard filters (> 0.1 μm) used when making cell culture reagents (Chi, 2013). The detection of mycoplasma requires extensive laboratory cleaning and disposal of all materials currently in use in the culture. Mycoplasma contamination can thus pose a serious biological and financial risk. Therefore, it is paramount to evaluate the potential risk of passing cultures between different facilities and to institute a routine screening process (Young et al., 2010). Standardized screening practices for mycoplasma detection are preferred as the range of different available assays produces variable results and conclusions (Nubling et al., 2015).

Unfortunately, even with the advances in detection methods mycoplasma infection rates have not changed noticeably since they were first found in cell cultures. Strict management against mycoplasma contamination must be the central task for any cell culture laboratory contamination and quality control program.

Bacterial contamination

Bacterial contamination can arise from a variety of sources in the laboratory environment, such as water bath, fridges, sinks, etc., and the operator. It is often easily detected within a few days of infection by visual inspection of the culture. Infected cultures usually appear cloudy (i.e., turbid) and a sudden drop in the pH of the culture medium is frequently observed as a change in color of the indicator. Under a low-power microscope, bacteria appear as tiny, moving granules between the cells, and observation under a high-power microscope can resolve the shapes of individual bacteria. However, visual observation of cultures may not always reveal low level contamination and it is recommended to perform regular inoculation of bacteriological and fungal growth media of samples from cell banks. Contamination is most effectively avoided with good aseptic techniques, correct use of class II biological safety cabinets (BSC II) and maintenance of a clean and tidy cell culture laboratory. The use of antibiotics in culture medium may be necessary during derivation of cell lines, but is not recommended for preparing cell banks and for routine use as it affects cell physiology and induces a false feeling of safety.

In general, from a laboratory safety perspective, it is recommended that end users should request documentation from the supplier on testing for contamination with serious human blood-borne pathogens as identified in the relevant guidance documents (Andrews et al., 2009).

Cross-contamination

Cross-contamination is the contamination of the cell culture of interest with other cell lines. There are strong demands on researchers to generate large numbers of iPSC lines. This challenge could create a legacy of large numbers of unqualified cell lines if demand and the required steep learning curve do not meet. Cross-contamination or creation of a significant number of lines that turn out not to have pluripotent potential can occur if the appropriate quality controls are not applied. It could take decades to resolve any ongoing issues from published work on misidentified or contaminated lines alone (Stacey et al., 2013). Certifying cells, and appropriate good cell culture practice minimize the risk of cross-contamination.

4.6 Reprogramming and cell line derivation

Various primary cell types have been successfully used for somatic reprogramming (Raab et al., 2014). Fibroblasts derived from skin punches have been the primary choice of somatic cells but blood has emerged as an appealing alternative. Peripheral blood mononuclear cells or specific blood cell types such as T cells and $CD34^+$ cells have been used for iPSC generation (Loh et al., 2009; Serwold et al., 2007; Merling et al., 2013; Ye et al., 2013). Recently a single drop of blood was successfully reprogrammed (Tan et al., 2014), indicating a trend towards smaller volumes and minimal manipulation.

Methods for reprogramming

The method selected to initiate reprogramming is a delivery system to shuttle previously defined transcription factors into a somatic cell to initiate reprogramming. A range of transcription factors has been used with Oct4 and Sox2 being most crucial and any combination of others like C- or L-Myc, Klf4, Lin-28 and Nanog. Delivery systems include Retro/Lentivirus, Sendai Virus, mRNA, piggybac, and episomal-based approaches (Yu et al., 2007, 2009; Shi et al., 2008; Mack et al., 2011; Woltjen et al., 2009; Stadtfeld et al., 2008). These methods may be performed

alone or in combination with additional factors, such as small molecules, to enhance the efficiency of reprogramming.

Retroviruses and lentiviruses typically demonstrate the highest efficiencies compared to other methods of reprogramming but require host cell integration to be effective. Non-integrating modes of reprogramming, however, are preferred to minimize disruption of the host genome and facilitate progress towards therapeutic use.

Sendai virus replicates through an RNA intermediate making integration an unlikely event. As the iPSCs are passaged, the viral RNA will be lost over time. Elimination and/or silencing of reprogramming vectors should be confirmed by a sensitive method such as qPCR.

Methods involving oriP-based plasmids are equipped with features extracted from the Epstein-Barr Virus and contain both the origin of replication (oriP) and encode the transcript for Epstein-Barr nuclear antigen 1 (EBNA1). Plasmids are stable, easily scalable, and amenable to GMP-grade manufacture, making them a desirable choice for reprogramming for therapeutic applications. Furthermore, only a single transfection is required to obtain iPSCs. The oriP-based components assist in maintaining plasmids within transfected cells until innate gene expression initiates. The plasmids replicate extrachromosomally and will eventually be lost as iPSCs are passaged.

The messenger ribonucleic acid (mRNA) based approach involves *in vitro* transcribed mRNAs and bypasses the concerns of genome integration. Cultures must be supplemented to limit stimulation of the immune response to foreign nucleic acids. However, mRNA is not as stable as plasmids and, therefore requires multiple transfections to be effective. Self-replicating mRNA overcomes this constraint, requiring fewer transfections of mRNA (Yoshioka et al., 2013), but its consistency of successfully reprogramming across various samples is still untested.

iPSC generated using integrating retroviral vectors and non-integrating Sendai virus and synthetic mRNAs show that none of these methods lead to significant mutations (Bhutani et al., 2016), suggesting that current methods of generating iPSC are less prone to generating genetically unstable and potentially malignant cell types.

The definition of success of reprogramming is variable and should be considered more carefully if method selection depends on it. For example, efficiency may be calculated based on the amount of host tissue or total cell numbers (e.g., iPSCs per input cell number and/or per ml of blood). In a high-throughput setting, however, success may be determined by the number of donors needed to successfully yield a specified number of clones. For example, up to 3 clones may be sufficient to meet the deliverables if attempting to generate iPSCs across hundreds of donors. Therefore, it is important to clarify the definition of success and identify what is the target goal.

Challenges

There is general interest in understanding not only the donor-to-donor variability across clones but also clone-to-clone variability from the same donor. If clonality is of importance, then it is key to understand how the iPSCs were derived to determine the likelihood that the cells at hand are actually clonal. It is much simpler to evaluate genome integrity from a clonal cell line than from a mixed cell line. In this way, several clonal cell lines can be tested to confirm a phenotype arising from a patient's unique genotype.

Some protocols call for pooling iPSCs at the end of reprogramming, then expanding them for banking. In this scenario, it cannot be discerned whether the banked clone represents a polyclonal or monoclonal population. Alternative methods rely on multi-well plating strategies and/or cell sorting to increase the probability of expanding and banking clonal populations.

It is difficult to draw conclusions when making assessments across lines derived by different methods and from different tissue types. When generalizing conclusions from experimental results, it is important to be sure the stem cells discussed have been derived from the same starting material and handled in similar culture conditions to minimize the number of variables that contribute to the analysis.

4.7 Differentiation

The reference method for verifying pluripotency potential is the teratoma assay. However, these tests are costly, time consuming, present some reproducibility problems and require special expertise (see appendix in Andrews et al., 2009, and further information in Sections 5.1 and 5.6). The need for the assay has been challenged (Buta et al., 2013) and alternatives are emerging.

Embryoid body (EB) formation now is commonly used to verify pluripotency of human ESCs and iPSCs by assessing the expression of specific genes and proteins characteristic of the three germ layers (De Miguel et al., 2010; Sathananthan and Trounson, 2005; Trounson, 2006; Pistollato et al., 2012). Amongst these, analyses of SRY (Sex Determining Region Y)-box 1 (Sox1), paired box 6 (Pax6), neural cell adhesion molecule (NCAM) and neuroectodermal stem cell marker (Nestin) might be suitable to characterize ectodermal commitment; α-fetoprotein, cytokeratins, somatostatin, bone morphogenetic protein 4 (BMP4), GATA binding protein 4 (GATA4) and hepatocyte nuclear factor-4 are commonly expressed in the endoderm; brachyury, α-cardiac actin, and the atrial natriuretic factor are expressed at the mesoderm level, as reviewed by Pistollato et al. (2012).

Additionally, hESCs and hiPSCs can be differentiated towards specific lineages by means of defined differentiation protocols, applying a wide range of differentiation media, matrices (Nagaoka et al., 2015; Tsai et al., 2015), scaffolds (Chen et al., 2015), in some cases the modulation of oxygen tension *in vitro* (Millman et al., 2009), and the use of suspension (e.g., matrix-free, 3D) culture conditions with small molecules/pathway modulators (Chen et al., 2015; Kempf et al., 2015). Importantly, monolayer/2D and suspension/3D cultures may show significant differences in biology and responses of differentiated cells (Bose and Sudheer, 2016; Ruan et al., 2015). In order to design reproducible differentiation protocols, defined media and matrix components should be preferentially used, avoiding elements that may introduce uncontrolled variables, such as serum and co-culturing conditions.

It is important to consider that methods of passaging and culturing undifferentiated PSCs may impact their differentiation efficiency (Pistollato et al., 2012) and that a transcriptional memory of the cells of origin may be retained in iPSCs at low-passages, which may affect their propensity to differentiate into specific lineages (Ohi et al., 2011).

4.8 Microphysiological systems and organ-on-a-chip technologies

Scientists have long tried to reproduce biological functions in a dish in order to understand the molecular mechanisms involved in toxic and disease processes. However, it is very challenging to simulate the complexity of the *in vivo* situation *in vitro*. Recently, the development of more organo-typical cell cultures (Alepee et al., 2014; Marx et al., 2016) has enabled the generation of more complex models to study human toxicity and disease. These models are often called microphysiological systems (MPS) (Andersen et al., 2014). MPS are three-dimensional (3D) cultures and co-cultures of more than one cell type that mimic the function of a tissue or organ (Alepee et al., 2014). In many cases, MPS are presented together with other new technologies such as microfabrication, microfluidics, microelectronics and/or biomaterials, calling this combination organ-on-a-chip technologies. Because these new systems aim to create models that better predict the human response, they often employ iPSC.

Some research initiatives, such as the projects initiated by the National Institutes of Health, the US Food and Drug Administration (FDA) and the Defense Advanced Research Projects Agency (DARPA) to develop human-on-a-chip tools to assess the safety and efficacy of countermeasures to biological and chemical terrorism and warfare (Hartung and Zurlo, 2012) and the European project to address the long-term strategic target of "Safety Evaluation Ultimately Replacing Animals Testing" (SEURAT-1[11]) have promoted the fast rise of these new culture systems. The uses of novel 3D *in vitro* models are emerging in parallel in different areas (such as regenerative medicine, disease studies, drug discovery, toxicology). However, there are still many challenges to overcome, not only with regard to the generation of the *in vitro* models but also in linking to the novel bioengineering technologies. The main challenges are: 1) Lack of detailed understanding of some human organs and tissues, 2) complexity of protocols, 3) expensive technologies, 4) requirement of precise cellular manipulation, 5) reproducibility of the systems.

4.9 Gene-editing and gene reporter lines

Gene-editing consists of the use of artificially engineered nucleases to insert, replace or remove parts of the cell genome, often introducing exogenous DNA. These technologies are common tools used to study gene and protein function by deletion or silencing of specific genes. After DNA damage, cells can be repaired through two mechanisms: nonhomologous end joining (NHEJ) and homologous recombination (HR). Modern gene-editing technologies use these natural mechanisms to modify specific genes in cells. There are 4 main families of (engineered) nucleases used for this purpose:

1. Zinc finger nucleases (ZFNs) are generated by the fusion of zinc finger DNA-binding domain to a DNA-cleavage domain. ZFN can target specific DNA sequences in a very efficient manner in complex genomes.
2. Transcription Activator-Like Effector Nucleases (TALENs) are generated by fusing a TAL effector DNA-binding domain to a DNA cleavage domain. The combination of engineered TALEN with a DNA cleavage domain can be used for genome editing *in situ*.
3. CRISPR/Cas system is a prokaryotic immune system with two novel features: CRISPRs (short palindromic repeats) confer resistance to foreign genetic elements such as plasmids and phages and Cas proteins recognize and cleave foreign genetic material.
4. Meganucleases are large recognition site endodeoxyribonucleases. They are divided into 5 families based on sequence and structure motifs: LAGLIDADG, GIY-YIG, HNH, His-Cys box and PD-(D/E)XK (Orlowski et al., 2007; Zhao et al., 2007, 2014). Meganucleases have been used for many years to replace, eliminate or modify sequences. Their recognition sequence can be altered through protein engineering (both on a small and large scale) to change the targeted sequence.

Gene-editing is commonly used to generate reporter cell lines. Reporter genes are artificial sequences introduced into an organism to create a property of interest, normally easy to identify and to measure (e.g., GFP, luciferase). Reporter cell lines commonly combine a reporter gene and gene of interest with the same promoter. In this form, due to the same transcriptional gene activation, it allows easy quantification or detection of the expression of genes of interest. Reporter cell lines can be used to study specific genes and monitor cell differentiation in PSC; therefore, these lines have been used in a wide variety of studies (Wu et al., 2016; Lai et al., 2016; Zhang et al., 2016). It should be noted that genetic modification of stem/progenitor cells can alter their biological properties and differentiation characteristics. For that reason, it is recommended to characterize polyclonal parental properties in multiple clones in order to obtain the desired cells[12]. It is important to assess phenotypic homogeneity and mature cell phenotype[11] and also to check that the culture does not already contain the gene-edited sequences without presence of the target sequence.

5 Principle 2: Assurance of the quality of all materials and methods, and of their use and application, in order to maintain the integrity, validity, and reproducibility of any work conducted

5.1 Cells and tissues

A laboratory should have specific protocols or SOPs for the receipt of new or incoming cells and tissues, and for the handling,

[11] http://www.seurat-1.eu

[12] https://eurl-ecvam.jrc.ec.europa.eu/about-ecvam/archive-publications/publication/hESC_%20010711.pdf (last accessed 23 Nov 2015)

maintenance and storage of all cells and tissues, with regular monitoring for compliance. The following are among the factors to be considered:

– viability, growth rate, passage number and/or population doubling;
– morphological appearance, marker expression;
– functionality, differentiation state;
– performance controls specific to the application;
– contamination and cross-contamination, authenticity.

For stem cells, several assays are utilized to confirm functional pluripotency via confirmation of marker expression and tri-lineage differentiation potential (summarized in Tab. 3 and see also Section 4.9). Besides *in vivo* teratoma formation or (though rarely used) chimera formation in embryos after injection into mouse embryos, none of the *in vitro* tests are definitive on their own and therefore they are often done in combination or as a panel of tests. However, the utility of teratoma assays as the "gold standard" is questioned with rising popularity of alternate low-burden, high-throughput molecular methods (Buta et al., 2013). There is only one experimental test that could be claimed to "verify pluripotency" and that is the germline complementation test, which can be done in mouse but the equivalent human test is not ethically acceptable and illegal in many countries. There are challenges (e.g. expensive, more complex) with the alternate approaches too, given the wide variety of analysis platforms, including next generation technologies like RNASeq, ChIP-Seq and whole genome bisulfate sequencing. Resources such as Embryonic Stem Cell Atlas[13] (from Pluripotency Evidence (ESCAPE) database that compiles published high-content data on human and mouse ESC), eagl-i[14] and the European Database of Pluripotent Human Stem Cell Lines[15] are important resources. The ultimate application of these assays in a regulatory context is the standardization and ultimate acceptance of large-scale datasets (Buta et al., 2013; Xu et al., 2013).

Additional tests, such as cell line-identification, karyo- and HLA-typing, and microbial testing all ensure the safety and quality of the cells. In general, a combination of several of these tests is carried out for both research-grade and clinical grade cells, albeit with greater rigor in the latter case.

Additional tests

a) Cell line identity

As stem cells are generated from diverse sources and conditions, it is extremely important to confirm the identity of the cell line, since any switching or cross-contamination can have adverse effects, especially for clinically relevant samples (Markovic and Markovic, 1998; Nelson-Rees et al., 1981; Stacey, 2000). In order to accurately identify cell lines, analysis of highly polymorphic DNA sequences via DNA fingerprinting is commonly employed (Thompson et al., 2012). The highly polymeric short tandem repeat (STR) loci are amplified using PCR and the products analyzed at high resolution through capillary gel electrophoresis (Butler, 2006). The Federal Bureau of Investigation (FBI) Laboratory has selected 13 independently inherited core STR loci for use in CODIS, the national US DNA databank (Budowle et al., 1998). ISCBI recommends using the core 13 loci from the field of forensics for stem cell identification without providing specifics on the number of loci to be used (Xu et al., 2013). There is also an ANSI standard for performance of STR profiling[9].

b) Karyotype

Certain mutations may confer a growth advantage and result in abnormal cells under certain conditions, thus necessitating regular confirmation of normal karyotype although this alone would detect more subtle changes (ISCBI, 2012). ISCBI guidelines (ISCBI, 2009) for release criteria of banked lines are based on best practices in clinical cytogenetics and recommend G-banding (Bickmore, 2001; Loring et al., 2007) and counting at least 20 metaphase spreads with greater than 95% of the cells confirmed to possess normal karyotype. Recent studies have employed higher resolution analyses, such as comparative genome hybridization (CGH) microarrays, single nucleotide polymorphism (SNP) arrays and whole genome sequencing (Cheng et al., 2012; Elliott et al., 2010, 2012; Gore et al., 2011; Hussein et al., 2011; Maitra et al., 2005; Martins-Taylor et al., 2011). Next-generation sequencing can be used to achieve nucleotide level resolution. These higher resolution genomic methods provide a lot of data but are yet to be routinely used as it is not yet defined what differences may constitute a threat to cell safety for cell therapy applications or impact on reproducibility of research (Kleensang et al., 2016). Given the complexity of these methods, current guidelines by ISCBI (2009) recommend the use of G-banding. It is also wise to check genetic stability by a suitable method every 5-10 passages. While these methods may emerge as valuable for clinical-grade cells, their routine use has been hindered by the costs and complexity associated with these methods.

c) HLA analysis

There has been a recent trend towards the development of HLA haplotype banks of clinical grade PSCs that address the needs of the majority of the population within a geographical location, while minimizing risk of allograft rejections (Gourraud et al., 2012; Nakatsuji et al., 2008; Taylor et al., 2012; Zimmermann et al., 2012). There are many commercially available assays for low-resolution typing that are generally faster and cheaper or for high-resolution typing with an extended set of molecular assays to characterize the alleles in more detail. ISCBI recommends performing HLA-typing, but does not prescribe specific guidelines on the resolution and loci that should be used for PSCs. Hematopoietic stem cell transplantations involve high resolution (Petersdorf, 2008) while solid organ transplants only require low resolution typing (Johnson et al., 2010; Opelz and Dohler, 2007, 2010). PSC HLA haplotype banks currently utilize low resolution typing (Gourraud et al., 2012; Nakatsuji

13 http://www.maayanlab.net/ESCAPE/

14 https://www.eagle-i.net/

15 https://www.hpscreg.eu/

Tab. 6: Assessment of the quality of reagents used in PSC

Reagent	Parameter	Quality assessor	
		Supplier	End user
Serum	Sterility testing	+	
	Physical and biochemical analysis including endotoxin testing and mycoplasma	+	
	Functional testing	+ (general)	+ (specific, e.g., plating efficiency)
Defined media (e.g., mTeSR, Essential 8)	Sterility testing	+	
	Physical and biochemical analysis including endotoxin testing	+	
	Functional testing	+ (general)	+ (specific, e.g., growth of in-house cell line)
Supplements (e.g., B27, Stempro)	Sterility testing	+	
	Physical and biochemical analysis	+	
	Functional testing	+ (general)	+ (specific tests added for defined media)
Growth factors (e.g., EGF, FGF)	Sterility testing	+	
	Physical and biochemical analysis	+	
	Functional testing	+	+ (difficult to assess but testing new batches with in-house cells may be helpful)
Detachment solution (e.g., Accutase™, Gentle Cell Dissociation Reagent™)	Sterility testing	+	
	Physical and biochemical analysis	+	+
	Functional testing	+	+
Surface coating (e.g., Matrigel™, Vitronectin™)	Sterility	+	
	Physical and biochemical analysis	+	+
	Functional test	+	+

et al., 2008; Taylor et al., 2012; Zimmermann et al., 2012) but further guidelines may emerge as more cells enter the clinical space. Specific requirements for such testing to enable immunological matching will be subject to regional variations in haplotype incidence and may also need to include analysis for other types of polymorphic cell surface molecules.

d) Mycoplasma and microbial testing

To avoid the alteration of cell behavior as well as to safeguard researchers and patients, cells are continually tested for sterility and for mycoplasma (Weber, 2006). At minimum, it is recommended to screen for mycoplasma at cell receipt, establish a regular testing interval (optimally every 3 months) and perform daily observation (see 1.5 and 2.5).

5.2 *In vitro* culture conditions

In vitro culture conditions require quality control of the media and supplements; however, this is time-consuming and expensive. Nevertheless, most of these materials are obtained commercially and suppliers should supply relevant quality control documentation (Tab. 6).

The GCCP task force report introduces laboratory user responsibilities and the appropriate procedures necessary for the purchase, installation, commissioning, correct use, performance monitoring and maintenance (Coecke et al., 2005). These procedures are identical for *in vitro* PSC culture conditions.

European norms and ISO standards can be adopted for these areas, and in some cases compliance may be a legal requirement (for example, for pressurized gases, such as carbon dioxide/air for cell cultures, where there are ISO standards and national or regional regulation for the gases, and safety standards for the cylinders and pressure regulators).

5.3 Handling and maintenance

It is important to consider the culture workflow, especially when carrying multiple ESC or iPSC lines. This includes passaging formats, split schedule, and determining how culturing vessels will be labeled and maintained (see Section 4.3). These

steps become increasingly important when culturing multiple clones from multiple donors in multi-well plates. Each laboratory should evaluate solutions that work best to minimize cross-contamination. For example, it is advisable to maintain clones from only one donor on one plate at a time where feasible rather than clones from multiple donors on one plate. A segregation system across incubators allows better tracking of material. Barcode labeling and colorimetric labelling may help to track cultures. When receiving iPSCs or ESCs, understanding what measures are in place in the laboratory of origin can provide insight into the magnitude of risk and the quality of the lines received.

Both qualitative and quantitative assessments may be made to determine the quality of iPSCs. Naturally, qualitative assessments are difficult to standardize but are required when colonies are passaged in clumps. Qualitative assessments include observations based on confluency, plating efficiency, and the general appearance of the culture. Cultures are typically passaged when they have reached roughly 70-85% of the cell culture surface area, although it should be recognized that evaluation of confluency may vary between labs and between individuals in the same lab (Stacey et al., 2016). A culture is considered relatively healthy when there are few or no signs of cell death, peeling from the culture surface or contamination by visual inspection. A predictable growth rate is also anticipated when a culture is maintained at a consistent split ratio and passaging occurs at regular intervals. More quantitative parameters can be implemented by assessing viability and growth rates based on cell counts and live/dead staining during maintenance.

Karyotypic instability may arise for a number of reasons including time in culture, stressful passaging techniques, and the nature of the source material among other factors. If cultures representing different donors are maintained in parallel, it is important to consider the possibility of cross-contamination (see Section 5.1). Therefore, a periodic screen for karyotype (or an equivalent molecular assay) and identity are paramount to ensure the identity of the cells. A more extensive evaluation of the gene expression profile and pluripotent capacity of the cells can also be employed periodically to confirm the quality of the iPSCs and ESCs in culture.

5.4 Cryopreservation

Cells and tissues are banked and stored by cryopreservation. They are diluted in a cryoprotectant solution before freezing. The freezing, storage and recovery process has a number of key technical elements that should be considered, such a type of cryoprotectant, additives, cooling rate, storage conditions or recovery methods (Coecke et al., 2005; Stacey et al., 2016). However, due to the specific characteristics of pluripotent stem cells, some processes need special attention.

Biological considerations

The quality of the culture prior to cryopreservation plays a critical role and cultures selected for preservation should have low levels of differentiated cells, high viability and optimal growth rate (i.e., exponential phase). The addition of ROCK inhibitors has demonstrated benefit when thawing cells by improving plating efficiency (Rizzino, 2010). However, it is best practice not to use these reagents *in lieu* of standardization of culture conditions.

Method

A commonly accepted method for handling cryopreservation of iPSCs and ESCs is a slow freeze and rapid thaw approach using a cryoprotectant (e.g., 10% DMSO) while vitrification (e.g., high levels of cryoprotectant and very rapid freezing) has also been applied (Hunt, 2007; Ji et al., 2004; Heng et al., 2005; Ware et al., 2005; Holm et al., 2010). When done properly, both approaches can result in successful cryopreservation. Cryopreserved cells may demonstrate a wide recovery range post thaw that can be improved by using a controlled-rate freezer (Ware et al., 2005) to create a more reproducible cooling rate and thus a more reliable recovery. Such devices can maintain temperature cooling rates ranging from 0.3 to 5°C per minute (depending on the method used). Alternatives are simple passive freezing devices (e.g., Mr Frosty, Biocell) stored in a -80°C freezer overnight before transferring vials to a liquid nitrogen (LN_2) tank.

Cryoprotective agent (CPA)

Careful consideration of the CPA is important to maximize cell recovery following the osmotic effect at the time of addition and elution from cells. An equilibration phase is recommended upon CPA addition while considering temperature, concentration, and time to minimize toxicity (Andrews et al., 2015). For an explanation of the cryopreservation process see Stacey et al. (2017).

Storage

Vapor-phase liquid nitrogen is recommended for storage as it minimizes the risk of cross-contamination and hazards associated with pressure build up when liquid nitrogen seeps into leaky cryovials. Electric freezers which maintain storage at < -100°C are also used but often require LN_2 or "cardice" emergency backup. Storage at -80°C is suitable for short term storage or shipment (i.e., on "cardice") of cryopreserved cells but vitrified cells will usually devitrify at this temperature and rapidly lose viability. Long-term storage at -80°C is not recommended as it may lead to progressive loss of viability.

Pre/post-harvest evaluation

An extended culture period post-thaw is recommended to evaluate the recovery of cells. An immediate assessment at thaw may lead to false conclusions about the competency of the cells to replicate that may not be readily apparent until further culture, see Section 4.4. Recovery is also dependent on the procedure used to thaw vials. Employing devices that enable consistency upon thaw is preferred.

Record keeping/tracking

To ensure accurate tracking of passage number, it is important to determine how an organization assigns passage numbers as interpretations differ. For example, some laboratories will note the passage number at the time of cryopreservation then add a

passage when thawing to account for the doubling. Other laboratories might note an additional passage when freezing rather than after thawing cells. Ideally, cell numbers should be determined at preservation and recovery to identify suboptimally preserved cultures.

5.5 Microbial, viral and cellular cross-contamination

The large resources required to generate hiPSCs dictate the most stringent quality control standards that should be applied to the cells before they are made widely available and used in a laboratory for different applications (see also Principle 1). The assurance of the quality control should include well-standardized methodology to confirm the absence of microbial, viral or cellular contamination. It is recommended to perform at least regular screens for mycoplasma and daily observation.

It is good cell culture practice to systematically perform mycoplasma, bacterial and viral contamination screening. Contamination with mycoplasma is a common problem when using "gifted" cell cultures. This can be avoided by purchasing cultures only from reputable cell repositories that have vigorous testing programs and certify their cell lines as authenticated and free of microbial contamination. Cultures obtained from other sources should be kept quarantined in a separate incubator until mycoplasma test results are available. If a separate incubator is unavailable then the culture should be grown in a sealed flask and kept inside a container such as a plastic box with a cover or lid. The suspect cultures should only be handled at the end of the workday after all other cell culture work is complete or in a dedicated quarantine lab or safety cabinet. All media, solutions and plastic ware that are used for these cultures should also be segregated from the other culture materials and supplies. The BSC II as laminar flow hood should be carefully disinfected before and after use.

Pharmacopoeia methods are established for detection of microbial contamination but, whilst still representing the industry standard, those used for cell culture samples rely on traditional culture media and conditions, which will not enable all microorganisms to grow (e.g., use of antibiotics). A range of rapid detection techniques have also been developed including non-specific methods (e.g., ATP bioluminescence, laser particle detection), detection of microbial products (e.g., bacterial endotoxin, fungal glycans) and specific detection methods including RT-PCR amplification of ribosomal RNA gene sequences (for more information see Tab. 1 of Young et al., 2010). Use of these rapid techniques is currently a subject of investigation and at this stage they have value when at least two methods are used in combination. However, at this time established sterility testing methods by broth inoculation remain the accepted test for vials from banks of cell lines.

Viral contamination

A major concern when using virally infected cell cultures is the potential health hazard they might have for laboratory personnel. Special safety precautions should always be taken when working with tissue or cells from humans or other primates to avoid possible transmission of viral infections (HIV, hepatitis B, Epstein-Barr, simian herpes B virus, among others), therefore the cells and all cell culture reagents should be purchased from certified sources only. Sendai virus has been used as a reprograming vector for iPSC. Sendai virus does not integrate into the genome. Although, there is no known human pathology for Sendai virus, infection can be produced in humans via aerosol and contact. Therefore, workers require adequate protection (BSL-2). However, strains used for reprogramming are deficient in the expression of fusion protein and should not be capable of spreading infection.

Cross-contamination

With the progress made in karyotyping methods it became apparent that some cell lines are cross-contaminated by cells even of other species. Human cell lines are most frequently contaminated by HeLa cells but also by a number of other rapidly growing cell lines. Often the invading cells are better adapted to the culture conditions and grow faster than the original cells. Because of the morphological similarities of iPSC and ESC lines, it is impossible to rely only on microscopic observations to screen for cross-contamination of cultures, particularly when growing a number of lines simultaneously (Pistollato et al., 2012).

DNA profiling (fingerprinting) should be carried out such as a short tandem repeat method, and the International Stem Cell Banking Initiative (ISCBI, 2009) guidance recommends that key profile loci should be shared between the stem cell bank and researchers in order to enable detection of cross-contamination while not releasing full profiles into the public domain, as these may permit identification of donors (Andrews et al., 2009) by testing cell bank samples using standard methodologies. Publication of full STR data places donors at risk of de-anonymization (ISCBI, 2012; Isasi et al., 2014) and the ANSI standard for STR profiling recommends that limited STR allele information should be shared to permit resolution of instances of cross contamination.

Mycoplasma contamination

Pharmacopoeia tests for these organisms have been established including broth/agar culture, assays for mycoplasma-characteristic enzyme activities, and DNA staining. For such industry standards sensitivity of detection should be defined and the testing regime used should also identify contamination with strains that will grow in cell culture only. A combination of methods is often recommended to achieve these requirements. However, such techniques require from several days (cell culture inoculation and DNA stain) up to three weeks (broth culture) incubation for a final result.

Commercial kits (including RT-PCR) are available to rapidly monitor for mycoplasma. Nucleic acid amplification is emerging as an alternative to the established official mycoplasma test methods for assurance of biopharmaceutical product safety. Since 2007, European Pharmacopeia (Sec.2.6.7) provides guid-

ance on the validation requirements for mycoplasma detection tests, including the nucleic acid amplification method (Ph. Eur., 2012). Novel and rapid test systems should be tested for their sensitivity and specificity for detection of different mycoplasma strains and absence of cross reactions with other organisms, so their performance is understood by users in comparison to standard, existing methods. It is also important to include controls for inhibition of PCR amplification by cell culture components.

The leading cause of culture loss in most laboratories is microbial contamination resulting from poor or insufficient aseptic technique. Developing successful aseptic techniques requires good training (GCCP principle 6) and knowledge of the nature and potential sources of contamination (GCCP principle 1).

5.6 Reprogramming and cell line derivation

Clearance of residual reprogramming elements is of significant interest for iPSCs generated via non-integrating reprogramming methods to ensure no interference with the host chromosomes. Many of the molecular-based delivery systems such as miRNA, RNA, and plasmids result in loss of exogenous genetic material following successive passaging. For example, cells transfected with the oriP/EBNA1-based system will typically loose plasmid from resulting iPSCs that are passaged over time. Other factors affecting timing of plasmid loss include transfection efficiency of the starting material. The likelihood of chromosome integration events is low and this can be ruled out using molecular based assays such as PCR.

The derivation of iPSCs from multiple donors increases the potential for contamination, especially in the absence of automation, when multiple cultures are handled in parallel. Therefore, it is good practice to regularly screen for mycoplasma, sterility and cross-contamination. Additional quality checks should be in place to screen karyotype, but it is important to consider the possibility that an abnormal karyotype might be reflective of the original host material. Working with one cell line at a time in the BSC II gives important protection from cellular cross-contamination.

It has become widely accepted to confirm the quality of newly derived iPSCs by way of gene expression and pluripotency. However, there is a need to standardize these measures considering the range of tests available with varying sensitivities. For gene expression, both flow cytometry and PCR have been used, but, ideally, a common panel of reprogramming genes against which clones are screened should be established so that clones can be compared to each other. Pluripotency is typically not determined by gene expression but rather by the ability of the iPSC to differentiate into cell types representative of all three germ layers (ectoderm, endoderm, and mesoderm). The gold standard for pluripotency testing has historically been the ability for clones to form teratomas when injected into immunodeficient mice. However, these tests are too costly and time consuming to execute for a large number of clones. Other approaches like the formation of embryoid bodies and/or differentiation into specific lineages have also been employed for confirmation but, like gene expression, a standardized test is preferable.

5.7 Differentiation

The routine use of PSC-derivatives for toxicology and other biomedical applications requires the development of harmonized quality control standards, allowing reproducibility, scalability and inter-laboratory comparisons. Both individual investigators and private companies developing differentiation protocols for PSCs should provide detailed phenotypic characterization of the differentiated cells, and ideally perform side-by-side comparisons between the PSC-derivatives and the adult cells they should resemble, such as primary hepatocytes, primary cardiomyocytes, and cortical neurons, representing ideal benchmarking cell models.

The suitability of a specific PSC-derivative for (toxicology) studies should be determined by the analysis of differentiation related markers, such as CYP3A4, CYP2B6, and CYP1A1/2 expression; the analysis of urea synthesis, glycogen uptake, albumin secretion/synthesis suitable to characterize PSC-derived hepatocytes; the analysis of tropomyosin, troponin I, actinin, atrial natriuretic peptide, and desmin, suitable for PSC-derived cardiomyocytes; the analysis of β-III-tubulin, MAP2, neurofilament 200, synapsin-I, MAPT, FoxA2, and En-1, for PSC-derived neuronal cells; and the analysis of K5, K14, DeltaNP63, and K10 for PSC-derived keratinocytes (Pistollato et al., 2012). Besides assessing the phenotypic identity of PSC-derivatives through analysis of genes and proteins/markers, other assays, such as multi-electrode array analysis suitable to evaluate neuron and cardiomyocyte electrical activity and contractility (Riedel et al., 2014; Illes et al., 2014; Kanda et al., 2016), are essential to evaluate the functional properties of PSC-derivatives.

Moreover, appropriate and realistic thresholds of the level of expression of these markers and functional endpoints should be defined in order to assess the applicability of individual cell preparations for studies. These should be based on practical local experience in characterization as culture systems will vary depending on local reagents and conditions. The definition of well-defined and practically qualified quality control metrics is mandatory to support a reduction of both intra- and inter-laboratory variability (Pistollato et al., 2012).

Additionally, "omics" technologies, such as transcriptomics, metabolomics, phospho-proteomics, and epigenomics, in combination with systems biology, while allowing the identification of the molecular mechanisms (i.e., mode of actions) underlying (toxicant) effects in a high-throughput manner, are also helping to expand current knowledge of PSC profiles, which has special relevance when PSC-derivatives are intended to be used for clinical applications (Silva et al., 2015).

5.8 Microphysiological systems and organ-on-a-chip technologies

The quality control of MPS and organ-on-a-chip technologies is challenging. MPS are complex models that require in most

cases complex protocols. To be able to generate good MPS, practice is required to have the personnel well trained in the protocols used and able to produce reproducible MPS structures reliably. Quality controls have to be set up for different aspects:
– MPS phenotypic properties,
– structural morphology,
– quantification of cell population (if possible),
– and functionality.

Moreover, the use of specific technologies (such as biomaterials or microfluidics) requires specific quality controls. The quality of the materials used in the scaffolds, surface coatings, microgeography of the scaffold surface, microfluidic flow, toxicity of the materials used, sterility of re-used equipment should be regularly monitored.

In many cases, these products are not yet commercialized and it is difficult to generate good quality controls. Therefore, each laboratory should pay special attention to internal quality controls, allowing production of reproducible and high-quality materials and maintain awareness of batch-to-batch changes. As to this, the laboratory has to be responsible:
– to ensure that all the materials are used adequately for their intended purposes;
– to ensure that the laboratory workers have the training required to handle the materials and their waste appropriately.
– to store the materials under adequate conditions.

Moreover, the same issues should be considered as for other cell cultures (Coecke et al., 2005).

5.9 Gene editing and gene reporter lines

As mentioned before, gene editing and gene reporter lines are useful tools commonly used in PSC research. However, there are some aspects that should be taken into consideration before starting to use them. Gene editing technologies present, depending of the methods chosen, some associated advantages and disadvantages: Zinc finger nucleases (ZFNs), which are normally used for disabling a mutant allele, inserting genes for gene therapy and repairing allele damage may present problems such as off-target cleavage and immunological response. Transcription Activator-Like Effector Nucleases (TALENs) are commonly used to knock down genes and to correct genetic defects. However, current delivery mechanisms of limited efficiency, unknown immunogenic responses, and certain non-specificity of binding limit their use. The CRISPR/Cas system is commonly used for gene editing and gene regulation (Xue et al., 2016). One of the main advantages is its dependence on RNA and not DNA sequence (in comparison to ZFN and TALEN) as the RNA machinery base-pairing rules are simpler between an engineered RNA and the target DNA site (Sander and Joung, 2014). Meganucleases, are used for gene correction, insertion of therapeutic genes, targeted mutagenesis and virus clipping among others (Silva et al., 2015).

However, efficiency of gene editing is still really low, requiring, single cell clones to be isolated, studied separately and expanded before use, which can increase costs and time. The creation of a nuclease reagent is time-intensive (approximately 10 weeks) and costs can vary widely.

6 Principle 3: Documentation requirements

In order to permit the repetition of cell culture studies, to track the materials and methods used, and to enable the target audience to understand and evaluate the work, it should be clearly and accurately documented. This includes accurate records of cell type, origin, authentication and characterization, and of the culture techniques performed along with the materials used.

As originally described in Coecke et al. (2005) and not different for PSC, all documentation should be retrievable, and should include:
– the objective of the work;
– the rationale for the choice of procedures and materials used;
– the materials and equipment used;
– the origin and characterization of the cells and/or tissues;
– the laboratory records, including results, raw data and quality control records;
– cell and tissue preservation and storage procedures; and
– the protocols and SOPs used, and any deviations from them.

In some circumstances, formal procedures for the retrieval and review of documentation are necessary (e.g., GLP or Good Manufacturing Practice (GMP) requirements). Such rigorous documentation is also useful for resolving any questions or disputes that may arise (Coecke et al., 2005).

Paramount to ensuring that reproducible results can be obtained with pluripotent stem cells is routinely verifying the origin, differentiation status, and pluripotency of the subject cell line. In October 2007, the International Stem Cell Banking Initiative (ISCBI) established a dialogue between stem cell distribution centers and national and international stem cell research funding bodies to develop a consensus on best practices for ESC banking, testing and distribution (ISCBI, 2009). Although the focus was on ESC, many aspects are also applicable to iPSC lines. Among the recommendations included in this document are general principles for ensuring informed consent, traceability, and governance of stem cells derived from human tissues. The unique tools and methods used for generating hESC and iPSC lines also need to be careful documented. This activity has also now been extended to the requirements for PSCs for clinical use (ISCBI, 2009; Andrews et al., 2015).

6.1 Cell and tissue origins

When working with mammalian cells or tissues, whether primary cells, immortalized cell lines, or PSC, a minimal set of information is essential to clearly define the specific cell type and its associated origin (see Tab. 7).

6.2 Handling, maintenance and storage

Records must be kept for all critical details associated with the handling, maintenance and storage of mammalian cell cultures to ensure that they maintain consistency across studies. All solutions (e.g., cell culture media) should include identifying details of supplier, batch, storage requirements, and expiration date. Components of the cell culture media should also be described and include all supplements and additives, as well as methods of preparation of the media, procedures for preparation

Tab. 7: Examples of requirements for documentation concerning the origins of pluripotent stem cells

	Isolated organs/ organ cultures (non-human)	Primary cell cultures (non-human)	All materials of human origin (adult)	All materials of human origin (fetal)
Safety information	+	+	+	+
Ethical issues	+	+	+	+
Species	+	+	+	+
Strain	+	+	NA	NA
Source	+	+	+	+
Sex	+	+	+	+
Age	+	+	+	+
Race			+	+
Number of donors	+	+	+	+
Health status	+	+	+	+
Tissue of origin	+	+	+	+
Cell type(s) isolated	+	+	+	+
Date of isolation	+	+	+	+
Isolation technique	+	+	+	+
Operator	+	+	+	+
Supplier	+	+	+	+
Informed consent	NA	NA	+	+
Material transfer agreement	NA	NA	+	+
Medical history of donor	NA	NA	+	+
Pathogen testing	+	+	+	+
Shipping conditions	+	+	+	+
Condition of material on arrival	+	+	+	+
Identification and authentication	+	+	+	+
Mycoplasma testing	+	+	+	+

NA, not applicable

or use of cells or tissues. Such procedures may be detailed in SOPs, but for specific standards required for regulatory compliance and/or clinical work, the traceability of each procedure may be required to ensure the use of appropriate reagents. The culture substrate and recording of the coating procedures, where applicable, should be defined. This could include the type and supplier of coating material.

The type and origin of culture-ware can also have profound effects on cell culture viability and reproducibility. Accordingly, the types and suppliers of flasks, Petri dishes, roller bottles, etc., should be detailed. The incubator conditions should also be routinely monitored and conditions reported to ensure that any issues associated with humidity (if appropriate), temperature and CO_2 levels can be ruled out. Likewise, documentation associated with laminar airflow and safety cabinet testing, calibration, maintenance and repair ensure that study results are not confounded by environmental effects.

With regard to the cell culture system, critical information must be recorded to permit tracing the history of the biological material, its characteristics, and the treatments, manipulations, measurements and procedures applied to it, including statistical procedures used to analyze the obtained results. For human cells, traceability should also include the informed consent obtained from the original donor of cells used to derive the cell line.

Cell and tissue preservation and storage details should include type of cell or tissue, passage/identity number, cryoprotectant used, number of cells per cryovial, position in storage container, viability and plating efficiency after thawing, and

date and operator. Storage processes should also be strictly defined and documented to provide confidence in the viability of sub-passaged cultures. This includes monitoring of refrigerator and freezer temperatures, liquid nitrogen level and/or temperature in storage containers, sterility controls (e.g., autoclaving, sterility tests), and regular maintenance and calibration of critical apparatus. The extent of testing and monitoring can vary, from alarms for research and development work to continuous monitoring of calibrated monitoring systems for critical work.

Cell banks should be maintained in facilities that are accredited, designated, authorized or licensed by an appropriate authority. It is strongly recommended to operate both a formal and documented Master Cell Bank and Working Cell Bank system to ensure that a supply of reproducible cells at the same passage level is maintained over extended periods (ISCBI, 2009; FDA, 2010a). Any changes in storage location should be formally recorded and relevant notification should be given for example (e.g., to the local health and safety officer where pathogenic, highly toxic, radioactive or genetically modified materials are involved). The disposal procedures and associated compliance for culture laboratory waste must also be documented.

There are specific requirements for documentation concerning the handling, maintenance and storage of cells and tissues used for deriving PSCs, regardless of the origin species or age (i.e., fetal or adult). These include the following:

- Safety information
- Ethical issues
- Purity of isolation
- Phenotype
- Differentiation state
- Type of culture, e.g., monolayer, suspension, spheroid
- Culture medium, i.e., type, supplements and other additives used
- Feeding cycles
- Growth/survival characteristics, e.g., cell survival, expression of cell-specific markers, ageing, initial density at plating, doubling time
- Initial passage number
- Confluency at subculture
- Subculturing details, e.g., date of sub-culture, subculture intervals, split ratios; seeding densities, perfusion rate
- Reprogramming method
- Identification and authentication
- Morphological characterization including silencing of ectopic genes and elimination of non-integrating reprograming vectors
- Molecular characterization
- Functional characterization
- Mycoplasma testing
- Life expectancy
- Special properties (characteristics or use)

A number of key phenotypic markers for ESC cell line characterization are recommended for use by a stem cell bank (Adewumi et al., 2007). For any pluripotent stem cell line, it is essential that the bank provides evidence for pluripotency. Several functional tests, which are generally applicable to both ESCs and iPSCs, can be used to define pluripotency (e.g., DNA methylation analysis, *in vivo* teratoma formation, tetraploid complementation) (Sohn et al., 2012).

6.3 Reporting

Careful attention should be given to the reporting procedures used since the report format will depend on the target audience (e.g., internal staff, client/sponsor, regulatory authority, general public). Regardless, the person(s) responsible for the report should be identified and where appropriate, the report should be formally authorized for its intended purpose.

A high-quality scientific report should cover the objective of the work, the protocols and SOPs used, planning and experimental design, execution of the study, definition of the test conditions, test procedure, test acceptance criteria, data collection and analysis as well as a discussion of the outcome. The extent to which the study adheres to relevant standards, regulations, guidelines or guidance documents should be stated, along with adherence to safety and quality assurance procedures. This could also include a statement of compliance with the GCCP principles. Reports on cell and tissue culture work should address a minimum set of information that covers the origins of the cells, characterization, maintenance, handling, and traceability of the cells, and the procedures used (see Tab. 7).

7 Principle 4: Establishment and maintenance of adequate measures to protect individuals and the environment from any potential hazards

National and local laws, based on moral and ethical principles, govern safety in the workplace in most countries. Many countries also issue guidelines on occupational health and laboratory safety, and individual laboratories may also have rules, which reflect local circumstances. Thus, the guidance on safety in the cell culture laboratory given here in no respect replaces these laws and regulations, but rather draws attention to certain aspects of them and highlights issues specific to the *in vitro* culture of animal and human PSC. In many countries, each laboratory is required to appoint a biological safety officer, and this individual should have suitable training or advice available and be involved in the safety evaluation of any cell culture procedures.

7.1 Risk assessment for human pathogens and general rules for hiPSCs and hESCs

Identifying and evaluating risks, and taking appropriate action to avoid or minimize them, are foundations on which safety is built. The laboratory environment contains hazards that are often complex and require specialist knowledge and experience. Key stages in the management of such risks are robust risk identification, establishment of procedures to control risk, and evaluation of residual risk to check it has been reduced to an acceptable level. These assessments should be documented and reviewed at regular intervals to take into account any changes in local practice, national or international regulations, or increases

in scientific knowledge. Risk assessments should also provide a reference document for other individuals performing the work and awareness of them should be a key element in laboratory training (see section 6 of Coecke et al., 2005).

It is important to pay particular attention to risks, which may be specific to, or more significant in, certain groups of workers. For example, women carrying a (possibly undiagnosed) pregnancy and would be at greater risk from the effects of certain chemicals, such as teratogens or biological agents. Similarly, persons with a diminished immune response (e.g., due to medication or a medical condition) should seek expert medical advice before they are allowed to work in a laboratory where cell and tissue culture is performed.

The safety conditions highlighted below relate not only to the safety of laboratory staff carrying out cell culture work but also to ancillary staff handling or disposing the materials used. Furthermore, there may be theoretical risks of laboratory workers becoming infected and transmitting disease outside the laboratory or recombinant organisms, pathogens or hazardous chemicals escaping the laboratory or failing to be dealt with correctly on disposal. In such cases the impact on the general public and the environment must be considered. General laboratory safety issues, where it may be appropriate to apply risk assessment, are shown in Table 6 of Coecke et al., 2005. Hazards of particular concern in the cell or tissue culture laboratory are further discussed in Sections 7.2 and 7.3 below.

7.2 Hazards related to cell and tissue culture work

Physical hazards

PSC and microphysiological systems are not typically associated with physical hazards different to other cell cultures. In general, physical hazards in the cell and tissue culture laboratory are constrained to movement and use of pressurized gases, for which there will be specific safety regulations (EU, 1997). However, incorrect use of devices, and particularly those using extreme heat (e.g., autoclaves, incinerators), irradiation and mechanically hazardous components (e.g., centrifuges, "sharps", potentially explosive components) is a major source of hazard and should be managed under the appropriate legislation and local safety rules for use and maintenance. Laboratories and workspaces should always be kept clean and tidy. It is wise to avoid storage of heavy objects or large glass vessels above typical head height or storage of material on the floor or anywhere where it can cause risk to other people. Any equipment or apparatus used should meet national safety guidelines. Equipment such as autoclaves, centrifuges and microbiological safety cabinets should have a program of maintenance and checks on correct operation for safe use. Such checks may be prescribed in legislation and local rules, but typically would be carried out annually as a minimum. Special attention, including formal staff training, should be in place to assure staff can safely use equipment connected with special hazards, such as ultra-violet light, lasers, radioisotopes, liquid nitrogen and extreme temperatures and pressures (e.g., autoclaving, use of pressurized gas).

Chemical hazards

The cell and tissue culture laboratory is not a particularly dangerous place to work with regard to chemical hazards. This is not different for laboratories using PSC or microphysiological systems, but for completeness some basic aspects are recapitulated here. Some chemicals have ill-defined or unknown biological effects, so general safety standards should always be maintained to protect workers against these uncertain hazards. Material Safety Data Sheets (MSDS) for all chemicals used in the laboratory should be requested from the suppliers and used in risk assessment. For any substances that are potentially hazardous to health (e.g., mutagens, cryoprotectants, labelling dyes), MSDS data should form the basis of a risk assessment for the use of this chemical. However, the level of risk will vary, depending on, for example, the quantities being used, their formulation and how they are used in the laboratory. This may be covered by national legislation in some countries. Approved waste disposal procedures should always be followed. Particular care should be taken with certain kinds of materials, such as teratogens where there are female workers of reproductive age.

Materials being tested in *in vitro* toxicity tests represent a particular problem, particularly if the study requires that they be anonymously coded and supplied via an independent, external source. Although the concentrations used in the final test solutions may be very low, the storage of the bulk material and its handling can represent a significant potential hazard particularly if blinded. It should always be possible to break the code quickly in the event of an accident.

Biological hazards

Many different issues related to potential biological hazards (e.g., infectious agents, mitogens, allergens, cytotoxins) must be considered and, in certain cases, may need to be monitored and recorded in the cell and tissue culture laboratory. Risk assessments for primary tissues/cells and cell lines should include special infectious hazards that could arise from the species of origin. In general, human and primate cells, thus also especially hESC and iPSC, are considered of highest risk (Doblhoff-Dier and Stacey, 2006), although it is important to bear in mind that cells from other species can also harbor serious human pathogens. The health status and geographical origin of human cell or tissue donors should also be considered and donor-screening procedures, such as virological screening for key pathogens and life-style questionnaires can be useful to assist risk assessment. For all sources of cells, the availability of data from microbiological screening tests will help to mitigate risk and the culture and storage history may be useful in flagging up potential hazards from reagents and co-stored materials (Frommer et al., 1993). Although not usually dangerous to the laboratory user, cells and tissues have the potential to permit the replication of viruses potentially pathogenic to humans (occasionally with tragic consequences (Lloyd et al., 1984), and should therefore be routinely treated as if they are a potentially infectious (Tab. 7 of Coecke et al., 2005).

In the case of cells intended for transplantation there are established requirements for donor selection, processing, testing,

storage and supply (EU, 2006a; FDA, 2001), however, cells intended for more complex therapies and particularly involving cell culture and application in large numbers of patients, it is wise to consider additional microbiological risks[16]. It may not be possible to screen for all potential contaminants for practical reasons of time and costs and whilst new molecular techniques such as massive parallel sequencing may offer more economic solutions, they have yet to be standardized for routine use. However, approaches that may be employed in risk assessment include post-donation donor assessment for acute infections and consideration of factors (see above) that elevate risk of contamination by viruses, which may replicate in cell culture and/or may cause human cell transformation[20].

In a number of cell culture procedures, the cell type of interest is cultured on a "feeder layer", i.e., another cell type that supports its growth. Feeder cells may be primary cell cultures derived from tissue or a cell line, which are treated to inhibit their division (e.g., mitomycin C, γ-irradiation). Such cell cultures are a potential source of contamination and should be prepared as cryopreserved cell stocks and subjected to quality control and safety testing according to the same principles applied to other cell cultures.

All cells and tissues new to the laboratory should be handled under a strict quarantine procedure, including suitable precautions to prevent the spread of potential contamination, according to the general guidance given in Table 7 of Coecke et al. (2005), with additional controls as necessary (such as the use of separate dedicated media and equipment, and work by dedicated staff). Microbiological horizontal laminar flow cabinets should not be used when handling cells, as such cabinets are designed to protect only the work area and the airflow is directed toward the worker and would expose them to any contaminants in the cell culture.

Where the nature of the work involves a significant risk of a biological hazard, special precautions must be taken in accordance with national requirements. Where infectious organisms are concerned, these are often based on the World Health Organization classification for human pathogens (Appendix 3 of Coecke et al., 2005).

If the cells or tissues originate from a certified source, such as a recognized cell bank, which provides certification of freedom from certain contaminants, this documentation may suffice for risk assessment, provided that the cells have not been exposed to potential sources of contamination since leaving the bank. However, it is recommended that, as a minimum, mycoplasma testing should be carried out on all samples received.

Laboratory workers' immune systems may not protect them against the tumorigenic growth of their own cells, which may be altered by *in vitro* procedures such as transformation, immortalization, infection, or genetic modification. Accordingly, most national guidelines make it unacceptable for operators to culture cells or tissues derived from themselves or from other workers in the same laboratory, nor to genetically manipulate such cells or tissues, or treat them with potentially pathogenic organisms.

Many countries have national safety committees, which establish guidelines for work with genetically modified organisms (GMOs) and require scientists to classify and perform their work at the appropriate biosafety level. Recombinant cells, (i.e., those produced by genetic engineering or genetic modification [terms used to cover most techniques, which artificially alter the genetic make-up of an organism by mixing the nucleic acids of different genes and/or species together]) will generally fall within the requirements of such guidelines. Viral transformation that occurs *in vivo* may also be considered genetic manipulation when performed *in vitro*, such as Epstein-Barr virus transformation of blood cells. The classification and control of this kind of work differs between countries, and countries may decide to classify work at a higher or lower level when new information on a particular vector/host system becomes available (see EU GMO contained use regulations: EU, 2009). Risk assessment is clearly a dynamic process that has to take into account new developments and the progress of science. It is the responsibility of the scientists involved to keep up to date with developments in this expanding field of activity, and at all times to respect national and international guidelines and requirements.

With specific respect to iPSCs, it is important to note that where the recombinant vectors remain in the genome they will remain subject to GMO regulation when used but also when stored or disposed of in many countries. Where vectors are non-integrating there must be evidence to assure removal of the recombinant DNA components to enable them to no longer be subject to GMO regulatory controls for those vectors.

7.3 Risks to the environment

There are no special concerns with PSC and microphysiological systems with respect to the environment compared to traditional cell culture approaches. Some general aspects are summarized here for completeness. Pathogens or genetically modified organism may present a risk to the environment where they are able to survive. Such risks would be increased where there are poor sterilization and waste disposal practices, leading to contamination of water, air or soil, or escape from containment. The environment can also be contaminated by release of biological material resulting from accidents, including transport accidents, and systems should be put in place either to prevent or minimize the potential for such events. Support from the local biological safety officer should be sought, if available.

Waste disposal

Methods of waste disposal appropriate to the work in hand must be identified during the risk assessment process. These methods must not only protect the individual tissue culture workers themselves, but also their colleagues, the wider population, and the environment. Work with known pathogens and GMOs must be performed according to the relevant regulations (see above), including methods of waste disposal. Where methods are not

[16] SaBTO - Advisory Committee on the Safety of Blood Tissues and Organs (2014). Donation of Starting Material for Cell-Based Advanced Therapies. London, UK: Department of Health. http://bit.ly/2gXXY3O

[20] https://www.cbd.int/abs/about/

specified in these regulations, there is a requirement to assess and justify all proposed methods of waste disposal as part of the risk assessment. Similarly, the appropriate method of disposal of hazardous chemicals must be identified before work with them is undertaken.

In line with the above precautionary principle, the following minimum precautions should be taken when disposing of waste from the cell culture laboratory:

- all liquid waste, with the exception of sterile media or solutions, should be either chemically inactivated (using sodium hypochlorite or another suitable disinfectant) or autoclaved before disposal; and
- all solid waste contaminated with tissue culture liquid and/or cells should either be autoclaved at the laboratory site, or should be placed in rigid, leak-proof containers before being transported elsewhere for autoclaving or incineration.

Transport

The transportation of any biological materials, chemicals (including liquid nitrogen) or other materials (for example, dry ice) of potential risk to humans, animals, plants and/or the environment, must comply with national or international regulations (see, for example, http://www.iata.org/whatwedo/dangerous_goods). They should be packed so as to prevent spills in the case of breakage, be correctly labelled (with appropriate hazard symbols), and have the appropriate accompanying documentation (MSDS, import form, export form, and CITES permit, if applicable). A typical MSDS for a cell line is shown in Table 8 of Coecke et al. (2005).

A cell culture may fall into any one of the classes of biological material used for shipping purposes, namely:

- diagnostic specimens;
- infectious specimens;
- biological products; or
- GMOs.

Wherever appropriate, the International Air Transport Association (IATA) guidelines should be followed, as they are stringent and are recognized internationally (for regular updates, see http://www.wfcc.info). Before arranging transport, the various legal requirements for export and import into the recipient country should be considered, including ethical issues (such as the use of human cells or tissues of embryonic origin), disease transmission, endangered species regulations (http://www.cites.org/), and bioterrorism regulations (see http://www.bt.cdc.gov/).

8 Principle 5: Compliance with relevant laws and regulations, and with ethical principles

8.1 General considerations

From an ethical and legal point of view, it is important that high standards for cell and tissue culture should be established and maintained for the derivation and use of iPSC lines. Whilst GCCP is not in itself required under any national laws, various guidelines, regulations and laws are in place for the procurement, use and storage of donor cells and tissues, their genetic manipulation, other safety issues and development of biomedical products. Thus, any researcher proposing to generate hESC or iPSC lines must ensure that all national laws and regulations and local organization rules are complied with for the relevant jurisdictions of origin of the cells and where they are to be used.

8.2 Human tissues and ethical issues

All tissues or cells from human donors should be obtained using applicable ethical procurement procedures to assure they are obtained with appropriate and well-documented informed consent. The requirements for acceptable informed consent may vary from one legal jurisdiction to another and the researcher responsible for obtaining the donor material must ensure that they have complied with all applicable laws, regulations and local rules. It is also important to note that consent may need to be specific and include consent to carry out genetic testing, and some donors may have applied constraints on the use of their tissue, which may prohibit certain types of research. In some countries there are stringent legal requirements for procurement of tissues for research (e.g., Human Tissues Act (UK, 2004)); in the EU, legislation is under discussion[17].

For human embryonic stem cells, there may be controls and in some cases prohibition on both the procurement and the use of the original donor tissues/cells and on the generation and use of the cell lines. For further information see Andrews et al. (2015) and Seltmann et al. (2016).

In Europe, there is specific legislation for the import and export of tissues for clinical use (EU, 2006a,b,c, 2012), which also has technical annexes that prescribe aspects of cell and tissue procurement, processing, storage and testing. Requirements vary around the world. Competent couriers are critical to efficient shipment, and it is recommended to use couriers that have good knowledge of local requirements for import and to have service level agreements in place with couriers that identify standards of service and emergency procedures when cryogens become depleted.

Human tissue banks should be recognized as the most legally and ethically acceptable source of human tissue for research, as they are best equipped to deal with, and advise on, the complex issues involved, including ethics, consent, safety and logistics. However, many companies now provide human tissue on an international basis and researchers should ensure that any supplier meets national and local ethical procurement and personal data protection requirements (see above and 5.3 below).

Blood and skin cells are commonly used to derive new iPSC lines. The removal of such samples from human volunteers should only be performed by qualified personnel, and particular precautions should be followed to minimize any risks. Such volunteers should also be considered to be donors, and documented informed consent will be required.

[17] http://cordis.europa.eu/result/rcn/91320_en.html (last accessed 04 July 2013)

8.3 Regulation of human iPSC lines for use in human treatments

Where there is intent to use donor cells/tissues in humans or to establish a cell line for human application, these may be subject to legal requirements and regulations. Such regulation is in place for Europe under the European Union Tissues and Cells Directive (EU, 2012) and separate regulation may apply in other jurisdictions (e.g., FDA, 1997, 2001, 2013). Use of iPSC or any cell lines for the manufacture of cell therapies or cell products will be regulated in the European Union as an Investigational Medicinal Product (IMP) or an Advanced Therapy Medicinal Product (ATMP) (EU, 2007) and subject to market authorization by the European Medicines Agency (EMA), achieved following clinical trials (EU, 2001c). In the US, similar regulation applies under the FDA (2013, 2015a, b, c). For further specific consideration of the requirement of establishing hPSC stocks for clinical application see Andrews et al. (2015) and the appendices therein.

8.4 Donor-sensitive data

Data held on donors of tissue used to generate iPSC lines, depending on its nature, may also be subject to legally binding regulation, which in the European Union is the EU Directive on Data Protection (EUDDP) and in other jurisdictions similar regulation may apply, e.g., US (FDA, 2001; FDA, 2010b). At the time of writing the EUDDP and respective US regulation are undergoing revisions, which will apply to cell/tissue donors for iPSC generation. In general, where specific regulation is not applied, compliance with good practice in this area is recommended[18]. In some countries, additional controls on donor information may also apply, such as the Caldicott Principles in the UK (Caldicott, 2013) and in the UK best practice guidance has been established to allow compliance with EU regulation. Important ethical problems can be faced especially with the iPSC technology, where cell donors are often still alive and can possibly be identified.

8.5 Non-human cell cultures and materials

In general, the use of animals in experimentation should be subject to the 3R principles (reduction, refinement and replacement) (Russell and Burch, 1959). However, where non-human cells are used as feeder layers to support the growth of iPSCs, the original tissues (typically mouse embryo origin) should be obtained using good practice for the maintenance of laboratory animals (European Directive 2010/63/EU (EU, 2010); NRC, 2013), which includes colony screening to exclude presence of key pathogens and the use of ethically approved procedures. Such requirements will usually require the lab isolating the animal tissues to have a license for the procedures, staff and laboratory facility.

Reprogramming has been used to produce iPSCs from an ever-increasing range of non-human species. Researchers doing such work should ensure that the procurement of tissues from the particular indigenous species meets national laws and if relevant, any requirements of the international Convention on Biodiversity[19], which may involve additional legal requirements involved in the Nagoya Protocol[20]. Special safety measures relating to potential carriage of unusual pathogens may need to be addressed.

Other international treaties may also impact on the transfer of certain cell lines based on potential animal virus contamination, use in the manufacture of biowarfare agents, etc. Such constraints on shipment will need to be checked on a local basis in discussion with national or regional authorities.

Use of certain animal derived products also raises ethical and legal issues. For example, the manufacture of fetal calf serum is ethically questionable (see references in Coecke et al., 2005) and many non-sterilizable materials of animal origin may raise issues of infectious disease, which are controlled internationally and for which import restrictions apply to certain animals and materials including cell lines (see Festen, 2007).

8.6 Genetically modified organisms

The creation of iPSC lines involving the introduction of recombinant DNA vectors means that such cells are considered genetically modified organisms (GMOs) and their creation, storage, transport, use and disposal are subject to the requirements that apply to other GMOs. Even systems where the vectors are removed (e.g., baculovirus systems) or do not become integrated into the genome but may persist in other forms in the cell (e.g., Sendai virus vectors, episomal vectors, modified mRNA/miRNA) may still be considered to be genetically modified, as would cells modified by gene-editing techniques. However, purely chemical means of inducing pluripotency are unlikely to be included in this group. Any viral vectors used should be modified to prevent release of infectious virus from reprogrammed cells and this should be checked as part of normal laboratory risk assessment procedures. This is a rapidly expanding field, and since it involves manipulating genes and cells in ways that do not occur in nature, for which the long-term consequences are as yet unknown, it raises sensitive ethical and safety issues (Hinxton Group statement on gene editing of 2015[21]). Genetic manipulation experiments are regulated in the EU (EU, 2001a), USA (FDA, 2015a,b,c) and in many other countries, where, before any work is initiated, relevant approval must be sought.

8.7 Other considerations for the selection and use of iPSC lines

General considerations for the selection of iPSC lines have been reviewed by Stacey et al. (2016). In addition to the issues described above, the ownership of lines may mean that there are

18 http://www.wellcome.ac.uk/About-us/Policy/Spotlight-issues/Data-sharing/EAGDA/index.htm

19 http://www.cites.org/

20 https://www.cbd.int/abs/about/

21 https://www.crick.ac.uk/media/256630/hinxton-2015-statement-100915.pdf

restrictions on their use even for research purposes. Ownership of cell lines can be complicated with many parties involved in negotiation on their use, including the hospital authority and clinicians where the original tissue sample was taken, the scientists engaged in deriving and researching the cell line, the institution that hosted the research and the sponsors (e.g., funding bodies, collaborating commercial companies). Signing of material transfer agreements could leave the researcher personally exposed to legal action should they contravene the conditions of the agreement. Accordingly, signing of such documents should not be undertaken lightly and it is strongly recommended that the researchers should consult their local legal or technology transfer office, as a legal representative of the host organization may also be required to review and sign such agreements.

Application for patents on processes using iPSC lines may also require submission of stock of the lines for independent scrutiny under international agreements (Anon, 1980) and researchers should be prepared for this requirement to avoid delays in critical stages of exploitation.

Furthermore, iPSC lines, in being potentially capable of replicating tissue-like cells and structures, could be valuable in growing significant quantities of pathogenic organisms and may therefore become subject to international controls on materials of potential use in the manufacture of biological weapons.

9 Principle 6: Provision of relevant and adequate education and training for all personnel to promote high work quality and safety

The range of applications for cell culture is expanding rapidly and involves an ever-broadening range of technical manipulations (such as chemically induced and genetic modifications) for use in basic and applied science, manufacturing, diagnosis, and efficacy and safety testing procedures, as well as for providing therapeutic materials. Work with hESC, iPSC and MPS is especially demanding and creates even stronger training needs.

The competence of staff to perform their duties in a laboratory is central to ensuring that work is performed according to the standards of the organization in relation to its scientific, legal and safety requirements and obligations. This requires education and training, as well as the regular monitoring of performance (Tab. 9 of Coecke et al., 2005).

A good basic education should be given in the nature and purposes of cell and tissue culture, which is an essential basis for any further training program. The basic principles of *in vitro* work, aseptic technique, cell and tissue handling, quality assurance, and ethics should be included. It is also important that those working with material of animal or human origin should have a sufficient understanding of any additional laws or regulations that will apply.

Training should be seen as an ongoing process for improving and developing practical skills, and maintaining competence. Given its critical importance to the success of any laboratory work, there should be a formally documented training program for all members of staff, including training records and regular reviews of training needs. To ensure the quality of work in the long-term, it is also important to link training with a personal development program for technical and scientific staff, in order to ensure they are progressively trained and educated in line with changing laboratory activities and demands.

When new staff join a laboratory, their skills and experience should be assessed, and the need for further training procedures in relation to their new job should be identified. These needs may include a variety of general and specific procedures, covering SOPs, general laboratory maintenance, and safety and emergency procedures.

Training can be provided in-house by experienced members of staff and/or visiting experts, via accredited on-line programs and/or through attendance at external courses. For certain applications including product manufacture and testing, and processing of cells and tissues for clinical use, training must be formally recorded and reviewed.

In the following sections, a number of education and training needs specific for PSC and microphysiological systems are reviewed.

9.1 Colony identification and selection

Colony identification and selection is a tedious process. It usually takes three to four weeks to generate iPSC colonies by a reprogramming method. Human iPSC colonies have a unique morphology as they grow as compact colonies and exhibit high nucleus-to-cytoplasm ratios. Different tests that can be used to detect undifferentiated colonies; however, it is not always possible to perform these. Therefore, experience and training to identify colonies is required. In addition, iPSCs initially created in a feeder-dependent system may take several passages to fully adapt to a new feeder-free culture system. The training of colony identification and selection will focus on 1) colony morphology, 2) immunofluorescence microscopy, and 3) selecting colonies to minimize carryover of MEFs and/or parent cell type.

9.2 Minimizing differentiation in newly derived iPSC cultures

During the stabilization phase of iPSC line development, some iPSC colonies generate fibroblast- or endothelial-like cells. It is critical to remove differentiated cells early and prior to any passaging to prevent overgrowth of differentiated cells and to maintain the undifferentiated state of iPSCs. The training for elimination of differentiation in the PSC colonies should focus on: 1) identification of iPSC colonies, 2) identification of differentiated cells, and 3) removal technique for differentiated areas.

9.3 Quality of colonies, confluence and passage

During the iPSC culturing process, the quality of the colonies is one of the most relevant aspects that should be controlled. Undifferentiated iPSCs grow as compact colonies and exhibit high nucleus-to-cytoplasm ratios and prominent nucleoli. During the expansion and maintenance of iPSCs, differentiation of iPSCs may occur. It is easy to distinguish iPSCs from differentiated cells: Differentiated cells have less-defined edges, loose mor-

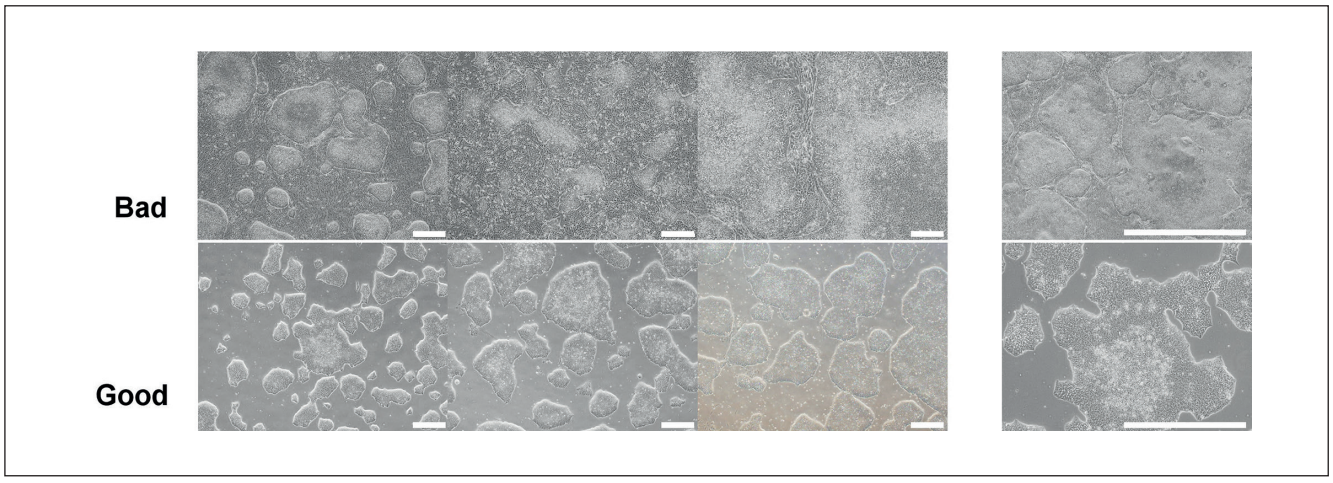

Fig. 2: iPSC examples colony morphologies which are unacceptable and acceptable for passage or preservation
A) shows examples of colony morphologies that would be unacceptable for passage or preservation, i.e., colonies with regions with spontaneous differentiation. B) Shows examples of optimal (panel B1-3 from left to right) and acceptable (4th panel left to right) morphology colonies. Bars represent 500 µm.

phology, dark areas, or exhibit fibroblast- or endothelial-like morphology. Identification and removal of the differentiated areas requires training and some experience. It is critical to change the medium and monitor cell growth daily, and to passage iPSCs regularly. Cell overgrowth will result in loss of pluripotency and differentiation potential, and trigger spontaneous differentiation of iPSCs (Fig. 2).

The most relevant points to focus on in the training will be: 1) colony morphology, 2) identification of differentiated areas, 3) removal technique for differentiated areas, 4) estimation of cell confluence, 5) making iPSC aggregates, and 6) timing of passaging iPSCs.

9.4 Photography and documentation of iPSC culture

Morphology and confluence of iPSCs needs to be followed and documented. This requires a portable microscope placed inside a biosafety cabinet or use of an inverted microscope with a camera on the lab bench. During development of iPSC lines, it is recommended to record media change logs and to photograph iPSC colonies at different magnifications over time. For creation of an iPSC batch file and documentation, the training will include: 1) creation of forms and working instructions for iPSC culture, 2) recording media change dates and media lots, 3) photographing iPSC colonies at 4x and 10x magnifications, and 4) adding pictures to iPSC documentation.

9.5 Viability and acceptability after recovery

Unlike standard cell culture, a single iPSC suspension does not survive well after seeding. Therefore, post-thaw recovery quality control is based on iPSC colony numbers after seeding instead of post-thaw viability. The training for thawing of cryopreserved iPSCs should focus on: 1) thawing procedures, 2) colony count, 3) seeding density.

9.6 Differentiation techniques

An iPSC may carry a genetic "memory" of the starting cell type and this "memory" may influence its ability to be reprogrammed and affect its efficiency of differentiation. Besides iPSC line-specific differentiation potential, not all iPSC clones from the same type of starting cells behave similarly regarding differentiation potential. For training of differentiation techniques, the focus will be on: 1) screening multiple iPSC lines with different parental cell types for differentiation studies, 2) optimization of differentiation protocols, 3) functional assays of differentiated cells.

9.7 Microphysiological systems

While *in vitro* cell-based systems have become an invaluable tool in biology, they often lack physiological relevance. The recent progress in microphysiological systems (Marx et al., 2016) has enabled manipulation of the cellular environment at a physiologically relevant length scale, which has led to the development of novel *in vitro* organ systems. The training on microphysiological systems will focus on 1) iPSC differentiation protocols, 2) co-culture of differentiated cells, 3) use of microfluidic devices.

9.8 Quality control (Q)C standards and reference iPSC lines

It is well documented how to create patient-specific iPSC lines by using different reprogramming methods and different starting cell types. However, there are pronounced differences in differentiation potential among iPSC lines. Therefore, there is a great need for establishment of QC standards and control of iPSC lines worldwide to ensure both reproducibility and consistency in basic research and clinical applications of iPSCs. The training in QC standards and reference iPSC lines will be on: 1) QC testing standards, 2) validation of iPSC culture media and reagents, 3) reference iPSC lines.

10 Conclusions

The development of GCCP (Coecke et al., 2005) has contributed to the quality assurance of cell culture work. The increasing use of stem cell-derived systems and more organotypic culture methods requires an update, especially as unique procedures and tests are required. The complexity of model systems and long-term culture needs further add to quality needs. They also imply higher costs and difficulties regarding extensive replicates as well as replication by others. Quality control is therefore paramount to ensure the validity of results.

The use of human cells implies a higher risk of human pathogens and necessitates strict adherence to the respective safety measures and assurance of ethical provenance. These are both especially important with iPSC technology, where increasingly blood samples are used for reprogramming and cell donors are often still alive and could possibly be identified through publication of certain data. Control of raw genetic data and other patient-sensitive information have to be carefully considered in the best interest of the donor and to assure the research is not discredited as unethical.

Proper training is mandatory for quality of work, to protect personnel, to avoid wasted time and resources and to help assure adherence to ethical and legal standards. Stem cell work is often more demanding in this respect than traditional cultures and might require additional training also for experienced researchers and technicians.

Many aspects of these new model systems are no different from traditional cell cultures. They were briefly summarized here for completeness of this report as a stand-alone document. A revised GCCP, working title GCCP 2.0, shall combine these aspects, update the original guidance and expand to other aspects such as the use of primary human tissues. This step toward GCCP 2.0 is paralleled by the establishment of an International GCCP Collaboration (GCCPC)[22], for which a secretariat is provided by CAAT at Johns Hopkins University. Interested parties are invited to contact the center (caat@jhsph.edu). This initiative aims for the development and implementation of cell culture quality standards in research and development as a prerequisite for reproducible, relevant research as an alternative to animal testing.

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Conflict of interest

No conflict of interest to declare.

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