Pamies et al.:

Guidance Document on Good Cell and Tissue Culture Practice 2.0 (GCCP 2.0)

Supplementary Data

Appendix 1: Examples of immortalization techniques and considerations for *in vitro* use[a]

Technique	Cell intervention	General features/examples
Culture of cancerous tissue or cells	Transformed cells emerge and further genetic variants may become established as a cell line.	Exhibit features of cancer cells and are often genetically unstable Numerous examples from leukemias (e.g., HL60, K562) and solid tumors of different kinds (e.g., HeLa, MCF7)
Bulk culture of non-diseased tissue or cells	Apparently non-malignant cells may emerge and become established as a cell line (NB not finite senescent cultures).	Uncertain origin and mechanism of immortalization Retain certain features of tissue cells but may also be prone to alteration. May or may not be genetically stable. A few examples include Vero, CHO, BHK, MDCK, NIH/3T3.
Exposure of cells and tissues to irradiation and/or treatment with chemical mutagens or carcinogens	Primary cells or finite cell lines exposed to irradiation or chemical mutagens undergo genetic mutation.	Relatively few human cell lines have been established by these techniques; irradiation appears to extend culture lifespan of human cells rather than create continuous cell lines. Examples include KD cells.
Isolation from cultures infected with viruses (for example, Epstein-Barr virus)	Cells are infected with native infectious virus that causes cell transformation and maintenance of cell replication.	The cells tend to show transformed phenotypes that may be similar to cancer cell lines. Probably the most common examples include EBV-induced lymphoblastoid cell lines. Isolated cancer cell lines may also harbor viral genes, which may be the cause of cell transformation such as the papilloma virus genes in HeLa cells, hepatitis B genes in HepG2 cells.
Genetic modification of cells by transformation with cloned genes	Specially designed recombinant DNA vectors or viral sequences are transformed into cells where they affect normal cell cycle mechanisms.	Numerous features of the primary cells may be lost. A commonly used cell line is HEK293. Examples of key cell transforming genes used are SV40 T, Adenovirius-5.
Transfection with telomerase to immortalize the cells	Recombinant DNA vector transformed into the cell expresses human telomerase reverse transcriptase (hTERT), which repairs telomeres and maintains chromosomal integrity and the ability of primary cells to replicate.	Can create stable cell lines with native features of the original cells. Some cases have required supplementary gene transformation such as SV40 T antigens. Although these are described as immortalized, culture collections usually describe them as "extended lifespan".[b] There are numerous examples of epithelial, endothelial, fibroblast and neural cells generated by hTERT held in culture collections.[b]
Isolation of embryonic stem cell lines from blastocysts	Typically, human embryonic stem cells are isolated from the epiblast of the inner cell mass of blastocyst cultures, or cells are taken from pre-blastocyst morula or parthenogenetic oocytes.	Provide stable cultures of pluripotent stem cells which in principle can be differentiated into any cell of the human body. Can self-organize into complex *in vivo*-like organoid structures. Can be technically challenging to obtain reproducible differentiated cultures. Differentiation can take months to complete. Many examples available.[c]
Single cell nuclear transfer (cloning)	Somatic cell nuclei are injected into an enucleated oocyte and cultured *in vitro* to generate an hESC-like culture.	This method in theory enables the bespoke production of cell lines from individuals with genotypes of scientific interest or personalized cell therapies. The efficiency of generation of hESC-like cultures is very low. Technical challenges are similar to those of hESC lines (see Tab. 2).

doi:10.14573/altex.2111011s

Reprogramming of somatic cells into stem-like cells	Somatic cells are returned to an embryonic epigenetic state by reprogramming factors introduced to the cells by recombinant DNA vectors using ectopic expression of embryonic transcription factors or protein inducers of reprogramming.	This method enables the bespoke production of cell lines from individuals with genotypes of scientific interest or personalized cell therapies. DNA vectors may become integrated in multiple locations and cause disruption to normal cell function. If cell lines retain modified genes or viral vectors, they are usually considered GMOs and systems designed to lose viral vectors (such as Sendai vectors) may have persistent virus. Multiple isolates, even from the same tissue sample, may show different functionality and more than one clone should be used to draw conclusions on the nature of the donor's disease or syndrome. Challenges with these cell types are similar to hESC lines (see Tab. 2)

[a] Some continuous cell lines are known to contain stem cell or precursor cell populations. For this guidance, these are not included as stem cell lines. The exact nature and significance of the apparent stem cell component in such lines remain to be determined in many cases. [b] https://www.atcc.org/ [c] https://www.wicell.org/ and https://www.nibsc.org/ukstemcellbank

Appendix 2: Challenges and issues in the culture and characterization of PSCs

Challenge/issue	Description
Scientific quality	– PSC lines typically express pluripotency and self-renewal genes (e.g., POU5F1, SOX-2 and NANOG) and also a common set of markers from early development which include SSEA-3, SSEA-4, TRA-1-60 and TRA-1-80. – There are currently no assays of pluripotency for PSC lines which can incontrovertibly confirm their pluripotent nature. The application of PluriTest or PluriTest in combination with a scorecard assay are used for pluripotency testing and are considered a powerful replacement of teratoma assays (International Stem Cell Initiative, 2018). However, for mouse iPSCs this can be confirmed in progeny by a germline complementation assay. PSC-related markers used to phenotypically characterize these cells identified to date may be necessary but are not sufficient to confirm pluripotency. Typically, *in vitro* cell differentiation assays (e.g., embryoid body formation, chemically induced differentiation) are still required to confirm pluripotency. – Continued expression of the reprogramming factor vectors after an iPSC line has been established may impact the pluripotent nature of the cells and their ability to differentiate. It is important to check that such expression does not persist once the iPSC has been established.
Potential safety issues	– Persistent expression of reprogramming vectors will require their continued control as GMOs. – Tumorigenicity and genetic variants would need to be considered for the development of a cell line for cell therapy products.
Technical challenges	– hPSC cultures often show morphological evidence of so-called "spontaneous" differentiation, and such differentiated cells may affect the quality of scientific data for study of both stem cell biology and cell differentiation protocols. – Some variation may be observed between iPSC lines from the same donor sample, and journals and reviewers may request to see data from a number of clones. – Currently, a range of serum-free and feeder-free culture methods are being used for hPSC culture. This can make comparison of data between publications difficult. Also, the relative benefits of different media and matrix combinations, particularly in relation to stem cell culture stability and quality, have yet to be fully resolved, so no single expansion system can be recommended.

Appendix 3: GCCP Acceptance criteria (GAC) for fundamental aspects of cell culture systems

Feature of the cell system	Fundamental scientific requirements	Approach to establish GACs	Examples of parameters for demonstration of GACs[a]
Viability	Understanding of the biological nature of the cell type and utility of the cell system	Multiparametric approach may be important to demonstrate comparability between "viable" cells and those required for the intended application (may be crucial for appropriate functional GACs).	Membrane function, cell death including apoptosis (e.g., Caspase 3 or Fas ligand activation) and necrosis, cell morphology, cell cycle state (combined membrane integrity, metabolic activity, and nucleic acid content)
Proliferation and growth rate	Understanding of the nature of the cell culture	Focus on parameters that identify the cells in an appropriate state of active cell proliferation and/or growth rate. Avoid reliance on cell enumeration alone or passage number. May need novel assays for cells expanded other than as adherent layers, suspensions, or colonies.	Population doubling time of viable cells % KI67 positive cells Incorporation of radiolabeled thymidine, bromodeoxyuridine (BrdU) or 5-ethynyl-2'-deoxiuridine (EdU) Early apoptosis markers (see viability above) Cell cycle state such as flow cytometry for DNA ploidy using nucleic acid content using dyes e.g., propidium iodide, 7-aminoactinomycin-D (7-AAD), Hoechst 33258
Identity, variability, and stability	Cell identity and origin must be known, and variability and stability must be understood.	Selection of most informative parameters for 1) cell identity, 2) variability, and 3) genetic stability and variant clones. Include negative GAC such as presence of unwanted populations.	1. Assays for cell line cross-contamination: DNA STR profiling, transcriptome and immunophenotype (flow cytometry, immunohistology) 2. Assays for cell antigens: flow cytometry, transcriptome 3. Karyology, single nucleotide polymorphism array, whole genome sequencing
Function	Understanding of the biological function.	Establish QC assays that replicate the desired function for final use. These may be performed in parallel with the experimental use of the cells or on seed stocks or passaged stock cultures to check for retained functionality.	Physiological function, e.g., transepithelial or transendothelial electrical resistance (TEER) and permeability of paracellular markers, neuron action potentials Pharmaco-toxicological function such as cytochrome P450 Temporal morphological development, e.g., cell migration, "neural sprout" length, differentiated cell function For other examples, see Stacey et al., 2016a and Section 2.2.
Microbial contamination	Understanding of the likely microbiological contaminants of the cell and media sources	1. Perform tests that are known to be generic for human and general animal cell cultures and for the laboratory environment. 2. Add any additional tests that may be applicable for particular cell cultures based on a risk assessment source and culture history.	1. Generic testing for mycoplasma, and a screen for bacteria and fungi. 2. Risk-based testing: human cell lines are often tested for serious blood-borne pathogens and bovine cell lines and bovine serum for bovine viruses.
Cell engineering	Understanding the nature of the engineering technology involved in techniques such as reporter systems, RNAi gene regulation (double-stranded RNA-mediated interference) and gene editing Understanding of the cell biology required to assure suitability for the selected technology	Each new technology or its application to a new culture system will require reconsideration of GAC. Particular attention is needed to establish GACs for the design and manufacture of core tools used in cell engineering and for the final quality of the cell substrate produced. GACs will also be needed for selection of specific host cell substrates such as those which will respond to RNAi techniques and provide stable knockdown cell lines or yield suitable cells following fusion.	Some examples[b] of issues to consider for GAC setting are: 1. What are the critical molecular tools which require GACs, e.g., DNA expression constructs (e.g., reporter molecules, Cas-9, shRNA, siRNA, pre-miRNA), guide molecules (e.g., guide ssRNAs, enzymes)? 2. What cell substrates are suited to the proposed engineering technique, for example, cells susceptible to RNAi (e.g., HEK293, certain mouse embryonic stem cells, mouse embryonal carcinoma cells) or suitable for hybridoma formation such as immunocytomas, which lack endogenous antibody expression, possess an enzyme defect to permit selection, have good fusion properties, and have an acceptable capacity for antibody secretion? 3. What GACs are required for RNAi knockdown in cell cultures (e.g., Northern blot, reverse transcription qPCR (RT-qPCR))? 4. Do genetic reporters correlate with or alter endogenous expression (qualitatively and

			temporally)? 5. Are genetic reporters affected adversely by cell differentiation or other *in vitro* changes in the cell system? 6. Have genetic constructs affected the desired locus in the intended way (e.g., has short distance and long distance sequencing been performed around the gene edit site)? 7. Have off-target effects in the genome occurred at critical sites? 8. Have on-target effects in the genome occurred at critical sites? 9. Can inducible genes been fully silenced when required? 10. Could the epigenetic status of key genes in the cells be altered significantly? 11. Do the engineered cells retain key features of the parent cells (e.g., differentiation potential, behavior as isogenic controls when gene is corrected, show the same toxicant sensitivity profiles).

[a] See also Table 1. [b] See also Table 2.

Appendix 4: Liquid nitrogen safety

Work with liquid nitrogen probably poses the greatest single threat to the safety of cell culture workers (as gauged by the number of individuals using it and the potential severity of any accident), and for this reason it is dealt with in greater detail here. Details of general hazards, precautions, and first aid can be obtained from the suppliers of liquid nitrogen and of liquid nitrogen vessels (see, for example, Bocgases document on cryogenics[1]). Such relevant information must be obtained, and its contents must be taken into account in the relevant laboratory risk assessments and rules. A printed version of these should be placed in a readily accessible location where it can be rapidly referred to *before* any work using liquid nitrogen is undertaken.

A serious hazard in the use of liquid nitrogen is the risk of asphyxiation due to the displacement of air by nitrogen gas within a confined area. Areas where liquid nitrogen is stored or handled must therefore be well ventilated. In addition, oxygen depletion monitors (wall-mounted and/or worn by staff), which can provide an early warning that the level of oxygen is declining below a safe level, should be used in areas where large numbers of storage vessels are held and/or significant amounts of liquid nitrogen are handled.

Liquid nitrogen is frequently stored in pressurized vessels. Many countries have regulations governing the design, construction, use, maintenance, testing, and other aspects of such pressurized vessels (for example, the UK Pressure Systems Safety Regulations[2]). In countries where no such regulations exist, similar precautions should be taken. In particular, cell culture workers should ensure that they know how to operate such vessels safely (see the user's manual) and must have their vessels maintained and tested on a regular basis. Further useful information can be found at the Health and Safety Executive[3].

Because of the ultra-low temperature of liquid nitrogen (–196°C), it can cause severe frostbite to exposed tissues, particularly if it is caught in loose clothing or shoes or spilled down the cuff of an insulated glove. Therefore, appropriate clothing should always be worn (open-toed footwear should not be worn, and clothing with loose cuffs, pockets and turn-ups should be avoided), with eye protection and insulated gloves (ideally, these should be loose-fitting for ease of removal, be made of impermeable material, and have close-fitting, elasticated cuffs).

Another hazard associated with liquid nitrogen is that it can enter storage vials (due to inadequate sealing) when they are immersed in the liquid phase, and this may cause the vials to explode upon thawing. Therefore, steps must be taken to protect workers from the effects of such an explosion. As a *minimum*, workers must wear a full-face visor, insulated gloves, and a long-sleeved laboratory coat when thawing vials from liquid nitrogen, and other individuals must be kept clear of the immediate area. The vessel containing the liquid in which the vial is being warmed, if judiciously chosen, can be used to further protect the worker by containing any flying debris and/or directing the force of the blast away from the worker.

Such explosions could be particularly dangerous if the vials contained pathogenic material. Therefore, material known to be pathogenic *must not* be stored in the liquid phase of liquid nitrogen, but instead should be stored in the vapor phase. Another reason for this is that transfer of pathogenic material between containers stored in the liquid phase has been documented (Tedder et al., 1995). Clearly, the greatest care must be taken to ensure that storage vessels containing pathogenic material are fully sealed before placing them in storage, and that they will stay fully sealed under the intended storage conditions.

Pressure vessels used to store liquid nitrogen represent a very high potential risk if they become over-pressurized and need to be maintained and monitored by appropriately trained staff.

[1] https://www.boconline.ie/en/images/care-with-cryogenics_tcm674-39400.pdf (accessed December 7, 2021)
[2] https://www.hse.gov.uk/pubns/books/l122.htm (accessed December 7, 2021)
[3] https://www.hse.gov.uk/pressure-systems/about.htm (accessed December 7, 2021)

Appendix 5: Key elements in assuring successful cryopreservation[a]

Variable	Important considerations
Biological and metabolic status	– Cell or tissue type (i.e., gross morphology or complexity of culture system). – Growth phase (i.e., cells harvested during exponential growth to increase trend to have a high nucleus:cytoplasm ratio, which can promote survival). – Status of cells regarding biochemical or morphological features such as low % viability, high levels of apoptosis, complex cell shape (membrane structures), high levels of secretory vesicles and other intracellular vacuoles.
Cryopreservation protocol	– Cryoprotectant (type, concentration and duration of pre-freezing exposure should be established to balance the degree of cryoprotection against any toxic effects, for example, 10% v/v DMSO and pre-freeze exposure for less than 10 minutes). – Additives to improve cell survival (e.g., oligosaccharides, serum, Rho kinase inhibitor). – Cooling rate (for example, freezing at controlled rate in the presence of the selected cryo-protectant: typically -1°C/minute (with 10% v/v DMSO). – Format of cells to be cryopreserved, e.g., single cell suspension or harvested as cell aggregates.
Storage conditions	– Sufficiently low temperature to eliminate biological changes, for example, liquid nitrogen vapor or liquid phase below -150°C. – Stable storage environment assured by regular monitoring and/or autofill for liquid nitrogen systems.
Recovery method	– Rate of warming to achieve thawing. – Gradual dilution to minimize osmotic shock. – Removal of cryoprotectant to avoid toxic effects.

[a] For general references, see Stacey et al. (2016b) and Awan et al. (2020).

Appendix 6: Precautions to promote aseptic handling in BSC Class II cabinets[a]

- Before using the BSC, ensure that it is working correctly. Check the airflow indicators or the negative pressure gauges. Most BSCs are fitted with alarms to indicate any unsafe operation conditions.
- Use appropriate disinfection to decontaminate surfaces before commencing work.
- Ensure that all essential materials and equipment are placed in the BSCs before work is started; this will reduce the risk of interruptions to the BSC air flow during use and will reduce the risk of contamination.
- Do not place too many items in the BSC at any one time, as cluttering the work area may affect the air flow.
- Ensure that a vessel of appropriate disinfectant is on hand, in case of spillages.
- Bear in mind that once the work has started, all materials within the BSC are potentially contaminated and should not be removed until after appropriate disinfection. This includes gloved hands.
- Do not subculture or otherwise manipulate more than one cell or tissue culture system in the BSC at any one time. This is essential to avoid mislabeling, cross-contamination and/or switching of cultures.
- Use separate bottles of growth medium for each cell or tissue culture system as this will prevent the transfer of microbial agents between culture systems or possible cross-contamination.
- Avoid rapid movements and high-energy processes[b], which may interrupt or disturb the air flow in the cabinet, especially at the operator's access point.
- When the work is completed, ensure that all materials and equipment are made safe. Place all materials that need to leave the BSC in appropriate transport containers and disinfect by either spraying or wiping. Disinfect the work area in case of spillage and splashes.
- Depending on the work being carried out, the BSC may need to be decontaminated prior to further work being undertaken[a].
- Leave the BSC running for at least 10 minutes before switching it off in order to remove any aerosols generated during the work.

[a] Class II safety cabinets should be sited, installed, and commissioned according to national regulations. [b] High energy processes may include extremely vigorous shaking and high-speed centrifuges.

Appendix 7: Considerations for quality of composite cell systems

Feature of the composite cell system	Fundamental scientific requirements	Approach to establish GCCP acceptance criteria (GAC)	Examples of parameters for demonstration of GACs
Co-culture of more than one cell type in direct contact or separated (e.g., membrane, filter)	Understanding of the proportion of the different cell types and their interactions Highly structured systems (such as self-organizing organoids, layered skin models and bioprinted models) will require understanding of the developmental process, plus environmental and nutritional needs.	Utilize relevant experiences in blood cell co-culture and tissue slice cultures where GACs have been developed. Special consideration of impact of starting cell materials and their preparation	Quantify ratios of cells in mixture ratios, e.g., microglia:neuronal cells, hepatocytes:non-parenchymal cells, blood leukocytes. Optimized cell ratios for seeding and assay-ready cells State of starting cell preparations
Differentiation of stem cells into mixed cell types	Understanding of the temporal profile of differentiation, its control by medium additives, and the nature and function of the populations arising (both desired and unwanted)	Special consideration of impact of starting cell materials and their preparation Optimize and control culture conditions. Consider functional GACs for composite cell preparation.	Requires fit-for-purpose functional GACs for each starting cell culture and batch of final cell composite preparation.
Modelling vascularization	A special form of co-culture where endothelial cells interact with other cells to vascularize organoids	Co-culture aspects as above are important. Vascular function parameters will need quality control and setting of GAC.	GACs may be needed for vascular resistance, tissue oxygenation, nutrient supply.
Modeling immune and inflammatory responses	Inflammatory reactions triggered by cell-cell interactions	Consider issues of cell compatibility (i.e., from different donors) that may require control and setting of GAC. Special consideration of potential inflammatory states induced purposefully or involuntarily Consider cell sources that may contain immune cells.	GACs for relevant inflammatory activation states as positive or negative GACs GACs for immune cells in starting cell materials (e.g., lymphocytes, activated cells of the innate immune system)

Appendix 8: Scientific Advisory Committee (SAC)

Name	Affiliation	Country
Alves, Paula M.	iBET and NOVA University Lisbon	Portugal
Askevik, Kaisa	The Swedish 3Rs Center	Sweden
Barenys, Marta	University of Barcelona	Spain
Bolognin, Silvia	Developmental and Cellular Biology	Luxembourg
Bunger, Maureen	Lonza	US
Chen, Kevin G.	NIH Stem Cell Unit, NINDS, NIH	US
Csontos, Lynn	Stemcell Technologies	UK
DeGeorge, George	MB Research Labs	US
Devos, John	Inserm	France
Ding, Jinfeng	Chinese Academy of Sciences	China
Fant, Kristina	RISE - Research Institutes of Sweden	Sweden
Fitzpatrick, Suzanne C.	US Food and Drug Administration	US
Fritsche, Ellen	IUF - Leibniz Research Institute of Environmental Medicine	Germany
Ghosh, Baishakhi	Johns Hopkins University	US
Giese, Christoph	ProBioGen AG	Germany
Gordon, John D.	U.S. Consumer Product Safety Commission	US
Grillari, Regina	Evercyte GmbH	Austria
Gutleb, Arno	Luxembourg Institute of Science and Technology (LIST)	Luxembourg
Hao, Jie	National Stem Cell Resource Centre, Chinese Academy of Sciences	China
Healy, Lyn	Francis Crick Institute	UK
Hill, Erin	Institute for In Vitro Sciences	US
Inutsuka, Takashi	Pharmacological Evaluation Institute of Japan	Japan
Jennings, Paul	Vrije Universiteit Amsterdam	The Netherlands
Kanda, Yasunari	NIHS Japan	Japan
Kandarova, Helena	Dept. of Tissue cultures and Biochemical Engineering	Slovakia
Kasper, Cornelia	University of Natural Resources and Life Science	Austria
Knezevic, Ivana	World Health Organization	Switzerland
Kurtz, Andreas	Fraunhofer-Institut für Biomedizinische Technik (IBMT)	Germany
Kopanska, Katarzyna	Competence Center TEDD, Zurich University of Applied Sciences	Switzerland
Lakshmipathy, Uma	Thermo Fisher Scientific	US
Lewinski, Nastassja	Virginia Commonwealth University	US

Lutolf, Matthia	EPFL	Switzerland
Mah, Nancy	Fraunhofer-Institut für Biomedizinische Technik (IBMT)	Germany
Maria Canals Coll, Josep	University of Barcelona	Spain
McCullough, Shaun	U.S. Environmental Protection Agency	US
Merten, Otto-Wilhelm	Miltenyi Biotec	France
Morales Pantoja, Itzy	Johns Hopkins University	US
Munic Kos, Vesna	Karolinska Institutet	Sweden
Neuhaus, Winfried	AIT Austrian Institute of Technology GmbH	Austria
Oredsson, Stina	Lund University	Sweden
Padhi, Bhaja Krushna	Health Canada	Canada
Pant, A. B.	CSIR-Indian Institute of Toxicology Research	India
Rimann, Markus	Zurich University of Applied Sciences	Switzerland
Riss, Terry	Promega Corporation	US
Sekino, Yuko	Division of Pharmacology, National Institute of Health Sciences	Japan
Settivari, Raja	Corteva Agriscience	US
Shoemaker, James T.	Lena Biosciences	US
Smirnova, Lena	Johns Hopkins University	US
Steeg, Rachel	Fraunhofer UK Research Ltd	UK
Ulrey, Amanda	Institute for In Vitro Sciences	US
Ungrin, Mark	University of Calgary	Canada
van den Bogaard, Ellen	Radboud university medical center	The Netherlands
Vemuri, Mohan C.	Thermo Fisher Scientific	US
Wages, Phillip	PepsiCo	US
Wang, Wen Bo	Cellular Dynamics International	US
Wiest, Joachim	cellasys GmbH	Germany