Chapter 5. Test systems

Key message: *With the advances in science and technology a variety of different cell and tissue culture-based test systems have been developed but only few have been used in regulatory-approved test guideline methods due to reliability issues caused by a variety of elements described in this chapter.*

Key content: *Elaborates on Good Cell Culture Practice logistics, cryostorage, handling, identification, containment, authentication and characterisation of the test system (e.g., cell lines, stem cells, primary cells, engineered tissues, etc.) already at the development stage.*

Guidance for improved practice: *Processes for checking test system identity and characteristics, comparison of ultra-low cryostorage methods and good subculture, cryopreservation and banking practices are given.*

Recommendations *are given for cell and tissue sourcing, contaminants screening, test system biomarkers and functional tests, since it may have influence on various aspects of the in vitro method.*

Data from *in vitro* cell and culture-based test systems are routinely used by industries and regulatory bodies in toxicity testing, safety assessment, and risk evaluation. The greatest use of *in vitro* test systems, however, is for elucidating mechanisms of toxicity and/or demonstrating the biological process involved, when exposing test systems to toxicants of various kinds.

In vitro methods utilise many types of test systems. The same biological source can be grown in different culture conditions, presented in different formats, and exposed to test item(s) through different means following different *in vitro* method procedures. For example, normal human keratinocyte cells can be cultured in a monolayer system for the neutral red uptake assay or cultured at the air liquid interface on a collagen matrix for the skin irritation test. These can be considered as two separate and distinct test systems and should be handled as such. Therefore, in this case it may be more appropriate to define the test system as the final preparation of those cells rather than normal human keratinocytes. As *in vitro* test systems become more sophisticated, the definition of the test system will need to cover the biological, chemical, or physical system in the finalised platform to be used for testing.

The need for more physiologically and human relevant *in vitro* test systems has led to a major effort to use microphysiological and microfluidic technologies in combination with advanced test systems including human stem cells (Watson, Hunziker and Wikswo, 2017[1]). With the advances in genetics and genetic screening approaches, routine *in vitro* methods already include the use of genetically altered cells, stem cells, stem-cell-derived models, organ-on-chip models (microphysiological systems; MPS) or other complex and sophisticated systems (Soldatow et al., 2013[2]). To date most of these novel methods are not yet ready for regulatory purposes, however rapid progress is being made with these new approaches.

The development process of such complex test systems requires characterisation in terms of viability, functionality, genotypic and phenotypic characteristics, which can be challenging. These extensive development efforts take place mainly in the *in vitro* method developer's laboratory. Moreover, reliability and performance of these novel *in vitro* methods will need to be determined before the method can be validated (Chapter 8).

5.1. Guidance on Good Cell Culture Practice

Good Cell Culture Practice (GCCP) identifies a set of core principles of best practice for working with simple but also with more complex cell and tissue culture systems (Good Cell Culture Practice (GCCP) and Good Cell Culture Practice for stem cells and stem-cell derived models). The principles of GCCP published in 2005 remain highly relevant to cell culture practice for *in vitro* methods today and may be applied to a broad set of applications, including research, manufacture of medicines, and laboratory based Good Laboratory Practice (GLP) testing. GCCP is a vital component of GIVIMP as it provides detailed and specific principles of best practice for the handling and management of cell and tissue culture systems.

As a result of a workshop organised in 2015 (Pamies, 2016[3]) scientists from European, Japanese and North American organisations identified new developments in cell and tissue cultures. The workshop report specifically addresses new technological developments in human pluripotent stem cell lines, stem-cell derived models and complex 3D cultures. Stem cells and their derivatives represent relevant *in vitro* toxicity models as they are characterised by unlimited self-renewal and the capacity to

differentiate into several human tissue-specific somatic cells such as liver cells, heart and brain cells.

5.2. Cell and tissue sourcing

A critical issue to consider when selecting a cell or tissue based test system is the source of the cells or tissues, as its history/handling may influence its characteristics and, consequently, the results of the *in vitro* methods conducted with this test system (Lorge et al., 2016[4]). Sourcing of cells and tissues from a certified provider, e.g., established cell banks with a high quality standard, commercial providers, or reputable culture collections (Table 1.1 Cell culture collections banks), who usually provide extensive documentation on the origins and characterisation of the test system is recommended[1]. If appropriate documentation is not provided, then each test facility will need to implement more rigorous processes for checking the identity and characterising the test system. Documentation of the absence of contamination by major classes of biological agents (e.g., mycoplasma, bacteria, fungi and viruses), genetic identity/consistency/traceability and stability of desired functionality should also be available. See Good Cell Culture Practice (GCCP) (Coecke et al., 2005[5]), GCCP principle 3[2] and Table 1.2 for examples of document requirements concerning the origins of cells and tissues.

Cell and tissue providers should be qualified by the test facility to assure appropriate documentation of cell and tissue origins and quality control key features (Section 2.4). An interesting example to mention is how the user community's joint efforts to define standardised cell sources in the field of genotoxicity made stocks of such mammalian cell lines available worldwide and issued recommendations for their handling and monitoring (Lorge et al., 2016[4]). In addition, the user should check that there is solid ethical provenance (e.g., the human Pluripotent Stem Cell Registry hPSCreg registry[3]) and safety assessment performed for the cells. Intellectual Property Rights (IPR) should also be checked to ensure that they do not impact on the use of the test system and future acquired data using the test system. For more detailed information on these issues see (Stacey et al., 2016[6]).

In the case of human tissues and primary cells, there is also a requirement to assure donor consent and to manage sensitive personal data. A broad range of issues in securing tissues for testing were addressed at the 32nd Workshop of the European Centre for Validation of Alternative Methods (Anderson et al., 1998[7]). Where tissues cannot be sourced via a qualified tissue bank, there should be an agreed testing method in place with clinical contacts regarding all aspects of harvesting, preparation, labelling, storage and transfer; for an example see (Stacey and Hartung, 2006[8]). It is also important to assess the risks of viral contamination of primary cells and tissues. More details on approaches for risk assessment of primary cells and tissues are described in (Stacey and Hartung, 2006[8]). Tissues should be obtained from tissue banks holding only materials from screened donors and this will significantly assist in managing viral safety issues. When working with human tissues and primary cells it is imperative to always follow national legislation.

Moreover, master and working cell banks, where applicable, should be established to guarantee a supply of constant quality and provide traceability to the original source (Section 5.5.1)

Where test systems used in *in vitro* studies are genetically modified, the Cartagena Protocol on Biosafety[4] provides a legal framework for international trade in Genetically

Modified Organism (GMOs) and provides Signatory State Parties with orientation and the framework for development of complementary national biosafety regulations (Bielecka and Mohammadi, 2014[9]). The Cartagena Protocol does not, however, address the risks and safe practices required when handling such organisms in the workplace. Therefore, specific measures for national implementation are necessary, e.g., Directive 2009/41/EC (EU, 2009[10]) in Europe.

5.3. Cell and tissue culture transportation

Many biological materials fall into the category of "dangerous goods" for shipping purposes and must comply with national regulations and/or international norms such as the International Air Transport Association (IATA) transport regulations[5] and/or the Dangerous Goods Regulations[6] (DGR). Diagnostic specimens of human or animal material including (but not limited to) blood and its components, tissue, tissue fluids or body parts are generally classified as Biological substance, Category B (UN3373[7]) for transport by air.

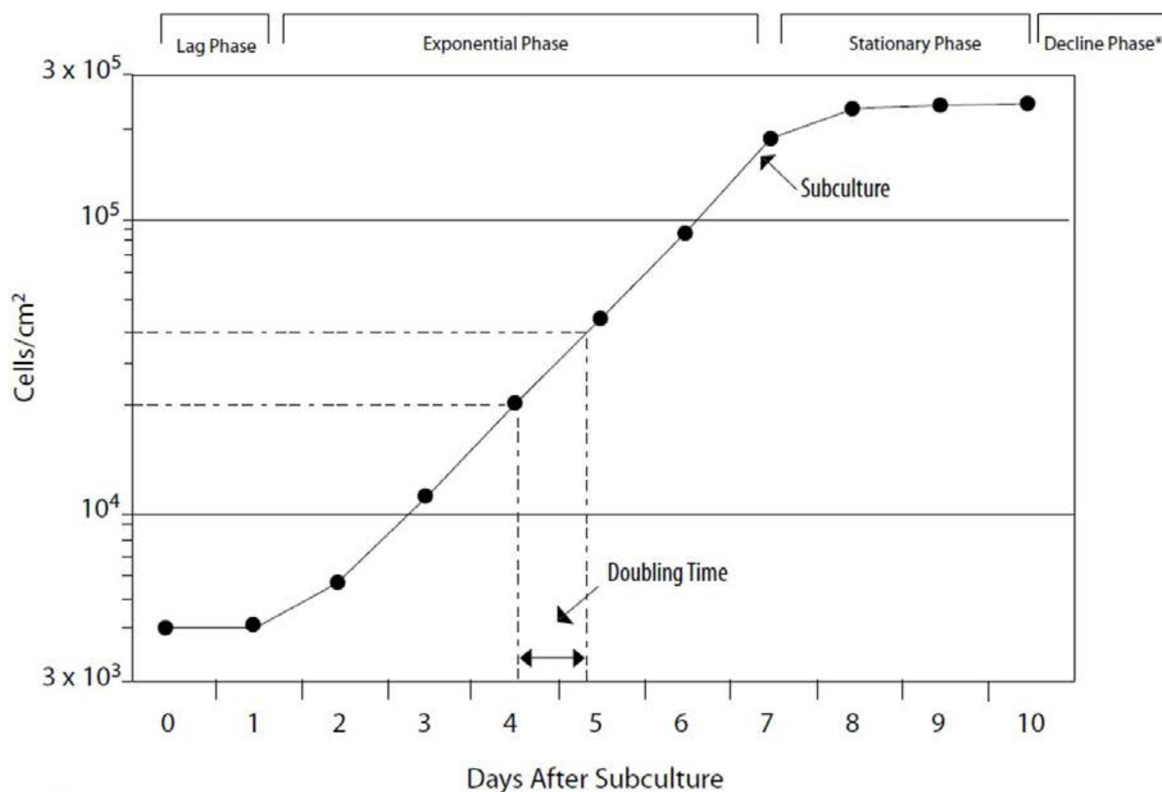
As cells and tissue in culture are often transported across the world, it is vital to keep these test systems as healthy as possible during the long transport times. Live cells and tissues may need to be shipped in a special temperature-controlled environment, such as that of a mini-cell culture incubator, where they are expected to reach their destination in good condition and are also less likely to become damaged during the transport process. Mini-cell culture incubators have limited space (2-3 plates or flasks) and require adequate sealing of plates to avoid leakage and may not always be an option or available. For short trips (e.g., arrive within one working day) it may be possible to ship cell lines in culture medium filled flasks.

Cell lines or cells are often shipped on dry ice. For shipment of some primary cells (e.g., primary liver cells) containers equilibrated with liquid nitrogen are used. Ideally, temperature should also be monitored (e.g., by using data-logger) during transportation, especially for long distance transport. When cells arrive at their destination, the conditions of the cell and tissue cultures should be examined and documented. Care should be taken when planning the shipment that the package does not sit over the weekend which may possibly compromise the test system integrity. Extra precautions must be taken for international shipping, as there is the possibility of samples being held up at customs. The fastest shipping times should be selected when long distances are involved.

5.4. Handling and maintenance of the test system

During routine handling and maintenance, growth and survival characteristics of the cell system (such as cell viability, doubling time, etc.) and subculturing details (e.g., date of subculture, subculture intervals, morphology, seeding density, passage number, etc.) should be recorded and documented, since they are required for the complete traceability of results. The documentation provided by the test system supplier (Table 1.2) should be taken into account together with historical data, when available, and used to establish acceptance criteria.

Figure 5.1. Growth curve for cells grown in culture



Source: (ATTC, 2014[11])

Figure 5.1 shows a typical growth curve for cells grown in culture. Whether cells grow and divide in a monolayer or in suspension, they usually follow the same characteristic growth pattern in which four different phases can be recognised: (a) a lag phase where the cells adapt to the new conditions; (b) a log or exponential phase of fast growth; (c) a plateau or stationary phase after the cells have completely covered the growing surface (are confluent) or saturated the suspension culture and (d) a decline phase where the cells begin to die. In order to ensure viability, genetic stability, and phenotypic stability, cell lines should ideally be maintained in the log or exponential phase, i.e. they need to be subcultured before a monolayer becomes 100% confluent or before a suspension reaches its maximum recommended cell density. The biochemistry of confluent/saturated cells may also be different from that of exponentially growing cells, and therefore, for most purposes cells are harvested or passaged before they become confluent or saturated. Some cell cultures can remain as confluent or saturated cultures for long periods, whereas others tend to deteriorate when they reach confluence. Some cell lines, particularly those derived from normal tissues such as human diploid fibroblasts, may be contact inhibited at confluence (Riss and Moravec, 2004[12]).

Two terms are commonly used to track the age of a cell culture: (i) passage number - indicates the number of times the cell line has been sub-cultured and (ii) the population doubling level[8] (PDL) - indicates the number of times a two-fold increase (doubling) in the total number of cells in culture has occurred since its initiation *in vitro*. PDL is not

determined for continuous cell lines as they are passaged at higher split ratios (ATTC, 2014[11]).

For diploid cultures, there is a correlation between PDL and passage number which in turn depends on the growth surface/volume area and the initial seeding density. Some test facilities prefer to use for tracking and reporting cellular age PDL and not passage number especially for cells where (1) growth may vary significantly between donors and between preparations, (2) to correlate directly PDL number with replicative senescence which can be linked to specific phenotypic characteristics (e.g., loss of potency in mesenchymal stromal cells), (3) to correlated PDL numbers directly with genomic instability and (4) to use PDL as a standard for the new cell preparations to compare and analyse different studies[9].

Passage number refers to the number of times the cell line has been re-plated (adherent cultures) or re-seeded (suspension cultures). For adherent or suspension cultures, each reseeding (dilution) of the cells increases the passage number by one. Cultures should be subcultured while still in the log phase, i.e. before reaching confluence/saturation.

Each test facility should have SOPs in place, where details are provided not only about how to thaw, handle, count, maintain, bank and store their cell cultures, as well as for screening for contamination, but also to univocally assign progressive passage numbers and how to determine the cell stock viability.

The frequency of passaging (transfer between flasks with or without cell dilution) depends on the growth rate of the cell culture (adherent or in suspension) and the seeding density at passage (split ratio). Many dividing primary human cell cultures have a split ratio of one in two (1:2), while continuous cell lines have much higher splitting rates, e.g., atypical split ratio is between 1:3 and 1:8. The cells can take much longer to resume exponential growth if they are split at higher dilution ratios. It should be remembered that passaging will initially result in a loss of cells. The proportion of cells lost is variable and depends on the type of cell culture, the expertise of the operator and the plating efficiency (the proportion of cells that reattach) in the case of adherent cultures.

Some cell lines require a fixed seeding density and subculturing scheme where counting the number of cells is required (Wilson et al., 2015[13]) A more specific example is 3T3-Swiss or NIH/3T3 cell lines which were established by the same subculturing scheme (3T3 is a designation that stands for passaging the cells 3 times/week at 1:3) which is important to maintain the cell characteristics[10]. To improve consistency across experiments, all routine cell culture should utilise a fixed and pre-determined seeding density as estimations of cell confluency are prone to error and contribute to variability in baseline cell physiology. Most commonly cell counting is performed using the Bürker Türk or Neubauer counting chambers. When automated cell counters are used, their correct functioning would need to be demonstrated (Cadena-Herrera et al., 2015[14]; Gunetti et al., 2012[15]; Phelan and Lawler, 2001[16]). Cell viability, using Trypan Blue stain or other nuclear counterstains (Annex I), is commonly performed so as to count only viable cells for accurate seeding density calculation.

Different cell lines have different growth rates which may depend on several environmental factors. Many diverse culturing techniques have been used to fully reproduce the various environments test systems normally encounter during development. Most of the work to date has been performed on solid plastic supports including high-throughput plastic supports. A plastic growth support has several limitations in its representation of the *in vivo* environment. As plastic is an impermeable smooth two-

dimensional surface, it forces the cells to exchange their gas and nutrients exclusively through the top side of the cultured cells while *in vivo* cell are exposed from several directions to factors from the blood, other cells, soluble factors, and liquid-air interfaces. Growth of cells in more physiological conditions such as air-liquid interface set-ups (Pezzulo *et al*., 2011) or on microporous membranes (Klein et al., 2013[17]), or by using a variety of biomaterial coated surfaces for specific cell attachment, propagation, differentiation, and migration requirements (Chai and Leong, 2007[18]; Tallawi et al., 2015[19]) has many advantages and may be applied when examining aspects such as:

- Permeability and transport of macromolecules, ions, hormones, growth factors, and other biologically relevant molecules
- Cell polarity e.g., sorting and targeting of macromolecules; and polarized distribution of ion channels, enzymes, transport proteins, receptors and lipids
- Endocytosis
- Tumour invasion and metastasis
- Chemotaxis and other cell motility studies including angiogenesis, phagocytosis
- Co-culture effects, including interactions of feeder layers with stem cell cultures and cell-to-cell/matrix interactions
- Microbial pathogenesis e.g., test item effects on microbial receptors
- *In vitro* fertilisation including small molecule transport studies

Another advantage of cells grown on porous membrane substrates is their ability to provide a surface that better mimics a three-dimensional *in vivo* setting important for tissue remodelling (e.g., wound healing). Porous membranes allow multidirectional exposure to nutrients and waste products. Membrane separation of dual chambers allows for the co-culture of cells of different origin, and is used to study how cells interact through indirect signalling or through providing a conditioned niche for the proper growth and differentiation of cell types. Permeable supports also permit culture of polarised cells (Sheridan et al., 2008[20]).

If required for the particular test system, justification of the method for differentiation should be described in the *in vitro* method, since potential of differentiation and the method used to induce differentiation will vary depending on the type of cells, and should include justification of the process in which the method was determined.

5.4.1. Influence of the quality of the feeder layer

The growth of stem cells in culture requires certain nutrients that support the cells in an undifferentiated state. In this case a feeder cell layer is often used. One consideration in minimising variability of *in vitro* testing using stem cells is to ensure standardised methodology in deriving, culturing, and inactivating feeder cells. There are many kinds of cell lines used as feeder cell layer. Fibroblasts like mouse embryonic fibroblast cells and mouse embryo derived thioguanine and ouabain-resistant cell lines are commonly used to establish and culture embryonic stem cells (ESCs). Cell lines derived from umbilical cord blood cells or adult bone marrow cells have been used as ESC feeder cell layers. The influence of the quality and type of feeder layer can affect some pluripotency marker genes and proteins in ESC cultures (Healy and Ruban, 2015[21]).

5.5. Cryopreservation and thawing

Cryostorage systems should ensure the long term preservation of the stored test system. For cryopreserved cell cultures, the viability of mammalian cells is progressively lost

within months at -80˚C, thus, long term storage below the glass transition point of water (-136˚C) is recommended. While true for mammalian cells, this is not the case for bacteria or yeast.

Improved technologies that allow cryopreservation of *in vitro* cell and tissue cultures at different stages of differentiation and their long-term storage has introduced new or more standardised *in vitro* test systems into the pipeline of potential *in vitro* methods to be used in human safety assessment. Controlled-rate and slow freezing, also known as slow programmable freezing has been used all over the world for freezing down cell and tissue cultures. New methods are constantly being investigated due to the inherent toxicity of many cryoprotectants. Depending on the type of cell culture, using dimethyl sulfoxide (DMSO) may not always be preferable, as DMSO shows relatively strong cytotoxicity to some cells types and affects differentiation of iPS and ES cells (Katkov et al., 2006[22]).

As described in GCCP Principle 1, 'Establishment and maintenance of a sufficient understanding of the *in vitro* system and of the relevant factors which could affect it' (Coecke et al., 2005[5]), it is essential to prepare preserved banks of cells intended for use, to assure that reliable stocks can be obtained for testing which are at a consistent passage level from the original 'seed stock'. This is in order to avoid the effects of changes or cross-contamination which may occur if cell lines are maintained indefinitely. Standard cryopreservation methods using DMSO (10%) and serum (20%) as cryoprotectants, combined with a slow cooling rate (e.g., -1°C/min) and standardised cell numbers per vial will usually be successful for most cells. However, it is necessary to check the viability of preserved stocks in case of freezing failure and also to try to assure consistency between individual vials in a cell bank regarding cell number, viability and desired functionality. Viability measurements made immediately post-thaw can give misleadingly high values as many cells can be lost during the 24h recovery phase post thawing.

When stored in liquid nitrogen, storage in the vapour phase (Table 5.1. Comparison of ultra-low cryostorage methods for cells) is generally advised for all cells and necessary for potentially infectious cells and tissues. This eliminates the chances of transfer of pathogenic material between vials which can occur in the liquid phase of nitrogen (Coecke et al., 2005[5]). It is also considered safer as liquid nitrogen can enter storage vials if they are stored in the liquid phase which may cause them to explode upon thawing. However, to accommodate storage in the vapour phase, the amount of liquid nitrogen needs to be reduced, which will require more frequent topping up of the liquid nitrogen so as to maintain the correct storage temperature. If vials need to be stored in the liquid phase, protection wrapping may be considered.

Cryostorage requires temperature and/or liquid nitrogen level monitoring to ensure that the test system stocks are at optimal storage temperature. Cryostorage vessels can be fitted with alarms and data loggers and liquid nitrogen levels recorded at regular intervals (e.g., weekly). Appropriate safety protection (e.g., wearing of safety glasses, gloves etc.) should always be used when working with liquid nitrogen as contact with the skin or eyes may cause serious freezing (frostbite) or other injury.

Table 5.1. Comparison of ultra-low cryostorage methods for cells

Method	Advantages	Disadvantages
Electric Freezer (-130°C or lower)	Ease of Maintenance Steady temperature Low running costs	Requires CO_2, liquid N_2 or electrical backup Mechanically complex Storage temperatures high relative to liquid nitrogen
Liquid Phase Nitrogen	Steady ultra-low (-196°C) temperature Simplicity and mechanical reliability	Requires regular supply of liquid nitrogen High running costs Risk of cross-contamination via the liquid nitrogen
Vapour Phase Nitrogen	No risk of cross-contamination from liquid nitrogen Low temperatures achieved Simplicity and mechanical reliability	Requires regular supply of liquid nitrogen High running costs Temperature fluctuations between -135°C and -190°C

Source: (ECACC , 2010[23])

Storing valuable test system stocks in more than one cryostorage location is recommended for security/backup purposes and off-site storage may also need to be considered in disaster recovery plans for the facility.

A number of factors may affect the viability of cells on thawing, including the composition of the freeze medium, the growth phase of the culture, the stage of the cell in the cell cycle, and the number and concentration of cells within the freezing solution (ATTC, 2014[11]). Another issue to take into consideration when using thawed cells is the possibility that the cells are stressed directly after thawing, which appears to involve apoptosis (Baust, Van Buskirk and Baust, 2002[24]). Most cells begin to recover after 24 hours and enter the log (exponential) growth phase soon afterwards. It is therefore necessary to remove DMSO and any dead cells as they might affect the seeding density calculation. It is also recommended not to use them straight away, but to passage them at least twice, so as to allow the cells to re-establish their normal cell cycle.

Optimum conditions should be defined in the *in vitro* method SOP(s) during the development phase. When the test system is sourced from a commercial supplier (Section 5.2) extensive documentation is usually provided including detailed information for handling the cells, including cryopreservation and thawing information. Batch-specific information such as the number of cells per vial, the recommended split or subcultivation ratio, and the passage number and/or population doubling level (PDL) when known are also provided.

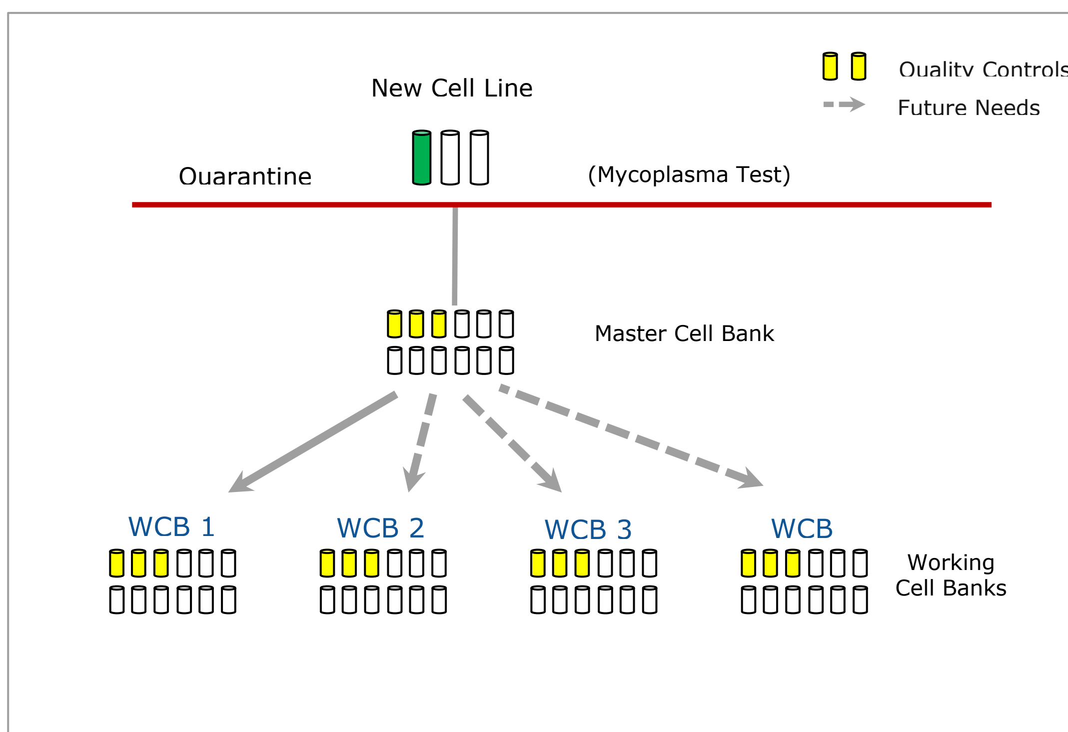
5.5.1. Cell Banking

Maintaining a cell line in continuous or extended culture is considered bad practice as there may be a higher risk of microbial contamination and/or cross contamination with other cell lines, a loss of characteristics of interest, genetic drift particularly in cells known to have an unstable karyotype or loss due to exceeding finite life-span[11]. It is therefore important to avoid subjecting cell lines to variable culture and passage conditions, and to establish cryopreserved stocks of early passage cells (Coecke et al., 2005[5]).

New cell lines should be quarantined (Section 3.5) until their origin has been authenticated and they are shown to be free of microorganisms (Geraghty et al., 2014[25]).

A two-tiered cell banking system consisting of a Master Cell Bank (MCB) and a Working Cell Bank (WCB) is recommended. Cells from the new cell line are placed in culture and harvested when they are at their maximum growth rate or almost confluent. These are then frozen to create a master cell bank, usually consisting of 10 to 20 vials of 1 ml, each usually containing 1–5 × 106 cells (Geraghty et al., 2014[25]). The MCB is not for distribution and should be protected from unintended use, however new working banks may be created from the original master bank when required (Figure 5.2). From this MCB, a single vial is thawed and cultured until there are enough cells to produce a working cell bank. The working cell bank should contain sufficient ampoules to cover the proposed experimental period plus sufficient ampoules for contingencies and distribution (UKCCCR, 2000[26]).

Figure 5.2. Flexible two tiered approach to cell banking



Quality controls procedures defined in SOPs should include checks on viability, mycoplasma and other microbial contaminants, cell line identity and any other relevant cell line characteristics (Coecke et al., 2005[5]), and should be applied systematically to the working cell banks. Quality Control (QC) tests for the absence of bacteria and fungi, and testing for mycoplasma should only be performed following a period of antibiotic-free culture. For primary cells, the state of cell differentiation should also be carefully observed during banking. Different passage of primary cells with different differentiation status will greatly influence results.

5.6. Cell line identity and genetic aberrations

Genetic, phenotypic and immunological markers are useful in establishing the identity of the cell(s). Genetic stability testing (also known as cell line stability) is a key component

in characterising cell banks and is especially critical in maintaining quality of mammalian cell cultures. For an engineered cell line, the inserted gene of interest should remain intact and at the same copy number, and be expressed. Furthermore, there should be traceability to the original provider of the cell culture and the related documentation. However, a frequent problem in the use of cell culture is the use of cells which have become cross-contaminated, misidentified (see International Cell Line Authentication Committee (ICLAC) database of cross contaminated or misidentified cell lines[12]), mixed-up, or present genomic instability (Allen et al., 2016[27]); (Frattini et al., 2015[28]); (Fusenig et al., 2017[29]); (Kleensang et al., 2016[30]); (Vogel, 2010[31]). This is not always detectable by cell morphology and/or culture characteristics. An example of a mistake from the past in an OECD test guideline method (OECD, 2016[32]) is BG1Luc4E2 cells which have been renamed VM7Luc4E2 cells. The reason being recent DNA analysis revealed that the original cell line used to generate the BG1Luc4E2 cells were not human ovarian carcinoma (BG1) cells but a variant of human breast cancer (MCF7) cells[13].

There are different genomic techniques for human and non-human cell line authentication (Table 5.2). Cell line authentication is an example of the kind of data that add confidence to the results of a scientific study. The lack of reporting of cell line authentication data reflects a broader failure to appreciate the need for more complete reporting of experimental details that qualify data and provide confidence in the scientific results (Almeida, Cole and Plant, 2016[33]); (Marx, 2014[34]).

Table 5.2. Current status of SNP, STR, and DNA barcode technologies as standard methods for assessing the identity of cell lines from different species

Species	Assays	Consensus Standard Method	Commercially Available Kit	Commercial Service	Comparative Data
Human	STR	ASN-0002	Yes	Yes	ATCC, DSMZ, JCRB, NCBI**
	SNP	No	Yes	Yes	(Liang-Chu et al., 2015[35]) (Yu et al., 2015[36]) NCBI
Mouse	STR*	No	No	Yes	Unpublished
	SNP	No	Yes	Yes	(Didion et al., 2014[37])
African green monkey	STR*	No	No	No	None
Chinese hamster ovary	STR*	No	No	No	None
Rat	STR*	No	No	No	None
Species-level identification	CO1 DNA barcode	ASN-0003	Yes	Yes	Barcode of Life Data System, NCBI**
	Species-specific primers	No	No	Yes	None needed

Note: These methods are currently the most developed for this application. There are extensive data on human cell lines, but while there are some kits and services for some nonhuman species, there is little available data for nonhuman species, except for DNA barcoding, which only distinguishes cell lines on the basis of species, not individuals.
* STR markers have been identified (Almeida, Hill and Cole, 2013[38]); (Almeida, Hill and Cole, 2011[39]). Markers for rat and Chinese hamster ovary cells are still under development by NIST.
** These sources contain a significant amount of data from multiple sources.
Source: (Almeida, Cole and Plant, 2016[33])

Establishing an early stock (or retention of a sample of original tissue) which is DNA fingerprinted will provide an important reference for future cell banks and for other centres. Short Tandem Repeat (STR) profiling is typically applied and has considerable background qualification for use in human samples (ISCBI, 2009[40]). STR profiling has been the subject of a comprehensive and definitive standard, ASN-0002[14], and can be performed in most laboratories that have the capabilities to execute molecular techniques. It is an easy, low cost and reliable method for the authentication of human cell lines. For non-human samples, Single Nucleotide Polymorphism analysis (aSNP), STR profiling, and DNA barcode technologies are available as methods for assessing the identity of cell lines from different species (Ono et al., 2007[41]). The field of genetic analysis is progressing rapidly and interested parties should maintain knowledge of current best scientific practice in this area as next generation sequencing begins to become a routine tool. Doing so problems can be avoided early in the process and not jeopardise the cell lines used for regulatory purposes as has happened for the Bhas 42 cell line in a cell transformation *in vitro* method (OECD, 2016[42]) where issues related to misidentification arose at a late stage.

Genetic instability is inherent in cell cultures and it is wise to minimise the number of passages over which cells are maintained (typically 15-20). Although passage number alone is not a reliable parameter to ensure good cell functioning, it is good practice to define a limit for the maximum number of passages, possibly in combination with defined performance characteristics. At that limit, new cultures should be restarted from a working cell bank. The use of cells at higher passage numbers must be justified and their integrity and functionality demonstrated. Where cells are known to be extremely unstable, some form of genetic testing, such as karyology or molecular analysis like aSNP or Comparative Genomic Hybridisation (aCGH) may need to be performed. In particular, this applies to recombinant cell lines including those maintained with antibiotic selection. Recombinant cell lines should be maintained in parallel with matched cells that were generated with the empty vector alone and were simultaneously subjected to antibiotic selection. Such matched cells will be a more suitable control than non-selected cells that do not express the same resistance marker as their modified counterparts.

There are special issues for stem cells. Stem cell lines may contain a mixture of diploid and aneuploid cells, which may be unavoidable, but genetic testing (see above) can be used to screen for progressive change (e.g., between master and working cell banks) which could impact on the suitability of the cell culture. Human induced Pluripotent Stem Cell (hiPSC) lines should also be tested for absence of ectopic expression of reprogramming genes and where produced by non-integrating vectors, for elimination of the vector. iPS/ESCs also need to be evaluated by their genotypes and differentiation capability by embryoid body formation, direct differentiation method and teratoma formation assays.

Acceptable intervals for periodic testing to confirm the genetic, phenotypic and immunological stability of the cell culture are highly case-dependent (Blázquez-Prunera et al., 2017[43]) (Daily et al., 2017[44]; Meza-Zepeda et al., 2008[45]). Therefore, this aspect should be included in the specific test system SOP(s).

5.7. Contaminants screening: sterility, mycoplasma, virus

Standard sterility tests are widely available[15] [16] and may be used for cell stocks and cultures; however, it is important to bear in mind that these tests are usually based on inoculation of broth cultures which may not support the growth of all contaminating

microorganisms. Alternative molecular methods such as identification by Polymerase Chain Reaction (PCR) and DNA sequencing of ribosomal RNA may be used.

Viruses may arise as contaminants of cell cultures via the original donor used to produce the cell line or feeder cells and other biological reagents used in cell culture. They may cause cytopathic effects, in which case the culture should be discarded, or they may have no effect and become diluted out when fresh uncontaminated reagents are used. In certain cases they may establish persistent infections, although this is believed to be rare. Whatever the outcome, their presence and influence on cell biology may be significant as amongst other effects they may modify transcription factor networks and alter the cells' biology. To assure laboratory worker safety, some organisations require testing of all human cell lines for serious human pathogens such as Human Immunodeficiency Virus (HIV) and Hepatitis B and C or evidence that the donors did not have these pathogens. However, such testing clearly does not cover more common human infections, and human pathogens may also be carried by cells from other species. Cell line testing may be initiated if there are special hazards associated with the work or with the cells. Workers should always follow local rules for performing cell culture work, maintain their competence in aseptic processing, as well as carry out regular and careful inspection of cells for any unusual effects or morphologies that might indicate infection. A robust testing regime for contamination should include procedures for managing positive results, whether to immediately discard or quarantine the affected cells until a means of action can be decided along with the detection of the root cause by supplementary testing (Stacey, 2011[46]).

It is crucial to routinely test cell cultures for the presence of mycoplasma. A range of test techniques are available and it is advised for critical tests to use methods which detect cultivable as well as non-cultivable mycoplasma species, e.g. PCR-based methods (Table Table 5.3). It is important to know what aspect of contamination the test is designed to detect, how well the test performs, its specificity (i.e., what strains of mycoplasma its detects and any likely causes of false positive reactions) and for detectable contamination, what level of sensitivity is achievable under the prescribed sampling and test conditions. Selection of test methods should be based on evaluation of the potential specificity and sensitivity of detection and the likelihood of inhibition of a positive result.

EMA has provided a general chapter on mycoplasma testing of cells which should be consulted (EMA, 2013[47]). All aspects of the test sample which are likely to influence the strains which may be isolated and any conditions which may affect detection such as inhibitory substances, should be evaluated before selecting a particular technique. Even where alternative detection kits are based on the same basic methodology, their specificity and sensitivity may vary considerably and even the same methods used in different laboratories may be influenced by local differences in raw materials, test conditions and the way the test is performed. Accordingly, any test method used should be subjected to the general evaluation indicated above and performance of testing should be accompanied by the inclusion of appropriate controls as below:

1. positive controls (including a reference sample close to the limit of detection),
2. negative controls to exclude false positives from reagents and test conditions and
3. positive controls spiked into test samples (or other approaches to control for sample inhibition) of positive test results.

All such testing should be performed only by a person trained and competent in the test. Records of performance, including positives and negative results, control performance

and any equivocal or anomalous results, and any trends in quantitative results for test and control samples (where applicable) should be kept, so as to enable ongoing evaluation.

Table 5.3. Mycoplasma detection methods, their sensitivity, and advantages and disadvantages

Method	Sensitivity	Advantages	Disadvantages
Indirect DNA stain (e.g., Hoechst 33258) with indicator cells (e.g., 3T3)	High	Easy to interpret because contamination amplified	Indirect and thus more time-consuming
Broth and agar culture	High	Sensitive	Slow and may require expert interpretation
PCR	High	Rapid	Requires optimisation
Nested PCR	High	Rapid	More sensitive than direct PCR, but more likely to give false positives
Enzyme-Linked Immunosorbent Assay (ELISA)	Moderate	Rapid	Limited range of species detected
Autoradiography	Moderate	Rapid	Can be difficult to interpret if contamination is at low level
Immunostaining	Moderate	Rapid	Can be difficult to interpret if contamination is at low level
Direct DNA stain (e.g., Hoechst 33258)	Low	Rapid, cheap	Can be difficult to interpret

Source: (Young et al., 2010[48])

5.8. Biomarkers and functional tests to confirm the required cell function state

It is important to recognise that cell quality can vary during passaging, and in particular the time point in the growth curve at which cells are harvested (ideally in the log or exponential phase) may affect performance (Section 5.4). Accordingly, each culture used to set up an *in vitro* method should be subject to a key control regime measuring or indicating functionality. Because the crucial function of the test system to be measured may be dependent on the last step in a long sequence of events (e.g., gene activation and gene-transcript-protein-reaction) it is of importance to ensure that a selected biomarker or a test is directly related to the crucial function to be measured. Acceptance criteria should be defined for functional tests and biomarkers that indicate the correct cell state. These may for example include: neuronal activity, competency of biochemical transformation, response to reference bioactive compounds when using metabolically competent cells, response to reference items in the particular *in vitro* method the cells are to be used for etc. In this way, each culture can be controlled, and consistency in *in vitro* methods is supported. For example, expression of self-renewal genes (e.g., Oct4, Nanog, Sox2) in stem cell cultures is crucial to the functionality of the cell population (further examples for stem cells are laid out in (Pistollato et al., 2014[49]; Stacey et al., 2016[6]). Additionally, key markers which are associated with poor performance may be identified for future improvement.

As stem cell-based (both hiPSC and hESC) *in vitro* models have and will be employed for regulatory use, not only key markers for cell state but also the maturation phase requires characterisation. For example, human pluripotent stem cell-derived cardiomyocytes have been shown to display morphological and functional properties typical of human foetal cardiomyocytes which may complicate their utilisation and interpretation of the obtained results (Robertson, Tran and George, 2013[50]; Snir et al., 2003[51]). Increased time in culture, electrical stimulation (Chan et al., 2013[52]) and 3D culture environment (Garzoni et al., 2009[53]; Schaaf et al., 2011[54]; Soares et al., 2012[55]; Valarmathi et al., 2010[56];

van Spreeuwel et al., 2014[57]) have been utilised in the production of more mature cardiomyocytes with adult-like properties. As such, the maturation phase of stem cells is deemed a critical quality parameter when the relevance of an *in vitro* test system is to be considered.

In a co-culture system, the use of stem cells provide the test system with multipotent differentiation capacity and can act as helper cells for ensuring homeostasis, metabolism, growth and recovery. Their inclusion in co-culture systems has shown benefits creating complex tissues, including orthopaedic soft tissues, bone, heart, blood vessels, lungs, kidneys, liver and nerves (Paschos et al., 2014[58]). In addition, it is necessary to evaluate both combination of biomarkers and cytometric analysis (e.g., flow cytometry or fluorescent microscopy) to check robustness of the stem cell culture.

Interactions within the same cell population (homotypic) and between different cell types (heterotypic or co-cultures) are essential for tissue development, repair, and homeostasis. Some cells cannot easily be mono-cultured *in vitro* or at least do not exhibit desired *in vivo* physiological behaviours, but the presence of another cell population may improve the culturing success or cell behaviour. Cell-cell interactions in co-cultures are strongly influenced by the extracellular environment, which is determined by the experimental set-up, which therefore needs to be given careful consideration (Goers, Freemont and Polizzi, 2014[59]). It is critical to identify biomarkers and functional tests to confirm the required co-culture system function state.

5.9. Metabolic activation

Metabolism is a bottleneck in *in vitro* toxicological method development since there is an inability of most mammalian *in vitro* cell and tissue cultures to predict the physiological effect of *in vivo* metabolism by the Phase I and Phase II biotransformation enzymes (Coecke et al., 2006[60]).

Currently no *in vitro* cell and tissue culture test system will mirror fully the complexity of *in vivo* metabolism, and the production of active metabolites may either not occur in non-metabolic competent test systems or be over or underestimated in metabolically-competent test systems. However, these considerations should not prevent the use of metabolic activation systems to mitigate this problem, provided the limitations and drawbacks are clearly understood and the results take into consideration these limitations.

The evolution of genotoxicity testing offers a good example of the use of metabolic activation mixtures to improve the physiological relevance of *in vitro* methods for genotoxicity testing[17]. In the case of the Ames test (OECD, 1997[61]) a metabolising system in the form of a cofactor-supplemented S9 fraction, containing microsomal and cytosolic fractions prepared from rat liver (usually) pre-treated with enzyme inducing agents such as Arochlor 1254 or a combination of phenobarbitone and ß-naphthoflavone, to induce metabolising enzymes, has been built into the method. In 1997 the OECD issued a detailed review paper (DRP) on the use of metabolising systems for *in vitro* testing of endocrine disruptors detailing different options how to produce the relevant metabolites of the test item under investigation when carrying out these types of tests. It is recommended that *in vitro* method developers take this aspect into consideration when designing *in vitro* method(s) (OECD, 2008[62]). Furthermore, there is a need for metabolically-active test systems both for toxicokinetics and toxicodynamics applications in regulatory testing (Chan et al., 2013[52]).

Possible strategies how to employ metabolic activation when designing *in vitro* methods remain a challenge even for well-established methods (Nesslany, 2017[63]). However, efforts are underway to introduce a metabolic component in OECD TG methods for the detection of chemicals with (anti)estrogenic potential (OECD, 2016[32]).

More and more integrated ways to predict the physiological effect of metabolism are being proposed in response to the open challenges for regulatory toxicology (Funk and Roth, 2016[64]; Wang et al., 2013[65]; Williams et al., 2013[66]).

Notes

1. See: http://wiki.toxbank.net/w/images/1/18/ToxBank_D4_6_final_10_04_13.pdf
2. GCCP Principle 3: Documentation of the information necessary to track the materials and methods used, to permit the repetition of the work, and to enable the target audience to understand and evaluate the work
3. See: https://hpscreg.eu/
4. See: http://bch.cbd.int/protocol/text/
5. See: http://www.iata.org/whatwedo/cargo/dgr/Documents/infectious-substance-classification-DGR56-en.pdf
6. See: http://www.iata.org/whatwedo/cargo/dgr/Pages/download.aspx
7. See: http://www.un3373.com/un3373-packaging/un3373/
8. See: https://www.atcc.org/en/Global/FAQs/B/C/Passage_number_vs_population_doubling_level_PDL-175.aspx
9. See: http://stemcellassays.com/2014/05/msc-pdl/
10. See: https://www.atcc.org/Global/FAQs/8/9/ATCC%20CCL92%20vs%20ATCC%20CRL1658-453.aspx
11. See: http://www.sigmaaldrich.com/technical-documents/protocols/biology/good-cell-banking.html
12. See: http://iclac.org/databases/cross-contaminations/
13. See: https://ntp.niehs.nih.gov/iccvam/methods/endocrine/bg1luc/bg1luc-vm7luc-june2016-508.pdf
14. See: http://webstore.ansi.org
15. See: http://www.who.int/medicines/publications/pharmacopoeia/TestForSterility-RevGenMethod_QAS11-413FINALMarch2012.pdf
16. See: http://medicaldesign.com/site-files/medicaldesign.com/files/archive/medicaldesign.com/Whitepapers/SterilityTestin_00000021071.pdf
17. See: https://www.oecd.org/chemicalsafety/testing/Genetic%20Toxicology%20Guidance%20Document%20Aug%2031%202015.pdf

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