Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks

Proposed replacement of TRS 878, Annex 1

Adopted by the 61st meeting of the WHO Expert Committee on Biological Standardization, 18 to 22 October 2010. A definitive version of this document, which will differ from this version in editorial but not scientific details, will be published in the WHO Technical Report Series.

Recommendations published by the WHO are intended to be scientific and advisory. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If a NRA so desires, these Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Recommendations be made only on condition that modifications ensure that the biological product is at least as safe and efficacious as that prepared in accordance with the recommendations set out below. The parts of each section printed in small type are comments for additional guidance intended for manufacturers and NRAs, which may benefit from those details.

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1. Introduction

Cell substrates are cells used to manufacture a biological product. It is well established that cell substrates themselves and events linked to cell growth can affect the characteristics and safety of the resultant biological products. Therefore, a thorough understanding of the characteristics of the cell substrate is essential in order to identify points of concern and to develop a quality control system that addresses those points.

Recent advances in the use and quality control of new animal cell substrates, particularly continuous cell lines (CCLs) and insect cells, led to the conclusion that an update of the current WHO Requirements (TRS 878) [1] should be prepared. In order to facilitate the resolution of regulatory / scientific issues related to the use of animal cell cultures, including human, as substrates for the production of biological products, WHO initiated this revision of its Requirements on cell substrates by establishing a Study Group (SG). Animal cells refer to cells derived from organisms classified in the animal kingdom. This document is the result of the SG effort, including wide consultations with individuals and organizations with expertise in this area. After receiving comments from this consultative process, as well as from invited reviewers, further revision of the draft recommendations was undertaken and presented to the ECBS in 2010. During the development of this document, guidances on this topic issued by other relevant organizations were considered. Effort was made to be compatible with the existing guidances, whenever possible.

These recommendations provide guidance to National Regulatory Authorities (NRAs), National Control Laboratories (NCLs) and manufacturers on basic principles and, in some cases, on detailed procedures that are appropriate to consider in the characterization of animal cells that are proposed for use in the manufacture of biological products. Although the decision-making authority lies with the NRA, it is advisable that NCL experts on this topic be consulted.

2. Historical overview

Historically, the major concerns regarding the safety of biological medicinal products manufactured in animal cells have been related to the possible presence of microbial contaminants and, in some cases, to the properties and components of the cells themselves such as DNA and proteins.

For example, in 1954, an experimental adenovirus vaccine was being developed, and human tumour cells (HeLa) were rejected as the cell substrate in favour of "normal" cells [2]. At that time, relatively little was known about the biological mechanism(s) that lead to human cancer, so that the risks to the recipients of a vaccine based on HeLa cells could not be assessed and quantified scientifically. Although "normal" cells were not defined, that decision led to the use of primary cell cultures (PCCs) from animals such as monkeys, hamsters, and embryonated eggs for vaccine research and development [3].

The first requirements for cell substrates were published by WHO in 1959 for the production of inactivated poliomyelitis vaccine in PCCs derived from the kidneys of clinically healthy monkeys [4]. Those requirements were revised and published in 1966 [5]. Subsequently, other PCCs were used for the production of other viral vaccines.

In the 1960s, human diploid cells (HDCs) were developed and proposed as an alternative to primary monkey kidney cell cultures for polio virus vaccine production as well as for other viral vaccines.

The rationale for using HDCs was based on the ability to: a) cryogenically preserve the cells at low population doubling levels (PDLs); b) establish and characterize cryopreserved banks of cells that later could be expanded to provide a standardized source of cells for many decades; c) extensively test recovered cells before use in vaccine production; and d) demonstrate that the cells were free from detectable adventitious agents and that they were unable to form tumors when inoculated into immunosuppressed animals. Thus, HDCs were normal by all of the then existing criteria. It was argued that because HDCs were normal and could be standardized, tested, and used for many years, they were a significant improvement over PCCs.

The pathway to acceptance of HDCs was difficult and lengthy, primarily because some members of the scientific community believed that HDCs might contain a latent and unknown human oncogenic agent, and that such a theoretical agent posed a risk to recipients of vaccines produced in HDCs. Numerous conferences and discussions of new data eventually led to the acceptance of HDCs as a substrate for viral vaccine production, and they continue to be used by many manufacturers for various viral vaccines that have a long history of safety and effectiveness. The concept of a master cell bank (MCB) and working cell bank (WCB) system and characterization of the cell substrate were introduced during that period [6,7].

Both our understanding of tumor cell biology and the technological tools that were available at that time were much more limited than they are today. As a result, the proponents of using HDCs for vaccine production based their argument that the cells were normal, and therefore safe to use, on four points: a) freedom from detectable adventitious agents; b) the finite life of HDCs; c) the diploid nature of HDCs; and d) the inability of HDCs to form tumors in various *in vivo* test systems.

In order to provide a high level of assurance that those four characteristics were stable, the initial lot-release tests for each batch of a vaccine derived from HDCs included tests of the cell substrate for adventitious agents, karyology, and tumourigenicity [8,9]. The main question that was being addressed by the routine use of tumourigenicity tests was whether or not the production cell culture had undergone a contamination or transformation event such that it contained a mixture of "normal" and tumourigenic cells. It was eventually agreed that tumourigenicity testing was not sensitive enough to detect a low level of tumourigenic cells, and that it was wasteful of animals and time in repeated testing of a cell line that had been well characterized and would be used in the context of a cell-bank system. Therefore, tumourigenicity tests eventually were required only for the characterization of a MCB (using cells at the proposed *in vitro* cell age for production or beyond) for both HDCs and CCLs [10,11].

In the 1970s, there was a clinical research need for more interferon alpha (IFNα) than could be produced from primary human lymphocytes. In response to that need, human tumour cells (Namalwa) grown *in vitro* were proposed as a cell substrate for the production of IFNα. The primary concerns about the use of Namalwa cells were that they contained the Epstein-Barr virus (EBV) genome integrated into the cellular DNA, and that either whole virus or DNA containing viral elements could be transmitted to the recipients of the IFNα product. Nevertheless, by the end of the 1970s, regulatory agencies had allowed human clinical studies to commence, and the product was eventually approved in several countries. Among the most important factors that contributed to those decisions was the fact that IFN, as opposed to live viral vaccines, was not a replicating agent, and IFNα was being used as a therapeutic rather than a prophylactic product, thus representing different risk/benefit considerations. In addition, technology had advanced significantly so that IFNα could be

highly purified and the purification process could be validated to demonstrate that EBV and cellular DNA were undetectable in the final product, within the limits of the assays then available, which permitted risk mitigation.

In the 1980s, advances in science and technology led to the development of recombinant DNA (rDNA) derived proteins and monoclonal antibodies (MAbs). Animal cells with the capacity to grow continuously *in vitro* (CCLs) were the substrates of choice for those products because of the ease with which they could be transfected and engineered. Also, in contrast to PCCs and HDCs, they grew rapidly to achieve a high density and expressed a variety of products at high concentrations. Chinese hamster ovary (CHO) cell lines became widely used for rDNA products, and hybridomas of various types were required for the production of MAbs. The use of such cells as substrates in the manufacture of a large array of potentially important biological medicinal products raised safety concerns once again. A scientific consensus emerged from numerous conferences that there are three major elements of potential concern related to animal cell substrates: DNA, viruses, and transforming proteins. In 1986, WHO established a SG to examine cell-substrate issues in greater depth.

The SG concluded that there is no reason to exclude CCLs from consideration as substrates for the production of biologicals, and that CCLs are in general acceptable when the manufacturing process is shown to eliminate potential contaminating viruses pathogenic for humans and to reduce DNA to acceptable levels and/or to eliminate its biological activity [12]. The SG's emphasis on infectious agents as the major risk factor was based in large part on actual experience in which virus transmission and disease had occurred through contaminated biological products (*e.g*., hepatitis B virus and human immunodeficiency virus (HIV) in Factor VIII). WHO Requirements for Continuous Cell Lines used for Biologicals Production were published in 1987 [13]. Based on a review of more recent data, those Requirements were revised in 1998 to raise the acceptable level of rcDNA to 10 ng per parenteral dose. In addition, it was pointed out that beta-propiolactone, a viral inactivating agent, may also destroy the biological activity of DNA. Use of this agent therefore provides an additional level of confidence even when the amount of DNA per dose may be substantial [1].

During the 1990s, and on into the 2000s, a variety of CCLs were explored as cell substrates for biological products in development because, like the cell lines already mentioned, they offered significant advantages during production (*e.g*., rapid growth and high expression). These include the following tumourigenic cell lines: HeLa for adeno-associated virus vectored HIV vaccines; PER.C6 for influenza and HIV vaccines; Madin-Darby Canine Kidney (MDCK) for influenza vaccines; and 293ORF6 for HIV vaccines. More recently insect cell lines and stem cell lines (SCLs) have been proposed for the manufacture of biological products, and such cells introduce a new set of challenges for their evaluation and characterisation.

The acceptability of a given cell type (primary, diploid, stem, or continuous) as a substrate for the production of a specific biological product depends on a variety of factors including an in depth knowledge of its basic biological characteristics. In that regard, it is important to recognize that the tumourigenic potential of a CCL is but one of many factors to consider such as the extent to which the manufacturing process reduces or eliminates cellular factors that may be of concern. An assessment of the totality of the data available is needed in order to determine whether a product manufactured in a given cell substrate is potentially approvable.

The following recommendations provide guidance to manufacturers and NRAs/NCLs on the evaluation of animal cell cultures used as substrates for the production of biological medicinal products, and for the characterization of cell banks.

The main changes from the requirements published in TRS 878, annex 1, include:

1. general manufacturing recommendations applicable to all types of cell culture production have been updated;
2. some considerations for the evaluation of new cell substrates such as insect cells and stem cells (SCs) have been added;
3. definitions have been updated and expanded in number and scope, and moved to an earlier point in the document;
4. the structure of the document has been modified to include more background information, and the applicability of various sections to different types of cell substrates is highlighted;
5. a new section on risk reduction strategies during the manufacture of biological products has been added;
6. a section on Good Cell Culture Practice has been added;
7. the section of selection of source materials has been updated, and the detailed methods used to test for bovine viruses in serum were added in Appendix 1;
8. tumourigenicity testing has been updated, and a model protocol for the nude mouse model was added in Appendix 2;
9. oncogenicity testing of tumourigenic cell lysates was added, and a model protocol was added in Appendix 3;
10. recommendations for acceptable levels of residual cellular DNA are product specific and not specifically addressed; and
11. recommendations for microbial agents testing have been updated.

3. Scope

These recommendations supersede previous WHO requirements or recommendations describing procedures for the use of animal cell substrates for the production of biological medicinal products [1,13].

Some of these recommendations also may be useful in the quality control of specific biological products during the manufacturing process, but it is beyond the scope of this document to recommend quality control release tests. Like-wise, risk-based assessments related to product approvals are beyond the scope of this document. Requirements or recommendations for individual products should be consulted in that regard.

Cells modified by recombinant DNA technology have been increasingly used in the manufacture of novel medicinal products, and specific considerations for those products are addressed elsewhere [1,10,14,15]. Nevertheless, there are a number of generic issues that apply to genetically modified as well as to other cell substrates.

These recommendations specifically exclude all products manufactured in embryonated eggs, microbial cells (*i.e.*, bacteria and yeast), and plant cells. Also excluded are whole, viable animal cells

such as SCs when they are used directly for therapy by transplantation into patients or when they are developed into SCLs for the purpose of using them as therapeutic agents by transplantation. In those cases, characterization tests should be discussed with the NRA/NCL. However, SCLs used for the production of biological products such as growth factors and vaccines should comply with these recommendations.

Some of the general recommendations given here (see sections A.1 – A.5) are applicable to all animal cell substrates. More specific guidance for PCCs can be found in the relevant documents published by WHO (*e.g.*, production of poliomyelitis vaccine in primary monkey kidney cells) [4,5].

Cell substrates should be developed and used in accordance with applicable requirements of the NRA/NCL.

In general, it is not a practice consistent with Good Manufacturing Practices to re-test materials that have already been released for further manufacture, so justification would be necessary before such re-testing is undertaken. Thus, the scope of this document is intended to cover cell substrates as new cell banks are established. Specific circumstances under which re-testing of already established and released cell banks would be appropriate should be discussed with the responsible NRA/NCL.

Recommendations published by WHO are intended to be scientific and advisory in nature. The parts of each section printed in normal type have been written in the form of recommendations so that, should a NRA/NCL so desire, they may be adopted as they stand as the basis of national or regional requirements. The parts of each section printed in small type are comments or additional points that might be considered in some cases.

4. Definitions
The definitions given below apply to the terms used in these recommendations. They may have different meanings in other contexts.

*Adventitious agent***:** Contaminating microorganisms of the cell culture or source materials including bacteria, fungi, mycoplasmas/spiroplasmas, mycobacteria, rickettsia, protozoa, parasites, transmissible spongiform encephalopathies (TSE) agents, and viruses that have been unintentionally introduced into the manufacturing process of a biological product.

> The source of these contaminants may be from the legacy of the cell line, the raw materials used in the culture medium to propagate the cells (in banking, in production, or in their legacy), the environment, personnel, equipment, or elsewhere.

Biological medicinal product: Biological medicinal product is a synonym for biological product or biological described in WHO Technical Report Series. The definition of a medicinal substance, used in treatment, prevention or diagnosis, as a "biological" has been variously based on criteria related to its source, its amenability to characterization by physicochemical means alone, the requirement for biological assays, or on arbitrary systems of classification applied by regulatory authorities. For the purposes of WHO, including the present document, the list of substances considered to be biologicals is derived from their earlier definition as "substances which cannot be fully characterized by physicochemical means alone, and which therefore require the use of some form of bioassay".

However, developments in the utility and applicability of physicochemical analytical methods, improved control of biological and biotechnology-based production methods, and an increased applicability of chemical synthesis to larger molecules, have made it effectively impossible to base a definition of a biological on any single criterion related to methods of analysis, source or method of production. Nevertheless, many biologicals are produced using in vitro culture systems.

> Developers of such medicinal products that do not fit the definition of biological medicinal product provided in this document should consult the relevant NRAs for product classification and licensing application pathway.

Biotherapeutic: For the purpose of this document, a biotherapeutic is a biological medicinal product with the indication of treating human diseases.

Cell bank: A cell bank is a collection of appropriate containers whose contents are of uniform composition stored under defined conditions. Each container represents an aliquot of a single pool of cells.

> The individual containers (*e.g.*, ampoules, vials) should be representative of the pool of cells from which they are taken and should be frozen on the same day by following the same procedure and by using the same equipment and reagents.

Cell culture: The process by which cells are grown *in vitro* under defined and controlled conditions where the cells are no longer organized into tissues.

Cell line: Type of cell population with defined characteristics that originates by serial subculture of a primary cell population that can be banked.

> Cloning and sub-coning steps may be used to generate a cell line. The term cell line implies that cultures from it consist of lineages of some of the cells originally present in the primary culture.

Cell seed: A quantity of well-characterized cells stored frozen under defined conditions, such as in the vapour or liquid phase of liquid nitrogen in aliquots of uniform composition derived from a single tissue or cell, one or more of which would be used for the production of a master cell bank. Also referred to as a pre-MCB or seed stock. May be made under Good Manufacturing Practices (GMP) conditions or under manufacturer's research and development conditions.

Cell substrate: Cells used to manufacture a biological product.

> The cells may be primary or cell lines, and may be grown in monolayer or suspension culture conditions. Examples of cell substrates include primary monkey kidney, MRC-5, CHO, and Vero cells.

> Cells used to generate essential components of a final product, such as Vero cells for the generation of "reverse genetics" virus for use in seeding vaccine production, are considered to be "pre-production" cell substrates. Whereas cells used to manufacture the bulk product (*e.g.*, packaging cell lines for gene therapy vectors; Vero cells for vaccine production; CHO cells for recombinant protein expression) are considered to be "production" cell substrates.

Continuous cell line (CCL): A cell line having an apparently unlimited capacity for population doublings. Often referred to as "immortal" and previously referred to as "established".

Diploid cell line (DCL): A cell line having a finite *in vitro* lifespan in which the chromosomes are paired (euploid) and are structurally identical to those of the species from which they were derived.

> While this definition is accurate for standard chromosome preparations, a given human diploid cell line may contain genetic variations that will be reflected in a Giemsa-banding pattern that differs from the standard. Gene expression differences also may be found.
> This definition is based on experience and current understanding of the *in vitro* behavior of human cells that are not of stem cell origin.

DNA infectivity: The capacity of cellular DNA to generate an infectious virus following introduction of that DNA into appropriate cells. The viral genome could be integrated or extrachromosomal.

Endogenous virus: A virus whose genome is present in an integrated form in a cell substrate. Endogenous viruses are present in the genome of the original animal from which the cells were derived. They may or may not encode an intact or infectious virus.

End of production cells (EOPC): Cells harvested at or beyond the end of a production (EOP) run.

> In some cases, production cells are expanded under pilot-plant scale or commercial-scale conditions.

Extended cell bank (ECB): Cells cultured from the MCB or WCB propagated to the proposed *in vitro* cell age used for production or beyond.

Functional integrity: The culture sustains the expected performance related to its intended use under specified conditions (*e.g.,* expression of secreted product at a consistent level; production of expected yield of virus).

Immortalized: having an apparently unlimited capacity for population doubling.

Indicator cells: Cells of various species used in the *in vitro* adventitious agent test that are intended to amplify adventitious viruses to promote their detection. Generally, this would include a human diploid cell line, such as MRC-5, a monkey kidney cell line, such as Vero cells, and a cell line of the same species and tissue as the cell bank. The purpose of these cell lines is to *indicate* a viral infection of the cell bank either through observation of cytopathic effect during and after an appropriate observation period or by hemadsorption and/or hemagglutination at the end of the observation period. Thus, they are referred to as *indicator* cells. The cell bank may be analyzed on such indicator cells either by co-cultivation or by passage of cell lysates or spent culture supernatants from the cell bank onto the indicator cells.

In vitro cell age: Measure of time between thaw of the MCB vial(s) to harvest of the production vessel measured by elapsed chronological time, by population doubling level of the cells, or by passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

Latent virus: A virus is considered to be latent when the viral genome is present in the cell without evidence of active replication, but has the potential to be reactivated.

Master cell bank (MCB): A quantity of well-characterized cells of animal or other origin, derived from a cell seed at a specific population doubling level (PDL) or passage level, dispensed into multiple containers, cryopreserved, and stored frozen under defined conditions, such as the vapour or liquid phase of liquid nitrogen in aliquots of uniform composition. The master cell bank is prepared from a single homogeneously mixed pool of cells. In some cases, such as genetically engineered cells, this may be prepared from a selected cell clone established under defined conditions. Oftentimes, however, the MCB is not clonal. It is considered best practice that the master cell bank is used to derive working cell banks.

*Oncogenicity***:** The capacity of an acellular agent such as a chemical, virus, viral nucleic acid, viral gene(s), or a subcellular element(s) to cause normal cells of an animal to form tumours.

> Oncogenicity is distinct from tumourigenicity (see Tumourigencity). The tumours that arise in an oncogenicity test are of host origin whereas in a tumorigenicity test, the tumors are derived from the inoculated cells.

*Parental cells***:** Cells that are manipulated to give rise to a cell substrate. For hybridomas, it is typical to also describe the parental cells as the cells to be fused.

> Manipulation may be as simple as the expansion of a primary cell culture to provide early passage cells, or a more complex activity such as developing a hybridoma or transfected clone, and both processes would provide a cell seed. The parental cells may refer to any stage prior to the preparation of the cell seed. Examples of a parental cell are: WI-38 and MRC-5 at very early passage; Vero at passage 121; and CHO before the introduction of a DNA construct to produce a recombinant cell. In certain situations (*e.g.*, myeloma cells), there may be a lineage of identified stable parental clones, thus, the term "parental cell" would normally refer to the cells used immediately prior to generation of the "cell seed".

Passage: Transfer of cells, with or without dilution, from one culture vessel to another in order to propagate them, and which is repeated to provide sufficient cells for the production process.

> This term is synonymous with "subculture". Cultures of the same cell line with the same number of passages in different laboratories are not necessarily equivalent because of differences in cell culture media, split ratios, and other variables that may affect the cells. This is a more important consideration for SCLs and CCLs than for DCLs. Population doubling is the preferred method of estimating cell line age, and whenever possible, it should be used instead of "passage". However, it also may be appropriate to quantify culture duration of CCLs by the number of subcultivations at a defined seeding density at each passage or time in days.

Population doubling: A two-fold increase in cell number.

Population doubling level (PDL): The total number of population doublings of a cell line or strain since its initiation *in vitro*. A formula to use for the calculation of "population doublings" in a single passage is: number of population doublings = $\text{Log}_{10}(N/N_o) \times 3.33$ where: N = number of cells in the growth vessel at the end of a period of growth. N_o = number of cells plated in the growth vessel [16]. It is best to use the number of viable cells or number of attached cells for this determination.

Primary culture: A culture started from cells, tissues or organs taken directly from one or more organisms. A primary culture may be regarded as such until it is successfully subcultured for the first time. It then becomes a "cell line" if it can continue to be subcultured at least several times.

*Production cell cultures***:** A collection of cell cultures used for biological production that have been prepared together from one or more containers from the working cell bank or, in the case of primary cell cultures, from the tissues of one or more animals.

Residual cellular DNA (rcDNA): cell substrate DNA present in the final product.

Specific pathogen free (SPF): Animals known to be free of specific pathogenic microorganisms and reared in an environment that maintains that state. SPF animals usually are raised in biosecure facilities, and their health status is monitored on an ongoing basis. The SPF status simply provides

an assurance that the stock is not infected with the specified pathogens. SPF animals are not disease free nor are they disease resistant. They may carry pathogens other than those from which they are specified to be free.

Stem cell line: A continuous cell line generated from stem cells, rather than normal or diseased differentiated tissue.

Transmissible Spongiform Encephalopathy (TSE): The transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative diseases which include classical and variant Creutzfeldt–Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI), and Kuru in humans, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in mule deer and elk, and scrapie in sheep and goats.

*Tumourigenicity***:** The capacity of a cell population inoculated into an animal model to produce a tumour by proliferation at the site of inoculation and/or at a distant site by metastasis.

Tumourigenicity is distinct from Oncogenicity (see Oncogenicity).

WHO Reference cell bank: A cryopreserved stock of cells prepared from a single, homogeneous pool of cells prepared under defined conditions and subjected to characterization tests. The purpose of such a bank is to serve as a well-characterized cell seed for the preparation of master cell banks that will be extensively characterized by manufacturers, and that has a high probability of meeting these recommendations.

*Working cell bank (WCB)***:** A quantity of well-characterized cells of animal or other origin, derived from the master cell bank at a specific PDL or passage level, dispensed into multiple containers, cryopreserved, and stored frozen under defined conditions, such as in the vapour or liquid phase of liquid nitrogen in aliquots of uniform composition. The working cell bank is prepared from a single homogeneously mixed pool of cells. One or more of the WCB containers is used for each production culture.

5. General considerations
Types of animal cell substrates
Primary Cell Cultures (PCCs)
PCCs have played a prominent role in the development of biology as a science, and of virology in particular. Cultures of PCCs from different sources have been in worldwide use for the production of live and inactivated viral vaccines for human use for many decades. For example, PCCs of monkey kidney cells have been used for the production of inactivated and oral poliomyelitis vaccines since the 1950s.

Major successes in the control of viral diseases, such as poliomyelitis, measles, mumps and rubella, were made possible through the wide use of vaccines prepared in PCCs, including those from chicken embryos and the kidneys of monkeys, dogs, rabbits and hamsters, as well as other tissues.

PCCs are viable cells of disaggregated tissues that are initiated as *in vitro* cell cultures usually as adherent cells. Many cell types will be present and a primary culture may be a complex mixture of cells that can be influenced by the process and conditions under which they were harvested, disaggregated and introduced to *in vitro* culture. Not all cells in a primary culture will have the capacity to replicate. Particular care should be applied to establishing highly reproducible procedures

for tissue disaggregation, cell processing and culture initiation and reproducible culture conditions and nutrition.

PCCs obtained from wild animals usually show a high frequency of viral contamination. For example, monkey-kidney cell cultures can be contaminated with one or more adventitious agents, including simian viruses.

If PCCs are necessary for the production of a given biological, then the frequency of contaminated cell cultures can be significantly reduced by careful screening of the source animals for the absence of such viruses. Viruses can be detected by molecular tests such as PCR, and by looking for the presence of circulating antibodies to those viruses in the source animals. The use of animals bred in a carefully controlled colony, especially those that are specific-pathogen-free, is strongly recommended. Nevertheless, as suitable alternative cell substrates become available, PCCs are less likely to be used in the future. WHO has promoted the replacement of animals for experimental purposes both from an ethical perspective [17] and in the interests of progressive improvement in product safety and quality.

Advantages: (a) they are comparatively easy to prepare using simple media and bovine serum; and (b) they generally possess a broad sensitivity to various viruses, some of which are cytopathic.

Disadvantages: (a) contamination by infectious agents is a higher risk than with DCLs and CCLs; (b) the quality and viral sensitivity of cultures obtained from different animals are variable; and (c) although cell cultures derived from nonhuman primates had been in wide use in the past, it has become increasingly difficult to obtain and justify the use of such animals for this purpose, (d) they cannot be tested as extensively as DCLs or CCLs.

Diploid Cell Lines (DCLs)
The practicality of using human DCLs for the production of viral vaccines was demonstrated in the 1960s. The experience gained with oral poliomyelitis and other viral vaccines in successfully immunizing billions of children in many countries has shown clearly that such substrates can be used in the production of safe and effective vaccines [3].

The essential features of DCLs of human (*e.g*., WI-38, MRC-5) and monkey (*i.e.*, FRhL-2) origin are: (a) they are cells passaged from primary cultures that have become established as cell lines with apparently stable characteristics over numerous PDLs; (b) they have a finite capacity for serial propagation, which ends in senescence, a state in which the culture ceases to replicate, the cells remain alive and metabolically active, but may show morphological and biochemical changes some of which begin to appear before replication ceases; (c) they are non-tumourigenic; and (d) they display diploid cytogenetic characteristics with a low frequency of chromosomal abnormalities of number and structure until they enter senescence. Substantial experience since the 1960s has been accumulated on the cytogenetics of WI-38 and MRC-5, and ranges of expected frequencies of chromosomal abnormalities have been published [18,19]. Similar data are available for FRhL-2 [20]. More sophisticated cytogenetic techniques (*e.g.*, high resolution banding, comparative genome hybridisation) [21,22] have demonstrated the presence of subtle chromosomal abnormalities that were previously undetectable. Recent studies have shown that subpopulations of human DCLs with such abnormalities may appear and disappear over time, and that they are non-tumourigenic and

undergo senescence in the same manner as the dominant population. Thus, possessing a stable karyotype might not be such an important characteristic as was previously thought.

Advantages: (a) they can be well characterized and standardized; (b) production can be based on a cryopreserved cell-bank system that allows for consistency and reproducibility of the reconstituted cell populations. A cell-bank system usually consists of cell-banks of defined population doubling or passage levels that generally include a MCB and a working cell-bank (WCB); and (d) unlike the CCLs and SCLs discussed below, DCLs are not tumourigenic and therefore do not raise the potential safety issues associated with CCLs and SCLs.

Disadvantages: (a) they are not easy to use in large-scale production, although they have been cultivated using bioreactor technology employing the microcarrier or multilayer method; (b) in general, they have more fastidious nutritional requirements than other cell substrates; (c) they may be difficult to adapt to serum-free growth; and (d) they are more difficult than CCLs to transfect and engineer, and require immortalization before they can be engineered; (*e.g*., they are not permissive for the production of vaccine vectors that require complementation, since they cannot be engineered readily to express complementing proteins).

Continuous cell lines (CCLs)
Some CCLs have been used for the production of safe and effective biotherapeutics and vaccines since the 1980s.

CCLs have the potential for an apparently indefinite *in vitro* life span and have been derived by the following methods: (a) serial subcultivation of a PCC of a human or animal tumour (*e.g*., HeLa cells); (b) transformation of a normal cell having a finite life span with an oncogenic virus or viral sequence (*e.g*., B lymphocytes transformed by EBV or transfected with viral sequences such as in PER.C6); (c) serial subcultivation of a primary-cell population derived from normal tissue that generates a dominant cell population having an apparently indefinite life span, often described as spontaneous transformation (*e.g*., Vero, BHK-21, CHO, MDCK, Hi5); (d) fusion between a myeloma cell and an antibody-producing B lymphocyte to produce a hybridoma cell line; or (e) use of ectopically expressed genes involved in the cell cycle such as hTERT telomerase gene to enable indefinite replication of normal human cells.

CCLs may display a consistent modal chromosome number (*e.g*., MDCK, Vero), and although the karyotype of individual cells in a culture at any one time-point may vary, the range of chromosome numbers per cell will usually show characteristic limits. However, other CCLs, such as highly tumourigenic cells including HeLa may show variation in modal number and a wider drift in the range of the number of chromosomes per cell.

In the early stage of establishing a cell line, significant diverse karyotypes and changes in karyotype may be observed, but a characteristic typical chromosome component may emerge with continued passage presumably as a dominant cell population develops.

Advantages: (a) they can be characterized extensively and their culture conditions standardized; (b) production can be based on a cell-bank system, which allows consistency and reproducibility of the reconstituted cell populations for an indefinite period; (c) as a rule, they grow more easily than

DCLs using standard media, (d) most can be adapted to grow in serum-free medium; (e) they usually can be grown on microcarriers for large-scale production in bioreactors; and (f) some can be adapted to grow in suspension cultures for large-scale production in bioreactors.

Disadvantages: (a) CCLs may express endogenous viruses, and some are tumourigenic in immunosuppressed animal models; (b) theoretical risks identified by the 1986 Study Group (*e.g.*, nucleic acids, transforming proteins, and viruses) need to be taken into account.

Stem Cell Lines (SCLs)
SCs differ from other types of cells because they sustain a predominant stem-cell population whilst simultaneously retaining the capacity to produce cell progenitors of differentiated cell types of almost all human tissues (*i.e.*, pluripotent). Pluripotent SCLs have an apparent capacity to generate cell types of all three human germlayers and may be capable of generating *in vitro* models of any tissue in the human body. At the time these recommendations are written (2010), two types of pluripotent SCLs, human embryonic stem cells and induced pluripotency stem cell lines have been isolated which may have the capabilities to prove useful for manufacturing biologicals. This property of pluripotency is sustained through numerous cycles of cell division. SCLs may be derived from early stage embryonic, fetal or adult tissues. Typically, specialized media and environmental conditions such as the attachment matrix are required for the growth of SCLs *in vitro* in order for them to maintain the undifferentiated state. While most stem cell research and development has been directed towards transplantation of SCs for therapeutic purposes, efforts also have gone into exploring a variety of SCLs as cell substrates for the production of biologicals.

Key considerations for the culture and control of such cell lines have been developed [23]. These include the fundamental issues common to the maintenance of all cell lines, but also emphasize the need for appropriate ethical governance regarding donor consent and careful attention to periodic confirmation of phenotype, absence of non-diploid cells, and sustained pluripotent capacity.

Recently, it has been shown that conditioned medium from SCLs can have regenerative properties, and such preparations produced from human embryonic SCs have shown regenerative capabilities including repair of myocardial infarction in animal models [24]. This raises the possibility of SCs being used as a substrate to produce a variety of biologically active molecules. SCLs can, in some respects, be considered as diploid cells, but they do not appear to have the finite life span characteristic of human diploid fibroblast cultures. In human embryonic stem-cell cultures, clonal variants with chromosomal abnormalities are known to arise. Whilst a diploid and non-transformed nature is to be considered a pre-requisite for cell therapy applications, transformed SCLs might be considered as a form of CCL for the manufacture of biologicals. Because they do not fall easily within any one category of substrate already discussed, SCLs are identified separately in this document.

Advantages
(a) they can be well characterized and their culture conditions standardized; (b) production can be based on a cell-bank system, which allows consistency and reproducibility of the reconstituted cell populations for an indefinite period; and (c) some may be adapted to grow in suspension cultures for large-scale production in bioreactors; (d) they may produce unique proteins of potential importance

as biotherapeutics; and (e) they have the potential to generate cells and tissue-like structures that may permit the expression of agents currently considered "unculturable" *in vitro*.

Disadvantages
(a) Subculture techniques commonly used for SCLs are laborious; (b) may produce growth proteins with undefined effects on adult cells/tissues; (c) usually require complex media that may have a TSE risk; (d) rapid development of differentiated cells also means that they are difficult to control *in vitro*; (e) there is little experience with their use as a cell substrate to manufacture biological products.

Potential risks and risk mitigation associated with biologicals produced in animal cell cultures
The main potential risks associated with the use of biologicals produced in animal cells are directly related to contaminants from the cells, and they fall into three categories: (a) viruses and other transmissible agents; (b) cellular nucleic acids (DNA and RNA); and (c) growth-promoting proteins. In addition, cell-derived inhibiting or toxic substances are theoretically possible. A summary of the risk assessment for each follows. More comprehensive information has been published elsewhere on the risks associated with contaminating DNA and growth-promoting proteins [25,26,27,28,29,30,31,32,33,34].

Early in 2010 NRA as well as WHO were made aware of new information regarding the presence of DNA sequences of porcine circovirus in live attenuated rotavirus vaccines. The detection of these sequences by the use of advanced analytical methods raised complex questions, *e.g.*, the evaluation of the potential risk, specific testing of vaccines and the general use of these methods for the characterization of vaccine cell substrates. The power of the new methodology that was used (*i.e.*, massively parallel (deep) sequencing) may uncover the presence of adventitious agents that might not be detected with current methods. While the implementation for routine use of such methods has benefits as well as challenges and risks, NRAs need to be prepared for similar situations. Considerations for making a risk assessment and potentially introducing risk mitigation strategies may need to be undertaken in such circumstances.

Viruses and other transmissible agents
There is a long history of concern regarding the potential transmission of viruses and other infectious agents that may be present in cell substrates. This area was reviewed most recently by WHO in 1986 by a SG who pointed out that, as described below, cells differ with respect to their potential for carrying viral agents pathogenic for humans.

Primary monkey kidney cells have been used to produce billions of doses of poliomyelitis vaccines since they were first developed in the 1950s, and although viruses such as SV40 were discovered in rhesus monkey kidney cells, control measures were introduced to eliminate or reduce as much as possible the risk of viral contamination associated with the manufacture of vaccines in cells containing those viruses. Additional controls may be needed as new viral agents are identified and technologies to detect them are developed.

Human and nonhuman primate lymphocytes and macrophages may carry latent viruses, such as herpesviruses and retroviruses. CCLs of non-haematogenous cells from human and nonhuman

primates may contain viruses or have viral genes integrated into their DNA. In either case, virus expression may occur under *in vitro* culture conditions.

Avian tissues and cells may harbour exogenous and endogenous retroviruses, but there is no evidence for transmission of disease to humans from products prepared using these substrates. For example, large quantities of yellow fever vaccines were produced for many years in eggs that contain avian leukosis viruses, but there is no evidence that these products have transmitted disease in their long history of use for human immunization. Nevertheless, the potential for transmitting avian retroviruses should be reduced as much as possible through manufacturing control measures.

Rodents may harbour exogenous and endogenous retroviruses, lymphocytic choriomeningitis virus, and Hantaviruses, and a range of other potentially zoonotic viruses. While contamination with the rodent viruses in the cell harvests of biotherapeutic products derived from CHO cell culture has been reported [34,35,36], there is no evidence that biological products released for distribution have been contaminated with rodent viruses, because, if present, they were detected during quality control testing in compliance with GMPs prior to release. In addition, it is important to note that there have been no reported cases of transmission of an infectious agent to recipients of recombinant protein products manufactured in animal cells.

Insect cells recently have been used for vaccine production, and various insect cell lines may be used for the production of biologicals in the future. Insect viruses tend to be ubiquitous in many insect cell lines, and are generally unknown and/or uncharacterized. Many insect cell lines have endogenous transposons and retrovirus-like particles, and some are positive in PERT assays.

HDCs have been used for vaccine production for many years, and although concern was initially expressed about the possibility of such cells containing a latent pathogenic human virus, no evidence for such an endogenous agent has been reported, and vaccines produced from this class of cell substrate have proven to be free from viral contaminants.

In light of the differing potential of the various types of cells mentioned above for transmitting viruses that are pathogenic in humans, it is essential that the cells being used to produce biological products be evaluated as thoroughly as possible with respect to infectious agents.

When DCLs, SCLs, or CCLs are used for production, a cell bank system should be used and the cell banks should be characterized as specified in this document. Efforts to identify viruses by testing for viral sequences or other viral markers, especially those not detectable by other means, constitute an important part of the evaluation of cell banks in addition to the standard tests that have been in place for many years.

When cell lines of rodent or avian origin are examined for the presence of viruses, the major emphasis in risk assessment should be placed on the results of studies in which transmission to target cells or animals is attempted. Risk to human recipients should not be assessed solely on ultrastructural or biochemical/biophysical evidence of the presence of viral or viral-like agents in the cells.

The overall manufacturing process, including the selection and testing of cells and source materials, any purification procedures used, and tests on intermediate or final products, should ensure the absence of detectable infectious agents in the final product. When appropriate, validation of purification procedures should demonstrate adequate reduction of relevant model viruses with a significant safety factor [14]. This is usually required for recombinant protein products.

There may be as yet undiscovered microbial agents for which there is no current evidence or means of detection. As such agents become identified, it will be important to consider whether to re-examine cell banks for their presence. In general, it is not a practice consistent with GMPs to re-test materials that have already been released, so justification would be necessary before such re-testing is undertaken. Positive findings should be discussed with the NRA/NCL. Whenever new data are developed with the potential for an impact on the quality, safety or efficacy of a biological product, it is the responsibility of the manufacturer to provide NRAs all relevant data and information currently available. This should include confirmation and evaluation of the finding, the manufacturer's own risk assessment as well as an investigational and action plan, in order to facilitate any regulatory action that might be necessary. In addition, new testing methods are likely to be developed, and as they become available and validated, they should be considered by manufacturers and NRAs/NCLs for their applicability to the characterization and control of new animal cell substrates.

Cellular DNA
The issue of rcDNA in biological products has been considered by many groups since the 1980s, and there has been an evolution of consensus on recommendations during that period. The most recent WHO recommendation (TRS 878) sets the upper limit of rcDNA at 10 ng per parenteral dose. As stated below, while this value has proved helpful in the past, it does not take into consideration important factors such as the size of the DNA fragments and any potentially inactivating steps in the manufacturing process. Therefore, the amount of rcDNA that might be acceptable for a specific product should take into consideration not only the limit of 10 ng per parenteral dose, but other factors as well when determining the acceptable level of rcDNA.

PCCs and DCLs have been used successfully for many years for the production of viral vaccines, and the rcDNA deriving from these cells has not been (and is not) considered to pose any significant risk. However, with the use of CCLs, which have an apparently indefinite life span, presumably due to the dysregulation of genes that control growth, and with the ongoing development of products from cells that are tumourigenic or were derived from tumors, the DNA from such cells has been considered to have the theoretical potential to confer the capacity for unregulated cell growth, and perhaps oncogenic activity, upon some cells of a recipient of the biological product. Although the risk of such DNA has been estimated based on certain assumptions and some experimental data, assessing the actual risk of such DNA has not been possible until recently when preliminary data generated from new experimental systems began to quantify those risks [37].

The potential risks of DNA arise from both of its biological activities: (a) infectivity, and (b) oncogenicity. Infectivity could be due to the presence of an infectious viral genome in the cellular DNA of the cell substrate [38,39,40]. The viral genome could be that of a DNA virus, whether integrated or extrachromosomal, or that of a proviral genome of a retrovirus. Both types of viral DNA have been shown to be infectious *in vitro* and in several cases, *in vivo* [37,38,39]. The

oncogenic activity of DNA could arise through its capacity to induce a normal cell to become transformed and perhaps to become tumourigenic. The major mechanism through which this could occur would be through the introduction of an active dominant oncogene (*e.g.*, *myc,* activated *ras*), since such dominant oncogenes could directly transform a normal cell. Other mechanisms require that the rcDNA transform through insertional mutagenesis, and have been considered less likely, since the frequency of integration of DNA in general is low [40]. The frequency of integration at an appropriate site, such as inactivating a tumor suppressor gene or activating a proto-oncogene, would be correspondingly lower [31].

The 1986 WHO SG addressed the risk posed by the oncogenic activity of rcDNA in biological products for human use [12]. Risk assessment based on a viral oncogene in an animal model suggested that *in vivo* exposure to one nanogram (ng) of rcDNA, where 100 copies of an activated oncogene were present in the genome, would give rise to a transformational event once in 10^9 recipients [26]. On the basis of this and other evidence available at that time, the 1986 SG concluded that the risk associated with rcDNA in a product is negligible when the amount of such DNA is 100 picograms (pg) or less per parenteral dose. Based on a review of more recent data, those requirements were revised in 1998 to raise the acceptable level of rcDNA to 10 ng per dose.

Studies in mice using cloned cellular oncogenes also suggest that the risk of neoplastic transformation by cellular DNA is probably very low [33,42]. However, more recent data have shown that cloned cellular oncogene DNA can induce tumors in selected strains of mice at levels below 1 ng. In addition, single oncogenes can also be biologically active [42] and initiate the tumour induction process. Because of these data, and the recent description that genes encoding for certain micro-RNA species can be oncogenic *in vitro* [44,45,46,47], thus increasing the number of potential dominant cellular oncogenes, the oncogenic risk of DNA needs to be considered when tumourigenic cells are considered for use in the production of biologicals. This would be especially important for live, attenuated viral vaccines where chemical inactivation of the DNA is not possible, and the only way the biological activity of DNA could be reduced would be by nuclease digestion and the reduction in the quantity of DNA.

In addition to its oncogenic activity, the infectivity of DNA should be considered. Since a viral genome once introduced could amplify and produce many infectious particles, the infectivity risk is likely greater than the oncogenic risk. The polyoma virus genome is infectious in mice at about 50 pg [48], and a recent report demonstrated that 1 pg of a proviral copy of a retrovirus is infectious *in vitro* [49]. Because such low levels of DNA may be biologically active, the amounts of rcDNA should be factored into safety evaluations when tumourigenic cell substrates are used, especially for live viral vaccines.

Therefore, considerations that need to be taken into account with respect to rcDNA are: (a) any reduction in the amount of the contaminating DNA during the manufacturing process; (b) any size reduction of the contaminating DNA during the manufacturing process; and (c) any chemical inactivation of the biological activity of contaminating DNA during the manufacturing process. A product might be considered by a NRA/NCL to have an acceptable level of risk associated with the DNA of the cell substrate on the basis of (a) and/or (b) and/or (c), when data demonstrate that appropriate levels have been achieved. For example, data have shown that nuclease digestion of DNA or chemical inactivation of DNA with beta-propiolactone, a viral inactivating agent, can

destroy the biological activity of DNA [37,49,50]. Therefore, the use of these procedures may provide an additional level of confidence with respect to DNA risk reduction.

For products such as monoclonal antibodies and subunit vaccines manufactured in tumourigenic cell substrates, it is necessary to demonstrate the clearance (removal and/or inactivation) of DNA by the manufacturing process, which may require the validation of the main inactivating or removal steps. For example, data should be obtained on the effects of DNA-inactivating agents under specific manufacturing conditions so that firm conclusions on their DNA-inactivating potential for a given product can be drawn.

There may be instances where CCL DNA is considered to pose a higher level of risk because it contains specific elements such as infectious retroviral proviral sequences. Under these circumstances, the steps taken to reduce the risks of rcDNA, such as reducing the size of DNA fragments, should be set in consultation with the NRA/NCL.

The 1986 WHO SG stated that the risks for rcDNA should be considered negligible for preparations given orally. This conclusion was based on the finding that polyoma virus DNA was not infectious when administered orally [48]. For such products, the principal requirement is the elimination of potentially contaminating viruses. Recently, additional data on the uptake of DNA *via* the oral route have been published [51]. These studies demonstrated that the efficiency of uptake of DNA introduced orally was significantly lower than that introduced intra-muscularly. Nevertheless, the specifics of the manufacturing process and the formulation of a given product should be considered by the NRA/NCL.

With respect to the efficiency of DNA uptake *via* the nasal route, no data have been published comparing the nasal route with parenteral routes. However, data presented publically show that uptake *via* the intranasal route is less efficient than by the intramuscular route. Limits for a specific product should be set in consultation with the NRA/NCL.

In general, acceptable limits of rcDNA for specific products should be set in consultation with the NRA/NCL taking into consideration the characteristics of the cell substrate, the intended use of the biological product, and most importantly the effect of the manufacturing process on the size, quantity and biological activity of rcDNA fragments. In general, it has been possible to reduce rcDNA in biotechnological products to <10 ng per dose, and in many cases <10 pg per dose, because they can be highly purified. Quantitative PCR for short amplicons has been used to determine total residual DNA levels as well as residual DNA fragment size distribution. It should be noted that other methods may give different results for small fragments or for DNA that has been treated with inactivating agents. Whatever methods are used should be validated. Some products, especially certain live viral vaccines, are difficult to purify without a significant loss in potency, so that the amount of rcDNA in those final products may be significantly higher than 10 ng per dose. Such cases are considered to be exceptional and should be discussed with the NRA/NCL.

Cellular RNA
While protein-coding RNA has not been considered to be a risk factor for biological products due to the unstable nature of RNA and the lack of mechanisms for self-replication, the recent description of small non-coding RNA molecules – microRNA (miRNA) – that are more stable and have the

capacity to modulate gene expression might necessitate a reassessment. Whether these miRNA molecules can be taken up by cells *in vivo* is unknown. However, as stated above, because certain miRNA genes can be oncogenic, DNA containing such sequences may need to be considered along with oncogenes when assessing the risk of rcDNA (see B.9 Oncogenicity). However, because this is an evolving area of research, no conclusions can be made regarding the risk of miRNA, and no recommendations are made to control miRNA at this time.

Growth-promoting proteins

Growth factors may be secreted by cells used to produce biologicals, but the risks from these substances are limited, since their growth-promoting effects are usually transient and reversible, they do not replicate, and many of them are rapidly inactivated *in vivo*. In exceptional circumstances, growth factors can contribute to oncogenesis, but even in these cases, the tumours apparently remain dependent upon continued administration of the growth factor. Therefore, the presence of known growth-factor contaminants at ordinary concentrations does not constitute a significant risk in the preparation of biological products manufactured in animal-cell cultures. However, some SCLs may secrete higher levels and more potent factors than CCLs. This should be taken into account when designing characterization studies, and the manufacturing process should be designed to address any safety issues that are identified.

Part A. General recommendations applicable to all types of cell culture production

A.1 Good manufacturing practices

The general principles of GMP for biologicals should be in place. Requirements or recommendations have been made by NRAs (*e.g*., EMA, FDA) and other groups (*e.g*., ICH). GMP should be applied from the stage of cell banking.

In the preparation of a cell substrate, it is considered best practice to establish tiered master and working cell banks (A.5.3) to ensure a reliable and consistent supply of cells that can be fully characterized and safety tested prior to use for production. By definition, primary-cell cultures cannot be subjected to such banking regimens. However, some manufacturers have utilized pooled and cryopreserved primary cultures, which enable completion of lot-release testing as in a tiered banking system. The strategy for delivery of primary cells or primary cells recovered from cryopreservation should be based on the quality and safety that can be assured for the final product according to the overall manufacturing and control processes involved.

A.2 Principles of Good Cell Culture Practices
A.2.1 *Understanding the cells and the culture system*
In all aspects of sourcing, banking and preparing cell cultures, the principles of Good Cell Culture Practices (GCCPs) should be observed (for example, 52, 53 for SCLs). Fundamental features to be considered in the development of cell cultures for production or testing are:
1. authenticity, including identity, provenance, and genotypic/phenotypic characteristics
2. absence of contamination with another cell line
3. absence of microbiological contamination, and
4. stability and functional integrity on extended *in vitro* passage

An important basic principle for all types of cells is that the donor should be free of transmissible diseases or diseases of uncertain etiology, such as CJD for humans and BSE for cattle. The NRA/NCL may allow specific exceptions concerning donor health (*e.g*., myeloma, other tumour cells).

Cells in culture may change their characteristics in response to changes in culture conditions or on extended passage under the same culture conditions. The four cell culture types (PCC, DCL, SCL, CCL) used in manufacture differ in their potential stability, and characterization approaches may need to be adapted in reflection of these differences.

Cell cultures grow in an *in vitro* environment that is substantially different from the conditions experienced by cells *in vivo*, and it is not unexpected that they may be susceptible to change or alteration as a result of *in vitro* culture and processing. It is important to be conscious of the variation that may arise in the cell-culture environment, as cells may undergo subtle alterations in their cell biology in response to such changes. It is therefore necessary to try to control key known variables that could have significant impact on the cell culture. Medium and specific additives (serum, growth factors, amino acids and other growth promoting compounds) should, where possible, be specified in

terms of chemical composition and purity. Where relevant, biological activity of the medium and additives should be determined before use. New batches of reagents for cell culture should be supplied with certificates of analysis and origin, which enable their suitability to be evaluated against the established specification. The use of serum or other poorly defined reagents is not recommended in the production of new biologicals from cell culture, and wherever possible chemically defined alternatives should be sought. However, given that our current understanding of cell biology is not complete, there is a balance to be made between the benefits that defined media bring in the form of higher reproducibility and reduced risk, and the potential effects of inadequacies of defined-culture systems that may not meet the full biological needs of cells. Where complex biological reagents, such as FBS remain necessary, they should be carefully controlled, whenever possible, by pre-use selection of batches. Such careful selection also should apply to cell-culture surfaces using specified culture vessels or surface coatings where relevant.

Variation in physical-culture parameters, such as pH, temperature, humidity, and gas composition, can significantly influence the performance and viability of cells and should be specified with established tolerances and the relevant equipment calibrated and monitored. In addition, any culture reagents prepared in the laboratory should be documented, quality controlled and released against an established specification.

A.2.2 *Manipulation of cell cultures*
In vitro processing of cells can introduce additional physical and biochemical stresses that could have an influence on the quality of the final product. Care should be taken to minimize manipulations, taking into consideration the specifics of the manufacturing process. In all cases, a consistent process should be demonstrated.

A.2.2.1 *Detachment and subculture*
Detachment solutions may adversely affect the cells if exposure is not minimized. Cell harvesting and passaging procedures should be carried out in a reproducible way ensuring consistency in the confluency of cells when harvested, incubation times, temperature, centrifugation speeds and times, and post-passage viable cell seeding densities.

A.2.2.2 *Cryopreservation* (see A.5.1)

A.2.2.3 *Introduction of contamination*
As already mentioned, the microbiological status of the donor individual, colony, herd, or flock is an important consideration in the establishment of PCCs. In order to avoid catastrophic failure of the production process and to avoid infectious hazards for recipients of products, it is important to minimize the opportunities for contamination of cell cultures. Therefore, cell manipulation and open processing steps should be minimized, taking into consideration the specifics of the manufacturing process. It is critical to adopt rigorous aseptic technique and provide appropriate environmental controls and air quality for cell-culture processing and preparation of growth media. The presence of any antimicrobial in a biological process or product is discouraged, although a notable exception is that antibiotic(s) and antifungal(s) may be required for primary-cell cultures. Additionally, antibiotics may be used in some cell-line selection systems. Where antibiotics have been used, sterility testing procedures should account for potential inhibitory effects of the antibiotic on

contaminating organisms. Penicillin or other beta-lactam antibiotics should not be present in production-cell cultures.

A.2.3 *Training and staff*
Training in all cell-culture processes is vital to ensure correct procedures are adhered to under GMPs. Staff should be trained in the underlying principles of cell-culture procedures to give them an understanding of cell-culture processes that will enable them to identify events and changes that could impact on the quality of cells [52]. Key procedures on which such GCCPs training should focus include passaging of cells, preparation of sterile media, maintenance and use of biological safety cabinets, incubators, and cryopreservation.

Cell cultures should be prepared by staff who have not, on the same working day, handled animals or infectious microorganisms. Furthermore, cell cultures should not be prepared by staff who are known to be suffering from a transmissible infection. The personnel concerned should undergo a "return to work" assessment to evaluate any residual risk.

A.2.4 *Cell line development and cloning*
Wherever a cell culture has passed through a process that may have a significant influence on its characteristics, such as tumourigenicity, it should be treated as a new (*i.e.*, different) cell line and should be renamed with a suffix or code to identify this. A MCB should then be prepared from the post 'treatment' culture. Treatments that may require such rebanking include cell cloning and genetic manipulation. Any change(s) to the cell-culture process should be demonstrated not to affect product quality and should be discussed with NRA. In the manufacture of monoclonal antibodies cloning of hybridoma cultures is particularly important to ensure that a single product is generated since inclusion of more than one hybridoma cell type could lead to a mixture of different antibody specificities and classes being present.

The details of cloning and selection may vary, depending on the practices of individual manufacturers and should be discussed with the NRA/NCL.

For example, in the early stages of cell-line development, a number of different recombinant vector systems and cell lines may be used. This will essentially be a research activity, but the cell lines and vectors should originate from well characterized and qualified sources and the cells from an appropriately qualified seed stock or MCB, which will usually be 'in-house' host cells and vectors. The most promising cell/vector combination will then be used to generate a large number of clones (100s -1000s) after transfecting the culture with rDNA. Typically, these clones will be screened based on their productivity, and a number with the highest productivity (10-50) will be taken forward for further evaluation. Further testing will then be used to select a small number (1-5) for establishment as small pre-master cell banks, and a final selection will be made, often based on stability characteristics and amenability to scale-up, before finally generating a MCB and WCB. Throughout the process, only well characterized and traceable growth media and other critical reagents will be used (usually the same as for the MCB), and cryopreserved stocks of all working clones will be made at appropriate stages in the development process (Figure 1).

Figure 1. Simplified Schematic of an Example of the Development of a Genetically Modified Cell Line
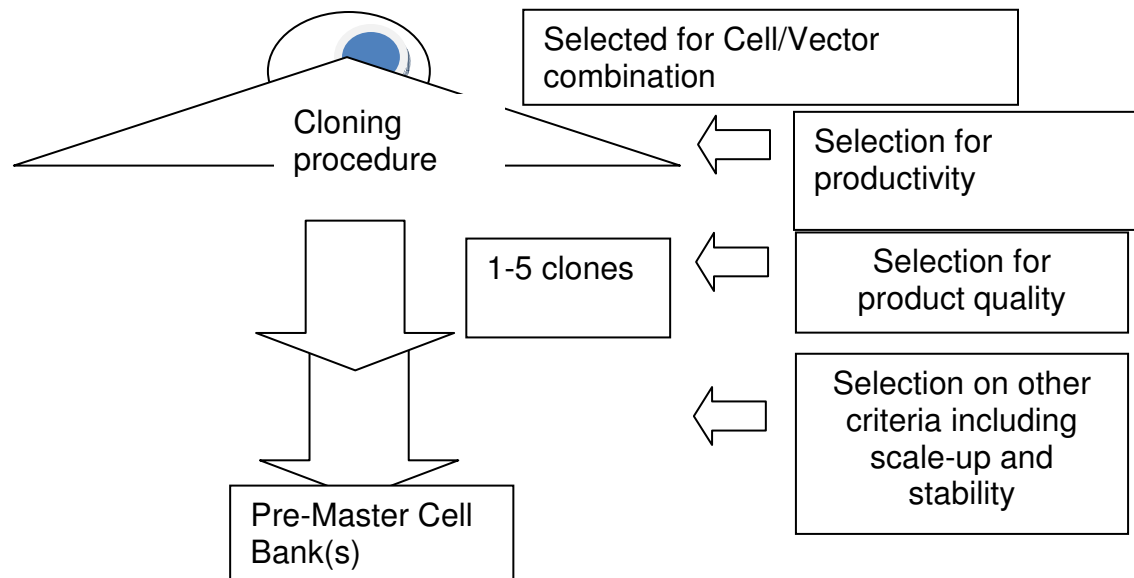
In the process of cloning a cell culture, single cells should be selected for expansion. The cloning procedure should be carefully documented, including the provenance of the original culture, the cloning protocol, and reagents used. Cloning by one round of limiting dilution will not necessarily guarantee derivation from single cells; additional subcloning steps should be performed. Alternatively or in addition to limiting dilution steps the cloning procedure can include more recent technology such as single cell sorting and arraying, or colony picking from dilute seeds into semi-solid media. In any case, the cloning procedure should be fully documented, accompanied by imaging techniques and/or appropriate statistics. For proteins derived from transfection with recombinant plasmid DNA technology a single, fully documented round of cloning is sufficient provided product homogeneity and consistent characteristics are demonstrated throughout the production process and within a defined cell age beyond the production process.

It is important to accurately document the establishment of each clone, which should also have a unique reference. Cryopreserved seed stocks of a significant number of clones should be established at an early stage. The clones can then be compared in parallel with the parental culture to establish candidate clones with the best overall characteristics for delivery of the desired product. The criteria used in the evaluation of the clone selected for production should include: genomic and phenotypic stability, growth rate, achievable product levels, and integrity/stability of the product. The evaluation of early candidate clones should generate sufficient information for the manufacturer to make an informed decision on the selection of the most promising clone(s) for further development. Where genetically engineered cell clones are under evaluation, these criteria also should include stability of integrated rDNA. The details of this process could vary depending on a number of factors including the nature of the host cell, desired characteristics of the product and the manufacturer's local procedures.

It is important to bear in mind that even following single cell cloning, epigenetic variation can result in a cloned culture showing evidence of heterogeneity (*i.e.*, more than one clone). This should not preclude the use of such a culture for production, unless there are indications of instability that could affect the quality and/or safety of the final product.

A.2.5 *Special considerations for neural cell types*
Agents causing transmissible spongiform encephalopathies have been propagated in certain cells. At the time of writing, the phenomenon has been observed only with very specific pairs of agents and cells; no cell line has been identified that will replicate all agents, although one has been described that seems to be infectable by many strains of scrapie. The phenomenon is unpredictable, except that if the line does not express the PrP protein, it may be assumed to be impossible to infect it, and experience to date suggests that infection is not commonly observed or easy to maintain. On the other hand, the cell types that can be infected include fibroblastic lines as well as neuronal cells. The cells have usually been of murine origin, because the infecting agents are usually mouse-adapted scrapie. The fact that certain cells can be infected with certain agents is proof of principle that cell lines may be infected so that exposure of cells to sources potentially contaminated with the agents is a concern. The scale of the risk is difficult to judge, and it is recommended that with respect to safety considerations and TSEs attention focuses on the selection and documentation of the cell culture reagents and other materials that come into intimate contact with the cells to provide assurance that they are not contaminated. Strategies to accomplish this are given in section B.11.4.

A.3 Selection of source materials
A.3.1 *Introduction*
All materials should be subjected to a risk assessment and testing when necessary, in particular, raw materials derived from humans and animals, which can be a primary source for the introduction of adventitious agents into the production of biologicals. Therefore, careful attention should be paid to their sourcing, production, handling, testing, and quality control. All cell-culture materials of biological origin that come into intimate contact with the cells during the establishment of cell cultures, derivation of a new cell line (if any), banking procedures (if any), and production, should be subjected to appropriate tests, as indicated by risk assessment, for quality and freedom from contamination by microbial agents to evaluate their acceptability for use in production. It is important to evaluate the microbiological risks represented by each individual human- and animal-derived reagent used in a cell culture production process, and it should address: (a) geographical origin; (b) species of origin; (c) general microbiological potential hazards including a consideration of the medical history for human-derived reagents; (d) husbandry/screening of donor animals; (e) testing performed on the product, including Certificates of Analysis (if any); and (f) the capacity for the preparation, purification, and sterilization procedures (if any) used to remove or inactivate contaminants [54]. Other reagents of biological, but of non-animal origin, may also present risks to product safety, and these are discussed further in section A.3.4

Recombinant protein technology now provides many materials formerly derived directly from animal or human sources. While this eliminates obvious virological risks from donors, the manufacturing process used for the recombinant proteins should be analyzed for any materials of biological origin and any associated hazards that may need to be addressed, as indicated in items a-f above.

The NRA/NCL should approve source(s) of animal-derived raw materials, such as serum and trypsin. These materials should comply with the guidelines on tissue infectivity distribution of TSEs [55]. They should be subjected to appropriate tests for quality and freedom from contamination by microbial agents to evaluate their acceptability for use in production. Their origin should be documented to ensure that the sources are from geographical regions with acceptable levels of microbiological risk (*e.g*., freedom from foot and mouth disease virus or bovine spongiform encephalopathies). In addition, documentation should be gathered on their manufacturing history, production, quality control, and any final or supplementary processing that could affect quality and safety, such as blending and aliquoting of serum batches. In addition, controls should be in place to prevent cross-contamination of one material with another (*e.g*., bovine material in a porcine product).

The reduction and elimination from the manufacturing process of raw materials derived from animals and humans is encouraged, where feasible.

For some human- and animal-derived raw materials used in the cell culture medium, such as insulin or transferrin, validation of the production process for the elimination of viruses can substitute for virus detection tests, when justified.

Animal-derived reagents, such as trypsin and serum, which would be substantially damaged or destroyed in physical sterilization processes, including heat and irradiation, present the most likely microbiological hazards to cell-culture processes. Batches of reagents, such as trypsin and bovine serum, have been known to contain Mycoplasma species and sometimes more than one viral contaminant. Certain contaminants also have been shown to infect cells in culture. The processing environment is also a common source of microbiological contamination and should be controlled to minimize this risk and prevent growth of contaminants.

A.3.2 *Serum and other bovine-derived materials used in cell culture media*

The source(s) of serum of bovine origin should be approved by the NRA/NCL. The responsibility for ensuring the quality of the serum used in the manufacture of cell banks and biologicals rests with the biologicals' manufacturer. This can be accomplished in more than one manner. The manufacturer might conduct adventitious agent testing and perform inactivation of the serum after purchase from the serum manufacturer. Alternatively, the manufacturer might qualify their serum vendor and only purchase serum from suppliers after conducting thorough and on-going audits of the serum supplier to ensure that they have properly performed the manufacture, quality control, and validation necessary to achieve the level of quality of the serum required for the biological it is being used to produce. In some cases, certificates of analysis may then be considered sufficient. Some combination of these approaches might be optimal, and the strategy taken should be considered in evaluating risk. Consultation with the NRA/NCL also might be advisable.

Serum and other bovine-derived materials should be tested for adventitious agents, such as bacteria, fungi, mycoplasmas, and viruses, prior to use in the production of MCBs and WCBs and in the manufacture of biologicals. Particular consideration should be given to those viruses that could be introduced from bovine-derived materials and that could be zoonotic or oncogenic (*e.g*., bovine viral

diarrhea virus, bovine polyoma virus, bovine circoviruses, rabies virus, bovine adenoviruses, bovine parvoviruses, bovine respiratory syncytial virus, infectious bovine rhinotracheitis virus, bovine parainfluenza virus type 3, reovirus 3, Cache Valley virus, bluetongue virus, and epizootic hemorrhagic disease virus). In addition, consideration should be given to risk-mitigation strategies, such as inactivation by heat or irradiation, to ensure that adventitious agents that were not detected in the manufacture and quality control of the serum would be inactivated to a degree acceptable to the NRA/NCL. If irradiation or other inactivation (*e.g.*, heat sterilization) methods are used in the manufacture of the serum, the tests for adventitious agents should be performed prior to inactivation to enhance the opportunity for detecting the contamination. If evidence of viral contamination is found in any of the tests, the serum is acceptable only if the virus is identified and shown to be present in an amount that has been shown in a validation study to be effectively inactivated. For serum that is not to be subjected to a virus inactivation / removal procedure, if evidence of viral contamination is found in any tests, generally, the serum would not be acceptable. If the manufacturer chooses to use serum that has not been inactivated, thorough testing of the serum for adventitious agents using current best practices should be undertaken. If any agents are identified, the cell banks made in this manner should be shown to be free of the identified virus(es).

> If irradiation is used, it is important to ensure that a reproducible dose is delivered to all batches and the component units of each batch. The irradiation dose must be low enough so that the biological properties of the reagents are retained while being high enough to reduce virological risk. Therefore, irradiation delivered at such a dose may not be a sterilizing dose.

If serum was used in the establishment or passage history of the animal-cell substrate prior to banking by the manufacturer, the cell bank (MCB or WCB) and/or cells at or beyond the level of production should be tested for adventitious agents of the species (*e.g.*, bovine) of serum used in the establishment and passage history of the cell substrate. If serum is not used in the production of the subsequent stages, then this testing would not need to be repeated on those subsequent stages once the cell bank has been tested and considered free of bovine (or whichever species of serum that was used) adventitious agents.

Methods used to test for bovine viruses should be approved by the NRA/NCL. Details of the methods are provided in Appendix 1. Infectivity assays are used as the primary screening method and have resulted in the detection of bovine viral diarrhea virus, reovirus 3, Cache Valley virus, bluetongue virus, and epizootic hemorrhagic disease virus amongst others. However, it should be noted that, in general, the infectivity screening assay methods described here do not readily detect some of the viruses (*e.g.*, bovine polyomaviruses) that can be frequent contaminants of serum. Additional methods may need to be considered, such as the nucleic acid amplification technique (NAAT), although the presence of viral genomic sequences is not necessarily indicative of infectious virus. In those cases, specific infectivity assays designed to detect the virus of concern (*e.g.*, bovine polyomavirus) may need to be considered.

A second factor in screening serum is the limited sample volume used compared with the batch size, which may be on the order of 1000 litres, which comes from the pooling of serum from many animals. Consequently, infectious viruses can be missed in the serum lot testing, and consideration should be given to direct screening of the cell bank for bovine viruses. These assays could include, in addition to the general screening procedure, NAAT for the presence of bovine viruses that may

infect the cell substrate but undergo abortive and/or transforming infections. Virus families of particular concern in this latter regard include polyomaviruses, herpesviruses, circoviruses, anelloviruses, and adenoviruses.

General screening assays for the detection of infectious viruses in serum or cell substrates involve the use of at least one indicator cell line, such as bovine turbinate cells, permissive for the replication of bovine viral diarrhoea virus (BVDV). A second cell line such as Vero also should be employed to broaden the detection range. Before initiating screening it may be necessary to evaluate the serum for the presence of antibody, particularly to BVDV, that could mask the presence of infectious virus.

Typically, indicator cells are cultured in the presence of the serum under test for 21 to 28 days, passaging the cells as necessary. During this period, the cells are regularly examined for the presence of cytopathic effect (CPE) indicative of virus infection. At the end of the observation period, which should not be less than 7 days after the last sub-culture, the cells are stained to detect CPE that may have been missed during observation of the living cells. Additional endpoint assays should include haemadsorbtion and haemagglutination at both 4°C and a higher temperature such as 20 to 25°C and also immunofluorescence assays (IFA) for specific viruses. Appropriate controls should be used for each assay, like bovine parainfluenza virus type 3 for heamadsorbtion. IFA is particularly important for BVDV, as non-cytopathic BVDV may be present in the serum. IFA endpoints are also used to detect other viruses that may be determined by geographical considerations like adenoviruses, bovine parvovirus, bluetongue virus, bovine syncytial virus, reovirus type 3 and unlikely, but serious contaminants like rabies virus.

If serum from another species is used (*i.e.*, other than bovine), the NRA/NCL should be consulted regarding acceptable testing methods for that species.

A.3.3 *Trypsin and other porcine-derived materials used for preparing cell cultures*
Trypsin used for preparing cell cultures should be tested for cultivable bacteria, fungi, mycoplasmas and infectious viruses, including bovine or porcine parvoviruses, as appropriate. The methods used to ensure this should be approved by the NRA/NCL.

> In some countries, irradiation is used to inactivate potential contaminant viruses. If irradiation is used, it is important to ensure that a reproducible dose is delivered to all batches and the component units of each batch. The irradiation dose must be low enough so that the biological properties of the reagents are retained while being high enough to reduce virological risk. Therefore, irradiation cannot be considered a sterilizing process.

The quality of the trypsin, like serum, is the responsibility of the biologicals' manufacturer (see section A.4.2). Recombinant trypsin is available and should be considered; however it should not be assumed to be free of risk of contamination and should be subject to the usual considerations for any reagent of biological origin (see A.4.1).

Like serum batches that are derived from many animals, trypsin batches are prepared from the pancreases of many animals. Most batches of porcine trypsin contain genetic sequences of porcine parvovirus 1 and porcine circoviruses and should be therefore treated to inactivate any virus

potentially present in a manner accepted by the NRA/NCL. It is acknowledged however that these viruses are relatively resistant to inactivation [56]. If trypsin from another species is used, the NRA/NCL should be consulted regarding acceptable testing methods.

General screening assays for the detection of infectious porcine viruses in trypsin or cell substrates involve the use of at least one indicator cell line, such as porcine testes cells or Vero cells, permissive for the replication of porcine viruses. Typically, indicator cell cultures would be incubated for 14 days with a sub-culture onto fresh test cells for an additional 14 days. Specific endpoint detection methods like IFA or PCR may be required in addition to observation for cytopathic effect periodically throughout the culture period and more general endpoint detection methods like hemadsorption and/or hemagglutination.

Furthermore, if trypsin was used in the establishment or passage history of the animal-cell substrate prior to banking by the manufacturer, the cell bank (MCB or WCB) should be tested for porcine parvovirus, or appropriate adventitious viruses relevant to the species of origin of the trypsin used in the establishment and passage history of the cell substrate. If trypsin is not used in the production of the subsequent stages, then this testing would not need to be repeated on those subsequent stages once the cell bank has been tested and considered free of porcine parvovirus (or relevant agents). Consideration should be given to screening for other agents such as porcine circoviruses. Molecular methods, such as PCR, may be used for such purposes.

Testing of cells exposed to trypsin or of other porcine-derived materials might entail testing for more than porcine parvovirus or porcine circoviruses. For example, testing for porcine adenovirus, transmissible gastroenteritis virus, porcine haemagglutinating encephalitis virus, bovine viral diarrhea virus, reoviruses, rabies virus, porcine anellovirus, porcine hokovirus, porcine bocavirus, porcine hepatitis E virus, porcine reproductive and respiratory syndrome virus, encephalomyocarditis virus, and potentially other viruses might be appropriate. Particular consideration should be given to those viruses that could be introduced from the porcine-derived material and that could be zoonotic or oncogenic. Additionally, tests for bacterial and fungal sterility and mycoplasmas depending on the type of porcine-derived material should be conducted. The NRA/NCL should be consulted in this regard.

A.3.4 *Medium supplements and general cell culture reagents derived from other sources used for preparing cell cultures*
Medium supplements derived from other species should be quality controlled from the perspective of adventitious agents. Consideration should be given to whether recombinant-derived medium supplements were exposed to animal-derived materials during their manufacture, and if so, evaluated for the potential to introduce adventitious agents into the manufacture of the cell banks and biological products. Testing for adventitious agents should assess viruses relevant to the species from which the supplement was derived. The NRA/NCL should be consulted in this regard.

Generally, medium supplements should not be obtained from human source materials. In particular, human serum should not be used. However, in special circumstances, and in agreement with the NRA/NCL, the use of human-derived supplements may be permitted. If human serum albumin is used at any stage of product manufacture, the NRA/NCL should be consulted regarding the requirements, as these may differ from country to country. However, as a minimum, it should meet

the revised Requirements for Biological Substances No. 27 [56], as well as the guidelines on tissue infectivity distribution of TSEs [55].

Recombinant human albumin is commercially available and should be considered; however, it should not be assumed to be free of risk of contamination and should be subject to the usual considerations for any reagent of biological origin (see A.3).

As for other cell-culture reagents, it is important to establish traceability and assess and reduce microbiological risks as described in section A.3.

A variety of cell-culture reagents of biological origin are available that are derived from non-animal sources, including a range of aquatic organisms, plants and algae. In such cases, the exact hazards involved may be uncertain and unfamiliar. The microbiological risks may be substantially different to those involved in animal-derived reagents (see section A.3.1-A.3.3), and other hazards may arise, such as immunogenic, mitogenic and allergenic properties of the reagent and its components. For example, plant-derived material may carry an increased risk of mycoplasma and mycobacteria contamination.

A.4 Certification of cell banks by the manufacturer
It is vital that the manufacturer has secured a body of information on the cell substrate that demonstrates clearly the origin or provenance of the culture and how the cell banks intended for production (MCB and WCB) were established, characterized, and tested. This should provide all the information required to demonstrate the suitability of that cell substrate and the established cell banks for the manufacture of biological products.

A.4.1 *Cell line data*
Each new cell line should have an associated body of data, which will increase as the cell line is established and developed for manufacturing purposes. This data set is vital to demonstrate the suitability for use of the cells and should provide information on cell provenance (donor information and any relevant details on ethical procurement), cell line derivation, culture history, culture conditions (including reagents), early stage safety evaluation data, banking and cell bank characterization, and safety testing. This information should be available to the NRA/NCL for approval of the cells used in manufacture.

A.4.2 *Certification by the manufacturer of primary cell cultures*
Full traceability should be established for PCCs to the animals of origin, husbandry conditions, veterinary inspection, vaccinations (if any), procedures for administering anaesthesia and cell harvesting, the reagents and procedures used in the preparation of primary cultures, and the environmental conditions under which they were prepared. Extensive testing should be performed, and this should be documented.

It is important to define the batch or lot of cells used in each individual manufacturing process. For production purposes, a batch or lot is a culture of primary cells derived from single or multiple animals that has been subjected to a common process of tissue retrieval, disaggregation, and processing leading to a single culture preparation of cells. Lots may be prepared by harvesting and pooling cells in different ways, but the cell-processing procedures should be reproducible, and it is

especially important to monitor cultures carefully for evidence of adverse change in the cell culture and microbiological contamination. Prior to any culture pooling, cells should be examined for acceptability for production. Acceptability criteria should be established and should include testing for microbiological contamination and the general condition of the cells (*e.g.*, morphology, number, and viability of the cells). Failure to detect and eliminate atypical (*i.e.*, potentially virally infected) or grossly contaminated cells will put the entire production run at risk and could compromise the safety of the product. Cells showing an unacceptably high proportion of dead or atypical cells should not be used, and ideally microbiological testing should be completed and passed before the cells are used.

The preparation of cell lots for manufacture should be carefully documented to provide full traceability from the animal donor(s) to production. Any pooling of cells should be clearly recorded, as should any deviation from standard operating procedures, as required by GMPs. In addition, any observations of variation between batches should be recorded, even where such observations would not necessarily lead to rejection of those batches. Such information may prove valuable in ongoing optimization and improvement of the production process.

A.4.3 *Certification by the manufacturer of diploid, continuous, and stem cell lines*
All cell lines used for biologicals production should have data available, as indicated in A.4.1.

The original PDL (or passage number, if the PDL is unknown) of the cell seed should be recorded.

For cell lines of human origin, if possible the medical history of the individual from whom the cell line was derived should be evaluated in order to better assess potential risks and the suitability of the cell line.

For SCLs, morphology continues to be an important characteristic, and representative images and immune-phenotypic profiles of undifferentiated and differentiated cells should be available for comparison.

A.5 Cryopreservation and Cell Banking
A.5.1 *Cryopreservation*
When cells are banked, the successful preservation of cells at ultra-low temperatures is critical to the efficient delivery of good quality cultures (*i.e.*, high viability cultures of the required characteristics). The need to prepare large stocks of frozen vials of cells for cell banks is especially challenging, and a number of key principles should be adopted:
- A method that meets current best practice for cell-culture preservation should be used (for example, see ref 58)
- The cooling profile achieved for the cells being frozen should be defined, and the same cooling process should be used for each separate preservation process (*i.e.*, an SOP should include documentation of the cooling process in the batch record.)
- Each preservation process should be recorded
- As a general guide, only cell cultures that are predominantly in the exponential phase of growth should be used. Cells in such cultures tend to have a low ratio of cytoplasm to nucleus (v/v) and should be more amenable to successful cryopreservation. It is unwise to use cells predominantly in the 'lag' phase very early after passage or in the 'stationary' phase when the culture has reached its highest density of cells

- For each bank, cells pooled from a single expanded culture (*i.e.*, not from a range of cultures established at different times post seeding or different PDLs) should be used and mixed prior to aliquotting to ensure homogeneity
- The number of cells per vial should be adequate to recover a representative culture (*e.g.*, 5-10 x 10^6 in a 1-mL aliquot)
- For new cell banks, antimicrobials should not be used in cell cultures to be banked, except where this can be justified for early PDL cultures which may carry contamination from tissue harvesting or recombinant cells which require antibiotic selection, and when necessary for the genetic stability of recombinant cell lines. In any case, if antimicrobials are used, they should not be penicillin or any other beta-lactam drug
- When a stock of cells has been frozen, a sample should be recovered to confirm it has retained viability and the results recorded. It is also important to establish the degree of homogeneity within the cell bank. Recovery of a sufficient percentage (*e.g.*, 1% or as recommended by the NRA) of vials representative of the beginning, middle and end of the cryopreservation process should be demonstrated to give confidence in the production process based on the use of that cell bank. Ultimately, stability (see B.3) and integrity of cryopreserved vials is demonstrated when the vials are thawed from production and demonstrated to produce the intended product at scale (see also B.7).
- Cell bank cryostorage vessels should be monitored and maintained to enable demonstration of a highly stable storage environment for cell banks. Access to such vessels may cause temperature cycling, which in extreme cases can cause loss of viability. It is therefore prudent to establish a stability-testing programme involving recovery of cells periodically where the frequency of recovery relates to risk of temperature cycling. New developments in remote monitoring of individual vials may in future eliminate the need for stability testing.

A.5.2 *Cell Banking*
When DCLs, SCLs, or CCLs are used for production of a biological, a cell-bank system should be used. Such banks should be approved by and registered with the NRA/NCL as part of the product approval process. The source of cells used in cell banking and production is a critical factor in biological product development and manufacture. It is highly desirable to obtain cells from sources with a documented history and traceability to the originator of the cell line.

After a sample of the original seed stock is obtained, an early stage pre-master bank of just a few vials should be established. One or more vials of that pre-master bank are used to establish the MCB. The WCB is derived by expansion of one or more containers of the MCB. The WCB should be qualified for yielding cell cultures that are acceptable for use in manufacturing a biological product.

When using early PDLs from primary cultures for production processes, the preparation of a cell bank should be considered on a case-by-case basis. This approach has significant benefits, as it gives great flexibility in the timing of the production process, permits quality control and safety testing to be completed prior to use, and reduces the overall burden of testing required in the production process.

Cell banks should be characterized as specified in Part B of this guidance and according to any other currently applicable and future guidance published by WHO. The testing performed on a replacement MCB (derived from the same cell clone or from an existing MCB or WCB) is the same

as for the initial MCB unless a justified exception is made. Efforts to detect contaminating viruses and other microbial agents constitute a key element in the characterization of cell banks.

Having been cryopreserved by qualified methods (see A.5.1), both the MCB and WCB should be stored frozen under defined conditions, such as in the vapour or liquid phase of liquid nitrogen. The location, identity, and inventory of individual cryovials or ampoules of cells should be thoroughly documented. It is recommended that the MCB and WCB each be stored in at least two widely separated areas within the production facility and/or in geographically distinct locations to assure continued ability to manufacture product in the event of a facility catastrophe. When cryopreserved cells are transferred to a remote site, it is important to use qualified shipping containers and to monitor transfers with probes to detect temperature excursions. All containers are treated identically and, once removed from storage, usually are not returned to the stock. The second storage site should operate under an equivalent standard of quality assurance as the primary site.

A.5.3 *WHO reference cell banks (RCBs)*
The principle of establishing RCBs under WHO auspices is one that offers potential solutions to future challenges for the development of vaccines and biotherapeutics in developing regions. However, WHO does not intend that cells supplied to manufacturers from any RCB be used as a MCB. The purpose of WHO RCBs is to provide well characterized cell seed material for the generation of a MCB by manufacturers with the expectation that such MCBs will comply with this guidance document and be fully characterized.

The WHO RCBs provide key advantages for vaccine development worldwide that include:
- Traceability to origin of cells and derivation of cell line and materials used in preparation of seed stock
- Subjected to open international scientific scrutiny and collaborative technical investigations into the characteristics of the cells and the presence of adventitious agents
- Results of characterization were peer reviewed and published
- Investigations evaluated under auspices of WHO expert review and qualified as suitable for use in vaccine production
- Supply of cells free of any adverse Intellectual Property 'reach through' on final products
- Single source of cells with growing and scientifically and technically updated body of safety-testing data and safe history of use, giving increasing confidence for manufacturers, regulators, and public policy makers

The Vero cell line is the most widely used continuous cell line for the production of viral vaccines over the last two decades. The WHO Vero RCB 10-87 was established in 1987 and was subjected to a broad range of tests to establish its suitability for vaccine production. This WHO RCB provides a unique resource for the development of future biological medicines where a cell substrate with a safe and reliable history of use is desired. A comprehensive review of the characterization of the WHO Vero 10-87 seed lot was conducted recently, and a detailed overview is provided on the WHO website at (http://www.who.int/biologicals/).

As concluded by an expert review in 2002, the WHO Vero RCB 10-87 is not considered suitable for direct use as MCB material. However, the WHO Vero RCB 10-87 is considered suitable for use as a

cell seed for generating a MCB, and its status has changed from "WHO Vero cell bank 10-87" to "WHO Vero reference cell bank 10-87" .

The WHO Vero RCB 10-87 is stored in the European Collection of Animal Cell Cultures (ECACC, www.hpacultures.org.uk), Salisbury, Wiltshire, UK, and the American Type Culture Collection (ATCC, www.atcc.org), in Manassas, Virginia, USA. These public service culture collections have distributed ampoules under agreements with the WHO to numerous manufacturers and other users. The WHO Vero RCB 10-87 is the property of WHO, and there are no constraints relating to intellectual property rights. The WHO Vero RCB 10-87 is available free of charge on application to WHO. However, due to the limited number of vials remaining, distribution of these vials is restricted solely for the production of vaccines and other biologicals. Potential replacement of the WHO Vero RCB 10-87 is currently under consideration.

WHO also has overseen the establishment of seed stocks of MRC-5 for the production of vaccines. The WHO MRC-5 RCB was established in 2007 because of stability issues associated with the original vials of MRC-5 cells, which dated to 1966. This RCB was prepared in a qualified cleanroom environment and subjected to specified quality-control testing endorsed by the ECBS.

Part B. Recommendations for the characterization of cell banks of animal cell substrates

B.1 General considerations

Since the 1986 Study Group report, advances in science and technology have led to an expanded range of animal cell types that are used for the production of biological products. In some cases, these new cell types provide significantly higher yields of product at less cost, while in other cases they provide the only means by which a commercially viable product can be manufactured. Many products manufactured in CCLs of various types have been approved, and some examples are listed in Table 1.

Table 1. Examples of Approved Biological Products Derived from CCLs

Product Class	Product (disease)	Cell Line
Therapeutic	Factor VIII (hemophilia)	CHO
	Factor VIIa (hemophilia)	BHK-21
	Monoclonal antibody (various disease)	CHO and murine myeloma (NS0 and SP2/ 0)
Prophylactic	Poliovirus vaccine	Vero
	Rotavirus vaccine	Vero
	Rabies vaccine	Vero
	JE vaccine	Vero
	Human papillomavirus vaccine	Sf-9
	Influenza vaccine	MDCK

Many more products are currently in development, and some use highly tumourigenic cells (*e.g*., HeLa; some banks of MDCK), and some involve sources previously unused in production such as insect cells. Examples are listed in Table 2.

Table 2. Examples of Biological Products in Development Derived from CCLs

Product Class	Product (disease)	Cell Line(s)
Therapeutic	> 50% of products in development use CHO or murine myeloma cells as the cell substrate [59]. Monoclonal antibodies are generally produced using CHO, SP2/0, PER.C6, and NS0 cells [60].	
Prophylactic	HIV vaccines	CHO, Vero, PER.C6, 293ORF6, HER96, HeLa
	Herpes Simplex Type 2 vaccine	CHO
	Influenza vaccines	SF9, Vero, PER.C6
	Rabies vaccine	S2

CCLs may have biochemical, biological, and genetic characteristics that differ from PCCs or DCLs and that may impose a risk for the recipients of biologicals derived from them. In particular, they may produce transforming proteins, and may contain potentially oncogenic DNA and viral genes. In some cases, CCLs may cause tumours when inoculated into animals. Non-tumourigenic cells (*e.g.*, PCCs and DCLs) had been thought to be intrinsically safer than tumourigenic cells. Where tumourigenic cells have been used in the past (*e.g*., CHO for recombinant proteins), high degrees of purity have been required with a special emphasis on reduction in quantity of DNA. When not possible to completely remove the amount of DNA to below the detection limit, emphasis has been on a reduction in size or other approaches to inactivate rcDNA and rcRNA (*e.g*., beta-propiolactone (BPL) for rabies vaccine).

Manufacturers considering the use of CCLs should be aware of the need to develop, evaluate, and validate efficient methods for purification as an essential element of any product-development programme. However, a minimally purified product, such as certain viral vaccines (*e.g*., polio), may be acceptable when produced in a CCL such as Vero when data are developed to support the safety of the product. Such data would include extensive characterization of the MCB or the WCB and of the product itself.

While tumourigenicity tests have been part of the characterization of CCLs, they comprise only one element in an array of tests, the results of which must be taken into account when assessing the safety of a biological produced in a given cell substrate. For example, if a CCL is positive in a tumourigenicity test, and if the CCL is to be used for the production of a live viral vaccine, an evaluation of the oncogenic potential of the cells might be requested by the NRA/ NCL to characterize the cellular DNA and to detect oncogenic viruses that might be present. Such studies should be discussed with the NRA/NCL.

Evidence should be provided for any animal-cell line proposed for use as a substrate for the manufacture of a biological product demonstrating that it is free from cultivable bacteria, mycoplasmas, fungi, and infectious viruses, including potentially oncogenic agents to the limits of the assay's detection capabilities. Special attention should be given to viruses that commonly contaminate the animal species from which the cell line is derived, and to cell-culture reagents of biological origin. The cell seed should preferably be free from all microbial agents. However, certain

CCLs may express endogenous retroviruses. Tests capable of detecting such agents should be carried out on cells grown under cell-culture conditions that mimic those used during production, and the levels of viral particles should be quantified. Viral contaminants in a MCB and WCB should be shown to be inactivated and/or removed by the purification procedure used in production. The validation of the purification procedure used is considered essential.

The characterization of any DCL, SCL or CCL to be used for the production of biologicals should include: a) a history of the cell line (*i.e.*, provenance) and a detailed description of the production of the cell banks, including methods and reagents used during culture, PDL, storage conditions, viability after thawing, and growth characteristics; b) the results of tests for infectious agents; c) distinguishing features of the cells, such as biochemical, immunological, genetic, or cytogenetic patterns, that allow them to be clearly distinguished from other cell lines; and d) the results of tests for tumourigenicity, including data from the scientific literature. Additional consideration should be given to products derived from cells that contain known viral sequences (*e.g.*, Namalwa, HeLa, 293, and PER.C6).

The recommendations that follow are intended as guidance for NRAs, NCLs, and manufacturers as the minimum amount of data on the cell substrate that should be available when considering a new biological product for approval. The amount of data that may be required at various stages of clinical development of the product should be discussed and agreed with the NRA/NCL at each step of the program.

B.2 Identity

Cell Banks should be authenticated by a cell-identification method approved by the NRA/NCL. Wherever practicable, methods for identity testing should be used that give specific identification of the cell line to confirm that no switching or major cross-contamination of cultures has arisen during cell banking and production. A number of the commonly used identity testing methods are compared in Table 3. In the case of human cells, genetic tests such as DNA profiling (*e.g.*, Short Tandem Repeat analysis, multiple Single Nucleotide Polymorphisms) will give a profile that is at least specific to the individual from whom the cells were isolated. Another test that might be used for human cells is HLA typing. Other tests that may be used but tend to be less specific include isoenzyme analysis and karyology, which may be particularly useful where there are characteristic marker chromosomes. However, where more specific genetic markers are available, they should be considered. It is not unexpected that a small proportion of cell lines, particularly those which are transformed, may show alterations to the expected identity profile. This has been observed in isoenzyme analysis where in rare cases a particular cell line may show a consistently different profile to that expected for the species of origin and is also a general issue relating to the effect of genetic instability for molecular identity testing techniques. Such effects in standard technologies are rare and may also arise with the implementation of new techniques. The implications of any unexpected results should be discussed with the NRA/NCL. For recombinant-protein products, cell line identity testing should also include tests for vector integrity, expression plasmid copy number, insertions, deletions, number of integration sites, the percentage of host cells retaining the expression system, verification of protein-coding sequences, and protein-production levels.

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Table 3. Identity Testing for Mammalian Cell Lines

Technique	Advantages	Disadvantages
Karyology (especially useful for DCLs and SCLs)	Gives whole chromosomal genome visualisation and analysis that can identify species of origin for a very wide range of species using the same methodology. Newer methods include, Spectral Karyotyping which involves the use of probes, labeled with fluorescent dyes. The probes paint the chromosomes yielding different colors in specific areas. Spectral karyotyping is able to detect translocations not recognizable by traditional banding methods.	Results are generally not specific to the individual of origin (*i.e.*, usually species specificity) although certain cell lines may have marker chromosomes that are readily recognised. Giemsa banding requires special expertise and is labour intensive. Standard analysis of 10-20 metaphase spreads is insensitive for detecting contaminating cells.
Isoenzyme analysis	Determination of species of origin within a few hours	Analysis for 4-6 isoenzyme activities will generally identify species of origin, but is not specific to the individual of origin
DNA profiling using variable number of tandem repeats (VNTR) analysis or other PCR method such as Exon Primed Intron Crossing-PCR (EPIC-PCR), or other techniques, such as restriction fragment length polymorphism (RFLP)	Short tandem repeats (STR) analysis by PCR is rapid and gives identity specific to the individual of origin. Commercial kits are available for a range of human populations. EPIC-PCR method is rapid and gives identity specific to the individual of origin. It provides the advantage of covering a broad spectrum of organisms and cell lines other than human cells	Some limited, but undefined, cross-reaction of human STR primers for primate cells

B.2.1 *Applicability*

Cell Banks: MCB and each WCB

Cell Types: DCL, SCL, CCL

B.3 Stability

The stability of cell banks during cryostorage and the genetic stability of cell lines and recombinant expression systems are key elements in a successful cell bank program.

B.3.1 *Stability during cryostorage*

Data should be generated to support the stability or suitability of the cell substrate and any recombinant expression system or necessary cell phenotype during cultivation to or beyond the limit of production, and to support the stability of the cryopreserved cell banks during storage. The latter may be demonstrated by successful manufacture of WCBs or production lots. Periodic testing for viability is not necessary if continuous-monitoring records for storage show no deviations out of specification, and periodic production runs are successful. If banks are used less than once every 5 years, then it may be prudent to generate data confirming suitability for manufacturing on a schedule that takes into account the storage condition once every 5 years.

B.3.1.1 *Applicability*
Cell Banks: MCB and WCB
Cell Types: DCL, SCL, CCL

B.3.2 *Genetic Stability*
Any form of genetic instability could potentially affect the quality of the final product and it will be important to know if the cells in culture are changing in a way that could affect the nature or safety of the product. Any features of the cell lines that might affect quality should be discussed with the NRA/NCL to ensure that tests used by the manufacturer to monitor genetic stability are adequate. The specific tests will vary according to the nature of the product, but the aim is to show consistency in the amount and characteristics of the product derived from cells within a few passages of the MCB or WCB with those derived from an ECB or EOPC. For recombinant protein products, emphasis will be on the protein sequence and post translational modifications.

For cell lines containing DNA expression constructs, the stability of these constructs between the MCB/WCB and an ECB or EOPC should be determined. The copy number of the construct and, if relevant, the sites of chromosomal insertion should be determined. The latter is accomplished by sequencing into the cellular flanking regions, but methods like fluorescent *in situ* hybridization may provide useful additional information, particularly where concatamers of the gene insert are present at individual chromosomal loci. The sequence of the construct within the cells should be determined. With conventional sequencing, a consensus sequence is obtained, but with massively parallel sequencing, it is possible to determine the sequence of individual gene inserts or their transcripts.

Where proteins are derived from non-genetically modified cells, consistency in the yield and properties of the protein should be evaluated together with the sequence of the mRNA encoding the protein of interest.

Additional characterization of the cell-biological processes and responses during cultivation (for instance using global or targeted gene expression, proteomic or metabolic profiles and other phenotypic markers) might be useful in further developing a broad understanding of the cell substrate.

Appropriate methods should be applied to assure that cell age is correctly assessed in the event that cell viability falls dramatically at any given step. Losses in viability are reflected in increased cultivation times to reach defined levels of growth.

The stability of cell function in terms of productivity within the production process also may need to be evaluated. Other stability studies may be performed where bioreactor methods are employed, especially where extended culture periods are involved.

B.3.2.1 *Applicability*
Cell Banks: MCB taken to EOPC/ECB
Cell Types: DCL, SCL, CCL

B.4 Sterility
(see B.11.3.1)

B.5 Viability
High level of viability of cryopreserved cells is important for efficient and reliable production. Typically, thawed cells should have viability levels in excess of 80%, though this is not always achieved and may depend on the cell line. Lower viabilities may still result in suitable growth recovery and in acceptable product qualities. In such cases, the data should be discussed with the NRA/NCL. A range of viability tests are available that measure different attributes of cell function (membrane integrity, metabolic activity, DNA replication). Under certain circumstances, such as pre-apoptotic cells excluding trypan blue, viability assays may give misleading results, and it is important to be aware of the exact information that a particular viability assay provides. Therefore, it is important to evaluate growth recovery of cryopreserved cells upon thawing.

> For certain cell cultures such as hybridomas, where a membrane-integrity test is used, additional cell markers such as indicators of apoptosis should be studied in order to avoid significantly overestimating viability.

A suitable viability test should be selected for the cell substrate in question and typical test values established for cultures considered to be acceptable (see B.6, B.7). It may also be necessary to select alternative viability assays that are better suited to providing in-process viability data required during production, *e.g.*, lactate dehydrogenase levels in bioreactor systems.

B.5.1 *Applicability*
Cell Banks: MCB and WCB
Cell Types: DCL, SCL, CCL

B.6 Growth Characteristics
For the development of production processes, the growth characteristics of the production-cell line should be well understood to ensure consistency of production. Changes in these characteristics could indicate any one of a range of events. Accordingly, data on growth characteristics, such as viability, morphology, cell-doubling times, cloning and/or plating efficiency, if applicable, should be developed. For certain cell substrates, it may be appropriate to apply such tests in homogeneity testing (see B.7). Experiments to demonstrate homogeneity and growth characteristics may be combined, although the analysis should be carried out separately.

B.6.1 *Applicability*
Cell Banks: MCB and WCB

Cell Types: DCL, SCL, CCL

B.7 Homogeneity Testing

Each cell culture derived from a container of the WCB should perform in the same way (*i.e.*, within acceptable limits and provide the same number of viable cells of the same growth characteristics). In order to assure this, it is important to recover a proportion of containers from each cell bank and check their characteristics, as indicated in B.6. The number of containers tested should be discussed with the NRA/NCL and be broadly in line with those normally sampled to establish product consistency. Recovery of a sufficient percentage of vials representative of the beginning, middle and end of the aliquotting process should be demonstrated to give confidence in the production process based on the use of that cell bank. Ultimately, stability and integrity of cryopreserved vials are demonstrated when the vials are thawed for production and demonstrated to produce the intended product at scale. Instead of testing a portion of containers at different stages of the banking process, an alternative strategy to ensure the homogeneity of the banks can be used based on the validation of the process method for filling and freezing. Assessment of growth characteristics (B.6) and homogeneity testing are commonly combined experimentally; however, the analysis and interpretation of each should be distinct. It may be appropriate to also test homogeneity of the MCB to assure future WCBs are consistent with the first WCB.

B.7.1 *Applicability*
Cell Banks: MCB, WCB
Cell Types: DCL, SCL, CCL

B.8 Tumourigenicity
B.8.1 *General considerations*
Several *in vitro* test systems, such as cell growth in soft agar [61] and muscle organ culture [62], have been explored as alternatives to *in vivo* tests for tumourigenicity; however, correlations with *in vivo* tests have been imperfect or the alternative tests have been technically difficult to perform. Therefore, *in vivo* tests remain the standard for assessing tumourigenicity.

Although WHO Requirements [1] have described acceptable approaches to tumourigenicity testing, a number of important aspects of such testing were not addressed. Therefore, a model protocol has been developed and is appended to this document. The major points included in the model protocol are listed below along with comments on each specific item.

A new diploid cell line (*i.e.*, other than WI-38, MRC-5, and FRhL-2) should be tested for tumourigenicity as part of the characterization of the cell line, but should not be required on a routine basis.

The tumourigenicity tests currently available are in mammalian species whose body temperatures and other physiologic factors are different from those of avian and insect species. Therefore, when the test is performed on avian or insect cells, the validity of the data is open to question unless a tumourigenic cell line of the species being tested is included as a positive control. The NRA/NCL may accept the results of an *in vitro* test such as growth in soft agar as a substitute for the *in vivo* test for avian and insect cell lines. However, as mentioned above, correlations of *in vitro* tests with *in vivo* tests are imperfect. This should be discussed with the NRA/NCL.

Many CCLs (*e.g*., BHK-21, CHO, HeLa) are classified as tumourigenic because they possess the capacity to form tumors in immunosuppressed animals such as rodents. Some CCLs become tumourigenic at high PDLs (*e.g*., Vero), even though they do not possess this capacity at lower PDLs at which vaccine manufacture occurs. A critical feature regarding the pluripotency of embryonic SCLs, even though they display a diploid karyotype, is that they form tumours in immunocompromised mice.

The expression of a tumourigenic phenotype can be quite variable from one CCL to another, and even within different sub-lines of the same CCL. This range of variability, from non-tumourigenic, to weakly tumourigenic, to highly tumourigenic, has been viewed by some as indicating different degrees of risk when they are used as substrates for the manufacture of biological products [10,11].

If the CCL has already been demonstrated to be tumourigenic (*e.g*., BHK-21, CHO, HEK293, Cl27), or if the class of cells to which it belongs is tumourigenic (*e.g*., hybridomas, SCLs), it may not be necessary to perform additional tumourigenicity tests on cells used for the manufacture of therapeutic products. Such cell lines may be used as cell substrates for the production of biological products if the NRA/NCL has determined, based on characterization data as well as manufacturing data, that issues of purity, safety, and consistency have been addressed. A new cell line (DCL, SCL, or CCL) should be presumed to be tumourigenic unless data demonstrate that it is not. If a manufacturer proposes to characterize the cell line as non-tumourigenic, the following tests should be undertaken.

Cells from the MCB or WCB propagated to the proposed *in vitro* cell age used for production or beyond should be examined for tumourigenicity in a test approved by the NRA/NCL. The test should involve a comparison between the cell line and a suitable positive reference preparation (*e.g*., HeLa cells) and a standardized procedure for evaluating results.

B.8.2 *Type of test animals*
A variety of animal systems have been used to assess the tumourigenic potential of cell lines. Table 4 lists several examples of such tests along with advantages and disadvantages of each. Because assessing the tumourigenic phenotype of a cell substrate requires the inoculation of xenogeneic or allogeneic cells, the test animal should be rendered deficient in cytotoxic T-lymphocyte (CTL) activity. This can be accomplished either by the use of rodents that are genetically immunocompromised (*e.g*., nude mice, severe combined immunodeficiency (SCID) mice) or by inactivating the T-cell function with anti-thymocyte globulin (ATG), anti-thymocyte serum (ATS), or anti-lymphocyte serum (ALS). The use of animals with additional defects in NK-cell function, such as, the SCID-NOD mouse, the SCID-NOD-gamma mouse, and the CD3 epsilon mouse, has not yet been explored for cell-substrate evaluation, but they might offer some advantages. In addition to these systems, several other *in vivo* systems, such as the hamster cheek pouch model and ATG-treated non-human primates (NHPs), have been used in the past, but rarely at present.

Table 4. *In vivo* tests to assess the tumourigenic potential of inoculated cells

Test & Brief Description	Advantage(s)	Disadvantage(s)	Refs
Adult Athymic mouse (Nu/Nu genotype): Animals inoculated by the i.m. or s.c. route with cells to be tested	• Animals readily available • No immunosuppression required	• Higher frequency of spontaneous tumors than in other animal models that are not genetically immunosuppressed • Low sensitivity for assessing the metastatic potential of the inoculated cells	65
Newborn athymic mouse Animals inoculated by the s.c. route with cells to be tested	• No immunosuppression required • More sensitive than adults	• Low sensitivity for assessing the metastatic potential of the inoculated cells • Since litters include heterozygous mice, twice the number of animals must be inoculated in order to be sure that a sufficient number of homozygous mice have been included. • Cannibalism of newborns by the mother	66
SCID mouse: Animals receive subcutaneous, intra-dermal. or intra-kidney capsule inoculation of test cells	• No immunosuppression required • Potentially increased sensitivity • Animals readily available	• Highly susceptible to viral, bacterial, and fungal infections • Infections can affect the results and reproducibility of studies • Spontaneous thymic lymphomas may occur	64, 67
Newborn rat: Animals immunosuppressed with ATG followed by i.m. or s.c. inoculation of cells to be tested	• Animals readily available • Sensitive model for detecting metastases • Very low frequency of spontaneous tumor formation	• Standardized ATG not available as a commercial product • Careful qualification and characterization of the ATG is required to find the balance between immunosuppressive capacity and toxicity	65, 68
Newborn hamster or mouse: Animals immunosuppressed with ATS followed by i.m. or s.c. inoculation of cells to be tested	• Animals readily available	• Cannibalism of newborns by mother • Standardized ATS not available and difficult to balance toxicity vs. immunosuppressive capacity	69
Newborn hamster or mouse: Animals immunosuppressed with ALS followed by i.m. or s.c. inoculation of cells to be tested	• Increased sensitivity compared to the HCP test • Animals readily available	• Cannibalism of newborns by mother • Standardized ALS not available and difficult to balance toxicity vs. immunosuppressive capacity	70
Hamster cheek pouch (HCP): Animals immunosuppressed with cortisone followed by inoculation of the cells to be tested into the cheek pouch	• Animals readily available	• Lower sensitivity than newer models	71
Nonhuman primates: Animals immunosuppressed with ATG followed by inoculation of cells to be tested into the muscle of the four limbs	• Species closer to human	• Standardized ATG not available • Animals not readily available • Expense and limited availability preclude using large numbers • Animal welfare principles mandate against use of NHP if same results can be obtained from lower specie	72

Although all of the animal models listed in Table 4 have been used to assess the tumourigenicity of cells, several sensitivity parameters from studies using positive-control cells should be considered when attempting to compare the various *in vivo* tumourigenicity models: i) frequency of tumour formation; ii) time to appearance of tumors; iii) size of tumours; iv) lowest number of inoculated tumour cells that result in tumour formation; and v) metastatic tumor formation. Factors i, ii, iii, and v usually depend on the number of cells inoculated (*i.e.*, they are dose-dependent). In addition, the rate of spontaneous tumour formation should be considered. Although comparisons of two or more assays have been reported in the literature [63,69,73] none of them takes into account all of these factors, nor do they use the same tumourigenic cell lines. Thus, it is not possible to draw definitive conclusions on the relative sensitivity of the various tumourigenicity assays. Nevertheless, the following points appear to be generally accepted: i) the ATS-treated newborn rat and the ATG-treated nonhuman primate systems are the most sensitive to assess the metastatic potential of inoculated cells; ii) ATS and ATG provide better immunosuppression than ALS; iii) the nude mouse has a more well-defined level of immunosuppression than those that depend on ALS, ATS, or ATG, and inter-laboratory comparisons of nude mouse data are more likely to yield valid conclusions.

The overall experience during the past 30 years, and taking into consideration the points mentioned above, has led to the conclusion that the athymic nude mouse is an appropriate test system for determining the tumourigenic potential of cells proposed for use in the production of biologicals. The major advantages of the athymic nude mouse system are that it is easier to establish and standardize and is generally available, while the newborn rat system is more sensitive for assessing the metastatic potential of tumourigenic cells. In some cases, it might be preferable to use newborn athymic nude mice, as these animals are more sensitive than adults for the detection of weakly tumourigenic cells [64]. A tumourigenicity testing protocol using athymic nude mice is provided as Appendix 2. The animal system selected should be approved by the NRA/NCL.

B.8.3   *Point in the life history of the cells at which they should be tested*
Investigation of tumourigenicity should form part of the early evaluation of a new cell substrate for use in production. Cells from the MCB or WCB, propagated to the proposed *in vitro* cell age used for production or beyond should be examined for tumourigenicity. The extra population doublings (*e.g.*, 3 to 10) ensure that the results of the tumourigenicity test can be used in the assessment of overall safety of the product even under the assumption of a "worst case" situation and therefore provides a safety buffer.

B.8.4   *Use of control cells*
The tumourigenicity test should include a comparison between the CCL and a positive control reference preparation such as HeLa cells from a reliable source. This source is preferred in order to standardize the test among laboratories, so that the cumulative experience over time can be assessed and made available to NRAs/NCLs and manufacturers to assist them in the interpretation of data. However, other sources for establishing positive-control cells may be acceptable. The purpose of the positive control is to assure that an individual test is valid by demonstrating that the animal model has the capacity to develop tumors from inoculated cells (*i.e.*, a negative result is unlikely to be due to a problem with the *in vivo* model). If the positive-control cells fail to develop tumours at the expected frequency, then this could be indicative of problems with the animals or at the testing facility, such as infections, which can reduce the efficiency of tumour development.

When the cell substrate has been adapted to growth in serum-free medium, which might contain growth factors and other components that could influence growth as well as detection of a tumourigenic phenotype, consideration should be given to processing the positive-control cells in the same medium. Whenever possible, both the test article and the positive-control cells should be resuspended in the same medium, such as phosphate-buffered saline (PBS) for inoculation.

In designing a tumourigenicity protocol, it is important to recognize that tumors arise spontaneously in nude mice and that the incidence of such tumors increases with the age of the mice. Therefore, databases (both published data and the unpublished records/data of the animal production facility that supplied the test animals) of rates of spontaneous neoplastic diseases in nude mice should be taken into account during the assessment of the results of a tumourigenicity test. Generally, negative controls are not recommended because the rates of spontaneous neoplastic disease in nude mice are low, and small numbers of negative control animals are unlikely to provide meaningful data. However, if negative control cells such as WI-38, MRC-5, or FRhl-2 are included, clear justification for including them should be provided. For example, if serum-free medium is used to grow the cell substrate, it is conceivable that growth factors may influence the appearance of spontaneous tumours; therefore, negative-control cells suspended in the same medium may be needed to interpret the test results.

B.8.5 *Number of test animals*
To determine whether the cells being characterized have the capacity to form tumors in animals, the cells being tested, the reference positive-control cells, and if any, the reference negative control cells should be injected into separate groups of 10 animals each. In a valid test, progressively growing tumors should be produced in at least 9 of 10 animals injected with the positive reference cells.

B.8.6 *Number of inoculated cells*
Each animal should be inoculated intramuscularly or subcutaneously [74] with a minimum of 10^7 viable cells. If there is no evidence of a progressively growing nodule at the end of the observation period, the cell line may be considered to be non-tumourigenic. If the cell line is found to be tumourigenic, the NRA/NCL might request additional studies to be done to determine the level of tumourigenicity. This can be done with dose-response studies, where doses of 10^7, 10^5, 10^3 and 10^1 are inoculated, and the data can be expressed as tumor-producing dose at the 50% endpoint (TPD_{50} value) [75].

B.8.7 *Observation period*
Animals are examined weekly by observation and palpation for evidence of nodule formation at the site of injection. The minimum observation period depends on the test system selected. In the case of the nude mouse, a minimum of 4 months is recommended. A shorter period is recommended for the ATS-treated newborn rat, because the immunosuppressive effect of the ATS declines after the final injection at two weeks.

B.8.8 *Assessment of the inoculation site over time (progressive or regressive growth)*
If nodules appear, they are measured in two perpendicular dimensions, the measurements being recorded weekly to determine whether the nodule grows progressively, remains stable, or decreases in size over time. Animals bearing nodules that are progressing should be sacrificed before the end of the study if the tumor reaches the limit set by the relevant authorities for the humane treatment of

animals. Animals bearing nodules that appear to be regressing should not be sacrificed until the end of the observation period. Cell lines that produce nodules that fail to grow progressively are not considered to be tumourigenic. If a nodule persists during the observation period and it retains the histopathological characteristics of a tumor, this should be investigated further and discussed with the NRA/NCL.

B.8.9 *Final Assessment of the inoculation site*
At the end of the observation period, all animals, including the reference group(s), are euthanized and examined for gross and microscopic evidence of the growth of inoculated cells at the site of injection and in other sites.

B.8.10 *Evaluation of animals for metastases*
Animals are examined for microscopic evidence of metastatic lesions in sites such as the liver, heart, lungs, spleen, and regional lymph nodes.

B.8.11 *Assessment of metastases (if any)*
Any metastatic lesions are examined further to establish their relationship to the primary tumor at the injection site. If what appears to be a metastatic tumor differs histopathologically from the primary tumor, it is necessary to consider the possibility that this tumor either developed spontaneously or that it was induced by one or more of the components of the cell substrate, such as an oncogenic virus. This may require further testing of the tumor itself or the tumourigenicity assay may need to be repeated. In such cases, appropriate follow-up studies should be discussed and agreed with the NRA/NCL (also see B.9 Oncogenicity).

B.8.12 *Interpretation of results*
A CCL is considered to be tumourigenic if at least 2 of 10 animals develop tumors at the site of inoculation within the observation period. However, the reported rate of spontaneous neoplastic diseases in the test animals should be taken into account during the assessment of the results. In addition, the histopathology of the tumors must be consistent with the inoculated cells, and a genotypic marker should show that the tumor is not of nude mouse origin.

If only one of 10 animals develops a tumor, further investigation is appropriate to determine, for example, if the tumor originated from the cell-substrate inoculum or the host animal and whether there are any viral or inoculated cell DNA sequences present. The NRA/NCL should be consulted in this regard.

The dose-response of the CCL may be studied in a titration of the inoculum as part of the characterization of the CCL. The need for such data will depend upon many factors specific to a given CCL and the product being developed. The NRA/NCL should be consulted in this regard.

B.8.13 *Applicability*
Cell Banks: Representative EOPC or ECB from the MCB or first WBC
Cell Types: DCL, SCL, CCL

B.9 Oncogenicity
B.9.1 *Tests for oncogenicity*

While tumourigenicity is the property of cells to form tumors when inoculated into susceptible animals, oncogenicity is the property of an acellular agent to induce cells of an animal to become tumor cells. As such, tumors that arise in a tumourigenicity assay contain cells derived from the inoculated cells, while tumors that arise in an oncogenicity assay are derived from the host. Oncogenic activity from cell substrates could be due to either the cell substrate DNA (and perhaps other cellular components) or an oncogenic viral agent present in the cells. Although there might be a perception that the cellular DNA from highly tumourigenic cells would have more oncogenic activity than the DNA of weakly or non-tumourigenic cells, at this time, it is not known if there is a relationship between the tumourigenicity of a cell and the oncogenicity of its DNA. Nevertheless, the NRA/NCL might require oncogenicity testing of the DNA and cell lysate from a new cell line (*i.e.*, other than those such as CHO, NS0, Sp2/0, and low passage Vero, for which there is considerable experience) that is tumourigenic in animal model systems (see below) because of the perception that a vaccine manufactured in such a cell line poses a neoplastic risk to vaccine recipients.

The major complication in assaying cellular DNA in animals arises from the size of the mammalian genome. Because the mammalian haploid genome is approximately 3×10^9 base pairs (bp) whereas the size of a typical oncogene could be $3\text{-}30 \times 10^3$ bp, the concentration of an oncogene in cellular DNA expression systems would be about 10^5 to 10^6 fold less concentrated than a plasmid containing the same oncogene. As a consequence, if 1 µg of an oncogene expression plasmid induces a tumor in an experimental animal model, the amount of cellular DNA that would contain a similar amount of the same oncogene is 10^5 µg to 10^6 µg (*i.e.*, 100 mg to 1 g). To date, three studies have indicated that between 1 and 10 µg of expression plasmids for cellular oncogenes can be oncogenic in mice (33, 42, 43). Therefore, more sensitive *in vivo* assays need to be developed before the testing of the oncogenic activity of cellular DNA becomes practicable. Recent results suggest that the sensitivity of the assay can be increased by several orders of magnitude with the use of certain immune-compromised strains of mice prone to develop tumours after inoculation with oncogenes. Thus, it may be possible to assess the oncogenic activity of cellular DNA in the future. However, at present there is no standardized *in vivo* oncogenicity test for cellular DNA. An example protocol is nonetheless provided in Appendix 3.

Several *in vitro* systems, such as scoring the neoplastic transformation of NIH 3T3 cells in a focus-forming assay following transfection of oncogenic DNA [75,77,78] have been used to assess oncogenicity, but it is not clear how these assays reflect the oncogenic activity of DNA *in vivo*, since they predominantly detect the oncogenic activity of activated *ras*-family members, and thus it is unclear how these assays can assist in estimating risk associated with the DNA or cell lysate from a cell substrate.

Based on experience with DCLs WI-38, MRC-5, and FRhL-2, testing of new MCBs of these cell lines for oncogenicity is not recommended. Other DCLs for which there is substantial experience also may not need to be tested. The NRA/NCL should be consulted in this regard. As stated in Section B.8.1, a new CCL should be presumed to be tumourigenic unless data demonstrate that it is not. If a manufacturer demonstrates that a new CCL is non-tumourigenic, oncogenicity testing on cell DNA and cell lysates might not be required by the NRA/NCL.

When appropriate, and particularly for vaccines, cell DNA and cell lysates should be examined for oncogenicity in a test approved by the NRA/NCL. An oncogenicity testing protocol is provided as Appendix 3.

B.9.2 *Applicability*
Cell Banks: MCB or first WCB taken to representative EOPC or ECB
Cell Types: CCL, SCL (recommended when tumourigenic cells are used in vaccine production)

B.10 Cytogenetics
B.10.1 *Characterization*
Chromosomal characterization and monitoring were introduced in the 1960s to support the safety and acceptability of human DCLs as substrates for vaccine production. Human DCLs differ from CCLs by retaining the characteristics of normal cells, including the normal human diploid karyotype. A significant quantity of data have been accumulated since then, and this has led to the conclusion that less extensive cytogenetic characterization is appropriate because of the demonstrated karyotypic stability of human DCLs used in vaccine production [79]. Thus, the use of karyology as a lot-by-lot quality-control test is unnecessary for well-characterized and unmodified human DCLs (*e.g.*, WI-38, MRC-5) and for FRhL-2.

Cytogenetic data may be useful for the characterization of CCLs, especially when marker chromosome(s) are identified. Such data might be helpful in assessing the genetic stability of the cell line as it is expanded from the MCB to the WCB and finally to production cultures (see B.3). The following recommendations are appropriate for the characterization of DCL and CCL cell banks.

Cytogenetic recharacterization of DCLs (*e.g.*, WI-38, MRC-5, and FRhL-2) should not be required, unless the cells have been genetically modified or the culture conditions have been changed significantly, since such data are already available (18,19,20). However, for each WCB generated, manufacturers should confirm once that the cells grown in the manner to be used in production are diploid and have the expected lifespan.

For the determination of the general character of a new or previously uncharacterized DCL, samples from the MCB should be examined at approximately four equally spaced intervals during serial cultivation from the MCB through to the proposed *in vitro* cell age used for production or beyond. The testing intervals should be agreed upon with the NRA. Each sample should consist of a minimum of 100 cells in metaphase and should be examined for exact counts of chromosomes, as well as for breaks and other structural abnormalities.

> Giemsa-banded karyotypes of an additional five metaphase cells in each of the four samples may provide additional useful information. ISCN [80] 400 band is the minimum acceptable level of Giemsa-banding analysis for human cells.

Stained slide preparations of the chromosomal characterization of the cells (*i.e.*, DCL, CCL), or photographs of these, should be maintained permanently as part of the cell-line record. Further recommendations have been proposed for SCLs [53].

B.10.2 *Applicability*

Cell Banks: MCB, ECB or representative EOPC
Cell Types: DCL, SCL, CCL (as a test for genetic stability, when appropriate),

B.11 Microbial agents
B.11.1 *General considerations*
While many biological production systems require human or animal-cell substrates, such cells are subject to contamination with and have the capacity to propagate extraneous, inadvertent, or so-called adventitious organisms, such as mycoplasma and viral agents. In addition, animal cells contain endogenous agents such as retroviruses that also may be of concern. Testing for both endogenous (*e.g*., retroviruses) and adventitious agents (*e.g*., mycoplasmas) is described in the succeeding sections. In general, cell substrates contaminated with microbial agents are not suitable for the production of biological products. However, there are exceptions to this general rule. For example, the CHO and other rodent cell lines that are used for the production of highly purified recombinant proteins express endogenous retroviral particles. Risk *versus* benefit must be considered when determining the suitability of a cell substrate for the production of a specific product. Further, risk-mitigation strategies during production, including purification (removal) and inactivation by physical, enzymatic, and/or chemical means, should be implemented whenever appropriate and feasible. Even though a cell substrate might be unacceptable for some products, such as for a live viral vaccine subjected to neither significant purification nor inactivation, that same cell substrate might be an acceptable choice for a different type of product, such as a highly purified recombinant protein or monoclonal antibody for which risk mitigation has been achieved by significant and validated viral clearance in the production process.

A strategy for testing cell banks for microbial agents should be developed. One strategy is to perform exhaustive testing at the MCB level and to perform more limited testing on the WCB derived from the MCB. This more limited testing would be selected on the basis of those agents that could potentially be introduced during the production of the WCB from the MCB. Testing would not need to be replicated for agents that could only have been present prior to the production of the MCB (*e.g*., an endogenous retrovirus, or BVDV from serum used for developing the cell seed or in the legacy of establishing the cell line).

However, if the number of vials of a MCB is limited, an alternative strategy would be to conduct the more exhaustive testing on the first WCB made from that MCB, and to limit testing on the MCB itself. An advantage to the strategy of performing more exhaustive testing on the first WCB is that it provides a greater opportunity for amplification of any agents that may have been introduced earlier and through to production of the WCBs. There are advantages and disadvantages to more extensive testing of the MCB or the WCB, and consideration should be given to what is more appropriate for the particular product(s) to be manufactured using a given cell bank. Consultation with the NRA/NCL should be considered prior to implementation to determine whether a proposed testing strategy is acceptable.

EOPC/ECB should be characterized once for each commercial production process. Testing of the ECB serves as further characterization of the MCB or WCB that was exhaustively tested and permits additional time/passages for amplification of low-level contaminants or reactivation of viral contaminants that may have been missed in the testing of the upstream bank.

B.11.2 *Viruses*

Manifestations of viral infections in cell cultures vary widely among the broad array of virus families that are potential contaminants; thus, the methods used to detect them vary. Lytic infections frequently are detected by the CPE they cause. However, in some cases such as non-cytopathic BVDV, no CPE is observed. Viruses also may be present latently (*e.g.*, herpesviruses) or endogenously (in the germline, *e.g.*, retroviral proviruses). Such inapparent infections might require specific techniques designed to reveal their presence, such as molecular and immunological methods, and electron microscopy. For new cell substrates, induction of a detectable infection by exposing the cells to special conditions (*e.g.*, chemical induction; heat shock) may be required, and special detection techniques like transcriptome sequencing or degenerate primer PCR may have utility.

The strategy developed to test cell substrates for viruses should take into consideration the families of viruses and specific viruses that may be present in the cell substrate. Consideration should be given to the species and tissue source from which the cell substrate originated, and to the original donor's medical history in the case of human-derived cell substrates or to the pathogen status of donor animals in the case of animal-derived cell substrates. In addition, consideration should be given to viruses that could contaminate the cell substrate from the donors or from animal- or human-derived raw materials used in the establishment and passage history (legacy) of the cell substrate prior to and during cell banking or production (*e.g.*, serum, trypsin, animal- or human-derived medium components, antibodies used for selection, or animal species through which the cell substrate may have been propagated), as well as laboratory contamination from operators or other cell cultures.

Tests should be undertaken to detect, and where possible identify, any endogenous or exogenous agents that may be present in the cells. Attention should be given to tests for agents known to cause an inapparent infection in the species from which the cells were derived, making it more difficult to detect (*e.g.*, simian virus 40 in rhesus monkeys).

Primary cells are obtained directly from the tissues of healthy animals and are more likely to contain adventitious agents than banked, well-characterized cells. In addition, recent vaccination of source animals should be considered, as they may be exposed to live vaccines. This risk with primary cells can be mitigated by rigorous qualification of source animals and the primary cells themselves. When feasible, animals from which primary cultures are established should be from genetically closed flocks, herds, or colonies monitored for freedom from pathogens of specific concern. Such animals are known as specific-pathogen-free (SPF). The term "closed" refers to the maintenance of a group (flock, herd, or colony) free from introduction of new animals (new genetic material that could introduce, *e.g.*, new retroviral proviruses). Many live viral vaccines are commonly produced in primary cells and undergo little purification during production. In such cases, and when feasible, the use of SPF animals is highly recommended. Documentation of the status of the source animals should be provided to the NRA/NCL. Animals that are not from closed flocks, herds, or colonies should be quarantined and thoroughly evaluated for a period sufficient to detect signs of disease or infection (*e.g.*, monkeys are generally quarantined for 6 or more weeks [81]). Such animals also should be screened serologically for appropriate adventitious agents to determine their suitability as a source for the primary-cell substrate. Animal-husbandry practices should be documented. Even so, viral contamination of the cells may not be excluded from all cultures. For example, contamination

of primary monkey kidney cells with foamy virus or simian cytomegalovirus is common in the absence of specific concerted efforts to prevent these contaminations.

For primary-cell cultures, the principles and procedures outlined in Part C, Requirements for Poliomyelitis Vaccine (Oral) [81], together with those in section A.4 of Requirements for Measles, Mumps and Rubella Vaccines and Combined Vaccine (Live) [82] may be followed.

The production of viral vaccines, such as those against smallpox or rabies, originally required the use of living animals, and the great range of possible viral contaminants only became apparent as cell-culture methods were developed. For example, human enteroviruses were not recognised until the development of monkey kidney cell cultures, in which they could produce cytopathic effects, because the disease produced in humans is either relatively mild or in some cases non-existent. It was also clear that viruses could be detected in some systems but not others; for instance, the polyomavirus SV40 does not produce a cytopathic effect in cultures from rhesus monkey kidney cells (in which much of the early polio vaccines were produced) derived from SV40-infected monkeys but will do so in cultures from cynomolgous or African green monkey kidney cells. The suspicion was therefore that there were many viruses in the culture systems of the time and that they were detected only if the assays were appropriate. This remains an accurate view and has lead to a range of different approaches to try and detect all contaminants.

Coxsackie viruses are named after the town in New York where they were first identified and were historically detected by their effects in mice. Coxsackie B viruses produce clinical signs and death in adult mice, while Coxsackie A viruses will affect only suckling mice. For many years, tissue-culture methods were a less reliable method of detection of Coxsackie A viruses than suckling mice, and the continued use of these animals in cell bank characterisation reflects this.

In the 1940s, embryonated chicken eggs were a popular substrate for the growth and assay of viruses such as influenza, measles, mumps, yellow fever and vaccinia. They therefore appear to have a wide range of susceptibility. Simian viruses such as SV5 or viruses such as Sendai virus also grow well in them. As many are paramyxoviruses with haemagglutinating (HA) activity, the egg-based assays include tests for HA activity as well as death of the embryos.

A range of tissue-culture cells is also used, typically including one human, one of the same species as the production cell, and one other (often, monkey origin). The hope is that the range will catch viruses not detected by other means, although in practice it is wise to assume that there is no such thing as a generic detection method; for example, cells from an inappropriate monkey species would not necessarily detect SV40 whereas cells from other species will (*e.g.*, Vero cells). In certain circumstances, where a virus is of particular concern, specific tests have been applied. For example, herpes B virus is a common infection of monkeys in the absence of precautions such as quarantine and clinical evaluation of the donor animals, and has very serious effects on infected humans. While herpes B virus was routinely detected by the use of primary rabbit kidney cell cultures, established rabbit cell lines are now acceptable for this purpose. Another example is Marburg virus, which in the 1970s caused a number of deaths in workers who handled monkeys that were to be used in a vaccine-production facility. The incident might have been avoided had the animals been adequately quarantined. A specific test in guinea pigs was introduced and maintained for a number of years to ensure the absence of the agent.

There is a disparate range of tests that have been or are still used with the aim of detecting any significant contaminant that may be present in cell cultures. Some, such as the rabbit kidney cell test, are very specific in intent while others may be expected to be more generic. In general, however, it is wise to assume that an assay will never be all encompassing whether based on historical virological approaches or more current methodologies. Thus, the consequences of deleting tests on the grounds of redundancy must be very carefully evaluated before any action is taken. On the other hand, it is difficult to justify maintenance of a test if it only detects viruses also detected by other methods of equivalent sensitivity, comparable ease of use, and cost. Each of these considerations should be given when developing an appropriate testing strategy for the given cell bank. Policies to minimize the use of animals in safety testing should also be considered, but must be considered in balance with the utility and necessity (sensitivity and ability to detect particular adventitious agents not readily detected by other means) of the test in which they are used.

B.11.2.1 *Tests in animals and eggs*
The cells of the MCB and WCB are unsuitable for production if any of the animal or egg tests shows evidence of the presence of any viral agent attributable to the cell banks.

Generally, MCBs are thoroughly characterized by the methods listed below. WCBs may be characterized by an abbreviated strategy, when appropriate. However, an alternative strategy to this general rule may be used, as discussed in section B.11.2 above. These tests may be performed directly on cells or supernatant fluids or cell lysates from the bank itself or on cells or supernatant fluids or cell lysates from passaged cells from the bank that have been passaged to the proposed *in vitro* cell age for production or beyond.

> In some countries, policies exist to minimize the use of animals in safety testing.

B.11.2.1.1 Adult mice
The original purpose of this test was for the detection of lymphocytic choriomeningitis virus (LCMV). The test in adult mice for pathogenic viruses includes inoculation by the intraperitoneal route (0.5 mL) with cells and culture fluids from the MCB or WCB, where at least 10^7 viable cells or the equivalent cell lysate are divided equally among at least ten adult mice weighing 15-20 g.

> In some countries, the adult mice are also inoculated by the intracerebral route (0.03 mL).

> In some countries at least 20 mice are required for each test.

The animals are observed for at least 4 weeks. Any animals that are sick or show any abnormality are investigated to establish the cause. Animals that do not survive the observation period should be examined for gross pathology and histopathology in order to determine a cause of death, and if a viral infection is indicated, efforts should be undertaken to identify the virus. Viral identification may involve culture and/or molecular methods. Further, each mouse that dies after the first 24 hours of the test, or is sacrificed because of illness, should be necropsied and examined for evidence of viral infection by subinoculation of appropriate tissue into at least five additional mice which should be observed for 21 days. The test is not valid if more than 20% of the animals in either or both of the

test and negative control groups (if used) become sick for non-specific reasons and do not survive the observation period.

> In some countries, the adult mice are observed for 21 days.

If the cell substrate is of rodent origin, at least 10^6 viable cells or the equivalent cell lysate are injected intracerebrally into each of ten susceptible adult mice to test for the presence of LCMV.

> In some countries, after the observation period, the animals are challenged with live LCMV to reveal the development of immunity against non-pathogenic LCMV contaminants resulting in otherwise unapparent infection.

B.11.2.1.1.1 Applicability
Cell Banks: MCB, WCB, or ECB or representative EOPC
Cell Types: PCC, DCL, SCL, CCL

B.11.2.1.2 Suckling mice
The original purpose of this test was for the detection of Coxsackie viruses. The test in suckling mice for pathogenic viruses includes inoculation by the intraperitoneal route (0.1 mL) with cells and culture fluids from the MCB or WCB; at least 10^7 viable cells or the equivalent cell lysate are divided equally between two litters of suckling mice, comprising a total of at least ten animals less than 24-hours old.

> In some countries, the suckling mice are also inoculated by the intracerebral route (0.01 mL).

> In some countries, 20 suckling mice are inoculated.

The animals are observed for at least 4 weeks. Any animals that are sick or show any abnormality are investigated to establish the cause. Animals that do not survive the observation period should be examined for gross pathology and histopathology in order to determine a cause of death, and if a viral infection is indicated, efforts should be undertaken to identify the virus, where this is practicable. Viral identification may involve culture and/or molecular methods. Further, such examination of viral infection should include subinoculation of appropriate tissue suspensions into an additional group of at least five suckling mice by intracerebral and intraperitoneal routes and observation daily for 14 days. In the case of suckling mice, it is often observed that those that perish are cannibalized by their mother and this renders determining a cause of death impossible (when they are fully cannibalized and no remains can be recovered). The test is not valid if more than 20% of the animals in either or both of the test group and negative control group (if used) do not survive the observation period.

> In some countries, the suckling mice may be observed for a period of 14 days followed by a sub-passage involving a blind passage (*via* intraperitoneal and intracerebral inoculation into at least 5 additional mice) of a single pool of the emulsified tissue (minus skin and viscera) of all mice surviving the original 14-day test.

B.11.2.1.2.1 Applicability

Cell Banks: MCB, WCB or ECB or representative EOPC
Cell Types: PCC, DCL, SCL, CCL

B.11.2.1.3 Guinea pigs
The original purpose of this test was for the detection of LCMV and Mycobacterium tuberculosis. When it is necessary to detect Mycobacterium species, a test in guinea pigs is performed and includes inoculation by the intraperitoneal route (5 mL) with cells and culture fluids from the MCB or WCB, where at least 10^7 viable cells or the equivalent cell lysate are divided equally among the animals.

> In some countries, tests in five guinea-pigs weighing 350-450 g are also inoculated by the intracerebral route (0.1 mL) and observed for 42 days to reveal *Mycobacterium tuberculosis* and other species.

The animals are observed for at least 6 weeks. Any animals that are sick or show any abnormality are investigated to establish the cause. Animals that do not survive the observation period should be examined for gross pathology and histopathology in order to determine a cause of death, and if a viral infection is indicated, efforts should be undertaken to identify the virus. Viral identification may involve culture and/or molecular methods. The test is not valid if more than 20% of the animals in either or both of the test and negative control (if used) groups do not survive the observation period.

The test in guinea-pigs for the presence of Mycobacterium may be replaced by an alternative *in vitro* method such as culture, or shortened culture with a PCR endpoint (also see B.11.3).

B.11.2.1.3.1 Applicability
Cell Banks: MCB, WCB or ECB or representative EOPC
Cell Types: PCC, DCL, SCL, CCL (the latter three are dependent on legacy and current use of media components of animal origin that could result in contamination with Mycobacterial species)

B.11.2.1.4 Rabbits
The original purpose of this test was for the detection of Herpes B virus. When it is necessary to detect simian herpes B virus, the test in rabbits for pathogenic viruses is performed and includes the inoculation by the intradermal (1 mL) and subcutaneous (>2 mL) routes with cells and culture fluids from the MCB or WCB, where at least 10^7 viable cells or the equivalent cell lysate are divided equally among the animals.

> In some countries, tests in five rabbits weighing 1.5-2.5 kg are inoculated by the subcutaneous route with either 2 mL or between 9 and 19 mL. Consultation with the NRA/NCL regarding acceptable methods should be considered.

The animals are observed for at least 4 weeks. Any animals that are sick or show any abnormality are investigated to establish the cause. Animals that do not survive the observation period should be examined for gross pathology and histopathology in order to determine a cause of death, and if a viral infection is indicated, efforts should be undertaken to identify the virus. Viral identification may involve culture and/or molecular methods. The test is not valid if more than 20% of the animals in either the test or the negative control (if used) groups do not survive the observation period.

The test in rabbits for the presence of herpes B virus is intended for primary simian cultures, and may be replaced by a test in rabbit kidney cell cultures.

B.11.2.1.4.1 Applicability
Cell Banks: MCB, WCB or ECB or representative EOPC
Cell Types: PCC, DCL, SCL, CCL

B.11.2.1.5 Embryonated chicken eggs
At least 10^6 viable cells or the equivalent cell lysate, along with culture fluids, from the MCB or WCB of avian origin, propagated to the proposed *in vitro* cell age for production or beyond are injected into the allantoic cavity of each of at least ten embryonated hens' eggs, and into the yolk sac of each of at least another ten embryonated hens' eggs. The eggs are examined after not fewer than 5 days of incubation. The allantoic fluids of the eggs are tested with red cells from guinea pig and chickens (or other avian species) for the presence of haemagglutinins. The test is not valid if more than 20% of the embryonated hens' eggs in either or both of the test group and negative control group (if used) are discarded for non-specific reasons.

> In some countries, the NRA/NCL also requires that other types of red cells, including cells from humans (blood group IV O) or monkeys should be used in addition to guinea-pig and chicken (or other avian species) cells. In all tests, readings should be taken after incubation for 30 minutes at 0-4°C, and again after a further incubation for 30 minutes at 20-25°C. For the test with monkey red cells, readings also should be taken after a final incubation for 30 minutes at 34-37°C.
>
> In some countries, inoculation by the amniotic route is used.
>
> In some countries, following incubation, allantoic fluids or a 10% suspension of yolk sacs, as appropriate, should be harvested, pooled, and blind passaged into an additional group of eggs.

Usually, the eggs used for the yolk sac test should be 5-7 days old. The eggs used for the allantoic cavity test should be 9-11 days old.

> Alternative ages for the embryonated chicken eggs and alternative incubation periods are acceptable if they have been determined to be equivalent or better at detecting the presence in the test samples of the adventitious agents this test is capable of detecting when performed as above.

Embryos that do not survive the observation period should be examined for gross pathology in order to determine a cause of death, and if a viral infection is indicated, efforts should be undertaken to identify the virus. Viral identification may involve culture and/or molecular methods.

B.11.2.1.5.1 Applicability
Cell Banks: MCB of avian origin, WCB of avian origin or ECB or representative EOPC
Cell Types: avian PCC, DCL, SCL, CCL (also recommended for novel cell substrates)

B.11.2.1.6 Antibody-production tests

Rodent cell lines are tested for species-specific viruses using mouse, rat and hamster antibody production tests, as appropriate. *In vivo* testing for lymphocytic choriomeningitis virus, including a challenge for non-lethal strains, is performed for such cell lines, as described in Section B.11.2.1.1. Avian cell lines may also be tested using a chick antibody-production test, *e.g.*, to detect chicken anemia virus. Further, if the cell substrate (even if not of rodent origin) has been exposed to rodent-origin materials, *e.g.*, selection using a monoclonal antibody, testing should be considered for the relevant-species viruses using an antibody-production test [83, 84].

> In some countries, consideration is being given to use of nucleic acid testing (NAT) in place of the *in vivo* antibody-production testing. In these cases, data should be provided to the NRA/NCL that justifies this practice.

B.11.2.1.6.1 Applicability
Cell Banks: MCB, WCB, or ECB or representative EOPC
Cell Types: DCL, SCL, CCL (recommended primarily for cells of rodent origin)

B.11.2.2 *Tests in cell culture*
Tests in cell culture are capable of detecting a broad array of viral families. Readouts include monitoring the cultures periodically for CPE and tests for haemadsorbing and haemagglutinating viruses, which are conducted at the end of the culture period. In addition to the indicator cells described below, it may be appropriate to expand the different types of indicator cells used (beyond 2 or 3) to enable the detection of viruses with differing host requirements. Decisions about which cell lines to use as indicator cells should be guided by the species and legacy of the production cell substrate taking into the consideration the types of viruses to which the cell substrate could potentially have been exposed and thus the viruses one would like to detect by this assay method. The cell substrate is unsuitable for production if any of the indicator cell cultures shows evidence of the presence of any viral agent attributable to the tested cell substrate.

B.11.2.2.1 Applicability
Cell Banks: MCB, WCB or ECB or representative EOPC
Cell Types: PCC, DCL, SCL, CCL

B.11.2.2.2 Indicator cells
Live cells or cell lysate, each with spent culture fluids of the MCB or WCB are inoculated onto monolayer cultures or cultivated with monolayer cultures of the cell types listed below, as appropriate.

> A lysate of the cells may be prepared by a method that avoids virus disruption while allowing maximal virus release (*e.g.*, typically three freeze/thaw cycles followed by low-speed centrifugation). If cells, lysate, or spent culture fluids are to be stored prior to testing, then they should be stored at $\leq$-70°C.

- Cultures (primary cells or CCL) of the same species and tissue type as that used for production.

- Cultures of a human DCL. The original purpose of this test, utilizing primary human cells, was the detection of measles virus. But, where the cell substrate is of human origin, a simian kidney cell line should be used as the second indicator cell line. The original purpose of the use of this cell type was the detection of simian viruses.

> In some countries, cultures of another (third) cell line from a different species are required.
>
> In many circumstances, more than two cell lines may be necessary to cover the range of potential viral contaminants and typically, a third cell line would be used that is of simian origin, if the cell substrate is not of simian origin.

For new cell substrates, additional cell lines to detect viruses known to be potentially harmful to humans could be considered (*e.g.*, for insect cell lines, if the cells selected for the above mentioned tests are not known to be permissive to insect viruses, an additional detector cell line should be included in the testing).

The cell bank sample to be tested is diluted as little as possible. At least 10^7 cells, or equivalent cell lysate, and spent culture fluids are inoculated onto each of the indicator cell types. The resulting co-cultivated or inoculated cell cultures are observed for evidence of viruses by cytopathic effect for at least 2 weeks. If the cell line is known to be capable of supporting the growth of human or simian cytomegalovirus, HDC cultures are observed for at least 4 weeks. Extended (4-weeks) cell culture for the purposes of detecting human or simian cytomegalovirus can be replaced by the use of NAAT to detect cytomegalovirus nucleic acid.

> In some countries, a passage onto fresh cultures for an additional 2 weeks is recommended for all indicator cultures. In some cases, it may be difficult to keep the cell cultures healthy for 2 weeks without subculturing. In those cases, it may be necessary to feed the cultures with fresh medium or to subculture after two weeks onto fresh cultures in order to be able to detect viral agents.

At the end of the observation period, samples of each of the co-cultivated or inoculated cell culture systems are tested for haemadsorbing and/or haemagglutinating viruses, as described in section B.11.2.1. 5.

B.11.2.2.3 Additional considerations to the tests in cell culture for insect viruses
Many insect cell lines carry persistent viral infections that do not routinely produce a noticeable CPE (*e.g.*, some clones of the Hi-5 cell line are persistently infected with an insect nodavirus). However, the viruses may be induced to replicate by stressing the cells using a variety of techniques such as increased/reduced culture temperature (above or below that routinely used for production), heat-shock for a short period, super-infection with other insect viruses, or chemical inducers. Therefore, the probability of detecting such low-level persistent infections may be increased by stressing the cells prior to analysis.

Intact cells and cell lysates from a passage level at or beyond that equivalent to the EOPC is co-cultivated with indicator cells from at least three different species of insect in addition to the

indicator cells as noted in section B.11.2.2.2. Cell lines should be selected on the following basis: one of the lines has been demonstrated to be permissive for the growth of human arboviruses, one has been shown to be permissive for the growth of a range of insect viruses, and the third has been derived from a species that is closely related to the host from which the MCB is derived (or another line from the same species). Duplicate cultures of indicator cells are typically incubated at two temperatures, such as 37 ± 1°C and a lower temperature, such as 28 ± 1°C, observed for a period of 14 days, and examined for possible morphological changes. The cell-culture fluids from the end of the test period are tested for haemagglutinating viruses, or the intact cells from the end of the test period are tested for haemadsorbing viruses. The cells comply with the test if no evidence of any viral agent is found.

Several mosquito cell lines are available that are permissive for the growth of some human arboviruses and could be considered for these tests. Alternatively, BHK-21 cells could be considered for this purpose. The most permissive insect cell lines characterised to date have been derived from embryonic Drosophila tissues. While the mosquito and Drosophila cell lines may be suitable for some aspects of the testing, it should be remembered that many insect cell lines are persistently infected with insect viruses that usually produce no obvious CPE. In addition, many insect cells may be infected with mammalian viruses, such as BVDV, that are known to replicate in insect cells. Demonstrating that the indicator cell lines are themselves free from adventitious agents is an important pre-requisite to their use in the testing outlined above. Consideration should also be given to risk-mitigation strategies as discussed above for highly purified products for which viral clearance can be achieved and validated.

B.11.2.3 *Transmission electron microscopy*
At least 200 cells from the MCB or WCB, and ECB are examined by transmission electron microscopy (TEM) for evidence of contamination with microbial agents. Methods include negative staining and thin section. A discussion of these methods is provided by Bierley *et al.* [85]. It may be appropriate to examine more cells in some cases, such as discussed below for insect cell lines, and the NRA/NCL should be consulted in this regard. Any unusual or equivocal observations that might be of microbiological significance should be noted and discussed with the NRA/NCL.

TEM can detect viral particles in a cell substrate, including certain endogenous retroviruses. While TEM is fairly insensitive (generally detecting gross contamination, but not necessarily low-level contamination), it is a generic assay that can detect microbial agents of many types.

B.11.2.3.1 Applicability
Cell Banks: MCB, WCB, or ECB or representative EOPC
Cell Types: DCL, SCL, CCL

B.11.2.3.2 Additional considerations to TEM for insect cells
For MCBs and WCBs derived from insect cells, the general screening test outlined above applies. In addition, cell lines should be subjected to stress conditions, such as described in section B.11.2.2.3, prior to examination by transmission electron microscopy. Further, increasing the number of cells examined may also improve the probability of detecting an agent (*e.g.*, errantiviruses and hemiviruses). The maintenance temperatures and treatments used should be agreed with the NRA/NCL along with the number of sectioned cells to be examined.

B.11.2.4 *Tests for retroviruses*
All vertebrate and insect cells that have been analysed possess endogenous, genetically acquired retroviral sequences integrated into chromosomal DNA in the form of proviruses. These sequences may be expressed, or be induced, as mRNA. In some cases, the mRNA is translated into viral protein and virus particles (virions) are produced. In many cases, these virions are defective for replication (*e.g*., avian endogenous retrovirus EAV, Chinese hamster ovary cell line gamma-retrovirus [86]) whereas in others (*e.g*., X-MuLV) the retroviruses may be capable of infecting cells of other species including human cells.

Consideration should also be given to the possibility that cell banks may be infected with non-genetically acquired retroviruses (exogenous retroviruses), either because the donor animal was infected or through laboratory contamination.

It should be noted that infection by retroviruses is not necessarily associated with any cytopathic effect on the cells and therefore it may require screening assays, like the Product-Enhanced RT (PERT) assay for reverse transcriptase or TEM to reveal their presence.

The cells of the MCB or WCB are unsuitable for production if the tests for infectious retroviruses, if required, show evidence of the presence of any viral agent attributable to the substrate that cannot be demonstrated to be cleared during processing. Generally, the downstream manufacturing process for products (*e.g*., monoclonal antibodies) made in cell substrates that produce retroviral particles (*e.g*., CHO cells) or infectious endogenous retrovirus (*i.e*., NS0, SP2/0 cells) is validated to provide adequate viral clearance [14]. The margin of viral clearance required should be agreed with the NRA/NCL.

Chick embryo fibroblasts (CEF) contain defective retroviral elements that frequently produce defective particles with reverse transcriptase activity. This has been the subject of many studies and WHO consultations because they are used for live viral vaccine production. If evidence is presented that the donor flock is free of infectious retroviruses and there is no evidence that the cultures are contaminated with infectious retroviruses, then the cultures can be considered acceptable with respect to retrovirus tests.

Rodent cell lines express endogenous retroviruses, and thus infectivity tests should be performed to determine whether these endogenous retroviral particles are infectious.

Cell lines such as CHO, BHK-21, NS0, and Sp2/0 have frequently been used as substrates for drug production with no reported safety problems related to virus contamination of the products and may be classified as "well-characterized" because the endogenous retrovirus particles have been studied extensively. Furthermore, the total number of retrovirus-like particles present in the harvest is evaluated quantitatively (TEM or QPCR) on a representative number of lots and retrovirus clearance is demonstrated with significant safety factors. Thus, in these situations testing for infectious retrovirus may be reduced; *e.g*., test one lot then discontinue testing, but repeat when there is a significant change in the cell-culture process such as a change in scale. Sponsors are encouraged to consult with the NRA.

B.11.2.4.1 Applicability
MCBs and cells that have been propagated to the proposed *in vitro* cell age for production or beyond. Alternatively, this testing could be performed on WCBs.
Cell Banks: MCB or WCB, and ECB or representative EOPC
Cell Types: DCL, SCL, CCL

B.11.2.4.2 Reverse Transcriptase (RT) Assay
Test samples from the MCBs or WCBs propagated to the proposed *in vitro* cell age for production or beyond are examined for the presence of retroviruses.

Culture supernatants are tested by a highly sensitive, quantitative, polymerase chain reaction (PCR)-based RT assay or PERT assay [87,88,89,90].

RTase activity is not specific to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete or infectious genome [91,92,93,94,95] or cellular DNA-dependent DNA polymerases [96,97]. Attempts to reduce the PERT activity associated with cellular DNA-dependent DNA polymerases have been reported [97,98,99], although no treatment can eliminate all activity, and thus the results of such highly sensitive assays need to be interpreted with caution. Use of appropriate controls in the assay assist in this regard. Since RT activity can be associated with the presence of defective retrovirus-like particles and since polymerases other than reverse transcriptase can result in apparent RT activity, a positive result in an RT assay is not conclusive evidence of the presence of infective retrovirus. Positive results may require further investigation, *e.g.*, carrying out infectivity assays (see Section B.9.1.4.4). Also, it may be useful to utilize the conventional RT assay in this investigation to determine whether the RT activity is Mg++ or Mn++ dependent. Such testing should be agreed in advance with the NRA/NCL.

CEFs and other cells of avian origin are known to express retroviral elements. With such cells, the appropriateness of this test should be discussed with the NRA/NCL. For example, it may be appropriate to direct testing strategies at the detection of infectious avian retroviruses, such as avian leukosis viruses and reticuloendotheliosis virus, including serological screening of flocks that are the source of the CEFs. Additionally, it is known that insect cells have retroviral elements that are detected by a PERT assay, and so they too can test positive by this assay.

B.11.2.4.3 PCR or other specific *in vitro* tests for retroviruses
When the PERT test gives unclear results, or when it is unavailable, it may be appropriate to screen the cell substrate for species-specific retroviruses by molecular methods, such as the PCR, immunofluorescence, ELISA, or other virus-specific detection methods. Molecular methods, such as PCR, also may be used for quantification of retrovirus like particles in the production harvests provided that the method is validated accordingly. Consultation with the NRA/NCL regarding the acceptability of this approach is recommended.

B.11.2.4.4 Infectivity test for retroviruses
When the test sample is found to have RT activity, it might be necessary to carry out infectivity assays to assess whether the activity is associated with replicating virus.

Because rodent cells generally express endogenous retroviruses, the infectivity and *in vitro* host range of such retroviruses should be assessed. Test samples from the MCB or WCB, propagated to the proposed *in vitro* cell age for production or beyond should be examined for the presence of retroviruses by infectivity assays. Cells to be used for these assays should be able to support the replication of a broad range of viruses; this might require using cells of various species and cell types. The testing strategy should be agreed with the NRA/NCL.

It is often possible to increase the sensitivity of assays by first inoculating the test material onto cell cultures that can support retroviral growth in order to amplify any retrovirus contaminant that may be present at low concentrations. For non-murine retroviruses, test cell lines should be selected for their capacity to support the growth of a broad range of retroviruses, including viruses of human and non-human primate origin [100,101].

For murine retroviruses, it is important to assess whether the cells release infectious retroviruses and, if so, to determine the host range of those viruses. The testing for murine retroviruses can be complex, and the NRA/NCL should be consulted for guidance. Murine and other rodent cell lines (CHO, NS0, SP2/0) or hybrid cell lines containing a rodent component should be assumed to be inherently capable of producing infectious retroviruses or non-infectious retrovirus-like particles. In such cases, the clearance (removal and/or inactivation) of such retroviruses during the manufacturing process should be quantified and provide a level of clearance acceptable to the NRA/NCL.

Any testing proposed by the manufacturer should be agreed with the NRA/NCL.

B.11.2.5 *Tests for particular viruses not readily detected by the tests described in sections B.11.2.1, B.11.2.2, B.11.2.3, and B.11.2.4 and their sub-sections*
Some viruses, such as hepatitis B or C viruses or human papillomaviruses, cannot be detected readily by any of the methods described above because they are not known to grow readily in cell culture or are restricted to human host range. Some animal viruses, *e.g.*, bovine polyomavirus and porcine circoviruses, are not readily detected by the routine tests previously described. In such circumstances, it may be necessary to include specific assays for such viruses. While broad general tests are preferable for detecting unknown contaminants, some selected viruses may be screened by using specific assays, such as molecular techniques (*e.g.*, nucleic acid amplification). Antibody-based techniques might also be employed, such as immunofluorescence assays.

Generally, once the MCB, WCB, or ECB has been demonstrated to be free of selected viruses, it might not be necessary to test the cells at later stages (*e.g.*, at the production level) if such viruses could not be introduced readily during culture.

Human cell lines should be screened using appropriate *in vitro* techniques for specific viruses that are the cause of significant morbidity, for those viruses that might establish latent or persistent infections and for viruses that may be difficult or impossible to detect by the techniques described in sections B.11.2.1, B.11.2.2, B.11.2.3, and B.11.2.4 and their sub-sections. Selection of the viruses to be screened should take into account the tissue source and medical history of the donor, if available, from whom the cell line was derived.

Under circumstances in which the cell origin or medical history of the donor, if available, would suggest their presence, it may be appropriate to perform specific testing for the presence of human herpesviruses, human retroviruses, human papillomaviruses, human hepatitis viruses, human polyomaviruses, or difficult-to-culture types of human adenoviruses.

Consideration should be given to screening insect cell lines for specific viruses that have been reported to contaminate that particular cell line (*e.g*., nodaviruses) or viruses that may be present persistently in insect cell lines and that are known to be infectious for humans.

B.11.2.5.1 Applicability
 The NRA/NCL should be consulted in regard to the specific pathogens or selected viruses that should be included in the testing strategy as these will be directed on a case-by-case basis depending on the species and origin of the cell and the medical history of the donor, if available.
Cell Banks: MCB, WCB, or ECB or representative EOPC
Cell Types: PCC (as needed), DCL, SCL, CCL

B.11.2.5.2 Nucleic acid detection methods
Tests for selected viruses usually are performed using nucleic acid amplification and detection methods. PCR can be performed directly on DNA extracted from the cells or on cell lysates or supernatant fluids by DNA amplification, or on RNA by reverse transcription followed by DNA amplification (RT-PCR). In this manner, both DNA and RNA viruses can be detected, as can the proviral DNA of retroviruses. PCR primers can be directed against variable regions of viral nucleic acids in order to ensure detection of a specific virus or viral strain, or against conserved regions of viral sequences shared amongst strains or within a family in order to increase the opportunity of detecting multiple related viruses. Standard PCR analysis can be coupled with hybridization methods to increase its versatility, sensitivity, and specificity. For example, the use of probes to various regions of the amplicon might be useful in identifying the virus strain or family. However, PCR methods have the limitation that viral genes might not be sufficiently conserved among all members of any particular viral family to be detected even when conserved regions are selected.

New, sensitive, molecular methods, with broad detection capabilities are being developed. These are not yet in routine use, but as they become widely available and validated, they will play an increasing role in the evaluation of cell substrates. The sensitivity of these methods as well as their breadth of detection should be considered when evaluating their applicability. One of the advantages of some of these new methods is that they have the potential to discover new viruses. These new approaches involve either degenerate PCR for whole virus families or random-priming methods, which do not depend on a known sequence. Analysis of the resulting amplicons has employed sequencing, hybridization to oligonucleotide arrays, and mass spectrometry [102, 103, 104]. The new generation of massively parallel (deep) sequencing (MPS) methods may have particular utility. They can be applied to detect virions after nuclease treatment to remove cellular DNA and unencapsidated genomes. Used in this mode, MPS has been used to discover new viruses in serum and other tissues and has revealed the contamination of human vaccines by porcine circovirus [102, 105, 106, 107, 108, 109]. MPS can also be employed to screen cell substrates for both latent and lytic viruses by sequencing the transcriptome. In this mode, enormous quantities of data are generated, and robust bioinformatic methods are required to detect viral sequences by either positive selection against viral databases or negative selection to remove cellular sequences [102, 109, 110].

Care is required to exclude false "hits" to viruses due to recognition of transduced cellular sequences present in some viral genomes or due to viral genes like virokines that have a close homology to cellular genes.[102,104, 110]

It is probable that application of methods of this type will be expected or required by regulatory agencies in future. At present they have not been evaluated for sensitivity and specificity and should be thought of as powerful investigational tools that can reveal issues to be explored by more established methods.

B.11.3 *Bacteria, fungi, mollicutes, and mycobacteria*
The most common contaminants of cell culture are non-viral. These can be introduced easily from the environment, materials, personnel, *etc*. Furthermore, many such organisms multiply rapidly and can be pathogenic for humans. It is also important in risk evaluation for the manufacturer to bear in mind that standard compendial tests for "sterility" are intended to give an indication of the effectiveness of aseptic processing to prevent general bacterial or fungal contamination and are not capable of isolating all potential bacterial and fungal contaminants. The manufacturer should consult with the NRA/NCL regarding any particular materials or environments where there may be an elevated hazard of contamination with particular types of fastidious organisms.

Biological starting materials, like cell substrates, should be characterized to ensure that they are free from adventitious infectious organisms such as bacteria, fungi, cultivable and non-cultivable mycoplasmas, spiroplasma (in the case of insect cells or cells exposed to plant-derived materials), and mycobacteria. For a substance to be considered free of such contaminants, the assays should demonstrate, at a predefined level of sensitivity, that a certain quantity of the substance does not contain detectable levels of that contaminant. Testing should be conducted in an aseptic environment under appropriate clean room conditions to avoid false-positive results. Testing should include a plan to account for the need for repeat testing to deal with potentially false-positive results and a prequalification plan for reagents used in the tests.

Mycobacterial testing might be applied to cell-bank characterization if the cells are susceptible to infection with *Mycobacterium tuberculosis* or other species. Such testing also should be performed on primary-cell cultures. It may be necessary to lyse the host cells in order to detect Mycobacteria, because some strains may be primarily intracellular.

Detection of mycoplasma/spiroplasma may require different growth conditions from methods used for mammalian cells - although at least one, Spiroplasma, can be cultivated at 30ºC. Positive controls for these tests (particularly for Spiroplasmas) are an issue that needs to be resolved. Spiroplasma have been reported as infectious agents in a number of insect species and insect cell lines, and in addition have been reported to cause pathogenic effects in mammals.

B.11.3.1 *Bacterial and Fungal Sterility*
Tests are performed as specified in Part A, section 5.2 [111] of the Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances), by a method approved by the NRA/NCL. Additional information can be found in national pharmacopoeias and ICH documents [10,112,113,114]. For the MCB and WCB, the test is carried out using for each medium 10 mL of supernatant fluid from cell cultures. In addition, the test is carried out on at least

1% of the filled containers (*i.e.*, cyropreservation vials) with a minimum of two containers. For supernatant fluid, the recommended method is the membrane filtration method. For cell bank vial testing, it may be necessary to use the direct inoculation method. Bacteriostasis and fungistasis should be excluded.

B.11.3.1.1 Applicability
Cell Banks: MCB, and each WCB
Cell Types: PCC, DCL, SCL, CCL

B.11.3.2 *Mollicutes*
Mollicutes are distinguished by an absence of a cell wall and includes mycoplasmas, acholeplasmas, spiroplasmas, and others. They are parasites of various animals and plants, living on or in the host's cells. They are also a frequent contaminant of cell cultures. In addition to their potential pathogenicity, mycoplasmas compete for nutrients, induce chromosomal abnormalities, interrupt metabolism and inhibit cell fusion of host cells. M. pneumoniae is pathogenic for humans, although there are no reported cases of human infections with this organism arising from exposure to cell cultures or cell-derived products. In any case, cell banks should be demonstrated to be free of such contamination in order to be suitable for the production of biologicals.

B.11.3.2.1 Mycoplasma and acholesplasma
Tests for mycoplasmas are performed as specified in Part A, sections 5.2 and 5.3 of the Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) [115], or by a method approved by the NRA/NCL. Both the culture method and the indicator cell-culture method should be used. NAAT alone, in combination with cell culture, or with an appropriate detection method, might be used as an alternative to one or both of the other methods after suitable validation and discussion with the NRA/NCL. In this case, a comparability study should be carried out. The comparability study should include a comparison of the respective detection limits of the alternative method and official methods. Specificity (mycoplasma panel detected, potential false-positive results due to cross-reaction to other bacteria) should also be considered. More details are available in the European Pharmacopoeia chapter 2.6.7 [116]. One or more containers of the MCB and each WCB are used for the test.

B.11.3.2.1.1 Applicability
Cell Banks: MCB and each WCB
Cell Types: PCC, DCL, SCL, CCL

B.11.3.2.2 Spiroplasma and others
Other mollicutes such as Spiroplasma may be introduced in cell substrates through contamination of raw materials (Peptons) or due to the nature and permissivity of the cells (*e.g.*, insect cells). According to the cell bank manufacturing process, *e.g.,* if the raw material exposure is at the level of MCB or before, it may be appropriate to test the MCB only. If further exposure is possible, then testing of WCB might be necessary too.

Detection of such mollicutes may require adapted culture conditions (medium and/or temperature) depending on the strain to be detected. To guarantee a broad detection of such mollicutes, it is

helpful to use NAAT after suitable validation with an appropriate model (*e.g.*, *Spiroplasma citri* or other strain according to the cell origin).

B.11.3.2.2.1 Applicability
Cell Banks: MCB, WCB (recommended for insect cells)
Cell Types: DCL, SCL, CCL (recommended for insect cell substrates and when raw materials of plant origin are used during the cell bank preparation or production process).

B.11.3.3 Mycobacteria
The test for mycobacteria is performed as described below or by a method approved by the NRA/NCL.

Inoculate 0.2 mL of the sample in triplicate onto each of 2 suitable solid media (such as Löwenstein-Jensen medium and Middlebrook 7H10 medium). Inoculate 0.5 mL in triplicate into a suitable liquid medium at 37°C for 56 days.

In some countries, the incubation period is 42 days.

An appropriate positive control test should be conducted simultaneously with the sample under evaluation, and the test should be shown to be capable of detecting the growth of small amounts of mycobacteria. In addition, the fertility of the medium in the presence of the preparation to be examined should be established by a spiking inoculation of a suitable strain of a Mycobacterium sp., such as BCG. If at the end of the incubation time no growth of mycobacteria occurs in any of the test media and the positive control and spiked control show appropriate growth, the preparation complies with the test.

Nucleic acid amplification techniques might be used as an alternative to this culture method, provided that such an assay is shown to be comparable to the compendial culture method. An appropriate comparability study should be carried out that includes a comparison of the respective detection limits of the alternative method and culture method. Specificity (mycobacteria panel detected, potential false-positive results due to cross-reaction to other bacteria) should also be considered. An *in vivo* method, as described in the test in guinea pigs, also may be used (B.11.2.1.3).

B.11.3.3.1 Applicability
Cell Banks: MCB or WCB
Cell Types: PCC, DCL, SCL, CCL

B.11.4 *Transmissible Spongiform Encephalopathies*
TSEs are a group of slowly developing fatal neurological diseases affecting the brain of animals and humans. The accepted view at present is that they are caused by non-conventional infectious agents known as prions (PrP^{tse}), which are made up of a normal host protein (PrP) in an abnormal conformation. TSEs include BSE of cattle, scrapie of sheep, CJD and its variant form (vCJD), GSS and FFI in humans, CWD in elk and deer, and transmissible mink encephalopathy (TME) [117,118]. Normal PrP (PrPc) protein may be expressed on cell surfaces, but *in vivo* this protein can mis-fold and become the abnormal disease-causing type PrP^{tse}, which is able to catalyse the conversion of

PrPc protein into the abnormal conformation. PrP^{tse} is relatively resistant to common proteolytic enzymes, such as proteinase K, compared with PrPc.

Bovine spongiform encephalopathy (BSE) was first described in the United Kingdom in 1984, and the numbers of clinical cases there reached a peak in 1992-93. Other countries were also affected. Currently, the number of new infections detected annually is low [119]. However, BSE remains a particular concern because cases still occur, albeit at a low rate, and there is a legacy arising from the prolonged incubation period of the disease, the life expectancy of cell banks, and the complexity of the processes by which they are established.

BSE in cattle has been transmitted to humans in the form of vCJD. Approximately 200 individuals have been affected either directly through exposure to BSE-infected material or through secondary transmission by non-leukocyte-depleted red blood cells. Classical CJD has also been transmitted by medical procedures, including administration of cadaveric growth hormone [120], corneal transplant, and the use of dura mater and vCJD may be transmissible by the same routes. vCJD has also been transmitted by human blood products. Although there is no evidence of vCJD transmission by plasma products, public health precautions have been implemented to minimize the possible risk of onward vCJD transmission by plasma products [121]. Cattle-derived proteins, including serum, have often been used in the growth of cells in culture and the production of biological products, including vaccines and recombinant products. Therefore, it is important to ensure that any ruminant-derived material used in biopharmaceutical manufacture is free of the agents that cause TSE. Moreover, as there is a possible but unquantifiable risk that cells can become infected by the agents of TSE, it is important that possibly contaminated ruminant material has been excluded from the start of the development of any cell line used. When there is insufficient traceability in the legacy of a cell line, a risk assessment should be undertaken to aid in decision-making about the suitability of the cell line for the intended use. There is currently no practical validated test that can be used for biological products or cell-line testing for the agents of TSE other than infection of susceptible species, where the experiments are very difficult because of the length of the incubation. More usable tests such as protein misfolding cyclic amplification (PMCA), which is analogous to PCR for nucleic acids, and epitope protection assays [121] are under investigation, but their performance characteristics when used to detect TSE agents in biological products or cell lines have not been defined. Strategies for minimising risk have therefore focused so far on sourcing materials from countries believed to be at very low risk of infection and on substituting animal-derived materials with non-animal-derived materials.

B.11.4.1 *Infectivity categories of tissues*
Ruminant tissues are categorized by the World Health Organization and other scientific bodies such as the European Medicines Agency into three Categories (Category A - High infectivity, Category B - Lower infectivity, and Category C - no detectable infectivity) [55, 123]. Category A includes brain and Category C includes materials such as testes and bile. Assays of improved sensitivity have shown infectivity in tissues, such as muscle, previously thought to be free of infectious agents, and the implication is that while certain tissues contain large amounts of infectivity, many other tissues may contain low levels that are difficult to detect [55,123, 124, 125].

B.11.4.2 *Control measures, sourcing and traceability*

Where effective alternatives to ruminant-derived material are available, they should be used in cell culture/manufacturing procedures. Examples include: 1) cell culture medium free of animal material, 2) polysorbate and magnesium stearate of plant origin, 3) enzymes, such as rennet, of microbial origin (used in lactose production), and 4) recombinant insulin and synthetic amino acids. It should be noted, however, that recombinant materials may themselves be exposed to animal materials, so this potential should be considered when choosing recombinant materials as alternatives. However, it is not always possible to use ingredients free of animal materials, and raw materials of non-ruminant origin. For example, foetal bovine serum might have to be used in the development of cell lines or for fermentation. Under these circumstances, the raw materials should be sourced from countries classified by the World Organisation for Animal Health (OIE) as negligible BSE risk or GBR I, as classified by the European Food Safety Authority (EFSA). Raw materials of Category C may be sourced from countries that are classified as controlled risk provided there is assurance that no cross-contamination with materials of Category A or B could have occurred during collection and processing, with the caveat that while they have undetectable levels of infectivity, it could conceivably be present. Manufacturers should maintain records, so that the finished product from any batch is traceable to the origin of any ruminant ingredient used in its manufacture that might pose a risk of exposure to a TSE agent, and each ruminant ingredient is traceable to the finished product. This includes ingredients used to develop and produce the MCB and WCB, and as far as is possible, to the derivation of the cell line itself. This traceability in both directions is important for appropriate regulatory action if new scientific research indicates that there is a TSE infectivity risk in the materials used, or if there is an association of the products' use with vCJD. Category A and B ruminant materials, originating from BSE-enzootic countries, should not be used in biologicals production under any circumstances. Because new BSE cases continue to occur despite feed bans, because suitable tests for TSE agents in raw-materials are not available, and because developments in scientific research indicate the presence of pathological prions in Category C materials, the best approach to TSE safety is not to use animal-derived protein. The next best approach is to source raw materials from countries classified as free of BSE, bearing in mind that cases may be detected in future.

B.11.4.3 *Tests*
Currently, there are no suitable screening tests available for TSE agents in raw materials of human/ruminant origin similar to serological or PCR assays for the screening for viral agents. Newer tests are being developed to screen for the presence of TSE agents in blood (such as PMCA, epitope-protection assay, *etc*.). Such tests, once validated, could eventually become suitable for the screening of raw materials and cell banks.

Approximately 15% of human TSEs are associated with inherited mutations in the PrP gene. These familial, but transmissible, TSEs are associated with around 30 known pathogenic mutations or with insertions and deletions in the octapeptide-repeat region of PrP [126]. The PrP gene of new human cell substrates should be sequenced to exclude the presence of these genetic changes.

B.12 Summary of tests for the evaluation and characterization of animal-cell substrates

The following sections provide an overview of tests that are recommended for the evaluation and characterization of animal-cell substrates proposed for use in the production of biological products. Not all of the tests are appropriate for all animal-cell substrates, but each of them should be

considered and a determination made as to its applicability for a given cell substrate in the context of its use to manufacture a specific product. In addition, the point(s) at which a test should be applied needs to be rationalized. The overall testing strategy should provide assurance that risks have been mitigated to reasonable levels for the product and its intended use. The testing strategy should be agreed with the NRA/NCL.

B.12.1 *Cell Seed*
The cell seed generally is derived from a cell or tissue source of interest because of its potential utility in the development of a biological product. In some cases, the cell may be expected to serve as the substrate for the production of multiple products. Reference Cell Banks (see also A.5.3) would be considered cell seeds. The cell seed is usually of limited quantity so that extensive testing is not feasible. Some of the seed is therefore used to produce a supply of cells in a quantity that allows more extensive testing as well as providing a long-term source of cells for use in manufacturing. This secondary cell source is usually termed the master cell bank (MCB). However, the cell seed also may be used to produce additional low-passage material that can be banked (pre-MCB) and used to generate MCBs that then are characterized as described in this document.

Tests on the cell seed can be done at any point before the establishment of the MCB. Usually, those tests are limited to information essential to make the decision to commit resources to the preparation of a MCB. Such tests typically include: viability, morphology, identity (*e.g.*, karyotype, isoenzymes), and sterility (*e.g.*, bacterial, fungal, mycoplasma). These data serve as important background information, but they cannot substitute for the full characterization of the MCB.

B.12.2 *Master Cell Bank (MCB) and Working Cell Bank (WCB)*
The MCB generally will be developed to generate a sufficient quantity of cells to supply enough vials of cells to produce many WCBs over an extended period (usually years). MCBs typically contain at least 200 vials and often 1000 or more. There also should be a sufficient number of vials in the WCB to provide material for the characterization of the cell line.

Some tests on the WCB are conducted on cells recovered directly from the bank itself; but other tests will be conducted on cells that have been propagated to a passage at or beyond the level that will be used for production. In addition, some tests may be appropriate to use as in-process control (IPC) tests. In such cases, they should be identified and described in the recommendations applicable to specific products.

Authors

The scientific basis for the revision of the Requirements published in WHO TRS 878 was developed at the meetings of the WHO Study Group on cell substrates in 2006 and 2007 attended by the following people:

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Since then, several draft recommendations were prepared by the drafting group Dr John Petricciani, Consultant, Palm Springs, USA; Dr Rebecca Sheets, NIH/NIAID, Bethesda, USA; Dr Glyn Stacey, NIBSC, Potters Bar, UK; and Dr Ivana Knezevic, Quality and Safety and Standards, IVB/ FCH/ WHO, and were reviewed by the WHO Study Group on cell substrates in 2008.

Following the meeting of the Study Group in April 2009, Bethesda, USA, draft recommendations were revised taking into account information on the current manufacturing and regulatory practice provided at the meeting attended by the following participants:

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Based on the comments received from a broad range of regulators, manufacturers of vaccines and other biologicals and other relevant experts in 2009, draft recommendations were updated by the drafting group and posted on the WHO biologicals web site for public consultation from 4 to 31 May 2010.

The WHO/BS/10.2132 document was prepared by the drafting group at its meeting held on 1-3 June 2010 at WHO, Geneva, taking into account comments received from the reviewers as well as from the following meeting participants:

Dr Christoph Conrad, Quality and Safety and Standards, IVB/ FCH/ WHO; Dr HyeNa Kang, Quality and Safety and Standards, IVB/ FCH/ WHO; Dr Ivana Knezevic, Quality and Safety and Standards, IVB/ FCH/ WHO; Dr John Petricciani, Consultant, Palm Springs, USA; Dr Rebecca Sheets, NIH/NIAID, Bethesda, USA; Dr Philip Minor, NIBSC, Potters Bar, UK; Dr David Onions, Invitrogen Corporation, Carlsbad, USA; Dr Keith Peden, CBER, Bethesda, USA; Dr JinHo Shin, Quality and Safety and Standards, IVB/ FCH/ WHO; and Dr Glyn Stacey, NIBSC, Potters Bar, UK.

Further changes were made to WHO/BS/10.2132 by the Expert Committee on Biological Standardization, resulting in the present document.

Acknowledgements

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Appendix 1

Tests for Bovine Viruses in Serum Used to Produce Cell Banks

Serum should be tested for adventitious agents, such as bacteria, fungi, mycoplasmas, and viruses, prior to use in the production of MCBs and WCBs. In addition, consideration should be given to risk-mitigation strategies, such as inactivation by heat or irradiation, to ensure that adventitious agents that were not detected in the manufacture and quality control of the serum would be inactivated to a degree acceptable to the NRA/NCL. If irradiation or other inactivation (*e.g.*, heat sterilization) methods are used in the manufacture of the serum, the tests for adventitious agents should be performed prior to inactivation to enhance the opportunity for detecting the contamination. If evidence of viral contamination is found in any of the tests, the serum is acceptable only if the virus is identified and shown to be present in an amount that has been shown in a validation study to be effectively inactivated. For serum that is not to be subjected to a virus inactivation / removal procedure, if evidence of viral contamination is found in any tests, generally, the serum would not be acceptable. If the manufacturer chooses to use serum that has not been inactivated, thorough testing of the serum for adventitious agents using current best practices should be undertaken. If any viruses are identified in the serum, the cell banks made in this manner should be shown to be free of the identified virus(es).

> If irradiation is used, it is important to ensure that a reproducible dose is delivered to all batches and the component units of each batch. The irradiation dose must be low enough so that the biological properties of the reagents are retained while being high enough to reduce virological risk. Therefore, irradiation delivered at such a dose may not be a sterilizing dose.

Factors to be considered in testing serum
Bovine serum can be contaminated by a wide range of viruses. Manufacturers typically produce very large pools of serum involving samples from up to a thousand animals. Consequently, many serum batches contain detectable, genomic sequences of viruses like bovine viral diarrhoea virus (BVDV) and bovine polyomavirus (BPyV) [1] although, this might represent contamination of the pool by one viraemic animal.

Other viruses are sporadic contaminants and may be regionally restricted like Cache Valley virus, bluetongue virus and epizootic haemorrhagic disease virus. In some cases, contamination has only been reported on a few occasions, as in the case of calicivirus 2117 [2].

Application of new methods, like massively parallel sequencing, has revealed new viruses, like parvoviruses, some of which are frequent and high level contaminants of serum [3, 4]. The importance and potential pathogenicity of these viruses requires further investigation.

An important factor in infectivity assays is that virions might be neutralised by antibody in the serum pool. It is advisable to set limits for the level of BVDV neutralising antibody in serum pools as this may mask the presence of potentially infectious virus.

There should be an awareness of the statistical limits of screening assays to detect viruses in large serum pools. For example, in an infection of a fermenter by Cache Valley virus it was estimated that less than 10 viruses per litre were present in the serum and, at this low level, the virus escaped detection by conventional screening methods [5].

General screening assay for infectious viruses
A general screening assay typically involves culturing indicator cells over 21 days with test serum at 15% in the medium. At least 2 sub-passages of the cells should be undertaken, usually at days 7 and 14. Detection of virus infection involves regular examination for the development of a cytopathic effect, haemadsorption assays, and immunofluorescence (or other appropriate immunological detection method) for specific viruses. Immunofluorescence (IF) is particularly important for the detection of BVDV as many isolates arenon-cytopathic. At the end of the assay, cytological staining (*e.g.,* with Giemsa stain) is used to reveal viral inclusions and other cytopathic effects not detected during the direct observation of the live cells.

Indicator cells should be selected that are permissive for a wide range of bovine viruses. Madin-Darby Bovine kidney Cells (MDBK) or, Bovine Turbinate cells, are often used and it is also of value to include additional cells like Vero cells.

The assay should be capable of detecting: bovine viral diarrhea virus (BVDV), bovine parainfluenza type 3 Virus (BPIV3), bovine parvovirus 1 (BPV), rabies virus (RV), reovirus 3 (REO3), infectious bovine rhinotracheitis Virus (IBR), bovine respiratory syncytial virus (BRSV), blue tongue virus (BTV), bovine adenovirus 5 (BAV5), and vesicular stomatitis virus (VSV). Separate, positive control bottles, of indicator cells should be infected with each of the viruses above, except rabies virus. In the case of rabies virus, slides of fixed, infected, cells should be used as a positive control for the immunofluorescent assay. Uninfected negative control cells should also be established.

A typical assay involves the use of 75cm^2 bottles containing the indicator cells and a total of ~250 ml of test serum, allowing for serum used during re-feeding the cells after passage.

Procedure
    Assay set up
Initially, negative control bottles and test article bottles are established. The test article bottles are inoculated and maintained with the test serum at 15% in the medium. The negative control bottles are mock infected with serum known to be free of detectable viruses. Passage of the cells is usually required on day 7.

Cells for the positive control are prepared from the negative control bottles on day 13 or 14 or when the cells are ≥70% confluent. The cells are subcultured into 25 cm^2 flasks (for IF) and 6-well plates [for haemadsorption (HAd) and cytological staining (CS)].

The following day, the remaining negative control and test article cells are subcultured to 75 cm² flasks for IF and to 6-well plates for haemadsorption (HAd) and cytological staining (CS).

Infection with positive controls

Coincident with the final subculture of test article and negative control cultures, flasks of bovine turbinate cells (BT) are inoculated with the IF positive control viruses BVDV, BAV5, BPV, BTV, BRSV, IBR, and BPIV-3. Plates of BT cells are inoculated with BPIV-3, the positive control for haemadsorption, and with cytopathic BVDV, the positive control for cytological staining. Likewise, Vero flasks are inoculated with REO3, the IF positive control, and plates are inoculated with BPIV-3, the haemadsorption and cytological staining positive control. All IF positive control viruses should be inoculated at 100-300 $TCID_{50}$.

Analysis

After a minimum of 21 days post-inoculation, and at least 7 days after the last subculture, (but earlier if CPE is observed), negative control and test article cultures are assayed for HAd and fixed for IF and CS.

Cells from the positive control flasks are transferred to multi-well slides and fixed for IF when CPE involving ≥10% of the monolayer is observed and stored at ≤ -60°C. Cells in the positive control 6-well plates are assayed for HAd and CS 7 days after inoculation or when CPE is apparent. HAd involves testing at least one 6 well plate with chicken and guinea pig erythrocytes at 2-8°C and at 20-25°C.

NAAT assays for viruses

Nucleic acid amplification technologies like PCR have utility in screening serum for sporadic contaminants and for those viruses where infectivity assays are not available. Nucleic acid extractions should be from a significant volume (*e.g.*, 25 to 50ml) and the statistical limits for detection in the serum pool should be calculated. The presence of genomic sequences does not necessarily indicate the presence of infectious virus, although encapsidated genomes can be identified by treatment of the sample with nucleases prior to amplification. Some virus inactivating or removal processes can be evaluated using NAAT, by determining if intact, full-length, amplifiable, genomes are present before and after treatment.

Specific in vitro infectivity assays

Bovine polyomavirus is an important contaminant because it is able to infect primate cells [6], belongs toan oncogenic family of viruses and expresses a T-antigen that can transform primary cells into tumour cells [7]. Furthermore, there is serological evidence of zoonotic infection [8]. Infectious virus is not easily detected in conventional assays, a long period of culture and a NAAT endpoint or, immunological endpoint like immunofluorescence, should be used.

Other viruses are not easily detected in standard infectivity assays. For instance calicivirus 2117 appears to be more permissive for replication in CHO cells than standard bovine cell lines used in *in vitro* infectivity assays. Similarly, while general screening methods will detect certain bovine adenoviruses, herpesviruses and parvoviruses, not all bovine viruses belonging to these families are detected.

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Appendix 2

Tumourigenicity Protocol Using Athymic Nude Mice to Assess Mammalian Cells

During the characterization of a MCB (or WCB), the cells should be examined for tumourigenicity in a test approved by the national regulatory authority (NRA) or the national control authority (NCA).

The following model protocol is provided to assist manufacturers and NRAs/NCLs in standardizing the tumourigenicity testing procedure so that the interpretation and comparability of data among various laboratories and regulatory authorities can be facilitated.

1. **Test Animals**
   The test article cell line and the control cells are each injected into separate groups of 10 athymic mice (Nu/Nu genotype) 4-7 weeks old.

   > Because male athymic mice often display aggressive traits against each other when housed together, loss of some mice during the observation period occurs often. Therefore, the use of only female mice should be considered.

2. **Test article cells**
   Cells from the MCB or MWCB that have been propagated to at least 3 population doublings beyond the limit for production are examined for tumourigenicity.

3. **Control cells**
   a. **Positive control cells**
      HeLa cells from the WHO cell bank are recommended as the positive control reference preparation. Portions of that bank are stored at the ATCC (USA) and NIBSC (UK).

      > Other cells may be acceptable to the NRA/NCL if HeLa cells from the WHO cell bank are not available.

   b. **Negative control cells**
      Negative control cells are not required. Databases (both published data and the unpublished records/data of the animal production facility that supplied the test animals) of rates of spontaneous neoplastic diseases in nude mice may be taken into account during the assessment of the results of a tumourigenicity test.

      If negative control cells are included, clear justification must be provided. In particular, the number of animals used must provide meaningful data, and the rationale for generating additional data must be persuasive to the NRA/NCL in the context of animal welfare regulations.

4. **Validity**
   In a valid test, progressively growing tumors should be produced in at least 9 of 10 animals injected with the positive control reference cells. At least 90% of the inoculated control and cells and test cells must be viable for the test to be valid.

5. **Inoculum**

   The inoculum for each animal is 10^7 viable cells (except as described in 11.b), suspended in a volume of 0.1 mL of PBS.

   > Cell culture media without serum has been used in the past to suspend the cell inoculum. However, many current media are serum-free and contain one or more growth factors that may affect the result of the tumourigenicity assay. Therefore careful consideration should be given to the choice of the liquid into which the cells are suspended.

6. **Injection route and site**

   The injection of cells may be by either the intramuscular (IM) or subcutaneous (SC) route. If the IM route is selected, the cells should be injected into the thigh of one leg. If the SC route is selected, the cells should be injected into the supracavicular region of the trunk.

   > Based on published studies, the intracerebral route may be more appropriate in some cases. For example, lymphoblastoid cells have been shown to proliferate best when inoculated by the intracerebral route.

7. **Observation period**

   All animals are examined weekly by observation and palpation for a <u>minimum</u> of 16 weeks (*i.e.*, 4 months) for evidence of nodule formation at the site of injection when the route of inoculation is IM or SC. Examinations need not be more frequent than twice a week for the first 3 to 6 weeks, and once a week thereafter.

   > In some countries, the observation period is 4 to 7 months, depending on the level of concern associated with the specific cell substrate in the context of the product being developed. Whether a longer observation period is needed should be agreed with the NRA/NCL. Also see 8 below.

8. **Assessment of the inoculation site over time**

   If a nodule appears, it is measured in two perpendicular dimensions, the measurements being recorded weekly to determine whether the nodule grows progressively, remains stable, or decreases in size over time. Animals bearing nodules that appear to be regressing should not be sacrificed until the end of the observation period. Cell lines that produce nodules that fail to grow progressively are not considered to be tumourigenic.

   > If a nodule that fails to grow progressively, but persists during the observation period and it retains the histopathological morphology of a neoplasm, this should be discussed with the NRA/NCL to determine whether additional testing will be required. Such testing could include extending the observation period or switching to a newborn nude mouse, ATS-treated newborn rat, or other *in vivo* model to assess the tumourigenicity of the cell substrate.

   > If the cells that are injected fail to form tumors or to persist during the 4-month observation period, it might be necessary to extend the observation period or switch to a newborn nude mouse, ATS-treated newborn rat, or other *in vivo* model to assess the tumourigenicity of the cell substrate. This will depend on the level of concern associated with the specific cell substrate in the context of the product being developed. Whether such additional testing is needed should be agreed with the NRA/NCL.

9. **Final Assessment of the inoculation site and other sites**

At the end of the observation period, or at an earlier time if required due to the death of an animal or other justifiable circumstances, all animals, including the reference group(s), are sacrificed and examined for gross and microscopic evidence of the proliferation of inoculated cells at the site of injection and in other sites such as the heart, lungs, liver, spleen, kidneys, brain, and regional lymph nodes since some CCLs may give rise to tumors at distant sites without evidence of tumor at the injection site. The tissues are fixed in 10% formol saline and sections are stained with hematoxylin and eosin for histological examination to determine if there is evidence of tumor formation and metastases by the inoculated cells.

10. **Assessment of metastases (if any)**
Any metastatic lesions are examined further to establish their relationship to the primary tumor. If what appears to be a metastasis to a distant site differs histopathologically from the primary tumor, consideration should be given to the possibility that the tumor either developed spontaneously, or that it was induced by one or more of the components of the cell substrate such as an oncogenic virus.

> If the histopathology or genotype of any tumors that develop are inconsistent with the inoculated cell type, or are of a histopathological type that has not been recognized as occurring spontaneously in the test species, additional tests should be undertaken to determine whether such tumors are actually spontaneous or are induced by elements within the cell substrate itself such as oncogenic viruses or oncogenic DNA sequences. In such cases, appropriate follow-up studies should be discussed and agreed with the NRA/NCL.

11. Interpretation of results
    a. The test in nude mice is considered positive if at least 2 of 10 animals inoculated with the test article cells develop tumors that meet all of the following two criteria:

        i. Tumors appear at the site of inoculation or at a metastatic site
        ii. Histologic or genotypic examination reveals that the nature of the cells constituting the tumors is consistent with that of the inoculated cells

> In the past, chromosomal markers have been useful to demonstrate that the tumor cells are of the same species as that from which the inoculated cells were derived. However, the use of cytogenetics for this purpose has largely been replaced by genetic and antigenic markers.

    b. If only one of 10 animals develops a tumor that meets the three criteria in 11.a, the cell line should be considered possibly tumourigenic and examined further. Such testing could include one or more of the following: a) repeating the test in an additional 10 nude mice, b) extending the observation period, c) increasing the size of the inoculum, or d) switching to the newborn nude mouse model, the ATS-treated newborn rat model, or other *in vivo* model. In such cases, appropriate follow-up investigations should be discussed and agreed with the NRA/NCL. For example, it may be appropriate to determine if the tumor is of nude mouse origin and whether there are any viral or inoculated cell DNA sequences present.

> Assessment of dose-response may provide additional information on the characteristics of the CCL. If such studies are undertaken, the design should be based on the *in vivo* titration of the inoculum in groups of 10 animals per dose level. For example, if 10 of 10 animals develop tumors with an

inoculum of 10^7 cells, the titration could be done with 10^5, 10^3, and 10^1 cells in groups of 10 animals each.

Appendix 3

Oncogenicity Protocol for the Evaluation of Cellular DNA and Cell Lysates

When appropriate, and particularly for vaccines, cell DNA and cell lysates from tumorigenic cell substrates should be examined for oncogenicity in a test approved by the NRA/NCL.

In some countries, the following testing strategy is used.

1. **Type of test animals**
   Newborn (*i.e.*, <3 days old) nude mice, newborn hamsters, and newborn rats have been used to assess the oncogenic potential of cell lines. At this stage, it is not possible to draw definitive conclusions on the relative sensitivity of the three animal assays for oncogenicity, and testing is recommended in each of them. When data on the ability of these models to detect oncogenic activity are obtained, this recommendation may change.

2. **Point in the life history of the cells at which they should be tested**
   Cells from the MCB or WCB, propagated to the proposed *in vitro* cell age for production or beyond, should be examined for oncogenicity. Three extra population doublings ensure that the results of the oncogenicity test can be used in the assessment of overall safety of the product even under the assumption of a "worst case" situation and therefore provide a safety buffer.

3. **Use of controls**
   The purpose of the positive control is to assure that an individual test is valid by demonstrating that the animal model has the capacity to develop tumors from inoculated cell components (*i.e.*, a negative result is unlikely to be due to a problem with the *in vivo* test). While an appropriate positive control for cell-lysate oncogenicity assay is not clear, the recent description of an oncogene-expression plasmid for activated H-*ras* and c-*myc* has been shown to induce tumours in animals [1]. As the test with cell lysates is designed primarily to detect oncogenic viruses rather than oncogenes, the use of DNA as a positive control might not be suitable, both because of the nature of the assay and because DNA might not be stable in a cellular lysate.

   Whether a negative control arm, such as PBS, is included should be discussed with the NRA/NCL. An advantage of including a negative-control arm is that the frequency of tumour induction with lysates is expected to be low and may approximate the spontaneous tumour frequency in the indicator rodent, providing an important comparison to the test article arm.

4. **Number of test animals**
   While the number of animals in a tumourigenicity test can be 10 per group, the number in an oncogenicity test should be larger due to the lower expected tumour incidence. The number per group should be discussed with the NRA/NCL.

5. **Inoculation of test material**

a. **Cell lysate.** A lysate of the cells should be prepared by a method that avoids virus disruption while allowing maximum virus release and ensuring all cells are lysed (*e.g*., three freeze/thaw cycles followed by low-speed centrifugation). Each animal should be inoculated subcutaneously above the scapula with a lysate obtained from 10^7 cells. Before inoculation, it should be determined that no viable cells are present, as development of tumours from cells would invalidate the test. The cell lysate is suspended in PBS and inoculated in a volume of 50-100 µL into newborn nude mice, newborn hamsters, and newborn rats [2]. If there is no evidence of a progressively growing tumour at the site of inoculation or at distant sites at the end of the observation period, the cell line may be considered not to possess oncogenic activity. If tumours are observed in this assay, the species of origin will need to be confirmed. The species of tumours that arise in a tumourigenicity assay will be that of the cell substrate, while the species of tumours that arise in an oncogenicity assay is that of the host (*e.g*., rodent). If the cells were not lysed properly, it may be that the tumours that arose were from the species of the cell substrate.

b. **DNA.** Total cellular DNA isolated from the cell substrate should be inoculated subcutaneously above the scapulae in PBS into newborn nude mice, newborn hamsters, and newborn rats. The amount of DNA inoculated should be ≥100 µg in 50 – 100 µL. Because of the concentrations necessary to achieve ≥100 µg of DNA, it might be necessary to shear the DNA; this can be done by sonication or by several passes in a needle and syringe. A positive-control plasmid with the test article DNA should be inoculated into a few mice to confirm that the cellular DNA is not inhibitory and that the animals are susceptible to tumour induction by DNA.

6. **Observation period**
   Animals are examined weekly by observation and palpation for evidence of nodule formation at the site of injection. The observation period should be at least 4 months.

7. **Assessment of the inoculation site over time (progressive or regressive growth)**
   If one or more nodules appear, they are measured in two perpendicular dimensions, the measurements being recorded weekly to determine whether the nodule grows progressively, remains stable, or decreases in size over time. Animals bearing nodules that are progressing should be sacrificed when the nodule reaches a size of approximately 2 cm in diameter, unless a lower limit has been established by authorities for the humane treatment of animals.

8. **Final Assessment of the inoculation site**
   At the end of the observation period, all animals, including the reference group(s), are sacrificed and examined for gross and microscopic evidence of tumour formation at the site of injection and in other sites. Any tumour that is identified is divided into three equal parts: a) fixed in formalin for histopathology; b) used to establish a cell line, when possible; and c) frozen for subsequent molecular analysis.

9. **Evaluation of animals for metastases**
   Animals are examined for microscopic evidence of metastatic lesions in sites such as the liver, heart, lungs, spleen, and regional lymph nodes.

10. **Assessment of metastases (if any)**

    All tumours are examined to establish their relationship to the primary tumour at the site of inoculation. If what appears to be a metastatic tumour differs histopathologically from the primary tumour, it is necessary to consider the possibility that this tumour developed spontaneously. This may require further testing of the tumour itself. In such cases, appropriate follow-up studies should be discussed and agreed with the NRA/NCL.

11. **Interpretation of results**

    If tumours arise in the cell lysate or DNA assay, then these could be induced by an oncogenic virus or oncogenic DNA. Because of the implications for the use of a cell substrate that contains an oncogenic agent or an oncogenic activity for a biological, the NRA/NCL should be consulted to consider additional experiments to identify the oncogenic agent/activity and to determine the suitability of the use of the CCL.

12. **Applicability**

    Cell Banks: MCB or WCB taken beyond EOPC level/ECB
    Cell Types: CCL, SCL (recommended when tumourigenic cells are used in vaccine production)

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List of Abbreviations

ALS	anti-lymphocyte serum
ATG	anti-thymocyte globulin
ATS	anti-thymocyte serum
BHK-21	baby hamster kidney
bp	base pairs
BPL	beta-propiolactone
BSE	bovine spongiform encephalopathy
BVDV	bovine viral diarrhoea virus
CCLs	continuous cell lines
CEF	chick embryo fibroblasts
CHO	Chinese hamster ovary
CJD	Creutzfeldt–Jakob disease
CPE	cytopathic effect
CTL	cytotoxic T-lymphocyte
CWD	chronic wasting disease
DCLs	diploid cell lines
EBV	Epstein-Barr virus
ECB	Extended Cell Bank
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EOP	End of production
EOPC	End of production cells
EPIC-PCR	Exon Primed Intron Crossing-PCR
FFI	Fatal Familial Insomnia
GMPs	good manufacturing practices
GSS	Gerstmann-Sträussler-Scheinker syndrome
HA	haemagglutinating
HCP	hamster cheek pouch
HDCs	human diploid cells
HEK	human embryonic kidney
HERVs	human endogenous retroviruses
HLA	human leukocyte antigen
ICH	International Conference on Harmonization
IFA	immunofluorescence assays
IFN	interferon
IM	intramuscular
IPC	in process control
LCMV	lymphocytic choriomeningitis virus
MAbs	monoclonal antibodies
MCB	master cell bank
miRNA	microRNA
MOI	multiplicity of infection
NAAT	nucleic acid amplification techniques
NCLs	National Control Laboratories

NRAs	National Regulatory Authorities
OIE	World Organisation for Animal Health
PCCs	primary cell cultures
PCR	polymerase chain reaction
PDL	population doubling level
PERT	product enhanced reverse transcriptase
PMCA	protein misfolding cyclic amplification
RCBs	Reference Cell Banks
rcDNA	residual cellular DNA
rDNA	recombinant DNA
RFLP	restriction fragment length polymorphism
RT	reverse transcriptase
s.c.	subcutaneous
SCs	stem cells
SCID	severe combined immunodeficiency
SCLs	stem cell lines
SG	Study Group
SIVB	Society for in Vitro Biology
SOP	standard operating procedure
SPF	specific-pathogen-free
STR	short tandem repeats
SV40	simian virus 40
TEM	transmission electron microscopy
TME	transmissible mink encephalopathy
TPD_{50}	tumor-producing dose at the 50% endpoint
TSEs	transmissible spongiform encephalopathies
TTV	transfusion-transmitted virus
VNTR	variable number of tandem repeats
WCB	working cell bank