

Annex 3

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

Replacement of Annex 1 of WHO Technical Report Series, No. 878

Abbreviations	81
1. Introduction	84
2. Historical overview	84
3. Scope	88
4. Definitions	89
5. General considerations	95
5.1 Types of animal cell substrates	95
5.2 Potential risks and risk mitigation associated with biologicals produced in animal cell cultures	100
Part A. General recommendations applicable to all types of cell culture production	107
A.1 Good manufacturing practices	107
A.2 Principles of good cell culture practice	107
A.3 Selection of source materials	112
A.4 Certification of cell banks by the manufacturer	118
A.5 Cryopreservation and cell banking	120
Part B. Recommendations for the characterization of cell banks of animal cell substrates	123
B.1 General considerations	123
B.2 Identity	126
B.3 Stability	128
B.4 Sterility	130
B.5 Viability	130
B.6 Growth characteristics	130
B.7 Homogeneity	131
B.8 Tumorigenicity	131
B.9 Oncogenicity	139
B.10 Cytogenetics	141
B.11 Microbial agents	142
B.12 Summary of tests for the evaluation and characterization of animal cell substrates	165

Authors	166
Acknowledgements	168
References	168
Appendix 1	
Tests for bovine viruses in serum used to produce cell banks	176
Appendix 2	
Tumorigenicity protocol using athymic nude mice to assess mammalian cells	180
Appendix 3	
Oncogenicity protocol for the evaluation of cellular DNA and cell lysates	184

Recommendations published by 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 an 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 the 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 these details.

Abbreviations

ALS	antilymphocyte serum
ATCC	American Type Culture Collection
ATG	antithymocyte globulin
ATS	antithymocyte serum
BAV5	bovine adenovirus 5
BCG	bacille Calmette–Guérin vaccine
bp	base pairs
BPIV3	bovine parainfluenza type 3 virus
BPV	bovine parvovirus
BRSV	bovine respiratory syncytial virus
BSE	bovine spongiform encephalopathy
BTV	bluetongue virus
BVDV	bovine viral diarrhoea virus
CCL	continuous cell line
CEF	chick embryo fibroblasts
CHO	Chinese hamster ovary
CJD	Creutzfeldt–Jakob disease
CPE	cytopathic effect
CTL	cytotoxic T-lymphocyte
CWD	chronic wasting disease
DCL	diploid cell line
EBV	Epstein–Barr virus
ECB	extended cell bank
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EOP	end of production
EOPC	end-of-production cell

EPIC-PCR	exon-primed intron crossing PCR
FBS	fetal bovine serum
FDA	Food and Drug Administration (USA)
FFI	fatal familial insomnia
GBR	geographical BSE-risk level
GMP	good manufacturing practices
GSS	Gerstmann–Sträussler–Scheinker syndrome
HCP	hamster cheek pouch
HDC	human diploid cell
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IBR	infectious bovine rhinotracheitis virus
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IFA	immunofluorescence assays
IFN	interferon
LCMV	lymphocytic choriomeningitis virus
MAbs	monoclonal antibodies
MCB	master cell bank
MDCK	Madin–Darby canine kidney
mRNA	messenger RNA
miRNA	microRNA
MPS	massively parallel sequencing
NAT	nucleic acid amplification technique
NCL	national control laboratory
NK	natural killer (cell)
NOD	non-obese diabetic
NRA	national regulatory authority

OIE	World Organisation for Animal Health
PBS	phosphate-buffered saline
PCC	primary cell culture
PCR	polymerase chain reaction
PDL	population doubling level
PERT	product-enhanced reverse transcriptase
PMCA	protein misfolding cyclic amplification
PrP	prion protein
RCB	reference cell bank
rcDNA	residual cellular DNA
rDNA	recombinant DNA
REO3	reovirus 3
RFLP	restriction fragment length polymorphism
RT	reverse transcriptase
SCID	severe combined immunodeficiency
SCL	stem cell line
SPF	specific-pathogen-free
STR	short tandem repeats
SV	simian virus
TCID ₅₀	median tissue culture infective dose
TEM	transmission electron microscopy
TPD ₅₀	tumour-producing dose at the 50% end-point
TSE	transmissible spongiform encephalopathy
vCJD	variant CJD
VNTR	variable number of tandem repeats
VSV	vesicular stomatitis virus
WCB	working cell bank

1. Introduction

Cell substrates are cells used to manufacture biological products. It is well established that both 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 these 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 to the WHO requirements (Requirements for the use of animal cells as in vitro substrates for the production of biologicals) (1) should be prepared. In order to facilitate the resolution of regulatory/scientific issues related to the use of animal (including human) cell cultures as substrates for the production of biological products, WHO initiated this revision of its requirements on cell substrates by establishing a WHO Study Group on Cell Substrates. Animal cells refer to cells derived from organisms classified as within the animal kingdom. This document is the result of the Study Group's work, which included a wide range of consultations with individuals and organizations with expertise in this area. After comments were received from this consultative process, and from invited reviewers, further revision of the draft recommendations was undertaken and presented to the WHO Expert Committee on Biological Standardization in 2010. During the development of this document, guidance on the topic issued by other relevant organizations was considered. An effort was made to make the recommendations compatible with existing guidance 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 it is appropriate to consider in the characterization of animal cells 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 should 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 instance, 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 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 and related to 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 production of polio virus vaccine, as well as for production of other viral vaccines. The rationale for using HDCs was based on the ability to:

- cryogenically preserve the cells at low population doubling levels (PDLs);
- establish and characterize cryopreserved banks of cells that later could be expanded to provide a standardized source of cells for many decades;
- extensively test recovered cells before use in vaccine production;
- demonstrate that the cells were free from detectable adventitious agents and that they were unable to form tumours 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 path to acceptance of HDCs was long and difficult, 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 the 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 the characterization of the cell substrate were introduced during that period (6, 7).

Both the understanding of tumour 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, namely: freedom from detectable adventitious agents; the finite life of HDCs; the diploid nature of HDCs; and the inability of HDCs to form tumours 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 tumorigenicity (8, 9). The main question that was being addressed by the routine use of tumorigenicity 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 tumorigenic cells. It was eventually agreed that tumorigenicity testing was not sufficiently sensitive to detect a low level of tumorigenic cells, and that it was a waste of animals and time to carry out repeated testing of a cell line that had been well characterized and would be used in the context of a cell bank system. Thus, tumorigenicity tests were eventually required only for the characterization of an 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 need in clinical research for more interferon alpha (IFN- α) than could be produced from primary human lymphocytes. In response, human tumour cells (Namalwa) grown *in vitro* were proposed as a cell substrate for the production of IFN- α . 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 IFN- α 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 contributing to those decisions was the fact that IFN, as opposed to live viral vaccines, was not a replicating agent, and IFN- α was being used as a therapeutic product rather than a prophylactic one, thus representing different risk–benefit considerations. In addition, technology had advanced significantly so that IFN- α 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, CCLs 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 WHO Study Group on Cell Substrates to examine cell substrate issues in greater depth.

The Study Group 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 that are pathogenic for humans and to reduce DNA to acceptable levels and/or eliminate its biological activity (12). The Study Group's emphasis on infectious agents as the major risk factor was based largely on experience of virus transmission and disease occurring through contaminated biological products (e.g. hepatitis B virus and HIV in factor VIII). WHO's Requirements for CCLs used for the production of biologicals were published in 1987 (13). On the basis of 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 into the 2000s, a variety of CCLs were explored as cell substrates for biological products in development because, like the cell lines referred to above, they offered significant advantages during production (e.g. rapid growth and high expression). They include the tumorigenic 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 with regard to their evaluation and characterization.

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 in-depth knowledge of the cell type's basic biological characteristics. It is important to recognize that the tumorigenic potential of a CCL is only one of many factors, including the extent to which the manufacturing process reduces or eliminates cellular factors that may be of concern, to be considered. 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, NRAs and 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 compared to the Requirements published in WHO Technical Report Series, No. 878, Annex 1, include the following:

- general manufacturing recommendations applicable to all types of cell culture production have been updated;
- some considerations for evaluating new cell substrates such as insect cells and stem cells have been added;
- definitions have been updated and expanded in number and scope, and are moved to an earlier point in the document;
- 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;
- a new section on risk-reduction strategies during the manufacture of biological products has been added;
- a section on good cell culture practice has been added;
- the section on selection of source materials has been updated, and the detailed methods used to test for bovine viruses in serum have been added in Appendix 1;
- tumorigenicity testing has been updated, and a model protocol for the nude mouse model has been added in Appendix 2;
- oncogenicity testing of tumorigenic cell lysates has been added, and a model protocol is added in Appendix 3;
- recommendations for acceptable levels of residual cellular DNA are product specific and are not specifically addressed;
- 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 the recommendations may also 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. Likewise, risk-based assessments related to product approvals are beyond the scope of this

document. Requirements or recommendations for individual products should be consulted for such assessments.

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, a number of generic issues apply to genetically modified and 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 stem cells 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. Nevertheless, 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 to 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 consistent with good manufacturing practices (GMP) to retest materials that have already been released for further manufacture, so justification would be necessary before such retesting is undertaken. Thus, the scope of this document is intended to cover cell substrates as new cell banks are established. The specific circumstances under which the retesting 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 an NRA/NCL so desire, this text may be adopted as it stands as the basis of national or regional requirements. The parts of each section printed in small type are comments or additional points that may be considered in some cases.

4. Definitions

The definitions given below apply to the terms used in these recommendations. The terms 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

encephalopathy (TSE) agents, and viruses that have been unintentionally introduced into the manufacturing process of a biological product.

The source of these contaminants may be 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 the 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 arbitrary systems of classification applied by regulatory authorities. For the purposes of WHO, including the current 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” (16). 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 the 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 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 subcloning 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 that are frozen and stored 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. Cell seed is also referred to as a pre-MCB or seed stock. It may be made under conditions of GMP or under the 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 (e.g. Vero cells for the generation of “reverse genetics” virus for use in seeding vaccine production) are considered to be “preproduction” cell substrates. Cells used to manufacture the bulk product (e.g. packaging cell lines for gene therapy vectors, Vero cells for vaccine production, or CHO cells for recombinant protein expression) are considered to be “production” cell substrates.

Continuous cell line (CCL): a cell line with an apparently unlimited capacity for population doubling. It is often referred to as “immortal” and was in the past referred to as “established”.

Diploid cell line (DCL): a cell line with 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 may also be found.

This definition is based on experience and the current understanding of the in vitro behaviour 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 may 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 (EOPCs): 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 and 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 (CPE) during and after an appropriate observation period or by haemadsorption and/or haemagglutination at the end of the observation period. Thus they are referred to as “indicator” cells. The cell bank may be analysed on such indicator cells either by co-cultivation or by passage of cell lysates or spent culture supernatants from the cell bank on to the indicator cells.

In vitro cell age: the measure of time between the thaw of the MCB vial(s) to the 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 with the potential to be reactivated.

Master cell bank: a quantity of well-characterized cells of animal or other origin, derived from a cell seed at a specific 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 MCB is prepared from a single homogeneously mixed pool of cells. In some cases, such as genetically engineered cells, the MCB may be prepared from a selected cell clone established under defined conditions.

Frequently, however, the MCB is not clonal. It is considered best practice for the MCB to be 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 subcellular element(s) – to cause normal cells of an animal to form tumours.

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

Parental cells: cells that are manipulated to give rise to a cell substrate. For hybridomas, it is usual also to 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 it may be a more complex activity such as developing a hybridoma or transfected clone. 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: the process of transferring 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, 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 twofold increase in cell number.

Population doubling level: 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:

$$\text{number of population doublings} = \log_{10} (N/N_0) \times 3.33$$

where N = number of cells in the growth vessel at the end of a period of growth and N_0 = number of cells plated in the growth vessel (17). 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 WCB or, in the case of PCCs, 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 are usually 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 from normal or diseased differentiated tissue.

Transmissible spongiform encephalopathy: 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.

Tumorigenicity: 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.

Tumorigenicity is distinct from oncogenicity (see “Oncogenicity”).

WHO reference cell bank (RCB): 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 MCBs that will be extensively characterized by manufacturers and that have a high probability of meeting these recommendations.

Working cell bank: a quantity of well-characterized cells of animal or other origin, derived from the MCB 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 WCB 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

5.1 Types of animal cell substrates

5.1.1 Primary cell cultures (PCCs)

PCCs have played a prominent role in the development of biology, 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 are present and a primary culture can be a complex mixture of cells that may 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 given to establishing highly reproducible procedures for tissue disaggregation, cell processing and culture initiation, as well as reproducible culture conditions and nutrition.

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

If PCCs are necessary for the production of a given biological, the frequency of contaminated cell cultures can be significantly reduced by screening the source animals carefully for the absence of such viruses. Viruses can be detected by molecular tests such as polymerase chain reaction (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 SPF, 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 for ethical reasons (18) and in the interests of progressive improvement in product safety and quality.

5.1.1.1 Advantages of PCCs

- PCCs are comparatively easy to prepare using simple media and bovine serum.
- They generally possess a broad sensitivity to a variety of viruses, some of which are cytopathic.

5.1.1.2 Disadvantages of PCCs

- Contamination by infectious agents is a higher risk than with DCLs and CCLs.
- The quality and viral sensitivity of cultures obtained from different animals are variable.
- Although cell cultures derived from non-human primates have been widely used in the past, it has become increasingly difficult to obtain and justify the use of such animals for this purpose.
- PCCs cannot be tested as extensively as DCLs or CCLs.

5.1.2 Diploid cell lines (DCLs)

The practicality of using human DCLs for the production of viral vaccines was demonstrated in the 1960s. 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:

- they are cells passaged from primary cultures that have become established as cell lines with apparently stable characteristics over numerous PDLs;
- 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;
- they are non-tumorigenic;
- they display diploid cytogenetic characteristics with a low frequency of chromosomal abnormalities of number and structure until they enter senescence.

Substantial experience has been accumulated on the cytogenetics of WI-38 and MRC-5 since the 1960s, and ranges of expected frequencies of chromosomal abnormalities have been published (19, 20). Similar data are available for FRhL-2 (21). More sophisticated cytogenetic techniques (e.g. high-resolution banding, comparative genome hybridization) (22, 23) 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, that they are non-tumorigenic and that they 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.

5.1.2.1 Advantages of DCLs

- DCLs can be well characterized and standardized.
- 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 an MCB and a WCB.
- Unlike the CCLs and SCLs discussed below, DCLs are not tumorigenic and therefore do not raise the potential safety issues associated with CCLs and SCLs.

5.1.2.2 Disadvantages of DCLs

- DCLs are not easy to use in large-scale production, although they have been cultivated using bioreactor technology employing the microcarrier or multilayer method.
- In general, they have more fastidious nutritional requirements than other cell substrates.
- They may be difficult to adapt to serum-free growth.
- DCLs are more difficult to transfect and engineer than CCLs, and DCLs 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).

5.1.3 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 lifespan and have been derived by the following methods:

- serial subcultivation of a PCC of a human or animal tumour (e.g. HeLa cells);
- transformation of a normal cell having a finite lifespan, with an oncogenic virus or viral sequence (e.g. B lymphocytes transformed by EBV or transfected with viral sequences such as in PER.C6);
- serial subcultivation of a primary cell population derived from normal tissue that generates a dominant cell population having an apparently indefinite lifespan, often described as spontaneous transformation (e.g. Vero, BHK-21, CHO, MDCK, Hi5);
- fusion between a myeloma cell and an antibody-producing B lymphocyte to produce a hybridoma cell line;
- 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 one point in time may vary, the range of chromosome numbers per cell will usually show characteristic limits. However, other CCLs, such as highly tumorigenic 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. However, a characteristic chromosome component may emerge with continued passage, presumably as a dominant cell population develops.

5.1.3.1 Advantages of CCLs

- CCLs can be characterized extensively and their culture conditions can be standardized.
- Production of CCLs can be based on a cell bank system, which allows for consistency and reproducibility of the reconstituted cell populations for an indefinite period.
- CCLs generally grow more easily than DCLs, using standard media.
- Most CCLs can be adapted to grow in a serum-free medium.
- CCLs can usually be grown on microcarriers for large-scale production in bioreactors.
- Some can be adapted to grow in suspension cultures for large-scale production in bioreactors.

5.1.3.2 Disadvantages of CCLs

- CCLs may express endogenous viruses, and some are tumorigenic in immunosuppressed animal models.
- Theoretical risks identified by the 1986 Study Group (e.g. nucleic acids, transforming proteins and viruses) need to be taken into account.

5.1.4 Stem cell lines (SCLs)

Stem cells differ from other types of cells because they sustain a predominant stem cell population while simultaneously retaining the capacity to produce cell progenitors of differentiated cell types of almost all human tissues (i.e. they are pluripotent). Pluripotent SCLs have an apparent capacity to generate cell types of all three human germ layers and may be capable of generating in vitro models of any tissue in the human body. At the time these Recommendations were written (2010), two types of pluripotent SCLs – human embryonic stem cells and induced pluripotency stem cell lines – had been isolated and were considered to possibly have the capabilities to prove useful for manufacturing biologicals. The 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 stem cells for therapeutic purposes, efforts also have been made to explore 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 (24). These include the fundamental issues common to the maintenance of all cell lines, but also include 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.

It has recently been shown that conditioned medium from SCLs can have regenerative properties. Such preparations produced from human embryonic stem cells have shown regenerative capabilities, including repair of myocardial infarction in animal models (25). This raises the possibility of stem cells 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 lifespan characteristic of human diploid fibroblast cultures. In human embryonic stem cell cultures, clonal variants with chromosomal abnormalities are known to arise. While a diploid and non-transformed nature is considered a prerequisite 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.

5.1.4.1 Advantages of SCLs

- SCLs can be well characterized and their culture conditions standardized.
- Production can be based on a cell bank system, allowing consistency and reproducibility of the reconstituted cell populations for an indefinite period.
- Some SCLs may be adapted to grow in suspension cultures for large-scale production in bioreactors.
- SCLs may produce unique proteins of potential importance as biotherapeutics.
- SCLs have the potential to generate cells and tissue-like structures that may permit the expression of agents that are currently considered unculturable in vitro.

5.1.4.2 Disadvantages of SCLs

- Subculture techniques commonly used for SCLs are laborious.
- SCLs may produce growth proteins with undefined effects on adult cells/tissues.
- SCLs usually require complex media that may have a TSE risk.
- Rapid development of differentiated cells also means that they are difficult to control in vitro.
- There is little experience with the use of SCLs as a cell substrate to manufacture biological products.

5.2 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. These risks fall into three categories, namely:

- viruses and other transmissible agents;
- cellular nucleic acids (DNA and RNA);
- 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 (26–35).

In 2010, NRAs and 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 (MPS)) may reveal 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. Consideration should be given to making a risk assessment and potentially introducing risk-mitigation strategies in such circumstances.

5.2.1 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. WHO reviewed this area in 1986 through a Study Group that pointed out that, as described below, cells differ with respect to their potential for carrying viral agents that are 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 non-human primate lymphocytes and macrophages may carry latent viruses such as herpesviruses and retroviruses. CCLs of non-haematogenous cells from human and non-human primates may contain viruses or have viral genes integrated into their DNA. In either case, virus expression may occur under conditions of *in vitro* culture.

Avian tissues and cells may harbour exogenous and endogenous retroviruses, but there is no evidence of 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 control measures during manufacture.

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 (35–37), there is no evidence that biological products released for distribution have been contaminated with rodent viruses. If present, such viruses were detected during quality-control testing in compliance with GMP 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 have recently 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 product-enhanced reverse transcriptase (PERT) assays.

HDCs have been used for vaccine production for many years. 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 should 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 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 are 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 GMP to retest materials that have already been released, so justification would be necessary before such retesting is undertaken. Positive findings should be discussed with the NRA/NCL. Whenever new data are developed with the potential for impact on the quality, safety or efficacy of a biological product, it is the responsibility of the manufacturer to provide NRAs with all the relevant data and information that are currently available. This should include confirmation and evaluation of the finding, the manufacturer's own risk assessment and an investigational and action plan, in order to facilitate any regulatory action that may 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.

5.2.2 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. The most recent WHO Recommendation (WHO Technical Report Series, No. 878) (1) 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. Thus, it is important to 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 lifespan, presumably due to the dysregulation of genes that control growth, and with the ongoing development of products from cells that are tumorigenic or were derived from tumours, 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 on the basis of 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 the risk (38).

The potential risk of DNA arises from both of its biological activities, namely infectivity and oncogenicity. Infectivity could be due to the presence

of an infectious viral genome in the cellular DNA of the cell substrate (39–41). The viral genome could be that of a DNA virus, whether integrated or extrachromosomal, or 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 (39, 40). The oncogenic activity of DNA could arise through its capacity to induce a normal cell to become transformed and perhaps to become tumorigenic. The major mechanism through which this could occur would be the introduction of an active dominant oncogene (e.g. *myc*, activated *ras*), since such dominant oncogenes could directly transform a normal cell. Other mechanisms would require that the rcDNA transforms through insertional mutagenesis, and have been considered less likely since the frequency of integration of DNA is generally low (42). The frequency of integration at an appropriate site, such as inactivating a tumour suppressor gene or activating a proto-oncogene, would be correspondingly lower (32).

The 1986 WHO Study Group 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 1 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 (27). On the basis of this and other evidence available at that time, the Study Group concluded in 1986 that the risk associated with rcDNA in a product is negligible when the amount of such DNA is 100 pg or less per parenteral dose. On the basis of 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 (34, 43). However, more recent data have shown that cloned cellular oncogene DNA can induce tumours in selected strains of mice at levels below 1 ng. In addition, single oncogenes can also be biologically active (44) and can initiate the tumour induction process. Because of these data and the recent evidence that genes encoding for certain micro-RNA species can be oncogenic in vitro (45–48), thus increasing the number of potential dominant cellular oncogenes, the oncogenic risk of DNA needs to be taken into account when tumorigenic 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 where the only way to reduce the biological activity of DNA 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 to be greater than the

oncogenic risk. The polyoma virus genome is infectious in mice at about 50 pg (49), and a recent report demonstrated that 1 pg of a proviral copy of a retrovirus is infectious in vitro (50). Because such low levels of DNA may be biologically active, the amounts of rcDNA should be factored into safety evaluations when tumorigenic cell substrates are used, especially for live viral vaccines.

Consequently, considerations that need to be taken into account with respect to rcDNA are: (i) any reduction in the amount of the contaminating DNA during the manufacturing process; (ii) any size reduction of the contaminating DNA during the manufacturing process; and (iii) any chemical inactivation of the biological activity of contaminating DNA during the manufacturing process. A product might be considered by an NRA/NCL to have an acceptable level of risk associated with the DNA of the cell substrate on the basis of (i) and/or (ii) and/or (iii) 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 virus-inactivating agent, can destroy the biological activity of DNA (38, 50, 51). Therefore, the use of these procedures may provide an additional level of confidence with respect to reduction of DNA risk.

For products such as monoclonal antibodies and subunit vaccines manufactured in tumorigenic cell substrates, it is necessary to demonstrate the clearance (removal and/or inactivation) of DNA by the manufacturing process. This may require 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 can be drawn on their DNA-inactivating potential for a given product.

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 agreed in consultation with the NRA/NCL.

The 1986 WHO Study Group stated that the risks for rcDNA should be considered to be negligible for preparations given orally. This conclusion was based on the finding that polyoma virus DNA was not infectious when administered orally (49). For such products, the principal requirement is the elimination of potentially contaminating viruses. Recently, additional data have been published on the uptake of DNA via the oral route (52). These studies demonstrated that the efficiency of uptake of DNA introduced orally was significantly lower than for that introduced intramuscularly. 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 this route with parenteral routes. However, data suggest that uptake via the intranasal route is less efficient than by the intramuscular route (53). 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 agreed 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 to <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 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.

5.2.3 Cellular RNA

While protein-coding RNA has not been considered to be a risk factor for biological products, owing 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 may necessitate a reassessment. Whether these miRNA molecules can be taken up by cells *in vivo* is unknown. However, as previously stated, 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 section B.9 on Oncogenicity). 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.

5.2.4 Growth-promoting proteins

Growth factors may be secreted by cells that are 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 may 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. 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. the European Medicines Agency (EMA), the United States Food and Drug Administration (FDA)) and other groups (e.g. the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)). GMP should be applied from the stage of cell banking onwards.

In the preparation of a cell substrate, it is considered best practice to establish tiered master and working cell banks (see section A.5.2), to ensure a reliable and consistent supply of cells that can be fully characterized and safety tested prior to use for production. By definition, PCCs 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 practice

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 practice should be observed (e.g. (54, 55) for SCLs). Fundamental features to be considered in the development of cell cultures for production or testing are:

- authenticity, including identity, provenance and genotypic/phenotypic characteristics;
- absence of contamination with another cell line;

- absence of microbiological contamination;
- 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. Thus, characterization approaches may need to be adapted to reflect 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 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, the biological activity of the medium and the additives should be determined before use. New batches of reagents for cell culture should be supplied with certificates of analysis and origin, to 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 chemically defined alternatives should be sought wherever possible. However, given that our current understanding of cell biology is not complete, the benefits that defined media bring in the form of higher reproducibility and reduced risk must be balanced against 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 fetal bovine serum (FBS) remain necessary, they should be carefully controlled whenever possible by pre-use selection of batches. Such careful selection also should apply, where relevant, to cell culture surfaces using specified culture vessels or surface coatings.

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, controlled for quality 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 that ensures consistency in the confluency of cells when harvested, in incubation times, temperature, centrifugation speeds and times, and in post-passage viable cell seeding densities.

A.2.2.2 Cryopreservation

(see section A.5.1)

A.2.2.3 Introduction of contamination

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 the 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 a rigorous aseptic technique and to provide appropriate environmental controls and air quality for cell culture processing and the 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 take into account the 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 that correct procedures are adhered to under GMP. 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 influence the quality of

cells (54). Key procedures on which training in good cell culture practice should focus include the passaging of cells, preparation of sterile media, and 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.

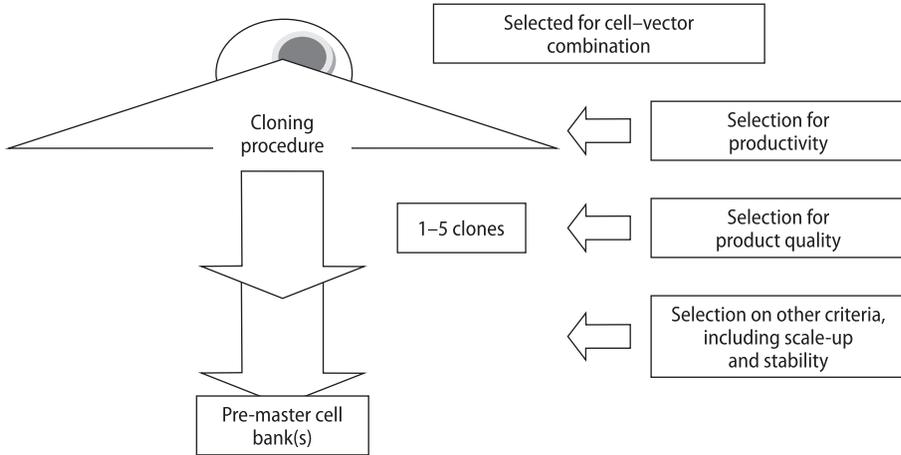
A.2.4 Cell line development and cloning

Wherever a cell culture has passed through a process that may have a significant influence on its characteristics, such as tumorigenicity, it should be treated as a new (i.e. different) cell line and should be renamed with a suffix or code to identify this. An 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 the NRA. In the manufacture of monoclonal antibodies, cloning of hybridoma cultures is particularly important in order 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 according to the practices of individual manufacturers and should be discussed with the NRA/NCL.

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 (from a few hundred to thousands) after transfecting the culture with rDNA. Typically, these clones will be screened on the basis of 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 an MCB and WCB are generated. 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 (see Figure A3.1).

Figure A3.1

Simplified outline 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, with details of 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 that 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 document accurately 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 should also include the stability of integrated rDNA. The details of this process could vary due to a number of factors, including the nature of the host cell, the 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 may 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 TSEs 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. Although the phenomenon is unpredictable, if the line does not express the prion protein (PrP), 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 the principle that cell lines may be infected; thus, exposure of cells to sources potentially contaminated with the agents is a concern. Since the scale of the risk is difficult to judge, it is recommended that, with respect to safety considerations and TSEs, attention should focus on the selection and documentation of the cell culture reagents and other materials that come into intimate contact with the cells, in order 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 risk assessment and testing when necessary – particularly raw materials derived from humans and animals, which can be a primary source for the introduction of adventitious agents into the production of biologicals. Careful attention should be paid to 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 subject to appropriate tests, as indicated by risk assessment, to establish

quality and freedom from contamination by microbial agents and to evaluate acceptability for use in production. It is important to evaluate the microbiological risks represented by each human- and animal-derived reagent used in a cell culture production process. The evaluation should address: (i) geographical origin; (ii) the species of origin; (iii) general microbiological potential hazards, including a consideration of the medical history for human-derived reagents; (iv) the husbandry/screening of donor animals; (v) testing performed on the product, including certificates of analysis (if any); and (vi) the capacity for the preparation, purification and sterilization procedures (if any) used to remove or inactivate contaminants (56). Other reagents of biological but 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 that were 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 analysed to identify any materials of biological origin and any associated hazards that may need to be addressed.

The NRA/NCL should approve the 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 (57). The materials 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 BSE). 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). 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 have also been shown to infect cells

in culture. The processing environment is a common source of microbiological contamination and should be controlled to minimize this risk and to 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 manufacturer of the biologicals. Quality can be ensured in more than one manner. The manufacturer may conduct testing for adventitious agents and perform inactivation of the serum after purchase from the serum manufacturer. Alternatively, the manufacturer may qualify the serum vendor and purchase serum from suppliers only after conducting thorough and ongoing audits of the serum suppliers to ensure that they have properly performed the manufacture, quality control and validation necessary to achieve the level of serum quality required for the biological being produced. 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 when evaluating risk. Consultation with the NRA/NCL may also 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 diarrhoea virus (BVDV), bovine polyoma virus, bovine circoviruses, rabies virus, bovine adenoviruses, bovine parvovirus (BPV), bovine respiratory syncytial virus (BRSV), infectious bovine rhinotracheitis virus (IBR), bovine parainfluenza virus type 3 (BPIV3), reovirus 3 (REO3), Cache Valley virus, bluetongue virus (BTV) and epizootic haemorrhagic disease virus). Consideration should also be given to risk-mitigation strategies, such as inactivation by heat or irradiation, to ensure that adventitious agents that are not detected in the manufacture and quality control of the serum will be inactivated to a degree that is acceptable to the NRA/NCL. If irradiation or other inactivation methods (e.g. heat sterilization) 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 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. If evidence of viral contamination is found in any tests on serum that is not to be subjected to a virus inactivation or removal procedure, the serum would not generally 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 must 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 to the component units of each batch. The irradiation dose must be low enough for the biological properties of the reagents to be retained, while being high enough to reduce virological risk. Thus, 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 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 BVDV, REO3, Cache Valley virus, BTV and epizootic haemorrhagic disease virus, among 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 (NAT), 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 of the order of 1000:1) that comes from the pooling of serum from many animals. Consequently, infectious viruses may 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, NAT 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 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, that is permissive for the replication of BVDV. A second cell line,

such as Vero, should also 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.

Indicator cells are cultured typically in the presence of the serum under test for 21–28 days, passaging the cells as necessary. During this period, the cells are regularly examined for the presence of CPE indicative of virus infection. At the end of the observation period, which should not be less than 7 days after the last subculture, the cells are stained to detect CPE that may have been missed during observation of the living cells. Additional end-point assays should include haemadsorption and haemagglutination at both 4 °C and a higher temperature such as 20–25 °C and also immunofluorescence assays (IFAs) for specific viruses. Appropriate controls should be used for each assay – such as BPIV3 for haemadsorption. IFAs are particularly important for BVDV, since non-cytopathic BVDV may be present in the serum. IFA end-points are also used to detect other viruses that may be determined by geographical considerations – such as adenoviruses, BPV, BTV, BRSV, REO3 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 about 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 do 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 for the biological properties of the reagents to be retained, while being high enough to reduce virological risk. Thus, 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 that recombinant trypsin is free from the risk of contamination and it should be subject to the usual considerations for any reagent of biological origin (see section A.4.1).

Like serum batches, which are derived from many animals, trypsin batches are also prepared from the pancreases of many animals. Most batches of porcine trypsin contain genetic sequences of porcine parvovirus 1 and porcine circoviruses and should therefore be treated in a manner accepted by the

NRA/NCL in order to inactivate any virus that may potentially be present. It is acknowledged, however, that these viruses are relatively resistant to inactivation (58). 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, that is permissive for the replication of porcine viruses. Typically, indicator cell cultures would be incubated for 14 days with a subculture on to fresh test cells for an additional 14 days. Specific end-point detection methods such as IFA or PCR may be required, in addition to periodic observation for CPE throughout the culture period and more general end-point detection methods such as haemadsorption and/or haemagglutination.

If trypsin has been 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 for appropriate adventitious viruses relevant to the species of origin of the trypsin used. 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 is 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 instance, it may be appropriate to test for porcine adenovirus, transmissible gastroenteritis virus, porcine haemagglutinating encephalitis virus, BVDV, 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. 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 should be conducted depending on the type of porcine-derived material. The NRA/NCL should be consulted on this issue.

A.3.4 Medium supplements and general cell culture reagents derived from other sources used for preparing cell cultures

The quality of medium supplements derived from other species should be 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, they should be 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 on this issue.

Medium supplements should generally 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. As a minimum, human serum albumin should meet the revised Requirements for biological substances no. 27 (59), as well as the Guidelines on tissue infectivity distribution of TSEs (57).

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 safety considerations for any reagent of biological origin (see section A.3.1).

As for other cell culture reagents, it is important to establish traceability and to assess and reduce microbiological risks as described in section A.3.1.

A variety of cell culture reagents of biological origin is available. These reagents 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 from those involved in animal-derived reagents (see sections A.3.1–A.3.3), and other hazards may arise – such as immunogenic, mitogenic and allergenic properties of the reagent and its components. Plant-derived material may, for instance, carry an increased risk of mycoplasma and mycobacteria contamination.

A.4 **Certification of cell banks by the manufacturer**

It is vital that the manufacturer secures a body of information on the cell substrate, demonstrating 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 dataset is vital for demonstrating 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 made 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 of PCCs should be established to the animals of origin, husbandry conditions, veterinary inspection, vaccinations (if any), procedures for administering anaesthesia and cell harvesting, 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 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. It is especially important to monitor cultures carefully for evidence of adverse change in the cell culture and of 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, 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 microbiological testing should ideally 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 GMP. 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 production of biologicals should have data available, as indicated in section 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, the medical history of the individual from whom the cell line was derived should, if

possible, 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. 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 with 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 (see, for example (60)).
- The cooling profile for the cells being frozen should be defined, and the same cooling process should be used for each separate preservation process (i.e. the standard operating procedure 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 aliquoting to ensure homogeneity.
- The number of cells per vial should be adequate to recover a representative culture (e.g. $5-10 \times 10^6$ 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. 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 that it has retained viability and the results have been 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 section B.3) and integrity of cryopreserved vials is demonstrated when the vials are thawed from production and shown to produce the intended product at scale (see also section 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 prudent to establish a stability-testing programme that involves periodic recovery of cells where the frequency of recovery relates to the risk of temperature cycling. New developments in remote monitoring of individual vials may, in the future, eliminate the need for stability testing.

A.5.2 Cell banking

When DCLs, SCLs or CCLs are used for the production of a biological, a cell bank system should be in place. Cell 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 with 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 the 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: 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 process.

Cell banks should be characterized as specified in Part B of these recommendations, and according to any other currently valid 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 section 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, in order to ensure continued ability to manufacture products 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, are not usually returned to the stock. The second storage site should operate under an equivalent standard of quality assurance to that at 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 should be used as an MCB. The purpose of WHO RCBs is to provide well-characterized cell seed material for the generation of an 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, which include:

- traceability to the origin of cells and derivation of the cell line and materials used in preparation of seed stock;
- being subject to open international scientific scrutiny and collaborative technical investigations into the characteristics of the cells and the presence of adventitious agents;
- the results of characterization are peer-reviewed and published;
- investigations are evaluated under the auspices of WHO expert review and qualified as suitable for use in vaccine production;
- supply of cells free of any constraint related to intellectual property rights on final products;
- a single source of cells with a growing and scientifically and technically updated body of safety-testing data and safe history of use, giving increased confidence to manufacturers, regulators and public policy-makers.

The Vero cell line has been the most widely used continuous cell line for the production of viral vaccines over the past 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 history of safe and reliable 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 web site (<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 an 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 (www.hpacultures.org.uk) in the United Kingdom and the American Type Culture Collection (ATCC, www.atcc.org) in the USA. These public service culture collections have distributed ampoules under agreements with 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, owing to the limited number of vials remaining, distribution is restricted to use in 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 from 1966. This RCB was prepared in a qualified cleanroom environment and was subjected to specified quality-control testing endorsed by the WHO Expert Committee on Biological Standardization.

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 being used for the production of biologicals. 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 A3.1.

Table A3.1
Examples of approved biological products derived from CCLs

Product class	Product (disease)	Cell line
Therapeutic	Factor VIII (haemophilia)	CHO
	Factor VIIa (haemophilia)	BHK-21
	Monoclonal antibody (various diseases)	CHO and murine myeloma (NS0 and SP2/0)
Prophylactic	Poliovirus vaccine	Vero
	Rotavirus vaccine	Vero
	Rabies vaccine	Vero
	Japanese encephalitis vaccine	Vero
	Human papillomavirus vaccine	Sf-9
	Influenza vaccine	MDCK

Many more products are currently in development. Some products use highly tumorigenic cells (e.g. HeLa, and some banks of MDCK) and some involve sources previously unused in production, such as insect cells. Examples are listed in Table A3.2.

Table A3.2
Examples of biological products in development derived from CCLs

Product class	Product (disease)	Cell line(s)
Therapeutic	More than 50% of products in development use CHO or murine myeloma cells as the cell substrate (61). Monoclonal antibodies are generally produced using CHO, SP2/0, PER.C6 and NS0 cells (62).	
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 the biologicals derived from them. In particular, CCLs 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-tumorigenic cells (e.g. PCCs and DCLs) had been thought to be intrinsically safer than tumorigenic cells. Where tumorigenic 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 the quantity of DNA. When it was not possible to reduce the amount of DNA to below the detection limit, emphasis has been placed on a reduction in size or other approaches to inactivate rcDNA and rcRNA (e.g. beta-propiolactone 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 if 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 tumorigenicity 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 tumorigenicity 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 may 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 that is proposed for use as a substrate for the manufacture of a biological product, to demonstrate, to the limits of the assay's detection capabilities that the CCL is free from cultivable bacteria, mycoplasmas, fungi and infectious viruses, including potentially oncogenic agents. 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 of 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 an 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 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;
- the results of tests for infectious agents;
- distinguishing features of the cells, such as biochemical, immunological, genetic or cytogenetic patterns, that allow them to be clearly distinguished from other cell lines;
- the results of tests for tumorigenicity, 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 programme.

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, in order 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 A3.3. In the case of human cells, genetic tests such as DNA profiling (e.g. short tandem repeat analysis, and 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 may be used for human cells is human leukocyte antigen (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. A small proportion of cell lines – particularly those that 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 from that expected for the species of origin; it is also a general issue relating to the effect of genetic instability on molecular identity-testing techniques. Such effects in standard technologies are rare but 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, percentage of host cells retaining the expression system, verification of protein-coding sequences, and protein-production levels.

Table A3.3
Identity testing for mammalian cell lines

Technique	Advantages	Disadvantages
Karyology (especially useful for DCLs and SCLs)	Gives whole chromosomal genome visualization and analysis that can identify species of origin for a very wide range of species using the same methodology.	Results are generally not specific to the individual of origin (i.e. there is usually species specificity), although certain cell lines may have marker chromosomes that are readily recognized.
Karyology (especially useful for DCLs and SCLs)	Newer methods include spectral karyotyping, which involves the use of probes labelled with fluorescent dyes. The probes paint the chromosomes, yielding different colours in specific areas. Spectral karyotyping is able to detect translocations that are not recognizable by traditional banding methods.	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 the species of origin but is not specific to the individual of origin.

Table A3.3 *continued*

Technique	Advantages	Disadvantages
DNA profiling using variable number of tandem repeats (VNTR) analysis or other PCR method such as exon-primed intron crossing-PCR (EPIC-PCR), or techniques such as restriction fragment length polymorphism (RFLP)	<p>Short tandem repeats (STR) analysis by PCR is rapid and gives identity specific to the individual of origin.</p> <p>Commercial kits are available for a range of human populations.</p> <p>The 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.</p>	Some limited, but undefined, cross-reaction of human STR primers for primate cells.

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 programme.

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, it may be prudent to generate data to confirm their suitability for manufacturing on a schedule that takes into account the storage condition once every 5 years.

Applicability

- *Cell banks:* MCB and WCB
- *Cell types:* DCL, SCL, CCL

B.3.2 Genetic stability

Any form of genetic instability can potentially affect the quality of the final product and it is 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 actual 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 also be determined. The latter is accomplished by sequencing into the cellular flanking regions, but methods such as 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 MPS it is possible to determine the sequence of individual gene inserts or their transcripts.

Where proteins are derived from cells that have not been genetically modified, consistency in the yield and properties of the protein should be evaluated, together with the sequence of the messenger RNA (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 ensure 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.

Applicability

- *Cell banks*: MCB taken to EOPC/ECB
- *Cell types*: DCL, SCL, CCL

B.4 Sterility

(see section B.11.3.1)

B.5 Viability

A high level of viability of cryopreserved cells is important for efficient and reliable production. Thawed cells should typically 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 is available to measure different attributes of cell function (e.g. 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 the 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 significant overestimation of 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 sections B.6 and B.7). It may also be necessary to select alternative viability assays that are better suited to providing the in-process viability data that are required during production (e.g. lactate dehydrogenase levels in bioreactor systems).

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, in order 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 – should be

developed. For certain cell substrates, it may be appropriate to apply such tests in homogeneity testing (see section B.7). Experiments to demonstrate homogeneity and growth characteristics may be combined, although the analysis should be carried out separately.

Applicability

- *Cell banks:* MCB and WCB
- *Cell types:* DCL, SCL, CCL

B.7 Homogeneity

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

Applicability

- *Cell banks:* MCB, WCB
- *Cell types:* DCL, SCL, CCL

B.8 Tumorigenicity

B.8.1 General considerations

Several *in vitro* test systems, such as cell growth in soft agar (63) and muscle organ culture (64), have been explored as alternatives to *in vivo* tests for tumorigenicity.

However, correlations with *in vivo* tests have been imperfect, or the alternative tests have been technically difficult to perform. Thus, *in vivo* tests remain the standard for assessing tumorigenicity.

Although WHO Requirements (1) have described acceptable approaches to tumorigenicity 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 (see Appendix 2). The major points included in the model protocol are listed below, along with comments on each item.

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

The tumorigenicity tests that are currently available are in mammalian species, whose body temperatures and other physiological 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 tumorigenic 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 tumorigenic because they possess the capacity to form tumours in immunosuppressed animals such as rodents. Some CCLs become tumorigenic at high PDLs (e.g. Vero), although they do not possess this capacity at the 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 tumorigenic phenotype can vary from one CCL to another, and even within different sublines of the same CCL. This range of variability, from non-tumorigenic, to weakly tumorigenic, to highly tumorigenic, has been viewed by some as indicating different degrees of risk when the CCLs are used as substrates for the manufacture of biological products (10, 11).

If the CCL has already been demonstrated to be tumorigenic (e.g. BHK-21, CHO, HEK293, Cl27), or if the class of cells to which it belongs is tumorigenic (e.g. hybridomas, SCLs), it may not be necessary to perform additional tumorigenicity tests on cells used for the manufacture of therapeutic products. Such cell lines may be used as cell substrates for the production of biologicals if the NRA/NCL has determined, on the basis of characterization data and 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 tumorigenic unless data

demonstrate that it is not. If a manufacturer proposes to characterize the cell line as non-tumorigenic, 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 tumorigenicity, 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 tumorigenic potential of cell lines. Table A3.4 lists several examples of such tests, along with advantages and disadvantages of each. Because assessing the tumorigenic 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 antithymocyte globulin (ATG), antithymocyte serum (ATS) or antilymphocyte serum (ALS). The use of animals with additional defects in natural killer (NK)-cell function – such as the non-obese diabetic (NOD)-SCID mouse, the NOD-SCID-gamma mouse and the CD3 epsilon mouse – has not yet been explored for cell-substrate evaluation, but they may offer some advantages. In addition to these systems, several other in vivo systems such as the hamster cheek pouch (HCP) model and ATG-treated non-human primates have been used in the past, but are rarely used at present.

Table A3.4
In vivo tests to assess the tumorigenic potential of inoculated cells

Test and brief description	Advantage(s)	Disadvantage(s)	References
<i>Adult athymic mouse (Nu/Nu genotype):</i> animals inoculated by the intramuscular or subcutaneous routes with cells to be tested	<ul style="list-style-type: none"> • Animals readily available • No immunosuppression required 	<ul style="list-style-type: none"> • Higher frequency of spontaneous tumours than in other animal models that are not genetically immunosuppressed • Low sensitivity for assessing the metastatic potential of the inoculated cells 	(65)

Table A3.4 *continued*

Test and brief description	Advantage(s)	Disadvantage(s)	References
<i>Newborn athymic mouse</i> : animals inoculated by the subcutaneous route with cells to be tested	<ul style="list-style-type: none"> • No immunosuppression required • More sensitive than adults 	<ul style="list-style-type: none"> • 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 neonates by the mother 	(66)
<i>SCID mouse</i> : animals receive subcutaneous, intradermal or intrakidney capsule inoculation of test cells	<ul style="list-style-type: none"> • No immunosuppression required • Potentially increased sensitivity • Animals readily available 	<ul style="list-style-type: none"> • Highly susceptible to viral, bacterial and fungal infections • Infections can affect the results and reproducibility of studies • Spontaneous thymic lymphomas may occur 	(66, 67)
<i>Newborn rat</i> : animals immunosuppressed with ATG, followed by intramuscular or subcutaneous inoculation of cells to be tested	<ul style="list-style-type: none"> • Animals readily available • Sensitive model for detecting metastases • Very low frequency of spontaneous tumour formation 	<ul style="list-style-type: none"> • 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)
<i>Newborn hamster or mouse</i> : animals immunosuppressed with ATS, followed by intramuscular or subcutaneous inoculation of cells to be tested	<ul style="list-style-type: none"> • Animals readily available 	<ul style="list-style-type: none"> • Cannibalism of neonates by the mother • Standardized ATS not available and difficult to balance toxicity versus immunosuppressive capacity 	(69)

Table A3.4 *continued*

Test and brief description	Advantage(s)	Disadvantage(s)	References
<i>Newborn hamster or mouse</i> : animals immunosuppressed with ALS, followed by intramuscular or subcutaneous inoculation of cells to be tested	<ul style="list-style-type: none"> • Increased sensitivity compared to the HCP test • Animals readily available 	<ul style="list-style-type: none"> • Cannibalism of neonates by the mother • Standardized ALS not available and difficult to balance toxicity versus immunosuppressive capacity 	(70)
<i>HCP</i> : animals immunosuppressed with cortisone, followed by inoculation of the cells to be tested into the cheek pouch	<ul style="list-style-type: none"> • Animals readily available 	<ul style="list-style-type: none"> • Lower sensitivity than newer models 	(71)
<i>Non-human primates</i> : animals immunosuppressed with ATG, followed by inoculation of cells to be tested into the muscle of the four limbs	<ul style="list-style-type: none"> • Species closer to human 	<ul style="list-style-type: none"> • Standardized ATG not available • Animals not readily available • Expense and limited availability preclude using large numbers • Animal welfare principles mandate against use of non-human primates if the same results can be obtained from lower species 	(72)

Although all the animal models listed in Table A3.4 have been used to assess the tumorigenicity of cells, several sensitivity parameters from studies using positive-control cells should be considered when attempting to compare the various *in vivo* tumorigenicity models. These sensitivity parameters are:

- (i) frequency of tumour formation;
- (ii) time to appearance of tumours;
- (iii) size of tumours;

- (iv) lowest number of inoculated tumour cells that result in tumour formation;
- (v) metastatic tumour 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 (68, 73, 74) none take all of these factors into account, nor do they use the same tumorigenic cell lines. Thus, it is not possible to draw definitive conclusions about the relative sensitivity of the various tumorigenicity assays. Nevertheless, the following points appear to be generally accepted:

- the ATS-treated newborn rat and the ATG-treated non-human-primate systems are the most sensitive for assessing the metastatic potential of inoculated cells;
- ATS and ATG provide better immunosuppression than ALS;
- the nude mouse has a better-defined level of immunosuppression than models that depend on ALS, ATS or ATG, and interlaboratory comparisons of data from nude mice are more likely to yield valid conclusions.

Overall experience during the past 30 years, 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 tumorigenic 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 tumorigenic cells. In some cases, it may be preferable to use newborn athymic nude mice, as these animals are more sensitive than adults for the detection of weakly tumorigenic cells (66). A tumorigenicity 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 **The point in cells' life history at which they should be tested**

Investigation of tumorigenicity 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 and used for production or beyond should be examined for tumorigenicity. The extra population doublings (e.g. 3–10) ensure that the results of the tumorigenicity test can be used in the assessment of overall safety of the product, even assuming a worst-case situation, and this therefore provides a safety buffer.

B.8.4 Use of control cells

The tumorigenicity 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 between laboratories, so that 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 tumours 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 (such as infections) in the animals or in the testing facility, which can reduce the efficiency of tumour development.

When the cell substrate has been adapted to growth in serum-free medium, which may contain growth factors and other components that could influence growth as well as detection of a tumorigenic 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 tumorigenicity protocol, it is important to recognize that tumours arise spontaneously in nude mice and that the incidence of such tumours 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 tumorigenicity test. In general, 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 FRh1-2 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; consequently, 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 tumours 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 tumours should be produced in at least 9 out of 10 animals injected with the positive reference cells.

B.8.6 Number of inoculated cells

Each animal should be inoculated intramuscularly or subcutaneously (75) 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-tumorigenic. If the cell line is found to be tumorigenic, the NRA/NCL may request additional studies to determine the level of tumorigenicity. This can be done with dose-response studies, where doses of 10^7 , 10^5 , 10^3 and 10^1 viable cells are inoculated, and the data can be expressed as tumour-producing dose at the 50% end-point (TPD₅₀ value) (76).

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 2 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 killed before the end of the study if the tumour 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 killed until the end of the observation period. Cell lines that produce nodules that fail to grow progressively are not considered to be tumorigenic. If a nodule persists during the observation period and retains the histopathological characteristics of a tumour, 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 killed and examined for gross and microscopic evidence of the growth of inoculated cells at the site of injection and 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 tumour at the injection site. If what appears to be a metastatic tumour differs histopathologically from the primary tumour, it is necessary to consider the possibility that this tumour either developed spontaneously or 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 tumour itself, or the tumorigenicity assay may need to be repeated. In such cases, appropriate follow-up studies should be discussed and agreed with the NRA/NCL (also see section B.9, “Oncogenicity”).

B.8.12 Interpretation of results

A CCL is considered to be tumorigenic if at least 2 out of 10 animals develop tumours 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 tumours must be consistent with the inoculated cells, and a genotypic marker should show that the tumour is not of nude mouse origin.

If only one of 10 animals develops a tumour, it is appropriate to investigate further in order to determine, for example, if the tumour originated from the cell substrate inoculum or from 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 on many factors specific to a given CCL and to the product being developed. The NRA/NCL should be consulted in this regard.

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 tumorigenicity is the property of cells to form tumours when inoculated into susceptible animals, oncogenicity is the property of an acellular agent to induce cells of an animal to become tumour cells. As such, tumours that arise in a tumorigenicity assay contain cells derived from the inoculated cells, while tumours that arise in an oncogenicity assay are derived from the host. Oncogenic activity from cell substrates could be due either to the cell substrate DNA (and

perhaps other cellular components) or to an oncogenic viral agent present in the cells. Although there may be a perception that the cellular DNA from highly tumorigenic cells would have more oncogenic activity than the DNA of weakly or non-tumorigenic cells, it is not currently known if there is a relationship between the tumorigenicity of a cell and the oncogenicity of its DNA. Nevertheless, the NRA/NCL may 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 tumorigenic 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 $10^5\text{--}10^6$ -fold less concentrated than a plasmid containing the same oncogene. As a consequence, if 1 μg of an oncogene expression plasmid induces a tumour in an experimental animal model, the amount of cellular DNA that would contain a similar amount of the same oncogene is $10^5\text{--}10^6$ 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 (34, 43, 44). 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 that are 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 of a 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 (77–79), have been used to assess oncogenicity. However, it is not clear how such 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 the 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 may also 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 tumorigenic unless data demonstrate that it is not. If a manufacturer demonstrates that a new CCL is non-tumorigenic, 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.

Applicability

- *Cell banks:* MCB or first WCB taken to representative EOPC or ECB
- *Cell types:* CCL, SCL (recommended when tumorigenic 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 has 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 (80). 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 may 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 section 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 (19–21). 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.

To determine 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 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. The ISCN (81) 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 (57).

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 may also be of concern. Testing for both endogenous (e.g. retroviruses) and adventitious agents (e.g. mycoplasmas) is described in the subsequent sections. In general, cell substrates contaminated with microbial agents are not suitable for the production of biologicals. 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. The balance of 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 a live viral vaccine subjected to neither significant purification nor inactivation, that same cell substrate may 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 carry out

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 an 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. It also 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 are frequently 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). Specific techniques such as molecular and immunological methods and electron microscopy may be required to reveal the presence of such inapparent infections. 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 such as transcriptome sequencing or degenerate primer PCR may be useful.

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. Consideration should

also 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, thereby making it more difficult to detect (e.g. simian virus 40 (SV40) 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 the animals may be exposed to live vaccines. The risk with primary cells can be mitigated by rigorous qualification of source animals and of the primary cells themselves. When feasible, animals from which primary cultures are established should be from genetically closed flocks, herds or colonies that are monitored for freedom from pathogens of specific concern. Such animals are known as specific-pathogen-free, or 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 new retroviral proviruses, for instance). 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 that is sufficient to detect signs of disease or infection (e.g. monkeys are generally quarantined for six or more weeks (82)). Such animals also should be screened serologically for appropriate adventitious agents, in order 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 such contaminations.

For primary cell cultures, the principles and procedures outlined in Part C of Recommendations for the production and control of poliomyelitis vaccine (oral) (82), together with those in section A.4 of Requirements for measles, mumps and rubella vaccines and combined vaccine (live) (83) 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 became apparent only as cell culture methods were developed. For example, human enteroviruses were not recognized until the development of monkey kidney cell cultures in which they could produce CPEs, 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 CPE 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 cynomolgus 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 led to a range of different approaches for trying to detect all contaminants.

Coxsackie viruses are named after the town in New York where they were first identified after being detected because of 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 characterization 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 activity, the egg-based assays include tests for haemagglutinating activity, as well as for the 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 of 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 will 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 in monkeys in the absence of precautions such as quarantine and clinical evaluation of the donor animals, and this 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 if the animals had 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 still are 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 the maintenance of a test if it detects only viruses that are also detected by other methods of equivalent sensitivity and comparable ease of use and cost. Each of these considerations should be borne in mind 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 balanced 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 show evidence of the presence of any viral agent attributable to the cell banks.

In general, 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, supernatant fluids or cell lysates from the bank itself, or on cells or supernatant fluids or cell lysates from 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 to detect 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 10 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 that 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 killed 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 the test group or the negative control group (if used), or in both, become sick for nonspecific 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 10 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 inapparent infection.

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 to detect Coxsackie viruses. The test for pathogenic viruses in suckling mice 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 10 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 the cause of death. If a viral infection is indicated, efforts should be undertaken to identify the virus where 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 determination of 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 the test group or the negative control group (if used), or in both, do not survive the observation period.

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

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 to detect 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 10⁷ viable cells or the equivalent cell lysate are divided equally among the animals.

In some countries, 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. 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 group or the negative control group (if used), or in both, 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 end-point (also see section B.11.3).

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 to detect herpes B virus. When it is necessary to detect simian herpes B virus, the test in rabbits for pathogenic viruses is performed and includes 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, 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. 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 group or the negative control group (if used) 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.

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

10 embryonated hens' eggs, and into the yolk sac of each of at least another 10 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 chicken (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 the test group or the negative control group (if used), or in both, are discarded for nonspecific 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 cells from guinea-pig and chicken (or other avian species). 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.

The eggs used for the yolk-sac test should usually 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 for detecting the presence, in the test samples, of the adventitious agents that the 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.

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 chicken antibody production test – e.g. to detect chicken anaemia virus. Further, if the cell substrate (even if not of rodent origin) has been exposed to materials of rodent origin (e.g. selection using a monoclonal antibody), testing should be considered for the species-relevant viruses, using an antibody production test (84, 85).

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

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 two or three) 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 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.

Applicability

- *Cell banks*: MCB, WCB or ECB or representative EOPC
- *Cell types*: PCC, DCL, SCL, CCL

B.11.2.2.1 Indicator cells

Live cells or cell lysate, each with spent culture fluids of the MCB or WCB, are inoculated on to 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, they should be stored at ≤ -70 °C.

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

Cultures of a human DCL may be used. The original purpose of this test, using primary human cells, was the detection of measles virus. 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 of simian origin would be used 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 on to each of the indicator cell types. The resulting co-cultivated or inoculated cell cultures are observed for evidence of viruses by CPE 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 week) cell culture for the purposes of detecting human or simian cytomegalovirus can be replaced by the use of NAT to detect cytomegalovirus nucleic acid.

In some countries, a passage on to 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 these cases, it may be necessary to feed the cultures with fresh medium or to subculture after 2 weeks on to 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.11.2.2.2 *Additional considerations regarding 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 with a variety of techniques such as increased/reduced culture temperature (above or below that routinely used for production), heat shock for a short period, superinfection 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 are co-cultivated with indicator cells from at least three different species of insect in addition to the indicator cells 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, a second line 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 $37 \pm 1^\circ\text{C}$ and a lower temperature such as $28 \pm 1^\circ\text{C}$ – 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 characterized 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 prerequisite 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 from the 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. (86). In some cases it may be appropriate to examine more cells, as discussed below for insect cell lines. The NRA/NCL should be consulted in this regard. Any unusual or equivocal observations that may 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.

Applicability

- *Cell banks:* MCB, WCB, or ECB or representative EOPC
- *Cell types:* DCL, SCL, CCL

B.11.2.3.1 Additional considerations on TEM for insect cells

The general screening test outlined above applies to MCBs and WCBs derived from insect cells. In addition, cell lines should be subjected to stress conditions, such as described in section B.11.2.2.3, prior to examination by TEM. 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, as should 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 may 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 (endogenous avian retrovirus), CHO cell line gamma-retrovirus (87)), 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 CPE on the cells. Therefore screening assays, such as the PERT assay for reverse transcriptase, or TEM may be required 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 production of live viral vaccine. 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, 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 quantitative PCR) on a representative number of lots, and retrovirus clearance is demonstrated with significant safety factors. 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.

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.11.2.4.1 Reverse transcriptase 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 PCR-based reverse transcriptase (RT) assay or PERT assay (88–91).

RT 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 (92–96) or cellular DNA-dependent DNA polymerases (97, 98). Attempts to reduce the PERT activity associated with cellular DNA-dependent DNA polymerases have been reported (98–100), although no treatment can eliminate all activity. Thus, the results of such highly sensitive assays need to be interpreted with caution. Use of appropriate controls in the assay can assist in this regard. Since RT activity can be associated with the presence of defective retrovirus-like particles, and since polymerases other than RT 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, such as carrying out infectivity assays (see section B.11.2.4.4). It may also be useful to utilize the conventional RT assay in this investigation to determine whether the RT activity is Mg^{2+} or Mn^{2+} 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. The appropriateness of this test with such cells should be discussed with the NRA/NCL. For example, it may be appropriate to direct testing strategies to 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 may test positive by this assay.

B.11.2.4.2 *PCR or other specific in vitro tests for retroviruses*

If 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 PCR, immunofluorescence, enzyme-linked immunosorbent assay (ELISA) or other virus-specific detection methods. Molecular methods, such as PCR, may also 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.11.2.4.3 *Infectivity test for retroviruses*

When the test sample is found to have RT activity, it may 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 with infectivity assays for the presence of retroviruses. Cells to be used for these assays should be able to support the

replication of a broad range of viruses; this may require the use of 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 on to 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 (101, 102).

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 should 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.11.2.4 and their subsections

Some viruses, such as hepatitis B or C viruses or human papillomaviruses, cannot be detected readily by any of the methods described above because these viruses 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 such as immunofluorescence assays may also be employed.

Generally, once the MCB, WCB or ECB has been demonstrated to be free of selected viruses, it may not be necessary to test the cells at later stages (e.g. at the production level) if such viruses cannot 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.11.2.4 and their subsections. 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 particular cell lines (e.g. nodaviruses) or that may be present persistently in insect cell lines and that are known to be infectious for humans.

Applicability

The NRA/NCL should be consulted with 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.11.2.5.1 Nucleic acid detection methods

Tests for selected viruses are usually 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 among strains or within a family, in order to increase the opportunity for 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 for identifying the virus strain or family. However, PCR methods have the limitation that viral genes may not be sufficiently conserved among all members of a particular viral family for the genes to be detected even when conserved regions are selected.

New and 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 (103–105). The new generation of massively parallel sequencing (MPS) methods may have particular utility. They can be applied to detect virions following nuclease treatment to remove cellular DNA and unencapsidated genomes. 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 (103, 106–110). 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 (103, 110, 111). 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 (103, 105, 111).

It is probable that application of methods of this type will be expected or required by regulatory agencies in future. At present the methods have not been evaluated for sensitivity and specificity and should be thought of as powerful investigational tools that can reveal issues that can 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 in preventing 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 of adventitious infectious organisms such as bacteria, fungi, cultivable and non-cultivable mycoplasmas, spiroplasmas (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 the 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 allow for repeat

testing to deal with potentially false-positive results and a prequalification plan for reagents used in the tests.

Mycobacterial testing may be applied to cell-bank characterization if the cells are susceptible to infection with *Mycobacterium tuberculosis* or other species. Such testing should also 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 or spiroplasma may require different growth conditions from methods used for mammalian cells, although at least one – spiroplasma – can be cultivated at 30 °C. Positive controls for these tests (particularly for spiroplasmas) are an issue that needs to be resolved. Spiroplasmas have been reported as infectious agents in a number of insect species, and insect cell lines have also 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 of the Requirements for biological substances no. 6 (112) by a method approved by the NRA/NCL. Additional information can be found in national pharmacopoeias and ICH documents (10, 113–115). 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. cryopreservation vials) with a minimum of two containers. For supernatant fluid, it is recommended to use 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.

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 include mycoplasmas, acholeplasmas, spiroplasmas and others. They are parasites of various animals and plants, living on or in the host's cells. Mollicutes 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 acholeplasma*

Tests for mycoplasmas are performed as specified in Part A, sections 5.2 and 5.3 of the Requirements for biological substances no. 6 (116), or by a method approved by the NRA/NCL. Both the culture method and the indicator cell-culture method should be used. NAT alone, in combination with cell culture or with an appropriate detection method, may 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 (117). One or more containers of the MCB and each WCB are used for the test.

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 into 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, 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, testing of the WCB may also be necessary.

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

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 on to each of two 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 bacille Calmette–Guérin (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.

NAT may 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, may also be used (see section B.11.2.1.3).

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 brains 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 (118, 119). Normal PrP (PrPc) protein may be expressed on cell surfaces, but *in vivo* this protein can misfold and become the abnormal disease-causing type PrP^{tse}, which is able to catalyse the conversion of PrPc protein into the abnormal conformation. Compared with PrPc, PrP^{tse} is relatively resistant to common proteolytic enzymes such as proteinase K.

BSE was first described in the United Kingdom in 1984, and the numbers of clinical cases there reached a peak in 1992–1993. Other countries were also affected. Currently, the number of new infections detected annually is low (120).

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 (121), 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 this route (122). 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. Thus, 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 should be 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 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 (123) 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 minimizing 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 WHO and other scientific bodies such as the EMA into three categories (category A: high infectivity; category B: lower infectivity; category C: no detectable infectivity) (57, 124). 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 that were

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 (57, 124–126).

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 and manufacturing procedures. Examples include: cell culture medium free of animal material; polysorbate and magnesium stearate of plant origin; enzymes, such as rennet, of microbial origin (used in lactose production); and recombinant insulin and synthetic amino acids. It should be noted 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 that are free of animal materials, and raw materials of non-ruminant origin. For example, fetal bovine serum may 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 (geographical BSE-risk level I, or GBR I), as classified by the European Food Safety Authority. 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 may 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, traceability should be 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 risk of TSE infectivity in the materials used, or if the use of the products is associated with vCJD. Category A and B ruminant materials originating from BSE-enzootic countries should not be used in the production of biologicals 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 materials of category C, 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

No suitable screening tests are currently available for TSE agents in raw materials of human/ruminant origin similar to serological or PCR assays for 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 and others). 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 (127). 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

This section provides 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 for its intended use. The testing strategy should be agreed with the NRA/NCL.

B.12.1 Cell seed

The cell seed is generally 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. RCBs (see also section A.5.3) would be considered cell seeds. The cell seed is usually of limited quantity, so 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 called the 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 carried out at any point before the establishment of the MCB. Usually, such tests are limited to obtaining information that is essential for making the decision to commit resources to the preparation of an 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 and working cell bank

In general the MCB 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 should also 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; other tests are 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 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 for the use of animal cells as *in vitro* substrates for the production of biologicals published in WHO Technical Report Series, No. 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 consisting of: Dr J. Petricciani, Consultant, Palm Springs, CA, USA; Dr R. Sheets, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; Dr G. Stacey, National Institute for Biological Standards and Control, Potters Bar, England; and Dr I. Knezevic, World Health Organization, Geneva, Switzerland. These recommendations were reviewed by the WHO Study Group on Cell Substrates in 2008.

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

Dr K.S. Ahn, Korea Food and Drug Administration, Seoul, Republic of Korea; Dr J.H. Blusch, Novartis, Basel, Switzerland; Dr da Silva Guedes Jr., Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil; Dr M. Deschamps, GlaxoSmithKline Biologicals, Wavre, Belgium; Dr G. Dong, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China; Dr B. Gauvin, Amgen Inc., Thousand Oaks, CA, USA; Dr H. Kavermann, Roche Diagnostics GmbH, Penzberg, Germany; Dr K. King, United States Food and Drug Administration, Bethesda, MD, USA; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr A. Lewis, Center for Biologics Evaluation and Research, Bethesda, MD, USA; Dr L. Mallet, Sanofi Pasteur, Marcy L'Etoile, France; Dr P. Minor, National Institute for Biological Standards and Control, Potters Bar, England; Dr P. Nandapalan, Therapeutic Goods Administration, Woden, ACT, Australia; Dr D. Onions, Invitrogen Corporation, Carlsbad, CA, USA; Dr K. Peden, Center for Biologics Evaluation and Research, Bethesda, MD, USA; Dr J. Petricciani, Consultant, Palm Springs, CA, USA; Ms E. Ika Prawahju, National Agency of Drug and Food Control, Jakarta, Indonesia; Dr R. Sheets, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; Dr G. Stacey, National Institute for Biological Standards and Control, Potters Bar, England; Dr R. Wagner, Paul-Ehrlich-Institute, Langen, Germany; and Dr D.J. Wood, World Health Organization, Geneva, Switzerland.

On the basis of the comments received from a broad range of regulators, manufacturers of vaccines and other biologicals and other relevant experts in 2009, the 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 on 1–3 June 2010 at WHO, Geneva, taking into account comments received from the reviewers and from the following meeting participants:

Dr C. Conrad, World Health Organization, Geneva, Switzerland; Dr H. Kang, World Health Organization, Geneva, Switzerland; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr J. Petricciani, Consultant, Palm

Springs, CA, USA; Dr R. Sheets, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; Dr P. Minor, National Institute for Biological Standards and Control, Potters Bar, England; Dr D. Onions, Invitrogen Corporation, Carlsbad, CA, USA; Dr K. Peden, Center for Biologics Evaluation and Research, Bethesda, MD, USA; Dr J. Shin, World Health Organization, Geneva, Switzerland; and Dr Glyn Stacey, National Institute for Biological Standards and Control, Potters Bar, England.

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

Acknowledgements

Acknowledgements are due to the following experts for their useful comments during several rounds of the draft recommendations:

Mr S.G. Bankar, Mr V.B. Vaidya, Serum Institute of India, Pune, India; International Federation of Pharmaceutical Manufacturers and Associations (Dr R. Field, Dr J. Kutza, Dr J. Liu, Dr D. Lindsay, AstraZeneca/MedImmune; Dr K. Duffy, Dr P. Duncan, Dr L. Plitnick, Dr L. Vromans, Dr J. Wolf, Merck; Dr R.W. Fedechko, Dr S. Pluschkell, Pfizer; Dr L. Scheppler, Crucell; Dr F. Mortiaux, GlaxoSmithKline Biologicals; Dr B. Mouterde, Sanofi Pasteur; Dr R. Krause, IFPMA); Dr J. Robertson, National Institute for Biological Standards and Control, Potters Bar, England; Parenteral Drug Association (Dr A. Cundell, Schering-Plough; Dr J-P. Gregersen, Novartis; Dr L. Hayflick, University of California San Francisco; Dr L. Hendricks, Centocor; Dr A. Khan, Center for Biologics Evaluation and Research; Dr R.W. Kozak, Bayer; Dr Z. Liu, Schering-Plough; Dr B. Potts, Consultant; Dr M. Ruffing, Boehringer-Ingelheim; Dr S. Seaver, Seaver Associates LLC; Dr D. Vacante, Centocor; Dr H. Willkommen, Regulatory Affairs and Biological Safety Consulting; Dr M. Wisher, BioReliance; Dr R. Wolff, Biologics Consulting Group); Dr K. Brusselmans, Dr G. Waeterloos, Scientific Institute of Public Health, Brussels, Belgium.

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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 will 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 will 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 to the component units of each batch. The irradiation dose must be low enough for the biological properties of the reagents to be 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 such as BVDV and bovine polyomavirus (1), although this may represent contamination of the pool by one viraemic animal.

Other viruses are sporadic contaminants and may be regionally restricted, such as Cache Valley virus, BTV and epizootic haemorrhagic disease virus. In

some cases, contamination has been reported only on a few occasions, as in the case of calicivirus 2117 (2).

Application of new methods such as MPS has revealed new viruses, like the 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 neutralized by antibody in the serum pool. It is advisable to set limits for the level of BVDV-neutralizing antibody in serum pools, as this may mask the presence of potentially infectious virus.

There should be awareness of the statistical limits of screening assays in detecting viruses in large serum pools. For example, in an infection of a fermenter by Cache Valley virus, it was estimated that fewer 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 two subpassages of the cells should be undertaken, usually at days 7 and 14. Detection of virus infection involves regular examination for the development of a CPE, haemadsorption assays and immunofluorescence (or other appropriate immunological detection method) for specific viruses. Immunofluorescence is particularly important for the detection of BVDV, as many isolates are non-cytopathic. At the end of the assay, cytological staining (e.g. with Giemsa stain) is used to reveal viral inclusions and other CPEs that were 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. MDCK cells or bovine turbinate cells are often used, and it is also of value to include additional cells such as Vero cells.

The assay should be capable of detecting the following: BVDV, BPIV3, BPV1, rabies virus, REO3, IBR, BRSV, BTV, bovine adenovirus 5 (BAV5), and vesicular stomatitis virus. 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 75 cm² bottles containing the indicator cells and a total of ~250 ml of test serum, allowing for serum used during refeeding of 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 $\geq 70\%$ confluent. The cells are subcultured into 25 cm² flasks (for immunofluorescence) and six-well plates (for haemadsorption and cytological staining).

The following day, the remaining negative-control and test article cells are subcultured to 75 cm² flasks for immunofluorescence and to six-well plates for haemadsorption and cytological staining.

Infection with positive controls

Coincident with the final subculture of test article and negative-control cultures, flasks of bovine turbinate cells are inoculated with the immunofluorescence positive control viruses BVDV, BAV5, BPV, BTV, BRSV, IBR and BPIV3. Plates of bovine turbinate cells are inoculated with BPIV3, the positive control for haemadsorption, and with cytopathic BVDV, the positive control for cytological staining. Likewise, Vero flasks are inoculated with REO3, the immunofluorescence positive control, and plates are inoculated with BPIV3, the haemadsorption and cytological staining positive control. All immunofluorescence positive-control viruses should be inoculated at 100–300 TCID₅₀ (median tissue culture infective dose).

Analysis

After a minimum of 21 days after 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 haemadsorption and fixed for immunofluorescence and cytological staining. Cells from the positive-control flasks are transferred to multiwell slides and fixed for immunofluorescence when CPE involving $\geq 10\%$ of the monolayer is observed, and stored at $\leq -60^\circ\text{C}$. Cells in the positive-control six-well plates are assayed for haemadsorption and cytological staining 7 days after inoculation, or when CPE is apparent. Haemadsorption involves testing at least one six-well plate with chicken and guinea-pig erythrocytes at 2–8 °C and at 20–25 °C.

Nucleic acid amplification assays for viruses

Nucleic acid amplification technologies such as 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. 25–50 ml) 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 NAT, by determining whether 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 to an 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 NAT end-point or immunological end-point such as 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

Tumorigenicity protocol using athymic nude mice to assess mammalian cells

During the characterization of an MCB (or WCB), the cells should be examined for tumorigenicity in a test approved by the NRA or the NCL.

The following model protocol is provided to assist manufacturers and NRAs/NCLs to standardize the tumorigenicity testing procedure so that the interpretation and comparability of data between 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 often occurs. Therefore, the use of only female mice should be considered.

2. Test article cells

Cells from the MCB or WCB that have been propagated to at least three population doublings beyond the limit for production are examined for tumorigenicity.

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 American Type Culture Collection (USA) and the National Institute for Biological Standards and Control (England).

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 tumorigenicity 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 tumours should be produced in at least 9 out 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, below), suspended in a volume of 0.1 ml PBS.

Cell culture medium 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 tumorigenicity 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 or the subcutaneous route. If the intramuscular route is selected, the cells should be injected into the thigh of one leg. If the subcutaneous route is selected, the cells should be injected into the supraclavicular region of the trunk.

On the basis of findings of 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 minimum of 16 weeks (i.e. 4 months) for evidence of nodule formation at the site of injection when the route of inoculation is intramuscular or subcutaneous. Examinations need not be more frequent than two times a week for the first 3–6 weeks, and once a week thereafter.

In some countries, the observation period is 4–7 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 killed until the end of the observation period. Cell lines that produce nodules that fail to grow progressively are not considered to be tumorigenic.

If a nodule fails to grow progressively but persists during the observation period and 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 tumorigenicity of the cell substrate.

If the cells that are injected fail to form tumours or to persist during the 4-month observation period, it may 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 tumorigenicity 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 killed and examined for gross and microscopic evidence of the proliferation of inoculated cells at the site of injection and at other sites such as the heart, lungs, liver, spleen, kidneys, brain and regional lymph nodes, since some CCLs may give rise to tumours at distant sites without evidence of tumour at the injection site. The tissues are fixed in 10% formol saline and sections are stained with haematoxylin and eosin for histological examination to determine whether there is evidence of tumour 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 tumour. If what appears to be a metastasis to a distant site differs histopathologically from the primary tumour, consideration should be given to the possibility that the tumour either developed spontaneously or 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 tumours 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 tumours 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 out of 10 animals inoculated with the test article cells develop tumours that meet the following two criteria:
 - i. Tumours appear at the site of inoculation or at a metastatic site.
 - ii. Histological or genotypic examination reveals that the nature of the cells constituting the tumours is consistent with that of the inoculated cells.

In the past, chromosomal markers have been useful to demonstrate that the tumour 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 1 out of 10 animals develops a tumour that meets the two criteria in 11.a, the cell line should be considered to be possibly tumorigenic and should be examined further. Such testing could include one or more of the following: repeating the test in an additional 10 nude mice, extending the observation period, increasing the size of the inoculum, or 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 whether the tumour 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 out of 10 animals develop tumours 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. The point in cells' life history 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 tumours 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 may not be suitable, both because of the nature of the assay and because DNA may 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 to 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 tumorigenicity test can be 10 per group, the number in an oncogenicity test should be larger, owing 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 that 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, at the end of the observation period, there is no evidence of a progressively growing tumour at the site of inoculation or at distant sites, 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 arises in a tumorigenicity assay will be that of the cell substrate, while the species of tumours that arises 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 $\geq 100 \mu\text{g}$ in 50–100 μl . Because of the concentrations necessary to achieve $\geq 100 \mu\text{g}$ of DNA, it may 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 last 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 killed when the nodule reaches a size of approximately 2 cm in diameter, unless a lower limit has been established by the 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 killed and examined for gross and microscopic evidence of tumour formation at the site of injection and at 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, 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.

Applicability

- *Cell banks*: MCB or WCB taken beyond EOPC level/ECB
- *Cell types*: CCL, SCL (recommended when tumorigenic cells are used in vaccine production)

References

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