| Micro-organism | Type of test |
|---|---|
| Newcastle disease virus | Haemagglutination inhibition |
| Turkey rhinotracheitis virus | Enzyme-linked immuno-sorbent assay |
| *Mycoplasma gallisepticum* | Agglutination and, to confirm a positive test, haemagglutination inhibition |
| *Mycoplasma synoviae* | Agglutination and, to confirm a positive test, haemagglutination inhibition |
| *Salmonella pullorum* | Agglutination |

SUBSEQUENT TESTING

*Subject to agreement by the competent authority, other types of test may be used provided they are at least as sensitive as those indicated and of appropriate specificity.*

| Micro-organism | Type of test |
|---|---|
| Avian adenoviruses | Enzyme-linked immuno-sorbent assay |
| Avian encephalomyelitis virus | Enzyme-linked immuno-sorbent assay |
| Avian infectious bronchitis virus | Enzyme-linked immuno-sorbent assay |
| Avian infectious laryngotracheitis virus | Serum neutralisation |
| Avian leucosis viruses | Enzyme-linked immuno-sorbent assay for the antibody |
| Avian nephritis virus | Fluorescent antibody |
| Avian reoviruses | Fluorescent antibody |
| Avian reticuloendotheliosis virus | Fluorescent antibody |
| Chick anaemia agent | Fluorescent antibody |
| Haemagglutinating avian adenovirus | Haemagglutination inhibition |
| Infectious bursal disease virus | Immunodiffusion against each serotype present in the country of origin |
| Influenza A virus | Enzyme-linked immuno-sorbent assay |
| Marek's disease virus | Enzyme-linked immuno-sorbent assay |
| Newcastle disease virus | Haemagglutination inhibition |
| Turkey rhinotracheitis virus | Enzyme-linked immuno-sorbent assay |
| *Mycoplasma gallisepticum* | Agglutination and, to confirm a positive test, haemagglutination inhibition |
| *Mycoplasma synoviae* | Agglutination and, to confirm a positive test, haemagglutination inhibition |
| *Salmonella pullorum* | Agglutination |

**01/2005:50203**

# 5.2.3. CELL SUBSTRATES FOR THE PRODUCTION OF VACCINES FOR HUMAN USE

This general chapter deals with diploid cell lines and continuous cell lines used for the production of vaccines for human use; specific issues relating to vaccines prepared by recombinant DNA technology are covered by the monograph on *Products of recombinant DNA technology (0784)*. Testing to be carried out at various stages (cell seed, master cell bank, working cell bank, cells at or beyond the maximum population doubling level used for production) is indicated in Table 5.2.3.-1. General provisions for the use of cell lines and test methods are given below. Where primary cells or cells that have undergone a few passages without constitution of a cell bank are used for vaccine production, requirements are given in the individual monograph for the vaccine concerned.

**Diploid cell lines**. A diploid cell line has a high but finite capacity for multiplication *in vitro*.

**Continuous cell lines**. A continuous cell line has the capacity to multiply indefinitely *in vitro*; the cells often have differences in karyotype compared to the original cells; they may be obtained from healthy or tumoral tissue.

For injectable vaccines produced in continuous cell lines, the purification process is validated to demonstrate removal of substrate-cell DNA to a level equivalent to not more than 10 ng per single human dose, unless otherwise prescribed.

**Cell-bank system**. Production of vaccines in diploid and continuous cell lines is based on a cell-bank system. The *in vitro* age of the cells is counted from the master cell bank. Each working cell bank is prepared from one or more containers of the master cell bank. The use, identity and inventory control of the containers is carefully documented.

**Media and substances of animal and human origin**. The composition of media used for isolation and all subsequent culture is recorded in detail and if substances of animal origin are used they must be free from extraneous agents.

If human albumin is used, it complies with the monograph on *Human albumin solution (0255)*.

Bovine serum used for the preparation and maintenance of cell cultures is tested and shown to be sterile and free from bovine viruses, notably bovine diarrhoea virus and mycoplasmas.

Trypsin used for the preparation of cell cultures is examined by suitable methods and shown to be sterile and free from mycoplasmas and viruses, notably pestiviruses and parvoviruses.

**Cell seed**. The data used to assess the suitability of the cell seed comprise information, where available, on source, history and characterisation.

*Source of the cell seed.* For human cell lines, the following information concerning the donor is recorded: ethnic and geographical origin, age, sex, general physiological condition, tissue or organ used, results of any tests for pathogens.

For animal cell lines, the following information is recorded concerning the source of the cells: species, strain, breeding conditions, geographical origin, age, sex, general physiological condition, tissue or organ used, results of any tests for pathogens.

Cells of neural origin, such as neuroblastoma and P12 cell lines, may contain substances that concentrate agents of spongiform encephalopathies and such cells are not used for vaccine production.

*History of the cell seed.* The following information is recorded: the method used to isolate the cell seed, culture methods and any other procedures used to establish the master cell bank, notably any that might expose the cells to extraneous agents.

Full information may not be available on the ingredients of media used in the past for cultivation of cells, for example on the source of substances of animal origin; where justified and authorised, cell banks already established using such media may be used for vaccine production.

*Characterisation of the cell seed.* The following properties are investigated:

(1) the identity of the cells (for example, isoenzymes, serology, nucleic acid fingerprinting);

Table 5.2.3.-1 – *Testing of cell lines*

| Test | Cell seed | Master cell bank (MCB) | Working cell bank (WCB) | Cells at or beyond the maximum population doubling level used for production |
|---|---|---|---|---|
| | 1. IDENTITY AND PURITY | | | |
| Morphology | + | + | + | + |
| Relevant selection of the following tests: biochemical (e.g. isoenzymes), immunological (e.g. histocompatibility), cytogenetic markers, nucleic acid fingerprinting | + | + | + | + |
| Karyotype (diploid cell lines) | + | + | +(1) | +(1) |
| Life span (diploid cell lines) | – | + | + | – |
| | 2. EXTRANEOUS AGENTS | | | |
| Bacterial and fungal contamination | – | + | + | – |
| Mycoplasmas | – | + | + | – |
| Tests in cell cultures | – | – | + | – |
| Co-cultivation | – | – | +(2) | +(2) |
| Tests in animals and eggs | – | – | +(2) | +(2) |
| Specific tests for possible contaminants depending on the origin of the cells (see above under Infectious extraneous agents) | – | – | +(2) | +(2) |
| Retroviruses | – | +(3) | – | +(3) |
| | 3. TUMORIGENICITY | | | |
| Tumorigenicity | – | – | – | +(4) |

(1) The diploid character is established for each working cell bank but using cells at or beyond the maximum population doubling level used for production.

(2) Testing is carried out for each working cell bank, but using cells at or beyond the maximum population doubling level used for production.

(3) Testing is carried out for the master cell bank, but using cells at or beyond the maximum population doubling level used for production.

(4) The MRC-5 cell line, the WI-38 cell line and the FRhL-2 cell line are recognised as being non-tumorigenic and they need not be tested. Tests are not carried out on cell lines that are known or assumed to be tumorigenic.

(2) the growth characteristics of the cells and their morphological properties (light and electron microscopes);

(3) for diploid cell lines, karyotype;

(4) for diploid cell lines, the *in vitro* life span in terms of population doubling level.

**Cell substrate stability**. Suitable viability of the cell line in the intended storage conditions must be demonstrated. For a given product to be prepared in the cell line, it is necessary to demonstrate that consistent production can be obtained with cells at passage levels at the beginning and end of the intended span of use.

**Infectious extraneous agents**. Cell lines for vaccine production shall be free from infectious extraneous agents. Tests for extraneous agents are carried out as shown in Table 5.2.3.-1.

Depending on the origin and culture history of the cell line, it may be necessary to carry out tests for selected, specific potential contaminants, particularly those that are known to infect latently the species of origin, for example simian virus 40 in rhesus monkeys. For cell lines of rodent origin, antibody-production tests are carried out in mice, rats and hamsters to detect species-specific viruses.

Cell lines are examined for the presence of retroviruses as described below. Cell lines that show the presence of retroviruses capable of replication are not acceptable for production of vaccines.

**Tumorigenicity**. For the preparation of live vaccines, the cell line must not be tumorigenic at any population doubling level used for vaccine production. Where a tumorigenic cell line is used for the production of other types of vaccine, the purification process is validated to demonstrate that residual substrate-cell DNA is reduced to less than 10 ng per single human dose of the vaccine, unless otherwise prescribed, and that substrate-cell protein is reduced to an acceptable level.

A cell line which is known to have tumorigenic potential does not have to be tested further. If a cell line is of unknown tumorigenic potential, it is either regarded as being tumorigenic or it is tested for tumorigenicity using an *in vitro* test as described below; if the result of the *in vitro* test is negative or not clearly positive, an *in vivo* test as described below is carried out. The tests are carried out using cells at or beyond the maximum population doubling level that will be used for vaccine production.

The MRC-5, the WI-38 and the FRhL-2 cell lines are recognised as being non-tumorigenic and further testing is not necessary.

**Chromosomal characterisation**. Diploid cell lines shall be shown to be diploid. More extensive characterisation of a diploid cell line by karyotype analysis is required if the removal of intact cells during processing after harvest has not been validated. Samples from four passage levels evenly spaced over the life-span of the cell line are examined. A minimum of 200 cells in metaphase are examined for exact count of chromosomes and for frequency of hyperploidy, hypoploidy, polyploidy, breaks and structural abnormalities.

The MRC-5, the WI-38 and the FRhL-2 cell lines are recognised as being diploid and well characterised; where they are not genetically modified, further characterisation is not necessary.

TEST METHODS FOR CELL CULTURES

**Identification**. Nucleic-acid-fingerprint analysis and a relevant selection of the following are used to establish the identity of the cells:

(1) biochemical characteristics (isoenzyme analysis),

(2) immunological characteristics (histocompatibility antigens),

(3) cytogenetic markers.

**Contaminating cells**. The nucleic-acid-fingerprint analysis carried out for identification also serves to demonstrate freedom from contaminating cells.

**Bacterial and fungal contamination**. The master cell bank and each working cell bank comply with the test for sterility (*2.6.1*), carried out using for each medium 10 ml of supernatant fluid from cell cultures. Carry out the test on 1 per cent of the containers with a minimum of two containers.

**Mycoplasmas** (*2.6.7*). The master cell bank and each working cell bank comply with the test for mycoplasmas by the culture method and the indicator cell culture method. Use one or more containers for the test.

**Test for extraneous agents in cell cultures**. The cells comply with the test for haemadsorbing viruses and with the tests in cell cultures for other extraneous agents given in chapter *2.6.16* under Production cell culture: control cells. If the cells are of simian origin, they are also inoculated into rabbit kidney cell cultures to test for herpesvirus B (cercopithecid herpesvirus 1).

**Co-cultivation**. Co-cultivate intact and disrupted cells separately with other cell systems including human cells and simian cells. Carry out examinations to detect possible morphological changes. Carry out tests on the cell culture fluids to detect haemagglutinating viruses. The cells comply with the test if no evidence of any extraneous agent is found.

**Retroviruses**. Examine for the presence of retroviruses using:

(1) infectivity assays,

(2) transmission electron microscopy,

(3) if tests (1) and (2) give negative results, reverse transcriptase assays (in the presence of magnesium and manganese) carried out on pellets obtained by high-speed centrifugation.

**Tests in animals**. Inject intramuscularly (or, for suckling mice, subcutaneously) into each of the following groups of animals $10^7$ viable cells divided equally between the animals in each group:

(1) two litters of suckling mice less than 24 h old, comprising not fewer than ten animals,

(2) ten adult mice.

Inject intracerebrally into each of ten adult mice $10^6$ viable cells to detect the possible presence of lymphocytic choriomeningitis virus.

Observe the animals for at least 4 weeks. Investigate animals that become sick or show any abnormality to establish the cause of illness. The cells comply with the test if no evidence of any extraneous agent is found. The test is invalid if fewer than 80 per cent of the animals in each group remain healthy and survive to the end of the observation period.

For cells obtained from a rodent species (for example, Chinese hamster ovary cells or baby hamster kidney cells), tests for antibodies against likely viral contaminants of the species in question are carried out on animals that have received injections of the cells.

**Tests in eggs**. Using an inoculum of $10^6$ viable cells per egg, inoculate the cells into the allantoic cavity of ten SPF embryonated hens' eggs (*5.2.2*) 9 to 11 days old and into the yolk sac of ten SPF embryonated hens' eggs 5 to 6 days old. Incubate for not less than 5 days. Test the allantoic fluids for the presence of haemagglutinins using mammalian and avian red blood cells; carry out the test at 5 ± 3 °C and 20-25 °C and read the results after 30 min and 60 min. The cells comply with the test if no evidence of any extraneous agent is found. The test is invalid if fewer than 80 per cent of the embryos remain healthy and survive to the end of the observation period.

**Tests for tumorigenicity *in vitro***. The following test systems may be used:

(1) colony formation in soft agar gels,

(2) production of invasive cell growth following inoculation into organ cultures,

(3) study of transformation activity using, for example, the 3T3 assay system for active oncogenes.

**Tests for tumorigenicity *in vivo***. The test consists in establishing a comparison between the cell line and a suitable positive control (for example, HeLa or Hep2 cells).

Animal systems that have been shown to be suitable for this test include:

(1) athymic mice (Nu/Nu genotype),

(2) newborn mice, rats or hamsters that have been treated with antithymocyte serum or globulin,

(3) thymectomised and irradiated mice that have been reconstituted ($T^-$, $B^+$) with bone marrow from healthy mice.

Whichever animal system is selected, the cell line and the reference cells are injected into separate groups of 10 animals each. In both cases, the inoculum for each animal is $10^7$ cells suspended in a volume of 0.2 ml, and the injection may be by either the intramuscular or subcutaneous route. Newborn animals are treated with 0.1 ml of antithymocyte serum or globulin on days 0, 2, 7 and 14 after birth. A potent serum or globulin is one that suppresses the immune mechanisms of growing animals to the extent that the subsequent inoculum of $10^7$ positive reference cells regularly produces tumours and metastases. Severely affected animals showing evident progressively growing tumours are killed before the end of the test to avoid unnecessary suffering.

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 proliferation of inoculated cells at the site of injection and in other organs (for example lymph nodes, lungs, kidneys and liver).

In all test systems, the animals are observed and palpated at regular intervals for the formation of nodules at the sites of injection. Any nodules formed are measured in two perpendicular dimensions, the measurements being recorded regularly to determine whether there is progressive growth of the nodule. Animals showing nodules which begin to regress during the period of observation are killed before the nodules are no longer palpable, and processed for histological examination. Animals with progressively growing nodules are observed for 1-2 weeks. Among those without nodule formation, half are observed for 3 weeks and half for 12 weeks before they are killed and processed for histological examination. A necropsy is performed on each animal and includes examination for gross evidence of tumour formation at the site of injection and in other organs such as lymph nodes, lungs, brain, spleen, kidneys and liver. All tumour-like lesions and the site of injection are examined histologically. In addition, since some cell lines may give rise

to metastases without evidence of local tumour growth, any detectable regional lymph nodes and the lungs of all animals are examined histologically.

The test is invalid if fewer than nine of ten animals injected with the positive reference cells show progressively growing tumours.

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# 5.2.4. CELL CULTURES FOR THE PRODUCTION OF VETERINARY VACCINES

Cell cultures for the production of vaccines for veterinary use comply with the requirements of this section. It may also be necessary that cell cultures used for testing of vaccines for veterinary use also comply with some or all of these requirements.

For most mammalian viruses, propagation in cell lines is possible and the use of primary cells is then not acceptable.

Permanently infected cells used for production of veterinary vaccines comply with the appropriate requirements described below. The cells shall be shown to be infected only with the agent stated.



CELL LINES

Cell lines are normally handled according to a cell-seed system. Each master cell seed is assigned a specific code for identification purposes. The master cell seed is stored in aliquots at − 70 °C or lower. Production of vaccine is not normally undertaken on cells more than twenty passages from the master cell seed. Where suspension cultures are used, an increase in cell numbers equivalent to approximately three population doublings is considered equivalent to one passage. If cells beyond twenty passage levels are to be used for production, it shall be demonstrated, by validation or further testing, that the production cell cultures are essentially similar to the master cell seed with regard to their biological characteristics and purity and that the use of such cells has no deleterious effect on vaccine production.

The history of the cell line shall be known and recorded in detail (for example, origin, number of passages and media used for multiplication, storage conditions).

The method of storing and using the cells, including details of how it is ensured that the maximum number of passages permitted is not exceeded during product manufacture, are recorded. A sufficient quantity of the master cell seed and each working cell seed are kept for analytical purposes.

The tests described below are carried out (as prescribed in Table 5.2.4.-1) on a culture of the master cell seed and the working cell seed or on cell cultures from the working cell seed at the highest passage level used for production and derived from a homogeneous sample demonstrated to be representative.

**Characteristics of culture**. The appearance of cell monolayers, before and after histological staining, is described. Information, if possible numerical data, is provided especially on the speed and rate of growth. Similarly, the presence or absence of contact inhibition, polynucleated cells and any other cellular abnormalities are specified.

**Karyotype**. A chromosomal examination is made of not fewer than fifty cells undergoing mitosis in the master cell seed and at a passage level at least as high as that to be used in production. Any chromosomal marker present in the master cell seed must also be found in the high passage cells and the modal number of chromosomes in these cells must not be more than 15 per cent higher than of cells of the master cell seed. The karyotypes must be identical. If the modal number exceeds the level stated, if the chromosomal markers are not found in the working cell seed at the highest level used for production or if the karyotype differs, the cell line shall not be used for manufacture.

Table 5.2.4.-1. − *Cell culture stage at which tests are carried out*

| | Master cell seed | Working cell seed | Cell from working cell seed at highest passage level |
|---|---|---|---|
| General microscopy | + | + | + |
| Bacteria and fungi | + | + | − |
| Mycoplasmas | + | + | − |
| Viruses | + | + | − |
| Identification of species | + | − | + |
| Karyotype | + | − | + |
| Tumorigenicity | + | − | − |

**Identification of the species**. It shall be shown, by one validated method, that the master cell seed and the cells from the working cell seed at the highest passage level used for production come from the species of origin specified. When a fluorescence test is carried out and the corresponding serum to the species of origin of cells is used and shows that all the tested cells are fluorescent, it is not necessary to carry out other tests with reagents able to detect contamination by cells of other species.

**Bacterial and fungal contamination**. The cells comply with the test for sterility (*2.6.1*). The sample of cells to be examined consists of not less than the number of cells in a monolayer with an area of 70 $cm^2$ or, for cells grown in suspension, an approximately equivalent number of cells. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

**Mycoplasmas** (*2.6.7*). The cells comply with the test for mycoplasmas. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

**Absence of contaminating viruses**. The cells must not be contaminated by viruses; suitably sensitive tests, including those prescribed below, are carried out.

The monolayers tested shall have an area of at least 70 $cm^2$, and shall be prepared and maintained using medium and additives, and grown under similar conditions to those used for the preparation of the vaccine. The monolayers are maintained in culture for a total of at least 28 days. Subcultures are made at 7-day intervals, unless the cells do not survive for this length of time, when the subcultures are made on the latest day possible. Sufficient cells, in suitable containers, are produced for the final subculture to carry out the tests specified below.

The monolayers are examined regularly throughout the incubation period for the possible presence of cytopathic effects and at the end of the observation period for cytopathic effects, haemadsorbent viruses and specific viruses by immuno-fluorescence and other suitable tests as indicated below.

**Detection of cytopathic viruses**. Two monolayers of at least 6 $cm^2$ each are stained with an appropriate cytological stain. The entire area of each stained monolayer is examined for any inclusion bodies, abnormal numbers of giant cells or any other lesion indicative of a cellular abnormality which might be attributable to a contaminant.