

## Annex 3

### **General requirements for the sterility of biological substances**

(Requirements for Biological Substances No. 6, revised 1973, amendment 1995)

#### **Introduction**

Since the General Requirements for the Sterility of Biological Substances were revised in 1973 (1), several developments have occurred. It is thus appropriate to modify as follows the existing General Requirements for the Sterility of Biological Substances.

#### **Sterility test for mycoplasmas** (section 5.3, page 52)

*Replace* the text of section 5.3 by the following:

“The test for mycoplasmas is carried out by culture in agar and broth or by means of an indicator cell culture and DNA staining.

Where a test for mycoplasmas is prescribed for a master cell bank, for a working cell bank, for a virus seed lot (or bank) or for cell cultures used as controls, the standard culture or the indicator cell-culture method can be used. Where a test for mycoplasmas is prescribed for a virus harvest, bulk vaccine or final lot, the standard culture method is used. The indicator cell-culture method can be used, where necessary, for the screening of media. Both the standard culture and indicator cell-culture methods are given in Appendix 3. Alternative methods may be used provided that they have been validated against the methods described here.”

#### **Tests for mycoplasmas** (Appendix 3, p. 56)

*Replace* the text of Appendix 3 by the following:

##### **“Standard culture method**

##### *Choice of culture media*

The test is carried out with a sufficient number of both solid and liquid media to ensure the growth, under the chosen incubation conditions, of small numbers of organisms that may be present in the product being examined. Liquid media must contain phenol red dye. The

ability of the media to support the growth of at least the following organisms shall be demonstrated:

*Mycoplasma orale* (vaccines for human use);

*Mycoplasma pneumoniae* (vaccines for human use);

*Mycoplasma hyorhinis* (non-avian veterinary vaccines, e.g. strain DBS 1050);

*Mycoplasma hyopneumoniae* (non-avian veterinary vaccines);

*Mycoplasma gallisepticum* and *Mycoplasma synoviae* (where avian material has been used during vaccine production or where the vaccine is intended for use in poultry).

Low-passage test strains are used and are stored frozen or freeze-dried. After cloning, the strains are identified as being of the required species by a suitable method.

In some countries, specific tests for insect mycoplasmas are performed.

#### *Incubation conditions*

Divide inoculated media into two equal parts; incubate one in aerobic conditions (an atmosphere of air containing 5–10% carbon dioxide and adequate humidity) and the other in anaerobic conditions (an atmosphere of nitrogen containing 5–10% carbon dioxide and adequate humidity).

#### *Demonstration of nutritive properties of media*

Inoculate the media with the appropriate test organisms; use no fewer than 200 and no more than 400 colony-forming units per plate of solid medium and no fewer than 20 and no more than 40 colony-forming units per container of the corresponding liquid medium; use a separate plate and container for each species. Incubate the media under the conditions that will be used for testing the product to be examined (aerobically, anaerobically or both, depending on the requirements of the test organism). The media comply with the test for nutritive properties if there is adequate growth of the test organisms (solid media) and an appropriate colour change (liquid media).

#### *Test for inhibitory substances*

Carry out the test for nutritive properties in the presence of the product to be examined. If growth of the test organisms is notably less than that found otherwise, the product contains inhibitory substances that must be neutralized (e.g. by dilution) before the test for mycoplasmas is carried out. The effectiveness of the neutralization process

is checked by repeating the test for inhibitory substances after neutralization.

#### *Test for mycoplasmas*

For solid media, use plates 60mm in diameter containing 9ml of medium. Inoculate each of at least 2 plates of each solid medium with 0.2ml of the product to be examined and inoculate at least 2 of each 100ml of liquid medium with 10ml of the product to be examined. If any significant pH change occurs when the product to be examined is added to the liquid media, restore the affected media to their original pH by the addition of a solution of either sodium hydroxide or hydrochloric acid. Incubate paired media at 35–38°C, one aerobically and one anaerobically, for 21 days and for the same length of time incubate an uninoculated 100-ml portion of each liquid medium as a control. On the first, second or third day after inoculation, make a subculture of each inoculated liquid medium by inoculating each of two fresh plates of each solid medium with 0.2ml of the product to be examined and incubating at 35–38°C, one aerobically and one anaerobically, for no less than 21 days. Repeat the procedure on the sixth, seventh or eighth day and again on the thirteenth or fourteenth day of the test. Inspect the liquid media every 2–3 days, and if any colour change occurs prepare a subculture as described above immediately. Inspect solid media once per week.

If any containers of liquid media show bacterial or fungal contamination, repeat the test. If at least 7 days after inoculation, no more than 1 plate of solid medium at each stage of the test has been contaminated with bacteria or fungi, or broken, that plate may be ignored provided that on immediate examination it shows no evidence of mycoplasmal growth. If, at any stage of the test, more than one plate is contaminated with bacteria or fungi, or broken, the test is invalid and must be repeated.

At the end of each incubation period, microscopically examine inoculated solid media for the presence of mycoplasmas. The product passes the test if growth of mycoplasmas has not occurred in any of the inoculated media. If growth of mycoplasmas has occurred, the test may be repeated once with twice the amount of inoculum and media; if growth of mycoplasmas does not occur when the test is repeated, the product passes the test.

#### ***“Indicator cell-culture method***

For the indicator cell-culture method, cell cultures are stained with a fluorescent dye that binds specifically to DNA. Mycoplasmas are

detected by their characteristic particulate or filamentous pattern of fluorescence on cell surfaces and, if contamination is heavy, in surrounding areas.

#### *Verification of the substrate*

With a Vero-cell substrate, pretest the procedure with an inoculum of no more than 100 colony-forming units (CFU) of a strain growing readily in liquid or solid media and demonstrate its ability to detect potential mycoplasma contaminants such as *M. hyorhinis* strain DBS 1050 and *M. orale* strain 1596 or other suitable strains. A different cell substrate may be used, e.g. the vaccine-production cell line, if it has been demonstrated that it provides at least equal sensitivity for the detection of mycoplasma contamination.

#### *Test for mycoplasmas*

Take no less than 1 ml of the material to be examined and use it to inoculate 2 or more indicator cell cultures grown to 50% confluence and representing no less than 25 cm<sup>2</sup> of cell culture in area; make at least one passage. The product of the last passage is incubated on coverslips placed in suitable containers or on some other suitable surface for the test procedure.

Include in the test a negative (non-inoculated) control and two positive controls inoculated with species such as *M. hyorhinis* and *M. orale*. Use an inoculum of not more than 100 CFU for the positive controls.

If for viral suspensions the interpretation of results is obscured by cytopathic effects, the virus may be neutralized by means of a specific antiserum that has no inhibitory effects on mycoplasmas. A cell culture substrate that does not allow growth of the virus may also be used.

#### *Procedure*

- (a) Seed the culture at a regular density ( $2 \times 10^4$  to  $2 \times 10^5$  cells per ml,  $4 \times 10^3$ – $2.5 \times 10^4$  cells/cm<sup>2</sup>) and incubate at  $36 \pm 1$  °C until about 50% confluence is reached. Inoculate the product to be examined and incubate to confluence.
- (b) Remove and discard the medium.
- (c) Rinse the monolayer with phosphate-buffered saline at pH 7.4, then with a mixture of equal volumes of phosphate-buffered saline at pH 7.4 and methanol, and finally with methanol.
- (d) Add methanol and allow to stand for 10 minutes.
- (e) Remove the methanol and discard.

- (f) If the monolayer is to be stained later, dry it completely.
- (g) If the monolayer is to be stained immediately, rinse it with sterile water and discard the wash.
- (h) Add working solution of bisbenzimidazole or some other suitable DNA staining agent and allow to stand for 10 min.
- (i) Remove the stain and rinse the monolayer with sterile water.
- (j) Mount each coverslip with a drop of a mixture of equal volumes of glycerol and phosphate–citrate buffer solution at pH 5.5; remove excess mountant from the edge of the coverslip by blotting.
- (k) Examine by epifluorescence (330 nm/380 nm excitation filter, LP 440 nm barrier filter) at 100–400× magnification or greater.
- (l) Compare the microscopic appearance of the test cultures with that of the negative and positive controls.

The product to be examined passes the test if there is no evidence of the presence of mycoplasmas in the test cultures inoculated with it.

#### *Reagents*

*Bisbenzimidazole* ( $C_{25}H_{27}Cl_3N_6O \cdot 5H_2O$  ( $M_r$  624)) 4-[5-[5-(4-methylpiperazin-1-yl)benzimidazol-2-yl]benzimidazol-2-yl]phenol trihydrochloride pentahydrate.

*Bisbenzimidazole stock solution:* To prepare stock solution, dissolve 5 mg of bisbenzimidazole in sterile water and dilute to 100 ml with the same solvent. Store in the dark.

*Bisbenzimidazole working solution:* To prepare working solution, dilute 10 µl of bisbenzimidazole stock solution to 100 ml with phosphate-buffered saline at pH 7.4. Use immediately.

*Phosphate–citrate buffer solution, pH 5.5:* To prepare buffer, mix 56.85 ml of a 2.84% (28.4 g/l) solution of anhydrous disodium hydrogen phosphate and 43.15 ml of a 2.1% (21 g/l) solution of citric acid.”

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## Reference

General Requirements for the Sterility of Biological Substances (Requirements for Biological Substances No. 6, revised 1973). In: *WHO Expert Committee on Biological Standardization. Twenty-fifth Report*. Geneva, World Health Organization, 1973, Annex 4 (WHO Technical Report Series, No. 530).