## Annex 4

# GENERAL REQUIREMENTS FOR THE STERILITY OF BIOLOGICAL SUBSTANCES

## (Requirements for Biological Substances No. 6) (Revised 1973) [1]



[1] These revised requirements have been derived from the document entitled "Sterility and Sterility Testing of Pharmaceutical Preparations and Biological Substances" (unpublished working document WHO/BS/73.1062 ; WHO/PHARM/73.474) which was prepared for consideration of the WHO Expert Committee on Biological Standardization and of the WHO Expert Committee on Specifications for Pharmaceutical Preparations ; additional information was obtained from a number of other sources. The names of those who prepared the original unpublished working document and the names of those who have submitted suggestions and comments to date on the document are given in Appendix 4 on p. 57 *et seq.*

## Introduction

General requirements for the sterility of biological substances (Requirements for Biological Substances No. 6) were formulated by a WHO Study Group in 1959.[1] These general requirements were applicable to any biological product from which the exclusion of microbial contamination is imperative, and they have been quoted in all the sets of requirements for individual biological substances that have since been formulated. The twenty-fourth Expert Committee on Biological Standardization [2] agreed that in view of recent developments in methods of sterility testing of biological products and the improvements in control measures that were now feasible, the general requirements for sterility should be revised. This task was therefore undertaken. Since the preparation of a set of amendments or alterations would be an unsatisfactory way of revising the requirements, it was decided to provide a complete document embodying all the requirements concerned. Where provisions formulated by the earlier Study Group were retained unchanged they have been embodied as such in the present revised requirements.

In preparing these revised requirements account has been taken of the opinions of consultants, the relevant regulations and requirements that have been formulated in a number of countries, as well as information from both published and unpublished reports. In addition, opinions and data relevant to these requirements have been received from a number of experts to whom grateful acknowledgement is made (see Appendix 4, page 57).

## General Considerations

The requirements in this document apply to all immunological biological substances, i.e., vaccines and sera, that must be sterile and are used for administration to man. Many of the provisions, however, may be used for the control of sterility of other biological preparations, or of blood and blood products, with suitable modifications, where necessary.

---

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, No. 200. The members of the Study Group were: Dr M. Weis Bentzon, Statens Seruminstitut, Copenhagen, Denmark; Dr P. H. Bonnel, Centre de Transfusion — Réanimation de l'Armée, Seine, France (*Rapporteur*); Dr P. de Góes, Institute of Microbiology, Rio de Janeiro, Brazil; Dr M. Pittman, Division of Biologics Standards, National Institutes of Health, Bethesda, Md., USA (*Chairman*); Dr G. Penso, Laboratory of Microbiology, Istituto Superiore di Sanità, Rome, Italy; Dr R. H. Regamey, Institut d'Hygiène de l'Université, Geneva, Switzerland; Dr Sumiatno, Pasteur Institute, Bandung, Indonesia (*Vice-Chairman*); Dr J. O'H. Tobin, Biological Standards Control Laboratory, Medical Research Council, Hampstead, London, England (*Rapporteur*); Dr G. V. Vygodchikov, N. F. Gamaleja Institute of Epidemiology and Microbiology, Moscow, USSR, *Secretariat*: Dr B. K. Bhattacharya, Medical Officer, Biological Standardization, WHO; Dr N. K. Jerne, Chief Medical Officer, Biological Standardization, WHO, acted as Secretary.

[2] *Wld Hlth Org. techn. Rep. Ser.*, 1972, No. 486, p. 19.

The Study Group on General Requirements for the Sterility of Biological Substances [1] (in 1959) pointed out that confidence in the sterility of biological preparations depended on two important considerations; first, adequate control tests for the sterility of biological preparations in their final containers, using a sufficient number of random samples, and secondly the use of suitable precautions with respect to source materials and manufacturing procedures. The Study Group considered available information on sampling procedures adopted in several countries and also discussed a suggested schedule for sampling of final containers to ensure an acceptably low probability of the release of contaminated final containers.

Theoretically, sterility may be defined as the absence of all micro-organisms capable of multiplying.[2] Sterility tests employing a reasonable number of samples are capable of detecting contamination only in a lot with a high percentage of contaminated units. Accordingly, it is not possible to detect, on the basis of acceptable confidence limits, a low percentage of contaminated units in a homogeneous lot of final product. If the finished product is a single final container of bulk material, then the control measures are applicable on the basis of an assumed homogeneity of material in such a single final container. Sterility, therefore, cannot be assured in the control laboratory, but must be built into the product during processing. Experience in many countries over the years has confirmed that greater reliance must be placed on appropriate techniques and procedures throughout the manufacture of the product (including in-process sterility testing at various stages) rather than simply depending on sterility tests made on a number of samples of the final batch as the sole basis for criteria of sterility. Notwithstanding the limitations of sterility tests, every effort should be made to use the most effective procedure and to strengthen and validate the tests in the light of the most recent knowledge and experience.

The tests for sterility mentioned in the previous general requirements have been revised in the present document, both in nature and in scope. They now include recommendations for test procedures for mycoplasmas and for tests using membrane filtration. Tests for viral contaminants are not dealt with in detail because requirements concerning such contaminants of biological products and appropriate tests for viral sterility have been included in the sets of Requirements for Biological Substances [3] that have been formulated for individual products. Similarly, information on when

---

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, No. 200, p. 4.

[2] In the case of a product consisting of living micro-organisms, e.g., certain vaccines, sterility consists of the absence of contamination by other micro-organisms. Practically, howerer, assertions regarding sterility relate to the probability that all units in the lot are sterile. For example, in some countries, a lot of a product is accepted as sterile if there be no more than one living micro-organism in one million units.

[3] See *Wld Hlth Org. techn. Rep. Ser.*, 1973, No. 530, p. 62, for a complete list.

and where sterility tests are to be made will be found in the requirements for individual products and this question is not discussed in detail in the present document.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in large type have been written in the form of requirements so that, if a health administration so desires, these parts as they appear may be included in definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance.

Should individual countries wish to adopt these requirements as the basis for their national regulations concerning sterility of biological products, it is recommended that a clause be included that would permit modifications of manufacturing requirements on the condition that it be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure a degree of sterility of the product at least equal to that provided by the requirements formulated below. It is desirable that the World Health Organization should then be informed of the action taken.

The terms " national control authority " and " national control laboratory " as used in these requirements, always refer to the country in which the product is manufactured.

## Part A. Manufacturing Requirements

### 1. Terminology

*Contamination :* The presence of live extraneous micro-organisms. The term extraneous applies not only to micro-organisms that may enter the product during processing, but also to those that may be present in the materials used for preparing the product, such as SV40 virus in tissues used to propagate certain viruses for vaccine production. Micro-organisms, as referred to in this document, include bacteria, fungi, viruses, rickettsias, mycoplasmas, and chlamydia.

*Bulk material* is a quantity of partly or wholly processed material, present in a single container at any stage prior to distribution into final containers.

*Final bulk material* is the homogeneous finished preparation present in a single container from which the final containers are filled, either directly or indirectly, through one or more intermediate containers.

In some cases a final bulk may constitute the manufacturer's final product.

*Product lot :* All finished material, in sealed final containers, that has been derived from the same final bulk, all of which, at the last stage of

processing capable of altering its composition, has been processed together and therefore has a uniform composition.

In the case, for example, of a vaccine such a lot may be termed a vaccine lot. The terms product batch and vaccine batch may be used as appropriate.

*Final lot :* A collection of sealed final containers derived from a single final bulk, which are homogeneous with respect to the risk of contamination during filling, sealing and, if applicable, during drying. A final lot must therefore have been filled from a single container, and, if applicable, dried in one continuous operation, e.g., in one working session, the processing being so arranged that each such operation yields a single homogeneous batch of the product.

A final lot may consist of the whole or part of a product lot.

In appropriate cases the terms final batch, filling lot, filling batch or drying lot may be used.

## 2. General precautions against microbial contamination in manufacture

Every manufacturing establishment should in addition have a separate and independent quality audit to which every batch of product would be subjected at the end of processing; the purpose of such an audit would be to ensure that the measures adopted for control of microbial contamination of the product have been carried out satisfactorily.

The general manufacturing requirements given in Part A of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)[1] shall apply which the addition of the following :

The requirements concerning buildings and equipment given in Part A, section 2, of the above requirements are applicable. The most desirable situation might be the provision of separate buildings for each product. This, however, is feasible or necessary only in special circumstances or for very large-scale manufacture. The situation usually encountered is one in which more than one product is manufactured in one building or laboratory.

If the manufacturing process requires that micro-organisms be cultured and processed to yield concentrated suspensions these may become the sources of aerosols or may contaminate equipment, clothing, and personnel. The potential for cross-contamination is high and barriers should be established to prevent contamination of the environment and cross-contamination.

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1966, No. 323, p. 13.

The various measures to avoid contamination are essentially barriers that may be spatial, temporal, or operational. The nature of these barriers depends on the risk of contamination and on the mechanisms by which contamination and cross-contamination may occur, and it will vary with the nature of the potential hazards involved. The barriers may include (*a*) measures to restrict certain organisms and/or operations to separate buildings or separate areas within the one building, (*b*) restriction of the movement of personnel and materials, (*c*) the treatment of air to remove or destroy aerosols containing micro-organisms and to control relative humidity, (*d*) the establishment of pressure gradients to control the direction of air movement, (*e*) the provision of special work stations such as laminar flow stations or biological safety cabinets, and (*f*) wearing of suitable clothing, including head-covering and footwear, by personnel.

If the same area is used for the successive manufacture of different products, the area as well as all equipment it contains shall be adequately cleaned and disinfected after the processing of one product has been completed and before the processing of another product is commenced.

Manufacturing procedures involving the handling of microbial spores shall be confined to a separate building or to a separate area within a building. Those involving live viruses shall be arranged so that at any time each distinct virus is confined to an area that is physically isolated by appropriate barriers. Virus product testing that requires the exclusion of adventitious agents shall also be subjected to this requirement.

The precautions against contamination given in Part A, section 3.4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply.

There should be careful control of the air filtration and routine microbial counts of the air in the manufacturing areas should be carried out during manufacturing operations or periodically using an appropriate method of air sampling. The results of such counts should be checked against criteria, established for the particular manufacturing area, and adequate records of the counts should be maintained. Where air filters are used in manufacturing areas, filters that remove 99.98% ± 0.01% of particles greater than 0.3 µm in diameter are suitable. The efficiency of the filters should be checked routinely.

The recommendations concerning filling and containers given in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [2] shall apply.

---

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1966, No. 323, p. 15.

[2] *Wld Hlth Org. techn. Rep. Ser.*, 1966, No. 323, p. 16.

Filling operations shall be conducted in such a way as to avoid any contamination or alteration of the product.

These operations should take place in controlled areas specially designed for the purpose, in which steps are taken to reduce to a minimum the number of micro-organisms to which the product being filled is exposed. The use of laminar flow work stations, disinfecting agents, air filters, and sterilized clothing, including head-covering and footwear for personnel conducting filling operations, is recommended. Germicidal lamps and disinfecting aerosols should not be used during filling operations.

The operations where liquid preparations are filled should be checked. This may be done, e.g., at least twice each year at the close of a working day, by filling not less than 1000 containers with a nutrient medium containing no antibiotics or bactericidal substances, and incubating the complete batch of filled containers at 30° – 36°C for at least 14 days. If containers filled and incubated in this manner show a contamination rate above 0.3%, some countries do not consider the procedure acceptable for the filling of sterile preparations with or without an antimicrobial agent.

The presence of micro-organisms in the air at the filling point should be monitored, for example, by means of settle plates or slit-sampling devices.

All material from an area in which virus is being processed shall be autoclaved before being sent to the washing and sterilizing departments, irrespective of whether each area has its own facilities or common facilities are employed.

A common washing and sterilization area for glassware and culture media may be used where two or more virus vaccines are being produced, provided that only heat-sterilized materials are issued for use in the production areas and that each production area has its own equipment suitably marked. All media that are sterilized by filtration alone should be prepared in a separate area with its own equipment for the preparation of such media.

In controlled areas, apart from specific organisms or cells being processed, all material introduced from the main washing and sterilization area should preferably be sterilized by heat. It is preferable that autoclaves or chambers used for the sterilization of materials passing in or out of an area be of the " double-ended " type, built into the wall in a suitable manner and subjected to control procedures such that the only passage way for materials between areas containing unsterilized and sterilized materials is through the autoclave or sterilization chamber itself.

Cell cultures for vaccine production shall be prepared in a special area separated from any area in which virus is being handled. Where different

cell lines are handled in an area measures shall be taken to prevent cross-contamination of one cell line with another.

The room and equipment used for filling a live virus vaccine into containers shall be used solely for this particular vaccine, unless it is adequately disinfected when it may be used later for the filling of other products.

When animals are used for obtaining tissues for cell culture, appropriate recommendations should apply.[1]

### 3. General precautions against microbial contamination from materials used for manufacture

Precautions shall be taken to avoid contamination from source or other materials used for manufacture that may affect the sterility of the final product.

All strains of micro-organisms used as seed materials for biological preparations shall be maintained in a manner that will assure freedom from contaminating micro-organisms.

As contamination of a seed lot introduces the possibility of subsequent contamination of a number of production lots, special precautions are necessary when preparing or manipulating seed lots.

All blood and blood-derived source materials shall be obtained under conditions that ensure a safe and effective final product. In the case of human blood material the requirements for source material given in Part A, section 3.1, of the Requirements for Human Immunoglobulin (Requirements for Biological Substances No. 14) [2] shall apply.

Particular attention should be paid to any regulations that are applicable to the manufacturer and/or organization that collects such human material.

### 4. General precautions against microbial contamination in sterility testing

The appropriate requirements for manufacture given in Part A, section 2, shall apply to those areas in which, and those procedures by which, sterility testing is done.

The risk of accidental contamination from the environment should be kept to a minimum by the use of disinfecting agents, germicidal lamps, and air filters. Germicidal lamps and disinfecting aerosols should not be used during actual testing opera-

---

[1] See, for example, *Health aspects of the supply and use of non-human primates for biomedical purposes, report of a WHO Scientific Group (Wld Hlth Org. techn. Rep. Ser.*, 1971, No. 470).

[2] *Wld Hlth Org. techn. Rep. Ser.*, 1967, No. 361, p. 49.

tions. The test manipulations should be carried out in a filtered air environment or under a laminar flow hood, and the operators should be dressed in sterilized, electrically neutral clothing, including head-covering and footwear. The air pressure in the testing room should be greater than the exterior area. The performance of the laminar flow hood should be monitored by settle plates or slit-sampling devices and the efficiency of the filters and germicidal lamps checked routinely.

Sterility testing shall not be carried out in production areas.

## 5. Sterility tests

Sterility tests shall be performed whenever specified in the requirements for individual products.

Tests for sterility are often performed advantageously at various stages of manufacture, in addition to those given in requirements. The tests described in these requirements may be used for this purpose.

Detailed rules and precautions for the sterility test procedures should be written and carefully followed.

The staff performing the sterility tests should have experience in the duties assigned. Supervision of the work and interpretation of sterility tests should be undertaken by an individual with training in scientific subjects, who should also have been trained in microbiology or be given training in that subject.

Where sterility tests for bacteria and fungi are required, such tests shall be made as described in Part A, section 5.2. Appropriate tests for viral and rickettsial sterility, when required, shall be made as specified in the requirements for the individual products. Tests for mycoplasmas, when required, shall be made as described in Part A, section 5.3.

### 5.1 *Sampling*

The specified number of suitable samples shall be taken from the product. Such samples shall be taken at least from each final bulk, as well as from each final lot.

#### 5.1.1 *Sampling from bulk*

A sample shall be taken from each bulk to be tested in such a manner as to be representative of the material to be tested. The amount taken shall be sufficient to perform the tests and any repeat tests that may be required. Such samples shall be taken so as to maintain intact the level of sterility of the material, or, if this is not possible, the samples shall be taken at the stage of further processing.

Since any microbial contaminants in a liquid may settle out, thorough mixing is required before the sample is taken.

### 5.1.2 *Sampling from final lot*

Samples of final containers from each final lot to be tested shall be taken in such a manner as to be representative of the lot to be tested. Appropriate periodic samples shall be taken, including samples at the beginning and the end of the filling operation.

If a product lot is filled through several outlets from a single bulk, samples should be taken from each outlet (filling lot) so as to be representative of the filling assembly.

The number of samples taken shall be at least that approved by the national control authority, provided that for final lots containing 500 or more containers, at least 20 samples shall be taken, including samples at the beginning and the end of the filling operation.

For final lots containing fewer than 500 containers, not less than 10 containers from the lot should be taken for a sample, except that for lots of less that 100 containers, only 10% of the lot need be tested.

A suggested rule[1] for calculating the number of samples is to take $0.4\sqrt{N}$ samples, where N is the number of final containers in the lot.

## 5.2 *Sterility tests for bacteria and fungi*

### 5.2.1 *Culture media*

The culture media used for sterility tests for bacteria and fungi shall be those approved by the national control authority. Such media shall have been shown to be capable of supporting the growth of a wide variety of micro-organisms, with both aerobic and anaerobic growth characteristics, including the types found in the environment of the manufacturing operations.

More than one culture medium, generally, will be needed to fulfil these criteria. The media used in many countries for sterility tests are fluid thioglycolate medium and soybean–casein digest medium.[2] Any other media that are used, however, should have been demonstrated to have at least equivalent growth supporting properties.

For testing the growth supporting qualities of each culture medium, strains of micro-organisms should be used with exacting nutritive and aerobic–anaerobic requirements, in an inoculum of only a small number of the organisms (less than 100).

---

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, No. 200, p. 31.

[2] The formulae of these culture media are given in Appendix 1.

The media should be incubated at the temperatures at which they will be used in the sterility test.

Each lot of dehydrated medium or each lot of medium prepared from basic ingredients should be tested for its growth supporting properties, since every lot may not support the growth of micro-organisms as well as desirable. It is desirable that lots so tested should be kept separate and reserved for use in sterility testing. This is a safeguard against occasional unsatisfactory components in a particular lot, or destruction of certain components by over-heating or over-sterilization, which may cause differences in growth response.

In some countries an additional test is made to verify the growth supporting properties of the medium, after the completion of a sterility test in which no growth has occurred in the medium, by inoculation with a small number of suitable organisms (less than 100).

### 5.2.2 *Performance of the test*

Prior to conducting a sterility test on any product, it shall have been determined wheter or not the material to be tested itself has the property of killing or inhibiting the growth of micro-organisms, or contains preservatives or other substances that have this effect. If such an effect is shown, the sterility test shall be made using a suitable procedure to counteract the effect.

The inhibitory effect in a preparation to be tested for sterility may be overcome by increasing the volume of culture medium used, so that the inhibitors are rendered ineffective, or if the preparation can be filtered, the inhibitors removed by membrane filtration.[1] The volume of medium required to overcome the inhibitory effect should be determined. Once established, these quantities may be used for subsequent sterility tests unless a change is made in the composition of the product.

#### 5.2.2.1 *Inoculum*

For sterility testing of bulk material, at least 5 ml must be used for inoculation into each culture medium and for each temperature of incubation.

For sterility testing of a final lot from each of the final containers that contain not more than 20 ml, an amount of at least 1.0 ml shall be inoculated into each culture medium and for each temperature of incubation. If the volume in each final container is less than 1.0 ml, the amount to be inoculated shall be the entire content of the container for each culture medium and for each temperature of incubation. If the volume in each final container is greater than 20 ml, but not more than 100 ml, the amount to be inoculated shall be at least 5 ml for each culture medium and for each temperature of incubation.

---

[1] See Appendix 2 for a description of the procedure using membrane filtration.

5.2.2.2 *Medium*

The quantity of medium put into each vessel for conducting the sterility test shall be sufficiently large to ensure that the volume of material inoculated does not impair the growth supporting properties of the medium by dilution.

All vessels of inoculated media shall be clearly identified by labelling that is adequate to identify the product being tested, each medium used, and each temperature of incubation.

5.2.2.3 *Incubation*

All vessels shall be incubated at the appropriate temperatures for the media. The temperatures selected shall be those approved by the national control authority and shall include 20°–25°C and 30°–36°C.

If only one temperature is selected for a particular medium, generally fluid thioglycolate medium is incubated at 30°–36°C and casein digest medium at 20°–25°C.

In some countries additional temperatures are used for psychrophilic and thermophilic organisms.

It is desirable that the incubation apparatus should have a continuous temperature recording device.

All vessels shall be incubated for a period of at least 14 days and shall be examined at regular intervals and on the last day of incubation for evidence of microbial growth.

If the preparation inoculated into the test vessels has clouded the medium to such an extent that it is difficult to recognize whether or not growth has taken place, subcultures of not less than 1.0 ml should be made from the cloudy medium between the third and seventh days after the start of the test. The original and transfer vessels should each be incubated and observed for a total of not less than 14 days.

5.2.3 *Membrane filtration of test samples*

The performance of sterility tests with the aid of membrane filtration of the test samples is a valuable method of improving results of such testing in certain situations.[1]

The extra manipulations involved in these procedures additional to those for the performance of the test described in section 5.2.2 above may, however, be a source of extraneous contamination. Hence the routine use of positive and negative controls is advisable for validating the results obtained. A suitable positive control is the occasional use of known con-

[1] See Appendix 2 for a description of a procedure for sterility tests using membrane filtration.

taminated solutions containing a few micro-organisms of differing types; if the product does not contain a preservative, approximately 10 viable microbial cells may be used in the total volume employed. If the product contains a preservative, the positive control may be added to the wash fluid.

### 5.2.4 *Interpretation of test results*

If no evidence of growth is found in any of the vessels inoculated for the test for sterility in Part A, section 5.2, the bulk material or final lot, whichever is applicable, meets the requirements for this test. If evidence of growth is found, the preparation tested fails to meet the requirements for the test for sterility, unless it can be demonstrated to the satisfaction of the national control authority, by retests or by other means, that the test was invalid.

The decision between failure of the product to pass the test and invalidity of the test procedure requires competent judgement of a trained individual.

### 5.3 *Sterility test for mycoplasmas*

For viral vaccines made by growing the virus in animal tissues or cell cultures, sterility tests for mycoplasmas of virus culture fluid and control fluid specified for the particular product shall be made by a method approved by the national control authority.[1]

The samples taken may include material prior to clarification or filtration in the case of live vaccines produced from *in vitro* living cell cultures or animal tissues. In the case of inactivated virus vaccines produced from such cell cultures or tissues, the samples may be taken prior to inactivation, from each virus harvest pool and from control pooled fluid.

Control cultures should be included in each test.

### 5.4 *Sterility test for viruses*

The sterility tests for the detection of extraneous viruses in products where their presence is unacceptable shall be performed as specified in the requirements for the individual products.

### 5.5 *Tests for specific micro-organisms*

The tests to ensure the non-survival of micro-organisms in inactivated vaccines and additional tests for the absence of specific extraneous micro-organisms in such products shall be performed as specified in the requirements for the individual products.

---

[1] A description of tests for mycoplasmas is given in Appendix 3.

## 6. Records

Records shall be permanent and clearly indicate all steps in the sterility testing of the product, including temperatures and incubation times, as well as the relationship of the samples under test to the manufacturing operations followed in the production of the lot under test. The records shall be of a type approved by the national control authority. Written records shall be kept of all tests performed, irrespective of their results. They shall be retained throughout the dating period of the product, and be available for inspection by the national control authority. Records shall be maintained of all cultures kept in the establishment. Such records shall include clear labelling for the identification of the cultures and the purposes for which the cultures are used.

For each sterility test performed a record shall be kept of the name and identifying numbers of the product under test, the quantities inoculated, the batch, type and tests of culture media used, the temperatures of incubation, the dates of inoculation and the results.

## 7. Additional samples

For each final lot for which sterility tests are made, additional samples shall be retained as reference material throughout the dating period of the product, in a manner that ensures the identity of the lot or batch of the product.

> It is desirable that, where possible, manufacturers should retain sufficient additional samples to permit repetition of the sterility tests.

# Part B. National Control Requirements

## 1. General

The general requirements for control laboratories given in Part B of the revised General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)[1] shall apply.

## 2. Release and certification

The requirements given in Part B, section 3.3, of the revised General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)[2] as well as the relevant provisions in the requirements for the individual products shall apply.

---

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1966, No. 323, p. 19.

[2] *Wld Hlth Org. techn. Rep. Ser.*, 1966, No. 323, p. 22.

## Appendix 1

## MEDIA FOR THE DETECTION OF AEROBIC AND ANAEROBIC BACTERIA AND FUNGI *

### 1. Fluid thioglycolate medium

| | |
|---|---|
| L-cystine | 0.5 g |
| sodium chloride | 2.5 g |
| glucose ($C_6H_{12}O_6.H_2O$) | 5.5 g |
| agar | 0.75 g |
| yeast extract, water soluble | 5.0 g |
| pancreatic digest of casein | 15.0 g |
| distilled water | 1000 ml |
| thioglycolic acid | 0.3 ml |
| (or sodium thioglycolate [1] | 0.5 g) |
| resazurin sodium (0.10% fresh solution) | 1.0 ml |
| Final pH=7.0–7.2 | |

**Preparation:**

Thoroughly grind the first six ingredients, in the order given above, in a mortar. Stir in some heated water, transfer to a suitable container, add the remainder of the water and complete the solution by heating in a boiling water bath, taking special care to ensure complete solution of the L-cystine. Add the thioglycolic compound, then 1 N sodium hydroxide so that the pH of the completed and sterilized medium will be 7.0–7.2. Reheat the solution, but do not boil, filter (if necessary) through a moistened filter paper and add the resazurin solution. Distribute into suitable vessels and sterilize by autoclaving for 18–20 minutes at 121°C. Cool promptly to 25°C and store at 20°–30°C, avoiding excessive light. If the uppermost portion of the medium has changed to a pink colour and this exceeds one-third of the depth of the medium, it is unsuitable for use, but may be restored once by heating in steam. Medium more than 3 weeks old should not be used.

### 2. Soybean-casein digest medium

| | |
|---|---|
| pancreatic digest of casein | 17.0 g |
| papaic digest of soybean meal | 3.0 g |
| sodium chloride | 5.0 g |
| dipotassium hydrogen phosphate | 2.5 g |
| glucose ($C_6H_{12}O_6.H_2O$) | 2.5 g |
| distilled water | 1000 ml |
| Final pH=7.1–7.5 | |

**Preparation**

Dissolve the solids in the water, warming slightly, then cool the solution to room temperature. Adjust the reaction with 1 N sodium hydroxide if necessary, so that the pH of the completed and sterilized medium will be 7.1–7.5. Filter, if necessary, to clarify, distribute into suitable vessels and sterilize in an autoclave for 18–20 minutes at 121°C.

---

* The quality of the components should be in accordance with *Specifications for Reagents mentioned in the International Pharmacopoeia*, Geneva, World Health Organization, 1963, unless otherwise stated.

[1] Reagent quality sodium thioglycolate is more stable than thioglycolic acid.

## Appendix 2

## PROCEDURE FOR STERILITY TEST USING MEMBRANE FILTRATION

### Apparatus

A suitable unit consists of a closed reservoir and a receptacle, separated from one another by a properly supported membrane of appropriate porosity. Membranes generally suitable for sterility testing have a nominal porosity of 0.22 μm or 0.45 μm, a diameter of approximately 47 mm, and a flow rate of 55–75 ml of water per minute at a pressure of 70 cm of mercury. Preferably assemble and sterilize the entire unit with the membrane in place, prior to use. Where the sample to be tested is an oil, it may be necessary to sterilize the membrane separately and, after thorough drying, assemble the unit, using aseptic precautions. If each entire membrane is to be cultured (see below), at least two filter units are set up.

### Diluting fluids

*Fluid A :* Dissolve 1 g of peptic digest of animal tissue [1] or the equivalent in distilled water to make one litre, filter or centrifuge to clarify, adjust to pH $7.1 \pm 0.2$, dispense into flasks in 100-ml quantities, and sterilize at 121°C for 18–20 minutes.

*Fluid B :* If the test sample contains lecithin or oil, use Fluid A to each litre of which has been added 1 ml of (*p-tert*-octylphenoxy)polyethoxyethanol, adjust to pH $7.1 \pm 0.2$, dispense into flasks, and sterilize at 121°C for 18–20 minutes.

*Note :* Any sterile diluent that does not manifest antimicrobial activity and does not affect the porosity of the membrane may be suitable for dissolving a preparation under test for sterility.

### Test procedure

*Liquids*

The number of sample containers or the volume of bulk sample that is specified for conducting the sterility test is taken. Aseptically transfer the required volumes from each container directly to a membrane filter previously moistened with sterile water or the diluting fluid used or to two sterile vessels for pooling prior to transfer to a moist membrane. Immediately draw each sample through the filter with the aid of vacuum.

If the substance is a viscous liquid or suspension not adaptable to rapid filtration, aseptically add a sufficient quantity of Fluid A to the pooled sample to increase the flow rate. Sterile enzyme preparations such as penicillinase or cellulase may be added to the diluting fluid to aid in dissolving insoluble substances. If the substance under test has inherent antimicrobial properties or contains a preservative, wash the filter with sufficient 100-ml portions of Fluid A. If the substance under test contains lecithin or oil, replace Fluid A by Fluid B. The number of portions of fluid used should be sufficient to allow growth of a small inoculum of organisms (approximately 50) sensitive to the antimicrobial substance in the presence of the residual inhibitory material on the membrane.

Upon completion of filtration, transfer each entire membrane to 100 ml of the culture medium (or approximately one-half of each membrane to 100 ml of each of two culture media) selected for the sterility test. The samples are then incubated at the selected

---

[1] Example : Peptone, dried, R, as described in *Specifications for Reagents mentioned in the International Pharmacopoeia*, Geneva, World Health Organization, 1963, p. 137. An autoclaved solution (2 in 100) is clear and is neutral or nearly so in its reaction.

temperatures and for the appropriate periods and examined as described in Part A, section 5.2 of these requirements. The period of incubation of membrane-filtered test samples used by some workers is shorter than the 14-day period prescribed in this section, since growth occurs more rapidly when micro-organisms concentrated on filters are inoculated.

## Appendix 3

## TESTS FOR MYCOPLASMAS

All varieties of mycoplasma are not capable of growth in the same medium. Some strains require special culture media. Suitable media should therefore be selected having regard to particular conditions, e.g., the tissue in which a virus is grown or source of animal serum, and the growth requirements of the potential contaminants, e.g., the need for yeast extract. Solid and semi-solid or liquid media containing serum fractions rich in certain lipids (sterols [1] and phospholipids) are used to provide the nutritive requirements. Control cultures on solid and semi-solid or liquid media of known fastidious strains of mycoplasma, both sterol-requiring and non-sterol-requiring, are included in each test to show that the media are capable of supporting the growth of mycoplasmas.

Samples of the material of not less than 6 ml for a single test (i.e., using one solid and one semi-solid or liquid medium) are stored either between 2° and 8°C for no longer than 24 hours or at –20°C or lower if stored for longer than 24 hours.

Not less than 2.0 ml of each sample are inoculated and evenly distributed over the surface of at least 10 plates of each solid medium used, and not less than 1.0 ml of each sample is inoculated into each of at least 4 tubes of each semi-solid or liquid medium used. Each tube should contain 10 ml of medium. Larger volumes of inoculum, e.g., making a total of 50 ml of material in 400 ml of medium, have been used advantageously. Ultracentrifugation before testing such samples has also been used.

The plates and tubes are incubated at 36°C ± 1.0°C for not less than 14 days. One half of each set is incubated aerobically in an environment of adequate humidity, and the other half is incubated anaerobically in an environment of 5–10% carbon dioxide in nitrogen, also of adequate humidity. At the end of 3 days and again at 14 days, 0.5 ml from each of 2 tubes being incubated aerobically are combined and inoculated on to not less than 4 plates. The 2 tubes being incubated anaerobically are treated likewise. Since some mycoplasmas may not grow on media in Petri dishes, such subcultures on solid media may also be made in tubes.[2]

All material seeded originally on solid media is subcultured on the day after the primary inoculation, on the third day and on the sixth day, on other suitable solid and liquid media, and these subcultures are incubated similarly, half aerobically and the other half anaerobically as described, and examined after 10 days, while the original cultures are examined at the end of the 14-day period. All plates (and tubes) after incubation for not less than 14 days are observed for the growth of mycoplasmas by appropriate procedures.

If no evidence is found of the presence of mycoplasmas the material, e.g., virus pool, meets the requirements of these tests. If evidence of growth of mycoplasmas is found, the mycoplasmas should be identified and the source traced.

---

[1] Non-sterol-requiring mycoplasmas will grow on media containing sterols.

[2] In order to induce mycoplasmas to grow in liquid media, similar subculturing could be done, but three or four passages may be needed.

For detection and identification of mycoplasmas the following procedures are suggested but other methods are available.

**1. For detection of mycoplasmas**

(*a*) typical colonies or specific coloration (blue) by Dienes's stain of colonies spread on blocks of agar in Petri dishes, and viewed under the microscope;

(*b*) phase contrast microscopy of colonies (strips or plaques) for globular bodies, filaments and granules;

(*c*) examination under the electron microscope of the pellet centrifuged from a semisolid culture;

(*d*) examination under the light microscope (× 1000) of a film stained by Giemsa.

**2. For separation of mycoplasmas into clasess**

(*a*) enzymatic effects; fermentation of glucose, breakdown of arginine and reduction of triphenyltetrazole, using decoloration of phenol red as indicator for the first two effects and red colour of the tetrazole derivative for the last;

(*b*) haemolysis in a 4% suspension of washed guineapig cells, added and incubated for 18–48 hours aerobically at 37°C;

(*c*) haemadsorption of a 4% suspension of guineapig cells poured over the growth surface or fixation of red blood corpuscles by the colonies after incubation at 37°C for 30 minutes.

**3. For specific identification**

Growth inhibition by specific anti-mycoplasma serum (prepared in the rabbit); such inhibition might be demonstrated by depositing paper discs impregnated with the serum on 2–4 hour growth cultures and observing them from the third day.

Immunofluorescence techniques for this purpose are being developed.

**Appendix 4**


## ACKNOWLEDGEMENTS

The unpublished working document WHO/BS/73.1062; WHO/PHARM/73.474 on which these requirements were based was prepared by the following experts and members of the WHO Secretariat:

Dr E. A. Christensen, Control Department, Statens Seruminstitut, Copenhagen, Denmark

Dr H. Cohen, Director, National Institute of Public Health, Bilthoven, Netherlands (*Consultant*)

Dr J. Desbordes, Director, Microbiology Section, National Public Health Laboratory, Paris, France (*Consultant*)

Dr L. F. Dodson, Director, National Biological Standards Laboratory, Commonwealth Department of Health, Canberra, Australia (*Consultant*)

Professor G. Edsall, Head, Department of Microbiology, London School of Hygiene and Tropical Medicine, London, England (*Consultant*)

Professor D. G. Evans, Director, National Institute for Biological Standards and Control, London, England (*Consultant*)

Mr J. W. Lightbown, Division of Biological Standards, National Institute for Biological Standards and Control, London, England (*Consultant*)

Dr T. J. Macek, 16541 S. Westland Drive, Gaithersburg, Md., USA

Mr A. P. Mechkovski, Pharmaceutical Officer, Pharmaceuticals, WHO, Geneva, Switzerland

Dr A. S. Outschoorn, Chief Medical Officer, Biological Standardization, WHO, Geneva, Switzerland

Dr M. González-Pacheco, Scientist, Biological Standardization, WHO, Geneva, Switzerland

Dr F. T. Perkins, Division of Immunological Products Control, National Institute for Biological Standards and Control, London, England (*Consultant*)

Dr H. D. Piersma, 2512 Patterson Ave. S.E., Grand Rapids, Michigan, USA (*Consultant*)

Dr M. Pittman, 3133 Connecticut Avenue N.W., Washington, D.C., USA (*Consultant*)

Mr K. O. Wallén, Chief Pharmaceutical Officer, Pharmaceuticals, WHO, Geneva, Switzerland

Miss A. Wehrli, Pharmaceutical Officer, Pharmaceuticals, WHO, Geneva, Switzerland

Grateful acknowledgement is made to the experts and institutions listed below for their comments and advice and for supplying additional data :

Dr F. E. André, Research Laboratories, Philips-Duphar B.V., Amsterdam, Netherlands

Dr W. R. Ashford, Assistant Director, Connaught Laboratories Limited, Willowdale, Ontario, Canada

Professor M. A. Attisso, Faculty of Pharmacy, Montpellier, France

Dr Sami Bağlum, P. Director, Refik Saydam, Central Institute of Hygiene, Ankara, Turkey

Dr M. Baharsefat, Razi State Institute for Serum and Vaccine Production, Teheran, Iran

Mr A. J. M. Bailey, Executive Officer, The Association of the British Pharmaceutical Industry, London, England

Professor A. J. Bandoni, Lima 1425, Buenos Aires, Argentina

Dr T. Bićan, Head, Chemistry Department, Institute for the Control of Drugs, Zagreb, Yugoslavia

Dr Carrol Brock, Food and Drug Administration, Washington, D.C., USA

Dr A. Brunzell, Associate Professor, The Galenical Section, Division of Pharmacy, Department of Drugs, National Board of Health and Welfare, Stockholm, Sweden

Dr J. R. Buriánek, State Institute for the Control of Drugs, Prague, Czechoslovakia

Professor A. Calò, Chief, Laboratory of Chemistry, Istituto Superiore di Sanità, Rome, Italy

Mr K. C. Chatterjee, Bombay College of Pharmacy, Kalina, Santa Cruz, Bombay, India

Dr E. A. Christensen, Control Department, Statens Seruminstitut, Copenhagen, Denmark

Dr V. F. Davey, Deputy Director (Technical), Commonwealth Serum Laboratories, Parkville, Victoria, Australia

Mr I. Davidson, Central Veterinary Laboratory, Weybridge, Surrey, England

Dr Nils Diding, Apotekens Centrallaboratorium, Solna, Sweden

Professor J. Dony, Scientific Director, Belgian Pharmaceutical Association, Drug Control Service, Brussels, Belgium

Mr J. Evans, Acting Chief, Drug Microbiology Branch, Division of Drug Biology, Office of Pharmaceutical Research and Testing, Washington, D.C., USA

Mr J. T. Faragher, Central Veterinary Laboratory, Weybridge, Surrey, England

Dr M. Fors, Secretary General, Nordic Pharmacopoeia Council, Stockholm, Sweden

Dr Ch. B. Gerichter, Director, Division of Laboratories, Ministry of Health, Jerusalem, Israel

Dr D. Ghosh, Director, Central Drugs Laboratory, Calcutta, India

Mr N. Goddard, Sartorius-Membranfilter GmbH, Göttingen, Federal Republic of Germany

Dr J. J. Graydon, Commonwealth Serum Laboratories, Parkville, Victoria, Australia

Dr F. Hartley, Dean, The School of Pharmacy, University of London, and Chairman, British Pharmacopoeia Commission, London, England

Dr L. Hayflick, Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California, USA

Dr John Hampton, National Biological Standards Laboratory, Canberra, Australia

Professor R. Hazard, Faculty of Medicine, Institute of Pharmacology, Paris, France

Professor G. Heymann, Director, Paul Ehrlich Institute, Federal Agency for Sera and Vaccines, Frankfurt-am-Main, Federal Republic of Germany

Dr L. Higy-Mandić, Final Control and Biological Standardization Section, Institute of Immunology, Zagreb, Yugoslavia

Dr N. W. Holm, Head, Accelerator Department, Danish Atomic Energy Commission, Research Establishment Risø, Roskilde, Denmark

Dr D. W. Howes, Assistant Director, National Biological Standards Laboratory (Viral Products Section), Parkville, Victoria, Australia

Mr E. C. Hulse, Deputy Director, Central Veterinary Laboratory, Weybridge, Surrey, England

Dr T. Inoue, Chief, Department of Drugs, National Institute of Hygienic Sciences, Tokyo, Japan

Dr S. Iwahara, Chief, Department of Microbiology, National Institute of Hygienic Sciences, Tokyo, Japan

Professor L. Jännes, State Serum Institute, Helsinki, Finland

Mr C. A. Johnson, Scientific Director, British Pharmacopoeia Commission, London, England

Mr S. C. Jolly, Director, Department of Pharmaceutical Sciences, The Pharmaceutical Society of Great Britain, London, England

Dr J. Kaneko, Medical Officer, The Vaccination Research Center, Tokyo, Japan

Dr J. C. Kelsey, Deputy Director, Public Health Laboratory Service, Central Public Health Laboratory, London, England

Dr K. Kihara, National Institute of Health, Tokyo, Japan

Mr E. Knoll, Chief, Sterility Testing Branch, National Center for Antibiotic Analysis, Office of Pharmaceutical Research and Testing, Washington, D.C., USA

Mr J. Kramer, Acting Director, Division of Drug Biology, Office of Pharmaceutical Research and Testing, Washington, D.C., USA

Professor K. G. Krebs, Director, E. Merck AG, Darmstadt, Federal Republic of Germany

Dr Hanne Kristensen, Statens Seruminstitut, Copenhagen, Denmark

Dr M. Kurokawa, Chief, Department of General Biologics Control, National Institute of Health, Tokyo, Japan

Dr A. Lafontaine, Director, Institute of Hygiene and Epidemiology, Brussels, Belgium

Dr E. Lang, Assistant Manager, CIBA-GEIGY Limited, Basle, Switzerland

Dr H. Lansberg, National Institute of Public Health, Bilthoven, Netherlands

Dr P. Laroux, Scientific Director, SPECIA, Paris, France

Dr F. J. Ley, Irradiated Products Ltd., Elgin Estate, Swindon, Wilts, England

Dr J. P. Lowenthal, Chief, Department of Biologics Research, Division of Communicable Disease & Immunology, Walter Reed Army Institute of Research, Walter Reed Medical Center, Washington, D.C., USA

Dr J. Lyng, Statens Seruminstitut, Copenhagen, Denmark

Mrs de Maeyer-Cleempoel, Chief, Bacteriology Section, Institute of Hygiene and Epidemiology, Brussels, Belgium

Mr A. G. Mathews, Chief of Quality Control, Commonwealth Serum Laboratories, Parkville, Victoria, Australia

Mr F. A. Maurina, 3485 Bedford, Detroit, Mich., USA

Dr J. A. McKiel, Director General, Laboratory Centre for Disease Control, Health Protection Branch, Ottawa, Ontario, Canada

Mr H. F. Meldahl, Kabi Ltd, Technical Department, Stockholm, Sweden

Dr H. Mirchamsy, Associate Director, Razi State Institute for Serum and Vaccine Production, Teheran, Iran

Dr R. Murray, Special Assistant to the Director, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., USA

Dr R. Netter, Director, Virology Section, National Public Health Laboratory, Paris, France

Dr E. Nieminen, Director, Laboratory for the Examination of Pharmaceutical Products, Helsinki, Finland

Miss M. Özsandik, Bacteriologist, Central Institute of Hygiene, Ankara, Turkey

Mr B. V. Patel, Dr Vikram Sarabhai Marg, Ahmedabad, India

Professor G. Penso, Istituto Superiore di Sanità, Rome, Italy

Professor M. Pernarowski, Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, Canada

Professor A. Puech, Director, Montpellier Section, National Laboratory for the Control of Drugs, Montpellier, France

Dr Chaloem Puranananda, Director, National Blood Centre, Thai Red Cross Society, Bangkok, Thailand

Dr J. D. van Ramshorst, Chief, Department of Biological Standards, National Institute of Public Health, Bilthoven, Netherlands

Mr M. K. Rangnekar, Commissioner, Food and Drug Administration, Maharashtra State, Bombay, India

Dr O. Ringertz, Associate Professor, Swedish National Bacteriological Laboratory, Sweden

Dr E. B. Seligmann, Jr, Director, Division of Control Activities, Bureau of Biologics, Food and Drug Administration, Rockville, Md., USA

Professor P. L. Senov, Flat 17, 16 Teplyi pereulok, Moscow, USSR

Dr J. Spaander, Director General, National Institute of Public Health, Bilthoven, Netherlands

Dr I. Suzuki, Chief, Department of Synthetic Chemistry, National Institute of Hygienic Sciences, Tokyo, Japan

Dr I. Di Tommaso, Head, Quality Control, Istituto Sieroterapico e Vaccinogeno Toscano " Sclavo ", Siena, Italy

Dr F. Tomiček, Pharmaceutical and Biochemical Research Institute, Prague, Czechoslovakia

Mr K. Topsy, Senior Government Chemist, Government Chemist Division, Candos, Mauritius

Professor A. Végh, Institute for Pharmaceutical Chemistry of Semmelweis University, Budapest, Hungary

Mr Voggel, Sartorius-Membranfilter GmbH, Göttingen, Federal Republic of Germany

Dr J. Volckringer, Honorary Inspector-General of Health, 3 rue Louis Rolland, Montrouge, France

Professor M. Welsch, Director, Faculty of Medicine, Chair of Microbiology, University of Liège, Belgium

Dr W. W. Wright, Acting Director, Office of Pharmaceutical Research and Testing, Bureau of Drugs, Food and Drug Administration, Washington, D.C., USA

Valuable suggestions were also obtained from the report of a discussion on recommendations for WHO Requirements that took place at the Symposium on Sterility and Sterility Testing of Biological Substances held from 9 to 12 April 1973 in Madrid, Spain, by the International Association of Biological Standardization.