WHO Expert Committee on Biological Standardization

Thirty-second Report

World Health Organization
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673

World Health Organization, Geneva 1982
The Committee established the histoplasmin as the International Reference Reagent of Histoplasmin and established the serum as the International Reference Reagent of Histoplasmin for H and M Immunodiffusion Test, and the International Reference Reagent of Histoplasmin Antiserum, Rabbit, for H and M Immunodiffusion Test.
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WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Thirty-second Report

GENERAL

The WHO Expert Committee on Biological Standardization met in Geneva from 22 to 28 September 1981. The meeting was opened on behalf of the Director-General by Dr Ch’en Wen-chieh, Assistant Director-General.

As so much of the Committee’s work is devoted to the establishment of standard preparations that are crucial to the quality control of biologicals throughout the world, the Committee gave its attention to matters affecting the increasing demand for, and the availability of, such preparations. In particular, the Committee considered the use of standards in countries in which national biological control programmes are still in an early stage of development.

The provision of international standards

The principle that international standards are distributed free of charge to national control authorities for the purpose of the calibration of national standards in international units has been adhered to since the establishment of an international biologicals programme (Bulletin of the Health Organization, League of Nations, 4, No. 4 (1935)). The programme has continually gained momentum and today more than 12,000 ampoules of standards and reference preparations are distributed to over 85 countries each year. Many of the countries receiving the standards, however, have been unable to establish national standards and have wished to use the international preparations to carry out routine tests. A few countries have set up national standards, thereby relieving the burden of a continuous distribution of the international standards to all Member States.

During the last 10 years there has been a greater understanding of the importance of standards, especially during the early stages of investigating the measurement of activity of a new biological substance. The unit of activity having been defined, a working standard of the substance is required for the measurement of activity of different lots.
of material, and the use of such working standards is now becoming essential in commerce.

It is a logical development for groups of countries in neighbouring geographical locations, or those with trade agreements, to make attempts to establish their own standards of biological substances. Thus, the question of regional standards, is under discussion and there are already European Pharmacopoeial standards and Commission of the European Communities standards. Such ventures are to be encouraged provided that the standard preparations have their biological activity calibrated in international units and that there is no wasteful duplication of work, for there would seem to be little point in groups of countries agreeing to prepare, assay, store, and distribute cooperative standards if, in addition, each country established its own national standards and only these had mandatory force. It is the latter point that has drawn the attention of legislative bodies to the importance of biological standards and has led to a debate on the need for cooperative rather than national standards. Under such circumstances the provision of cooperative working standards should be considered most carefully, since the cost of setting up national standards, both in monetary and technical manpower resources, is large and it has been argued that the setting up of cooperative standards would allow this burden to be shared. It should be emphasized, however, that such standards should be similar in quality and purity to the international standards and their calibrations should be carried out with comparable precision. Before starting a new venture, a very careful cost accounting should be done of all the factors involved in the procurement of the materials, the preparation of the standard, as well as in its calibration, storage and sustained distribution. Perhaps the most important point at the outset is a firm agreement by all parties on the acceptance of the cooperative standards as they become established. Without this the ultimate objective of the project would be lost.

If these conditions can be satisfied, the WHO Biologica ls Programme would continue to give enthusiastic support to the establishment of national, pharmacopoeial, regional, or cooperative standards in order that they may be established by direct comparison with the international standard for each new standard and its subsequent replacement.

The establishment of biologicals laboratories

For several years, discussions have been taking place concerning the most effective assistance that the Biologicals Programme can give
to countries wishing to become involved in the control of biologicals in which the use of standard preparations plays a major role. The Committee agreed that such countries should be given every assistance in order to work towards this goal, which can be achieved in a stepwise progression of activities, although starting in a modest way.

The first step is to establish a laboratory in which control tests can be carried out. This activity is likely to have the greatest success if done in association with the science faculty of a university. The setting up of such a control facility will involve the identification of staff who may require training in a well established national control laboratory. Such training would of necessity include the correct use of biological standards in the measurement of biological activity. Although it may take several years to assemble a nucleus of staff capable of controlling the important products used in health care or in the essential clinical laboratory services, this goal is an initial and integral part of the improvement of health care.

The reason for establishing a quality control laboratory with qualified personnel as a primary objective is that in all successive steps, whether they be the establishment of a facility for the finishing of the products when imported in bulk or the national production of biologicals, an essential proof of quality of the final product is the measurement of activity by comparison with a similar material calibrated in international units.

The provision of working standards

The availability of biological standards is therefore fundamental to this issue, and the Committee debated the economic consequences of countries setting up national standards or cooperative standards compared with the central provision of working standards. Brief mention of this has been made under general remarks in the thirtieth report of the Committee, in which it was recognized that the distribution of international working standards to all countries would be the most efficient use of the strictly limited technical and financial resources available today. It was recognized also that distribution of such working standards should be distinguished from the supply of international standards which are distributed free of charge to national control laboratories for the purposes of setting up national standards. The provision of international working standards would obviate the need for setting up national standards for those countries in which the necessary resources were severely restricted. The setting up and mainte-
nance of national standards is expensive and the alternative approach of paying towards the provision of international working standards would impose a much smaller financial burden and would be a worthwhile investment for any country. The Committee agreed that the need for financial resources in order to support the implementation, on a global scale, of the proposal to provide working standards from a central source is urgent and should be investigated.

The reconstitution and use of standards

In order to assist countries that are becoming involved in the use of standards for the first time, the Committee agreed that the Guidelines for the Preparation and Establishment of Reference Materials and Reference Reagents for Biological Substances (WHO Technical Report Series, No. 626, 1978, Annex 4) should have a section added to give guidance on the correct procedures to be used in the reconstitution of a biological reference material.

While most of the recently established international reference preparations and standards are in the freeze-dried form, with the unit defined on an ampoule-content basis, certain international standards continue to be distributed in the form of a dried powder, which must be weighed by the user. When either form of standard is used, great care is necessary in the preparation of a solution of the standard of known concentration. When ampoules with a defined content of international units are used, careful attention must be given to the instructions for use that accompany the ampoules. A particular solvent may be necessary to ensure complete solution and care must be taken to dissolve material adhering to all parts of the opened ampoule.

When weighing is necessary, extreme precautions must be taken in some cases to avoid errors arising from the uncontrolled change in water content during the process (WHO Technical Report Series, No. 638, 1979, p. 17). Certain national standards may be prepared in a form that necessitates the material being dried by the user before a precise weight is taken. Material dried in this way may also be extremely hygroscopic and special precautions will also be necessary in its use.

The assignment of units of activity

A basic precept for the conduct of assays intended to estimate the concentration of an analyte in a test specimen is that the analyte and the active substance in the standard should be identical, or similar to
the extent that they behave identically in the assay system. Where these conditions exist, then theoretically the same relative potency will be obtained with any assay system.

In practice, however, this rarely obtains and preparations of a medically important biological product are rarely identical. Moreover, improvements in analytical methods have revealed that many such products, previously considered to be homogeneous, actually consist of mixtures of similar but not identical molecules and that these individual components may have slightly different properties in the assay systems used to estimate them. When the materials are compared in biological assays such dissimilarities will be made evident in the non-parallelism of their log–dose responses or as a significant heterogeneity of results of repeated assays; moreover, distinctly different estimates will often be obtained when different assay systems are used.

Examples of such differing properties are the biological activity of “chorionic gonadotrophin(s)” and its immunoreactivity; the activities of preparations of follicle-stimulating hormone in in vivo and in vitro bioassays; the in vitro flocculating and in vivo toxin-neutralizing activities of diphtheria antitoxins; the antitumour and antimicrobial activities of preparations of antitumour antibiotics, and the differing activities of preparations of heparins in delaying the clotting of blood and in antagonizing factor Xa in “anti-Xa assays”.

For many biological preparations, however, the differences between analyte and the active principle of the standard preparation are minor and the associated differences in assay results are small and for practical purposes can be ignored. In most cases, the unitage of a replacement international reference material is assigned on the basis of the combined estimates from a wide variety of assay methods used in the international collaborative assay to calibrate the replacement material. The international reference material is then intended for application in the commonly used assay systems by which it was calibrated. When the differences between preparations are more marked, as often happens with preparations of heterogeneous substances (immunoglobulins, antibiotics, glycoprotein hormones, and preparations of heparins), they may give rise to variations in the estimates of relative potency that are too great to be ignored.

The implications of such dissimilarities are many and complex, as discussed in the Committee’s twenty-sixth report (see WHO Technical Report Series, No. 565, 1975, p. 21). One is that there is likely to be some discontinuity in the biological activity attributed to the international unit of a replacement standard in assays of heterogeneous
materials. Thus, if a national standard of such material is calibrated by only one particular assay method (e.g., the pharmacopoeial method), the unitage assigned to it may well be different from the unitage derived from a variety of different assays. Furthermore, the discrepancy that may occur with the introduction of a replacement international standard could be such as to necessitate the revision of the national unit by the recalibration of the national standard.

A second implication is that the recognition of dissimilarities of certain candidate materials may require the clear definition of criteria by which to select material for a replacement standard; this may be particularly important if the preparation is intended for the measurement of a natural component of the body, such as the closely related groups of glycoprotein molecules that are referred to as chorionic gonadotrophins, thyrotrophins, or follicle-stimulating hormones. Normally a hormone preparation would be selected for use as a standard in the form of the natural and unaltered material that showed the highest potency in a classical in vivo assay system generally recognized by the scientific community as defining that hormone.

A third implication derives from the increasing use of in vitro assays that are simple, rapid, inexpensive, and may be automated, such as the methods employing small molecular substrates (e.g., synthetic esters and peptides) for the estimation of certain enzymes (e.g., fibrinolytic and clotting factors) or the assay of anti-factor Xa activity of heparins. Such systems may not directly assay the same functional property of those substances as that which causes a desired biological activity in in vivo bioassays or in the patient treated. It has been shown, for example, that a partly degraded unstable enzyme can appear to be satisfactorily potent in an assay with small molecular substrate, but that it may have lost its activity when tested in an assay system involving its natural protein substrate. While such simpler assay systems are invaluable for many purposes, they cannot always be relied upon for control purposes to measure the important biological activity of a product or a reference material.

A fourth implication is therefore that a record of any assay result must include statements of the assay method used, the standard, and its unitage.

**Biological standards for clinical chemistry**

Throughout its history, the work of the Biologicals Programme has been concerned almost entirely with the establishment of standard
preparations of biological substances the activity of which cannot be measured by physical or chemical means and for which a biological evaluation is necessary. Indeed, standard preparations for some substances, notably certain antibiotics and vitamins that are now identified by chemical structure, have been transferred to the collection of International Chemical Reference Substances. It is now apparent, however, in the field of clinical chemistry that there is a need to have certain biological reference materials, such as serum, made available with declared contents of electrolytes. The body fluids in which the concentration of such electrolytes needs to be measured are biological substances and the reference materials used in the determinations need to be similar preparations; it is not satisfactory to have aqueous solutions of the individual electrolytes as the reference materials. The Committee considered that although the establishment of such materials as that for the comparison of electrolyte content was a departure from past practice it was appropriate for this new group of reference substances to be considered within the framework of the Biologicals Programme.

**Antibiotic susceptibility tests**

The Committee considered a proposed revision of the Requirements for Antibiotic Susceptibility Tests. I. Agar Diffusion Tests using Antibiotic Susceptibility Discs (Requirements for Biological Substances No. 26). Since these requirements were published in 1977, the WHO Secretariat has received a number of suggestions for changes. Some such changes were considered by the Committee in its thirty-first report; the most common suggestions concerned the codes to be imprinted on discs to identify them during use. It appeared that the three-letter codes recommended in the Requirements were not widely used. Further, different codes were used for a particular antibiotic, and the same code was used for different antibiotics. In order to obtain more information, the WHO Secretariat in 1980 corresponded with 19 individuals with knowledge of and interest in codes used for such antibiotic discs. The responses received included several tabulations of codes used in different countries. Several correspondents suggested that the requirements be expanded to cover synthetic antimicrobial discs in addition to antibiotic discs, since laboratories use both kinds side by side in testing the susceptibility of clinical isolates. When the Requirements were drafted, much of the information on synthetic antimicrobial discs, such as their performance characteristics and appro-
priate zone sizes for interpretation of susceptibility, was not available. In the light of the comments received, the WHO Secretariat has prepared a complete revision of the Requirements to include synthetic antimicrobials (and to retile them as Requirements for Antimicrobial Susceptibility Tests) and to provide identification codes for 131 antimicrobics. Of the 131 recommended codes, 125 are already used by one or more manufacturers, 5 were new because the codes being used conflicted with ones recommended for other antimicrobics, and 1 was for a substance that did not already possess a code.

Another suggestion received by the WHO Secretariat from several sources was that WHO should assign new codes for use on new antimicrobial discs. The Committee was informed that this function could be performed as part of the international nonproprietary names (INN) programme.

**Poliomyelitis vaccine (inactivated)**

There have been marked advances in the technology involved in the production of inactivated poliomyelitis vaccine. The growth of the cell substrate on microcarriers has markedly increased the yield of cells and thus the virus yield. Well characterized nontumorigenic cells may now be used as the cell substrate, thus avoiding the need for a large number of monkeys as formerly required in the production of this vaccine. Finally, the technology for the purification and concentration of the virus has now been developed to provide a more immunogenic vaccine while maintaining safety. The Committee agreed that the greater availability of such a killed poliomyelitis vaccine would be important in immunization programmes and the revision of requirements should ensure this.

**Requirements for Rift Valley fever vaccine**

It is usual for the formulation of requirements for a new vaccine to await its production and use in more than one country. The situation with respect to killed vaccine against Rift Valley fever virus for use in humans, however, is a special case. The virus causes a haemorrhagic disease which has hitherto occurred mainly in cattle and exclusively in countries in the south-east of Africa, but in 1977 it spread to Egypt, causing an epidemic in cattle which spread to man and was further disseminated from person to person. It is clear from this experience that special groups of personnel likely to come into contact with in-
fected humans or cattle should be protected against infection by the virus. Early attempts at the preparation of a killed vaccine have been successful and it has been shown to give protection in man. International requirements for the manufacture and control of such a vaccine, already considered for use in man in several countries, are needed urgently.

**Stability of measles vaccine**

The thirty-first report of the Committee drew attention to the need for more stable vaccines, especially for use in countries in which the ambient temperature is high and where there may be a limited cold chain. As measles is a serious disease in developing countries and the virus is sensitive to heat, the stability of this vaccine in the freeze-dried form has been studied. It was shown that there was marked variation in the stability of the different vaccines available throughout the world. Improvements have now been made by some manufacturers who produced the least stable vaccine and the majority of measles vaccines now available are stable. The Committee agreed that in order to ensure that only stable vaccine is provided to the developing world a heat-stability test should be added to the Requirements for Measles Vaccine (Live).

**Test for pyrogens**

The potential usefulness of a simple *in vitro* test for pyrogens is immense, as experience with the test based on the ability of endotoxins of Gram-negative bacteria to accelerate the clotting of the lysate of amoebocytes of *Limulus* crabs (the LAL test) has shown. Nevertheless, several studies have revealed some of the difficulties of standardizing batches of the substrate, which consists of a mixture of clotting factor enzymes and their precursors. A recent and extensive collaborative study, carried out at the request of the Committee was planned to assess the extent to which batches of lysate substrate are or could be standardized or calibrated with respect to their sensitivity to preparations of various endotoxins, including those used as international and national reference endotoxins. The preliminary summary of the statistical analysis of the international study indicated that standardization of current reagents had not yet been achieved, that various batches of lysate substrate varied widely in their sensitivity, and that interlaboratory differences in test results were large and irregular.
New biologicals prepared by gene control and other methods

The application of new technology has opened up possibilities for considerable changes in the production of vaccines and other biologicals.

There has been increasing interest in synthesis by microorganisms, made possible by DNA recombinant technology, directed towards obtaining proteins and peptides with biological activity. Proteins with hormone activity, such as insulin, growth hormone, and somatostatin have been produced and are under study. One preparation of human insulin, whose component chains were produced by Escherichia coli modified by recombinant DNA technology, is now being included in a WHO collaborative study. There have been many clinical investigations and basic scientific studies concerning proteins with interferon activities. Peptides, including those cross-reactive with surface antigens of hepatitis A and B, the haemagglutinin of influenza viruses, and the toxins and adhesive substances of bacteria are being investigated. Semisynthetic vaccines, composed of protective antigens chemically conjugated to components designed to increase their immunogenicity are under active study. Hybrid molecules, designed for specific cellular sites and composed of toxins (such as the A component of diphtheria toxin or ricin) have been assembled in vitro; in addition, monoclonal anticytotoxic antibodies derived from hybridoma cultures are being investigated for their antitumour activity. There is considerable activity also in the field of passive immunotherapy with the use of intravenous immunoglobulins from hyperimmune donors and monoclonal antibodies. The development of human hybridoma cultures capable of synthesizing antibodies, immunoregulatory molecules, and hormones will require new approaches to standardization.

SUBSTANCES

ANTIBIOTICS

1. Amikacin

The Committee noted that further studies of the trial fill of the preparation of amikacin referred to in its thirty-first report (WHO Technical Report Series, No. 658, 1981, p. 12) had confirmed its
stability and that the remainder of the material had been distributed into ampoules and freeze-dried (WHO/BS/81.1316). The Committee noted also that the variation in weight of contents from ampoule to ampoule was very small, and that a collaborative assay was being arranged.

2. Sisomicin

The Committee noted that studies of the trial fill of the preparation of sisomicin referred to in its thirty-first report (WHO Technical Report Series, No. 658, 1981, p. 12) had shown that the preparation was stable. The Committee noted also that additional material from the same batch had been obtained, distributed into ampoules, and freeze-dried (WHO/BS/81.1317) and that a collaborative assay was being arranged.

3. Tobramycin

The Committee noted that in accordance with the request made in its thirty-first report (WHO Technical Report Series, No. 658, 1981, p. 13) the National Institute for Biological Standards and Control, London, had reconstituted some of the International Reference preparation of Tobramycin and, after filtration, had distributed it into more suitable ampoules and freeze-dried it (WHO/BS/81.1318). The Committee was informed that the reprocessed material was stable. The Committee noted also that additional material from the same batch of tobramycin used to prepare the International Reference Preparation of Tobramycin had been obtained.

The Committee therefore requested the National Institute for Biological Standards and Control, London, to dispense the additional material into ampoules, to freeze-dry it, and to arrange a collaborative assay in terms of the International Reference Preparation of Tobramycin.

References prefixed “WHO/BS” are to unpublished working documents obtainable on request from Biologicals, World Health Organization, Geneva, Switzerland.
4. Bleomycin A₅

The Committee noted that two national standards of bleomycin A₅ had been received and studied (WHO/BS/81.1319). The Committee noted also that the two preparations differed in some respects and agreed that neither would be appropriate for defining the biological activity of both preparations. The Committee was informed that there was only limited clinical use of bleomycin A₅ in two countries and it therefore agreed that an international standard for bleomycin A₅ was not needed at present.

5. Nystatin

The Committee noted that the stocks of the International Standard for Nystatin were becoming exhausted (WHO/BS/81.1320). The Committee noted also that a suitable replacement preparation of nystatin had been obtained and dispensed into ampoules as a powder and dried. Samples of the international standard and of the proposed second international standard had been sent to nine laboratories in nine countries for comparison in a collaborative assay. The results had been received from all laboratories and were being analysed.

The Committee authorized the National Institute for Biological Standards and Control, London, to establish the preparation as the second International Standard for Nystatin, and, on the basis of the results of the collaborative assay and with the agreement of the participants, to define the international unit in terms of weight of the second International Standard for Nystatin.

ANTIBODIES

6. Clostridium botulinum Type B Antitoxin

The Committee noted that after five years' collaborative work between the State Serum Institute, Copenhagen, and the Chiba Serum Institute, Japan, it had been shown that the heterogeneity of the Clostridium botulinum Type B toxin produced by different strains of Cl. botulinum was not causing problems in the standardization of the type B antitoxin (WHO/BS/80.1290).
The Committee was informed that the collaborative assay of the proposed second International Standard for *Clostridium botulinum*, type B antitoxin, referred to in its thirty-first report was in progress.

7. *Clostridium welchii* (perfringens)  
**Types B and D Antitoxins**

The Committee noted that the activities of the International Standards for *Clostridium welchii* (perfringens) Type B Antitoxin, Equine, and for *Clostridium welchii* (perfringens) Type D Antitoxin, Equine, were measured in terms of the beta and epsilon toxins respectively (WHO/BS/80.1296). A change in names, therefore, consistent with the names of the international reference preparations of the respective toxin would describe the activities of the antitoxins more accurately.

The Committee noted also that the proposed changes had been sent to the Expert Advisory Panel and that as there had been no objections the Director-General had approved these changes in the names of the two antitoxins:

The International Standard for *Clostridium welchii* (perfringens) Type B Antitoxin, Equine, is renamed the International Standard for *Clostridium perfringens* Beta Antitoxin, Equine.

The International Standard for *Clostridium welchii* (perfringens) Type D Antitoxin, Equine, is renamed the International Standard for *Clostridium perfringens* Epsilon Antitoxin, Equine.

The Committee requested the Central Veterinary Laboratory, Weybridge, England, to make the necessary amendments in the labelling of these two international standards.

8. Anti-Poliovirus Sera Types 1, 2 and 3

The Committee noted that the stocks of the International Standard for Anti-Poliovirus Serum (Type 3) were almost exhausted (WHO/BS/81.1312). The Committee was informed that as the three international standards for types 1, 2 and 3 were established almost 20 years ago and as reference sera with a higher antibody content were now required for immunodiffusion techniques, it would be advisable to replace all three antisera to the three serotypes. The Committee noted also that suitable sera were being prepared and requested the Secretariat to arrange a collaborative study to determine their suitability for use in both neutralization and immunodiffusion tests.
9. Anti-Streptolysin O, Human

The Committee noted that the first International Standard for Anti-Streptolysin O, Human, was distributed as a solution, which was inconvenient and gave rise to stability problems (WHO/BS/81.1301). The Committee noted also that a pool of human serum with an adequate anti-streptolysin O content had been offered to WHO by the State Serum Institute, Copenhagen, as a replacement.

The Committee requested the State Serum Institute to distribute the serum into ampoules, freeze-dry them, and determine the suitability of the serum to serve as a second international standard for anti-streptolysin O. If the material was shown to be suitable, the Committee requested the State Serum Institute to arrange a collaborative assay.

10. Anti-Rubella Serum, Human

The Committee noted that the International Reference Preparation of Anti-Rubella Serum, Human, is a human immunoglobulin, and that it is used not only for the measurement of potency of rubella immunoglobulin preparations, as intended, but also for the control of diagnostic tests (WHO/BS/81.1302). For the latter purpose, however, it is important to have a preparation with defined contents of rubella IgM as well as IgG antibodies. The Committee, therefore, requested the State Serum Institute, Copenhagen, to investigate further the possibility of procuring a serum containing substantial amounts of IgM as well as IgG antibodies against rubella virus and to examine its usefulness as an international reference preparation.

The Committee also requested the State Serum Institute to arrange a collaborative study to determine the haemagglutinating inhibiting activity of the International Reference Preparation of Anti-Rubella Serum, Human.

11. Anti-Varicella Zoster Immunoglobulin, Human

The Committee noted that human plasma containing a high titre of anti-varicella zoster had been collected and that the immunoglobulin will be isolated from it; sufficient material is now available to prepare an international reference preparation of anti-varicella zoster immuno-
globulin (WHO/BS/81.1307). The Committee therefore requested the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, to distribute the material into ampoules, freeze-dry it, study its suitability to serve as an international reference preparation and, if it is shown to be suitable, arrange a collaborative assay.

12. Hepatitis A Immunoglobulin

The Committee noted the report of the Bureau of Biologics, Food and Drug Administration, Bethesda, MD, USA, (WHO/BS/81.1339), which showed that a suitable preparation of hepatitis A immunoglobulin had been obtained and that the collaborative assay referred to in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 22) had been carried out. The Committee noted also that the preparation had been shown to be stable and suitable to serve as a reference preparation by several methods of assay, such as radioimmunoassay, immune adherence haemagglutination, and immunoelectron microscopy.

The Committee established the preparation as the International Reference Preparation of Hepatitis A Immunoglobulin and on the basis of the results of the collaborative assay defined the activity of the contents of each ampoule as 100 International Units of Hepatitis A Immunoglobulin.

ANTIGENS

13. Tetanus Toxoid, Adsorbed

The Committee noted that, in the collaborative studies referred to in its twenty-ninth report (WHO Technical Report Series No. 626, 1978), different products containing an adsorbed tetanus toxoid component, had been compared with the International Standard for Tetanus Toxoid, Adsorbed, using several methods of assay in both guinea-pigs and mice (WHO/BS/81.1311), and that after a long series of such comparisons difficulties in assays had come to light that were not likely to be overcome for many years, if at all.

The urgency of replacing the international standard was very great, and therefore the Committee established TEXA 55/56, one of the
freeze-dried preparations examined in the study, as the Second International Standard for Tetanus Toxoid, Adsorbed, and, on the basis of the tests in guinea-pigs, defined the activity of the contents of each ampoule as 340 International Units of Tetanus Toxoid, Adsorbed.

The Committee requested the State Serum Institute, Copenhagen, to prepare a document explaining the difficulties that had been encountered in the assay of tetanus toxoid, adsorbed, and to distribute the document with the standard.

14. Purified Protein Derivative (PPD) of Mammalian Tuberculin

The Committee noted that the stocks of the International Standard for Purified Protein Derivative (PPD) of Mammalian Tuberculin were nearly exhausted (WHO/BS/81.1306). The Committee noted also that the State Serum Institute, Copenhagen, had reconstituted in a buffer solution the contents of several ampoules containing larger quantities of the standard and redistributed it in smaller equal quantities of about 5000 International Units into each ampoule. The ampouled material has been freeze-dried as the proposed second international standard for purified protein derivative (PPD) of mammalian tuberculin.

The Committee agreed that as the proposed second international standard was prepared from the first international standard there was no need to carry out a full collaborative assay in order to determine the biological activity of the contents of the ampoules, but that it was appropriate to compare the activity of the proposed second international standard with that of the first international standard in a limited study and also, if possible, to have an estimate of its activity in man. The Committee therefore requested the State Serum Institute to arrange a collaborative assay for this purpose.

15. Rabies Vaccine

The Committee noted that, in accordance with the request made in its thirtieth report (WHO Technical Report Series No. 638, 1979, p. 15), the WHO Secretariat had arranged a collaborative study on the relative potency of a proposed working reference preparation (CVR2) in comparison with the third International Reference Prepa-
ration of Rabies Vaccine (WHO/BS/81.1332). The study had also included a proposed fourth international reference preparation.

The Committee noted also that the results of the in vivo potency assays involving the immunization of mice with the reference materials and their subsequent challenge with virus were highly variable and had been unable to differentiate between the potencies of any of the three preparations. This was due to the inherent imprecision of such a test with the number of animals used.

The Committee was informed that when a single radial diffusion technique was used for the determination of the antigen content of the third international reference preparation and CVR2 there was a significant difference between the two preparations with the former containing the greater quantity of antigen.

The Committee requested the National Institute for Biological Standards and Control, London, to arrange a collaborative assay to compare the antigen contents of several different rabies vaccines, including the reference materials, by methods including immunodiffusion techniques.

16. Tetanus Toxin

The Committee was informed that the National Institute for Public Health, Bilthoven, Netherlands, had studied the irregular pattern of results for the potency of preparations of tetanus toxin as previously reported by the National Institute for Biological Standards and Control, London (WHO/BS/80.1279). The Committee agreed with the institutes in both London and Bilthoven that these data showed that it was impracticable to establish a single tetanus toxin preparation for international use in tests for the assay of tetanus antitoxins.

The Committee agreed also that the small improvements that may be gained from the study mentioned in its thirty-first report (WHO Technical Report Series No. 658, 1981, p. 17) would not justify the large effort required, and accordingly recommended that the study should not be continued.

17. Yellow Fever Vaccine

The Committee noted that the collaborative assay, referred to in its thirtieth report (WHO Technical Report Series No. 638, 1979, p. 19), of a number of yellow fever virus preparations had been com-
pleted (WHO/BS/81.1328). The results demonstrated that with all assay methods the reproducibility was improved when a reference preparation was included in each assay.

The Committee was informed that owing to the variation in sensitivity of the assay systems used in different laboratories it would not be feasible to assign an agreed virus content to a particular preparation that would have the same value in all laboratories. Nevertheless, the inclusion of a reference preparation in each titration for the purposes of comparison would serve a useful purpose.

The Committee was informed also that the National Institute for Biological Standards and Control, London, had received two preparations for consideration as an international reference preparation for yellow fever virus titration and that they had arranged a limited collaborative study to determine the suitability of one of these preparations for this purpose.

**BLOOD PRODUCTS AND RELATED SUBSTANCES**

18. **Haemoglobincyanide**

The Committee noted that in accordance with the authorization in its thirty-first report (WHO Technical Report Series 1981, No. 658, p. 22) the National Institute for Public Health, Bilthoven, had started the issue of batch No. 90500 of haemoglobincyanide solution as the third International Reference Preparation of Haemoglobincyanide (WHO/BS/81.1298). This batch will continue to be distributed until 31 December 1983.

19. **Plasmin**

The Committee was informed that a collaborative study of the freeze-dried plasmin referred to in its thirty-first report (WHO Technical Report Series, No. 658, 1981, p. 19) had been completed and that the results were being analysed (WHO/BS/81.1341). The preliminary results had shown that the preparation was satisfactory.

Since the first international standard was no longer available, the Committee authorized the National Institute for Biological Standards and Control, London, to establish this highly purified and stable preparation as the second International Reference Preparation of Plasmin.
and, on the basis of the results of the collaborative assay and with the agreement of the participants, to define the international unit.

The Committee recommended that, to preserve continuity of the international unit of activity as defined by the first International Reference Preparation of Plasmin, the second international reference preparation should be assigned a unitage on the basis of fibrinolytic assays. This second International Reference Preparation of Plasmin will also be suitable for use in amidolytic assays.

20. Blood Coagulation Factor VIII in Human Plasma

The Committee was informed that in accordance with the request made in its thirty-first report (WHO Technical Report Series No. 658, 1981, p. 19) the National Institute for Biological Standards and Control, London, had recently completed a collaborative study of a freeze-dried plasma which was being evaluated as a proposed reference preparation of blood coagulation factor VIII-related activities in plasma, and that the data were now being analysed. Preliminary studies had shown that the freeze-dried plasma had sufficient stability to serve as an international reference preparation of blood coagulation factor VIII, in human plasma. The Committee recognized the need for this international reference preparation to be used for the calibration of factor VIII-related activities in national and other plasma standards. The existing International Standard for Blood Coagulation Factor VIII, Human, which is a freeze-dried concentrate of blood coagulation factor VIII, will continue to be used for the calibration of national standards of therapeutic concentrates of factor VIII.


The Committee noted the results of a collaborative study of the haemagglutinating activities of (1) a proposed second international standard for anti-A blood typing serum, (2) a proposed third international standard for anti-B blood typing serum, and (3) a proposed international standard for anti-A,B blood typing serum (WHO/BS/81.1309).

The Committee was informed that the participants in the study had agreed on the proposed potencies of the preparations. The Committee, therefore, established the three preparations, respectively, as:
(1) the second International Standard for Anti-A Blood Typing Serum, the activity of the contents of each ampoule being defined as 470 International Units of Anti-A Blood Typing Serum;
(2) the third International Standard for Anti-B Blood Typing Serum, the activity of the contents of each ampoule being defined as 860 International Units of Anti-B Blood Typing Serum;
(3) the International Standard for Anti-A,B Blood Typing Serum, the anti-A,B activity of the contents of each ampoule being defined as 400 International Units of Anti-A activity and 240 International Units of Anti-B activity.

22. Fluorescein Isothiocyanate-Conjugated Sheep Anti-Human IgG (Anti-\gamma-Chain)

The Committee noted a report from the International Union of Immunological Societies on the results of the collaborative assay of the proposed international standard for fluorescein isothiocyanate (FITC) conjugated sheep anti-human IgG (anti-\gamma-chain) (WHO/BS/81.1297), which showed the material to be potent, immunologically specific, and stable.

The Committee established the preparation as the International Standard for Fluorescein Isothiocyanate-Conjugated Sheep Anti-Human IgG (Anti-\gamma-Chain) and, on the basis of the results of the collaborative assay, defined the activity of the contents of each ampoule as 100 International Units of Fluorescein-Isothiocyanate-Conjugated Sheep Anti-Human IgG (Anti-\gamma-Chain).

23. International Reference Preparation of Calcium, Chloride, Magnesium, Potassium, and Sodium in Serum

The Committee noted the results of the study coordinated by the Centers for Disease Control, Atlanta, GA, USA, of the measurement of electrolytes in a proposed reference preparation of bovine serum (WHO/LAB/BS/81.1330). The Committee agreed that it was important to have a reference material similar to those specimens that would be compared with it and that such a reference preparation would be of great assistance to laboratories in setting up working reference preparations.
The Committee established the serum studied as the International Reference Preparation of Calcium, Chloride, Magnesium, Potassium, and Sodium in Serum, and, on the basis of the results assigned:

- 2.2687 mmol Ca/l
- 100.2 mmol Cl/l
- 0.86809 mmol Mg/l
- 4.791 mmol K/l
- 142.42 mmol Na/l

as the concentrations of these electrolytes in this International Reference Preparation.

**ENDOCRINOLOGICAL AND RELATED SUBSTANCES**

### 24. Human Growth Hormone

The Committee noted that two candidate preparations (80/505 and 80/521) of human growth hormone had been assayed in a collaborative study against the International Standard for Growth Hormone, Bovine, for Bioassay, the stocks of which were almost exhausted (WHO/BS/81.1322). The Committee noted also that nearly all of the results of *in vivo* bioassays, radioimmunoassays, and receptor assays had been received. The Committee was informed that the statistical analysis of the results had shown that one of the preparations was suitable for use as a standard.

The Committee therefore authorized the National Institute for Biological Standards and Control, London, to establish the preparation as the International Standard for Growth Hormone, Human, for Bioassay, and on the basis of the results of the collaborative study and with the agreement of the participants, to define the international unit.

### 25. Follicle-Stimulating Hormone (FSH), Pituitary, Human, for Immunoassay

The Committee noted that, in collaboration with the Reproductive Endocrinology Research Unit, Karolinska Institute, Stockholm, the National Institute for Biological Standards and Control, London, had compared the *in vivo* and *in vitro* biological activities and immuno-reactivities of 12 preparations of highly purified human pituitary FSH
(WHO/BS/81.1314). The Committee noted also that the micro-
heterogeneous composition of the preparations revealed by electro-
focusing had also been studied and was shown to be different in each;
as a result of these studies a preparation had been selected that had
a high in vivo and in vitro biological FSH potency and was almost
free from other pituitary hormones.

The Committee therefore requested the National Institute for
Biological Standards and Control to arrange a collaborative assay of
this material.

26. Parathyroid Hormone, Human, for Immunoassay

The Committee noted that the National Institute for Biological
Standards and Control, London, had analysed the data obtained in a
collaborative study of a highly purified human parathyroid hormone
extract obtained from adenomas (WHO/BS/81.1315). The results
of the study confirmed that the ampouled material had biological
activities in in vitro bioassay systems. The Committee noted also that
the activity of the proposed international reference preparation had
been calibrated by immunoassays in terms of the unit of the Research
Standard of Human PTH (75/549) of the National Institute for
Biological Standards and Control, which has been used internationally.

The Committee therefore established the material as the Interna-
tional Reference Preparation of Parathyroid Hormone, Human, for
Immunoassay, and, on the basis of the results of the collaborative
assay defined the activity of the contents of each ampoule as 0.1 Inter-
national Unit of Parathyroid, Human, for Immunoassay.

27. Parathyroid Hormone, Bovine, for Bioassay

The Committee noted that the International Reference Preparation
of Parathyroid Hormone Bovine, for Bioassay, established in 1974,
has been shown (WHO/BS/81.1315) to contain impurities that make
it unsuitable for use in certain in vitro bioassay systems. The Com-
mitee noted also that it would be desirable to replace the international
reference preparation with a preparation suitable for both in vivo and
in vitro bioassays and distributed in amounts appropriate for in vivo
and in vitro assays, which require markedly different quantities of
material. The Committee recognized that the cost of obtaining a suf-
ficient number of bovine parathyroid glands to achieve this objective
is very high and that support or assistance will need to be sought from various sources.

The Committee requested the National Institute for Biological Standards and Control, London, to try to obtain such bovine parathyroid glands and to prepare highly purified bovine parathyroid hormone from them.

28. Insulin

The Committee noted that the National Institute for Biological Standards and Control, London, had received quantities of highly purified porcine and bovine insulins, human insulin derived by chemical modification of porcine insulin, and human insulin made by combining A and B chains made by recombinant DNA procedures (WHO/BS/81.1323). All these insulins had been distributed into ampoules and freeze-dried.

The Committee requested the National Institute for Biological Standards and Control to arrange a collaborative study of these four preparations and to examine their suitability for use as reference materials to replace the international standard for insulin.

29. Thyroid Stimulating Hormone, Human, for Immunoassay

The Committee noted that further to the preliminary investigation mentioned in its thirtieth report (WHO Technical Report Series No. 638, 1979, p. 25), the National Institute for Biological Standards and Control, London, had now selected two of five materials as candidate preparations for the replacement of the International Reference Preparation of Thyroid Stimulating Hormone (Pituitary TSH), Human, for Immunoassay (WHO/BS/81.1321). The Committee noted also that these materials had been distributed into ampoules and requested the National Institute for Biological Standards and Control to arrange a collaborative assay.

30. Chorionic Gonadotrophin, Human

The Committee noted that the International Reference Preparation of Chorionic Gonadotrophin, Human, for Immunoassay and the International Reference Preparation of Chorionic Gonadotrophin, Human,
Beta Subunit for Immunoassay were not convenient for the calibration of certain pregnancy detection kits and that proposals had been made by manufacturers to prepare a working standard for use in the control of such kits.

The Committee noted also, that urine from women in the first trimester of pregnancy had now been distributed into ampoules and freeze-dried (WHO/BS/81.1331). It was intended that this preparation should be assayed against preparations of human chorionic gonadotrophin of medium and high purity, the second International Standard for Chorionic Gonadotrophin, Human, for Bioassay, the International Reference Preparation of Chorionic Gonadotrophin, Human for Immunoassay, and the United States Pharmacopeia Human Chorionic Gonadotrophin Reference Standard by various immunoassay systems and also by in vivo bioassays.

The Committee noted that, in accordance with the request made in its thirty-first report (WHO Technical Report Series No. 658, 1981, p. 23) this work was being carried out by manufacturers in collaboration with the National Institute for Biological Standards and Control, London.

31. Prolactin, Human, for Immunoassay

The Committee noted that the supply of the International Reference Preparation of Prolactin, Human, for Immunoassay was almost exhausted (WHO/BS/81.1324) and that in spite of the scarcity of the material and the difficulties encountered in its extraction from human pituitaries a proposed replacement material had now been obtained, dispensed into ampoules and freeze-dried. The Committee noted also that a collaborative assay was being arranged.

In view of the urgent need for a replacement reference preparation the Committee authorized the National Institute for Biological Standards and Control, London, to establish the preparation as the second international reference preparation of prolactin, human, for immunoassay and on the basis of the results of the collaborative assay and with the agreement of the participants to define the international unit.

32. Tetracosactide for Bioassay

The Committee noted the results from two laboratories of the bioassay of a proposed international reference preparation of tetracosac-
tide for bioassay (WHO/BS/81.1313). Several physical methods were also included in the collaborative study in order to characterize the material.

The Committee noted also that the report of the study had been sent for comment to 21 laboratories in 10 countries, which agreed that this material was suitable to serve as an international reference preparation and that the proposed definition of the unit was acceptable.

The Committee therefore established the material (coded 80/590) as the International Reference Preparation of Tetracosactide, for Bioassay, and, on the basis of the results of the collaborative study, defined the activity of the contents of each ampoule as 490 International Units of Tetracosactide, for Bioassay.

33. Kininogenases

The Committee noted that the National Institute for Biological Standards and Control, London, had received ampouled and freeze-dried preparations of human kininogenase from urine and from plasma (WHO/BS/81.1326) in addition to the highly purified porcine pancreatic kininogenase (code 78/543) previously described (WHO/BS/80.1260).

The Committee noted also that a collaborative study was in progress to assess the suitability of the freeze-dried porcine pancreatic kininogenase to serve as an international reference material and that the study would also evaluate various methods of estimating kininogenase activity using bioassay as well as immunoassay and enzymatic methods; the study included the two preparations of human material. The Committee was informed that the proposed reference material had been shown to be stable and that the results of the study were being analysed.

REFERENCE REAGENTS

34. Adenovirus Antisera, Equine: Types 34, 35 and 36

The Committee noted that three adenoviruses had now been shown to be new serotypes designated 34, 35 and 36 (WHO/BS/81.1310 and WHO/BS/81.1333). Equine antisera to each of the serotypes had been prepared and the sera had been dispensed into ampoules,
freeze-dried, and shown to be specific (WHO/BS/81.1310 Add. 1 and WHO/BS/81.1333). The Committee was informed that the stability of the sera was satisfactory.

The Committee established the three specific antisera as the International Reference Reagents of Adenovirus Antisera, Equine for Types 34, 35 and 36 and noted that the sera will be distributed by the Centers for Disease Control, Atlanta, GA, USA.

35. Histoplasmin Antigen (H and M) and its Antiserum, Rabbit

The Committee noted that an international collaborative study of a proposed histoplasmin reference preparation containing H and M antigens and a proposed reference serum against them had been completed (WHO/BS/81.1308). The immunodiffusion test used showed that the serum was specific and the freeze-dried histoplasmin, when stored at −20 °C, was stable for 2 years for the H antigen but showed a loss of 20% of the M antigen activity over this period. The Committee noted that all the participants had found the antigen and the serum to be suitable as international reference reagents.

The Committee established the histoplasmin as the International Reference Reagent of Histoplasmin and established the serum as the International Reference Reagent of Histoplasmin for H and M Immunodiffusion Test, and

the International Reference Reagent of Histoplasmin Antiserum, Rabbit, for H and M Immunodiffusion Test.

The Committee noted that the reagents would be stored at −70 °C and distributed by the Centers for Disease Control, Atlanta, GA, USA. The Committee agreed that a description of the technique of using these reference reagents, to be known as the WHO method, should be distributed with the reagents.

**REQUIREMENTS FOR BIOLOGICAL SUBSTANCES**

36. Requirements for Pertussis Vaccine, Single or Combined

The Committee was informed that in the revised Requirements for Pertussis Vaccine and the revised Requirements for Diphtheria Toxoid,
Pertussis Vaccine, Tetanus Toxoid and Combined Vaccines (WHO Technical Report Series, No. 638, 1979, Annex 1), the manufacturers are not permitted to repeat the potency test of the pertussis component more than once. The Committee noted that this restriction was causing difficulties for manufacturers and that it had no sound statistical basis (WHO/BS/81.1335).

The Committee agreed that more than one repeat test should be permitted provided that all the potency values were taken into consideration in the calculation of the potency of the pertussis component.

The Committee adopted an addendum to these requirements and agreed that it should be annexed to this report (Annex 1).

37. The Dating Period of Vaccines containing a Pertussis Component

The Committee was informed that countries importing vaccines containing pertussis expected them to have a full shelf-life on importation. Under the present WHO requirements this was not possible where the expiry date is calculated from the start of the potency test (WHO/BS/81.1334).

The Committee agreed that some limited latitude should be given to the manufacturers. The Committee adopted an amendment to the requirements and agreed that it should be annexed to this report (Annex 1).

38. Requirements for Poliomyelitis Vaccine (Inactivated)

The Committee was informed that the advances in technology of production and test methods warranted a complete revision of the Requirements for Poliomyelitis Vaccine (Inactivated). Of particular importance was the recognition that the virus may be grown in well characterized nontumorigenic continuous cell lines, provided that the vaccine is purified and shown to contain no detectable cellular DNA and that tests for freedom from tumorigenicity of the cell lines have been included. Another important advance is the recognition of the need to ensure that the vaccine is sufficiently concentrated and is shown by both in vitro and in vivo methods to contain an adequate quantity of all three antigenic types.
The Committee was also informed that the test for the detection of virulent virus particles that may have escaped inactivation has been reviewed. The experience over the last 25 years has shown that the inoculation of cell cultures for this purpose is more sensitive than the inoculation of monkeys, and therefore the appropriate change for the detection of residual virulent virus by cell cultures has been made. The Committee was informed that there is a pressing need to make this purified and concentrated vaccine more readily available.

The Committee noted the proposed revisions to the Requirements for Poliomyelitis Vaccine (Inactivated) (WHO/BS/79.1242 Rev. 2). The Committee adopted the revised Requirements for Poliomyelitis Vaccine (Inactivated) and agreed that they should be annexed to this report (Annex 2).

39. A Review of Tests on Virus Vaccines

The Committee was informed that the experience in virus vaccine production and control over the last 25 years had shown that there was justification for a complete review of the tests for extraneous agents applied both during production and on the final product. During this time there had been a gradual shift from the use of tissues from free-living animals to tissues from colony bred animals or cell cultures with known characteristics. It had been shown that the latter tissues were rarely contaminated with extraneous agents. Furthermore, it had been shown that the tests in cell cultures had been more effective for the detection of extraneous agents than the inoculation of mice, guinea-pigs, and rabbits.

The Committee noted that in the review of the tests (WHO/BS/81.1305 and Addendum) it was suggested that greater control should be placed on the cells and culture fluids at that stage of production at which adventitious agents are most likely to be detected. They noted also that when the cell substrates were derived from a source that had been shown over many years to be free from contaminants, and when the virus seed had also been shown to be a pure culture of the virus for production, then the control tests could be confined to the detection of contaminants that may arise in the course of production of the vaccine. The Committee agreed that the document with the amendments suggested in the addendum would be a useful guide to national control authorities for the amendment of national requirements. The
Committee adopted the document and agreed that it should be annexed to this report (Annex 3).

40. Requirements for Rift Valley Fever Vaccine

The Committee was informed that in the past four years Rift Valley fever virus had spread to hitherto uninfected areas and that where there was contact between man and animals, particularly at the time of slaughter of the animals, the virus had been transmitted to man on a scale that had not occurred hitherto. Furthermore, there was evidence for the spread of the disease from man to man.

For many years, cattle in infected areas have been vaccinated with a live attenuated vaccine but the more recent transmission of the virus to man, with fatal results, had emphasized the urgent need for a vaccine for man. Accordingly, at a recent WHO meeting a request was made for the formulation of requirements for rift valley fever vaccine (inactivated) for use in man.

The Committee noted that such requirements had been formulated and distributed for comment (WHO/BS/81.1329). The tests included were based on those that had been successfully applied in the production and control of poliomyelitis vaccine (inactivated). In view of the urgent need for these requirements, the Committee adopted the Requirements for Rift Valley Fever Vaccine (Inactivated) for Human Use and agreed that they should be annexed to this report (Annex 4).

41. Requirements for Antibiotic Susceptibility Tests

The Committee noted that the Secretariat had sought additional information from a number of individuals in several countries concerning the codes for antibiotic discs included in the Requirements for Antibiotic Susceptibility Tests (WHO Technical Report Series, No. 610, 1977, p. 98; WHO/BS/81.1337). The Committee noted also that the replies received covered not only codes but also much broader issues, such as the possible inclusion of synthetic antimicrobials in the requirements and changes in certain labelling and manufacturing requirements, and that a completely revised and retitled

version of the requirements had therefore been prepared (WHO/BS/81.1337, Rev. 1). The Committee adopted the revised Requirements for Antimicrobial Susceptibility Tests and agreed that they should be annexed to this report (Annex 5).

In view of the recurring need for codes for new antimicrobics, the Committee requested the WHO Secretariat to develop appropriate codes as part of the programme of devising international nonproprietary names.

42. Stability of Measles Vaccine

The Committee noted that tests on the heat stability of freeze-dried measles vaccine (live) from some manufacturers had shown some vaccines to be unsatisfactory for distribution in countries with high ambient temperatures (Weekly Epidemiological Record, 1981, 56, No. 23., 177–178). In order to ensure that vaccines distributed in such countries would be satisfactory, a stability test had been proposed that, when satisfied, would ensure that the vaccine would be stable for at least seven days when held at 37 °C (WHO/BS/81.1338).

The Committee adopted this amendment to the Requirements for Measles Vaccine (Live) and agreed that it should be annexed to this report (Annex 6).

43. The Collection, Fractionation, Quality Control and Uses of Blood and Related Products

The Committee was informed that since the publication of the Requirements for the Collection, Processing and Quality Control of Human Blood and Blood Products (WHO Technical Report Series, No. 626, 1978, Annex 1) four subjects had been discussed in greater detail by consultative groups. These were: (1) plasmapheresis and immunization of donors; (2) indications and contraindications for the use of albumin solutions; (3) indications and contraindications for the use of coagulation factor concentrates; and (4) indications and contraindications for the use of normal and specific immunoglobulins.

The Committee noted that the consensus of these four consultative groups have been published together with the requirements under the title The Collection, Fractionation, Quality Control, and Uses of Blood and Blood Products, Geneva, World Health Organization, 1981.
ACKNOWLEDGEMENTS

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Annex I

REQUIREMENTS FOR DIPHTHERIA TOXOID, PERTUSSIS VACCINE, TETANUS TOXOID AND COMBINED VACCINES

(Requirements for Biological Substances Nos. 8 and 10)
(Revised 1978)
Addendum 1981

The WHO Expert Committee on Biological Standardization in 1978 adopted the revised Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid and Combined Vaccines and these were published in its thirtieth report (WHO Technical Report Series No. 638, 1979, Annex 1). For many years it has been considered satisfactory to allow one repeat test of the potency of the pertussis component of either the single or combined vaccine.

Several manufacturers have indicated that there is no statistical basis for restricting the total number of potency assays, to two.

Accordingly, in its thirty-second report, the WHO Expert Committee on Biological Standardization agreed that more than one repeat test should be permitted and adopted the following amendment to the potency test:

Revision to the Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid and Combined Vaccines with reference to the Requirements for the Pertussis Component

Part A, section 3.4.6 Potency test

Replace the last sentence of subparagraph (g), p. 71, by the following:

Additional tests may be made, and in this case the vaccine passes the requirements for potency only if the weighted geometric mean of all the potency values found in statistically valid tests is equal to or more than 4 IU in the volume recommended for a single human dose.

Expiry date

The need for a further revision of these requirements has come to light that concerns the expiry date.

Countries importing vaccine expect to have vaccine with a full shelf-life on importation. Under the present requirements for pertussis vaccine (Part A, section 10.2) this is not possible since they state “The expiry date shall be fixed with the approval of the national control authority and shall relate to the date of the last satisfactory potency test, the date of the potency test being that on which the test animals were inoculated with the vaccine”.

This difficulty would be overcome if the manufacturer was given some latitude in order to carry out a potency test and hold the vaccine for a short period under refrigeration (5 °C ± 3 °C) and the requirements are, therefore, amended as follows.

Part A, section 10.2, p. 75

Delete last sentence and replace with:

The manufacturer may, with the approval of the national control authority, issue pertussis vaccine single or combined with a full 2-year expiry period provided that the time from the inoculation of the animals for the potency test to the expiry date does not exceed 2 years and 6 months.
Annex 2

REQUIREMENTS FOR
POLIOMYELITIS VACCINE (INACTIVATED)

(Requirements for Biological Substances No. 2)
(Revised 1981)

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GENERAL CONSIDERATIONS

Since the Requirements for Poliomyelitis Vaccine (Inactivated) first formulated in 1959 (1) were revised in 1965 (2, Annex 2) there have been several advances in technology in vaccine production and control that make a further revision of the requirements necessary. Among the most significant changes has been the degree to which the vaccine itself can now be purified.

Additional important advances have been made in the use of different cell substrates and the large-scale production of cells for the propagation of the virus used in the vaccine. Three methods have been used, each of which has been employed in an attempt to avoid the contaminating viruses frequently present in monkeys caught in the wild and quarantined for 6–8 weeks.

The first method under consideration is the production of killed poliomyelitis vaccine in human diploid cells, which have been used successfully for many years for the growth of the attenuated polio-
myelitis viruses. This would not give rise to any unforeseen difficulties but preliminary data have shown that the yields of virus are not as high as with monkey kidney cells and that the large-scale cultivation of human diploid cells is still problematic. If these problems can be solved, then this step may be the one of choice.

The second method involves the use of primary monkey kidney cell cultures that have subsequently been passaged to produce secondary and tertiary cells. Such a procedure increases the cell yield from a pair of kidneys thereby increasing the virus yield. In order to be successful in the serial propagation of the monkey kidney cells, it is necessary to use monkeys bred in captivity. It has been shown that these monkeys harbour fewer viruses than those caught in the wild. Cells at the tertiary stage will have gone through 10–15 cell doublings. Since the further subcultivation of such monkey kidney cells has produced several continuous cell lines with abnormal chromosomes, a test for tumorigenicity on the production cultures has been included as a precautionary measure.

The third method rapidly under development is the use of non-tumorigenic continuous cell lines. The justification for this is the possibility of growth of much larger quantities of cells, possibly in cultures using microcarrier systems with a consequently higher yield of virus. This may decrease the cost of the vaccine to bring it within reach of the majority of countries. However, because such cells are usually chromosomally abnormal it is essential that the vaccine does not contain biologically active macromolecules derived from the cell substrate. It is necessary, therefore, that the continuous cell line used shall be approved by the national control authority and that the method of purification for the removal of cellular DNA and the method of concentration of the virus also have such approval. The approval for the use of vaccines prepared in continuous cell lines must be given by the national control authorities who are responsible for the health of the community.

The reformulation of these requirements has taken these three methods into consideration.

The test in monkeys for the detection of virulent virus that may have escaped inactivation and the part it may play in assuring the safety of the vaccine have been reviewed. The experience over the last 25 years has shown that the test procedure of inoculation of monkeys, even by the intraspinal route, is no more sensitive than that of inoculation of cell cultures. Furthermore, the volume of vaccine that can be added to cell cultures is much greater than can be injected in mon-
keys. There is a growing shortage of monkeys and it is important that their use should be restricted to tests for which there is no equally acceptable alternative. The decision to omit a test in monkeys for residual virus, however, must rest with the national control authority. In any event, if the test in monkeys is retained by the national control authority there does not appear to be any justification for the test to be done by both the manufacturer and the control authority.

The tests for extraneous agents have been reviewed also and although the present requirements do not include tests for extraneous agents that might be present in the cell substrate used for virus growth manufacturers are carrying out such tests. It would not be an additional burden, therefore, to include such tests and bring these requirements into line with those that have been formulated for other virus vaccines. The most sensitive and logical point in the manufacturing process at which to test for extraneous agents that may be present in the cell substrate is at the time of the inoculation of the production cultures, when a sample of the cells is set aside as uninoculated control for examination at the end of the observation period.

There are several in vitro methods under investigation for measuring the concentration of the immunizing antigen in the vaccine. The test measuring the concentration of the D antigen has been correlated with the ability of the vaccine to stimulate antibody production in man and animals. These in vitro tests can give a useful guide as an “in process” control to manufacturers, especially where purification and concentration steps are included in the production process. Nevertheless, it is important to retain a test in animals that measures the ability of the vaccine to give an antibody response and await the outcome of other in vitro tests. In recent investigations the rat has been shown to be useful for the in vivo test.

The pressing need is to make more generally available inactivated poliomyelitis vaccines that have uniformly high potency and that will reliably give an immunogenic response in children after one or two doses. Such vaccines will be useful particularly in the developing countries where poliomyelitis is largely uncontrolled. As the vaccine will be used in countries with high ambient temperatures it is important to ensure that the vaccine is stable under such conditions.

As with the requirements for vaccines that have been formulated or revised during the last five years, a protocol for reporting the results of all tests has been included (see Appendix). Completion of such a protocol, which is based on the present revised requirements, enables a control authority to check whether all the tests have been completed.
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 of their national regulations concerning poliomyelitis vaccine (inactivated), it is recommended that a clause should be included permitting modifications of manufacturing requirements on the condition that it can be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure that the degree of safety and the potency of the vaccine are at least equal to those provided by the requirements formulated below. 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 vaccine is manufactured.

PART A.
MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be “Vaccinum poliomyelitidis inactivatum”. The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

1.2 Descriptive definition

Vaccinum poliomyelitidis inactivatum shall consist of an aqueous suspension of poliovirus types 1, 2 and 3 grown in cell cultures and
inactivated. The preparation shall satisfy all the requirements formulated below.

1.3 International standards or reference preparations and international units

The International Reference Preparation of Poliomyelitis Vaccine (Inactivated) (established in 1962) is stored frozen in ampoules containing 10 ml of trivalent inactivated poliomyelitis vaccine. This reference preparation is intended for the determination of the relative potencies of preparations of poliomyelitis vaccines (inactivated).

A proposed replacement international reference preparation of trivalent vaccine is under study.

The International Standards for Antipolio virus Sera type 1, type 2 and type 3 (established in 1962) are stored in ampoules each containing 10 IU of dried hyperimmune monovalent serum per ampoule. These standards are intended for calibration of national standards for antipolio virus sera.

The above standards and reference preparation are in the custody of the International Laboratory for Biological Standards, State Serum Institute, Copenhagen. Samples are distributed free of charge on request to national control laboratories.

1.4 Terminology

*Virus seed lot.* A quantity of virus processed together and of uniform composition. In each manufacturing establishment a *primary virus seed lot* is that from which further *virus seed lots* are prepared. Vaccine is one passage removed from the seed lot, i.e., 2 passages from the primary seed lot.

*Cell seed.* A quantity of cells derived from normal tissue and stored frozen at −70 °C or below in aliquots, one or more of which would be used for the production of a manufacturer's working cell bank (MWC B).

*Manufacturer's working cell bank (MWC B).* All of the cellular material derived by serial subculture from one or more ampoules of cell seed. After serial subculture the cells are combined into a pool and preserved cryogenically to form the MWC B. One or more ampoules from a pool would be used for the production of a single harvest.

*Single harvest.* A virus suspension of one virus type harvested from cell cultures that have been derived from a single batch of cells and processed together.
Monovalent pool. A virus suspension of a single virus type processed at the same time.

Purified and concentrated monovalent pool. A purified and concentrated virus suspension or vaccine of a single virus type processed at the same time.

Trivalent bulk. A pool of a number of monovalent pools and containing all three virus types.

Final bulk. The finished biological preparation present in the container from which the final containers are filled.

Filling lot (final lot). A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling. A filling lot must, therefore, have been filled in one working session, from a single final bulk.

Tissue culture infective dose 50% (TCID_{50}). The smallest quantity of a virus suspension that will infect 50% of inoculated cell cultures.

Plaque-forming unit (PFU). The smallest quantity of a virus suspension that will produce a plaque in cell cultures.

2. GENERAL MANUFACTURING REQUIREMENTS

The general manufacturing requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirement for Manufacturing Establishments and Control Laboratories) (2, Annex 1) shall apply to establishments manufacturing poliomyelitis vaccine (inactivated), with the addition of the following directives:

Production areas shall be decontaminated before they are used for the manufacture of poliomyelitis vaccine.

The production of poliomyelitis vaccine shall be conducted by staff who have not handled other infectious microorganisms or animals in the same working day. The staff shall consist of persons who shall be examined medically and found to be healthy. Steps shall be taken to ensure that all such persons in the production areas are immune to all three poliomyelitis viruses.

No culture of any microorganisms other than the cultures approved by the national control authority for the production of poliomyelitis vaccine shall be introduced or handled in the production area at any time during manufacture of the vaccine.

Persons not directly concerned with the production processes shall not be permitted to enter the production area.
3. PRODUCTION CONTROL

3.1 Control of source materials

3.1.1 Virus strains and seed lot system

Strains of poliovirus used in the production of vaccine shall be approved by the national control authority. They shall be identified by historical records, by infectivity tests, and by immunological methods. Any strain that will yield a vaccine meeting the requirements set forth in the present document may be used. Production of vaccine shall be based on the seed lot system; the seed virus used for vaccine production shall not have passed more than 10 subcultures, counted from a seed lot used for the production of the vaccine on which the original laboratory and field tests were done.

Samples of the strains used shall be deposited in the national control laboratory.

3.1.2 Cell cultures for virus propagation

3.1.2.1 Monkeys used for obtaining kidney cell cultures. If monkey kidney cell cultures are used, monkeys that are in good health and of a species approved by the national control authority and that have not previously been used for experimental purposes shall be used as the source of kidney tissue for the production of virus.

An exception can be made in the case of monkeys used for the safety or potency tests if these have given negative clinical findings.

The monkeys shall be kept in well-constructed animal rooms in adequately ventilated cages spaced as far apart as possible. Adequate precautions shall be taken to prevent cross-infection between cages. Not more than 2 monkeys shall be housed in each cage and cagemates shall not be interchanged. The monkeys shall be kept in the country of manufacture of the vaccine in quarantine groups for a period of not less than 6 weeks before use. If at any time during the quarantine period the overall death rate of a shipment consisting of one or more groups reaches 5% (excluding deaths from accidents or causes specifically determined not to be due to infectious diseases) monkeys from that entire shipment shall continue in quarantine from such time for

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2 A quarantine group is a colony of selected, healthy monkeys kept in one room, with separate feeding and cleaning facilities and having no contact with other monkeys during the quarantine period.
a further period of not less than 6 weeks. Only monkeys that have reacted negatively to tuberculin at the start of the quarantine period and again within 2 weeks prior to use shall be used in the manufacture of vaccine. The groups shall be kept continually in isolation, as in quarantine, even after completion of the quarantine period, until the monkeys are used or discarded. After the last monkey of a group has been taken, the room that housed the group shall be thoroughly cleaned and decontaminated before being used for a fresh group.

In some countries the test for sensitivity to tuberculosis in monkeys may be omitted because the monovalent pools are subjected to two stages of filtration through 0.22 μm filters.

All actions of working personnel shall be based on the premise that there is a great potential hazard at all times in the quarantine area. Personnel shall be provided with protective clothing, including gloves, footwear, and masks or visors. Street clothes shall not be permitted in the animal rooms. Personnel shall be forbidden to smoke, eat or drink while they are in the animal rooms.

Responsibility shall be assigned to a supervisor for reporting illness among employees and ensuring that all injuries are properly treated. No worker who has cuts or abrasions on exposed areas of the body shall enter the animal area. Any unexplained febrile illness while off duty shall be considered as potentially related to the employee's occupation.

Monkeys from which kidneys are to be removed shall be thoroughly examined at necropsy, particularly for evidence of tuberculosis and simian herpesvirus (B virus) infection. Monkeys prepared for removal of kidneys shall be anaesthetized.

A monkey that shows any pathological lesion relevant to the use of its kidneys in the preparation of a seed lot or vaccine shall not be used, and none of the remaining monkeys of the quarantine group concerned shall be used unless it is evident that their use will not impair the safety of the product.

All operations described in this section shall be conducted outside the areas where vaccine is made.

If the monkey kidney cells are passaged in series, the number of cell doublings permitted before the growth of the poliomyelitis virus shall be approved by the national control authority.

It has been shown that a “primary” cell culture may have undergone about 5 cell doublings, “secondary” cells about 10 cell doublings, and “tertiary” cells about 15 doublings. Prefer
ably, records of the cell doublings should be kept. It has been shown also that because of their decreased exposure to other animals, monkeys bred in captivity may have fewer extraneous agents than monkeys caught in the wild. If the cells are passaged in series, therefore, monkeys bred in captivity may be preferred.

If the kidney cells are serially passaged, then the cells at the production level shall be tested for tumorigenicity (see Part C, section 3.1.2).

In some countries karyology is also required. The extent of such tests should be determined by the national control authority.

3.1.2.2 Cell banks used for providing cell cultures. If human diploid cells are used for the propagation of poliomyelitis virus they shall be those approved by and registered with the national control authority. The cells shall have been characterized with respect to their genealogy, growth characteristics, viability during storage, and karyology, and they shall have been shown, by tests in animals and eggs, to be free from detectable adventitious agents (see Part C).

If continuous cell lines are used for the propagation of poliomyelitis virus they shall be those approved by and registered with the national control authority.

The cells shall have been characterized with respect to their genealogy, growth characteristics, and viability during storage, and they shall have been shown to be free from detectable adventitious agents (see Part D).

3.1.3 Cell culture medium

Serum used for the propagation of cells for vaccine production shall be tested to demonstrate freedom from bacteria, fungi and mycoplasma, according to the requirements given in Part A, sections 5.2 and 5.3 of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (3) as well as freedom from pathogens of the species of the origin of the serum by methods approved by the national control authority.

In some countries sera are examined for freedom from phage.

Penicillin shall not be used at any stage in the production.

Suitable other antibiotics in minimum concentrations required for sterility may be used. Nontoxic pH indicators may be added, e.g., phenol red in a concentration of 20 mg/l.
3.1.4 Tests on virus seed lots

Each virus seed lot shall be subjected to all tests applicable to a monovalent pool (see Part A, sections 3.2, 3.4.1, 3.4.2, 3.4.4, 3.4.5, and 3.4.6). The national control authority shall approve the virus seed lots.

3.2 Control cells

A cell sample equivalent to at least 500 ml of the cell suspension, at the concentration employed for vaccine production cultures, shall be used to prepare control cell cultures.

In some countries in which the technology of large-scale production has been developed, the national control authority should determine the size of the sample of cells to be examined at the time at which the control cells should be taken from the production culture, and the monitoring of the control vessels.

These control cell cultures shall be incubated for at least 2 weeks and shall be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures may be discarded for nonspecific, accidental reasons.

At this time, the control cell cultures shall be examined for any evidence of degeneration caused by an extraneous agent. If human diploid cells or continuous cell lines are used, tests on control cells shall be carried out according to the requirements described in Part C, section 3.2 or Part D, section 3.2, respectively. If primary or subcultured monkey cells are used, the control cells and the supernatant fluid from such cells shall also be tested for extraneous agents by the tests described in Part A, sections 3.2.1, 3.2.2 and 3.2.3. If this examination or any of the tests required in this section show evidence of the presence in a control culture of any adventitious agent, the poliomyelitis virus grown in the corresponding inoculated cultures shall not be used for vaccine production.

In some countries in which primary cell cultures are prepared from the kidneys of monkeys caught in the wild, “foamy virus” is an inherent contaminant. Such cultures may be used for vaccine manufacture at the discretion of the national control authority.

3.2.1 Tests for haemadsorbing viruses

At the end of the observation period or at the time the virus is harvested from the production cultures, whichever is the later, 25% of the control cells shall be tested for the presence of haemadsorbing
viruses using guinea-pig red cells. If the guinea-pig red cells have been stored, the duration of storage shall not have exceeded 7 days, and the temperature of storage shall have been in the range of 2–8 °C.

This test is usually made using guinea-pig red cells. In some countries the national control authority requires that tests for haemadsorbing viruses should be made in addition using other types of red cells, including those from humans (blood group O), monkeys, and chicken (or other avian species).

All tests should be read after incubation for 30 min at 0–4 °C and again after a further incubation for 30 min at 20–25 °C. The test with monkey red cells should be read once more after yet another incubation for 30 min at 34–37 °C.

3.2.2 Tests in rabbit kidney cell cultures

A sample of at least 10 ml of the pooled supernatant fluid from the control cultures shall be tested for the presence of simian herpesvirus (B virus) and other viruses in rabbit kidney cell cultures. Serum used in the nutrient medium of these cultures shall have been shown to be free from B virus inhibitors.2 The sample shall be inoculated into bottles of these cell cultures, in such a way that the dilution of the harvest suspension in the nutrient medium does not exceed 1 in 4. The area of the cell sheet shall be at least 3 cm²/ml of harvest suspension. At least one bottle of the cell cultures shall remain uninoculated and serve as a control.

The cultures shall be incubated at a temperature of 37 °C and shall be observed for a period of at least 2 weeks.

For the test to be valid, at least 80% of the culture vessels shall be available and suitable for evaluation at the end of the test period.

If the presence of B virus is demonstrated, the manufacture of poliomyelitis vaccine shall be discontinued and the national control authority shall be informed. Manufacturing shall not be resumed until a thorough investigation has been completed and precautions have been taken against reappearance of the infection, and then only with the approval of the national control authority.

3.2.3 Test in Cercopithecus kidney cell cultures

A sample of at least 10 ml of the pooled supernatant fluid of the control cultures shall be tested for the presence of SV40 virus and

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2 On account of the danger of handling B virus, herpes simplex virus has been used as the indicator for freedom from B virus inhibitors.
other extraneous agents by inoculation on to cell cultures prepared from the kidneys of *Cercopithecus* monkeys by the method described in Part A, section 3.2.2. The cell cultures shall be incubated at 37°C and observed for a period of at least 2 weeks.

A cell culture shown to be equally sensitive to SV40 virus may be used.

For the test to be valid, at least 80% of the culture vessels shall be available and suitable for evaluation at the end of the observation period.

If there is any cytopathogenic effect attributable to the supernatant fluid, the virus grown on the same batch of cells shall not be used for vaccine production.

### 3.3 Production precautions

The general production precautions as formulated in Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2, Annex 1) shall apply to the manufacture of poliomyelitis vaccine (inactivated). If animal serum is used for the growth of cell cultures, the serum concentration in the final vaccine shall be not more than 1 µl/l. The serum concentration shall be reduced to this level by rinsing the cell cultures with serum-free medium and/or purification of the virus harvests.

In some countries, control tests are carried out to detect the residual animal serum content in the final vaccine (see Part A, section 5.5).

Penicillin shall not be used in the cell or virus culture medium.

### 3.4 Control at the monovalent stages of the product

When the tests on the control cells have shown the cells to be satisfactory, the single virus harvest from such cells may be pooled and samples taken for the following tests.

#### 3.4.1 Test in *Cercopithecus* cell cultures (applied to all seeds and to all harvests grown in monkey kidney cells except for continuous cell lines)

A sample of at least 25 ml of each monovalent pool produced in primary or serially passaged monkey kidney cell culture shall be tested for the presence of SV40 virus or other adventitious agents.
The monovalent pool shall be neutralized by a high-titred antiserum against the specific type of poliovirus.

The immunizing antigen used for the preparation of the antiserum should be shown to be free from extraneous agents and grown in cell cultures free from extraneous microbial agents that might elicit antibodies that could inhibit the growth of any adventitious agents present in the monovalent poliovirus pool.

This sample shall be tested in primary *Cercopithecus* kidney cell cultures or cells that have been demonstrated to be of equal susceptibility to SV40 virus. The tissue cultures shall be incubated at 37°C and observed for 14 days. At the end of this observation period, at least one subculture of fluid shall be made in the same tissue culture system and both primary cultures and subcultures observed for an additional 14 days.

If necessary, serum may be added to the primary cultures at this stage, provided that the serum does not contain SV40 antibody or other inhibitors.

The virus pool passes the test if there is no evidence of the presence of SV40 virus or other adventitious agent attributable to the virus pool.

3.4.2 *Test in rabbits* (applied to all seeds and to all harvests grown in monkey kidney except for continuous cell lines)

A sample of at least 100 ml of each monovalent pool shall be tested as soon as possible after pooling by inoculation into 10 healthy rabbits, each weighing between 1.5 and 2.5 kg; proportionately larger volumes shall be used if more animals are inoculated. The inoculations shall be made at multiple sites, each rabbit being given a total of 1 ml of the monovalent pool by intradermal injection and 9 ml by subcutaneous injection. The animals shall be observed for at least three weeks. All rabbits that die after the first 24 h of the test or that show signs of illness shall be examined by autopsy, with removal of the brain and organs for detailed inspection.

The monovalent pool passes the test if at least 80% of the rabbits remain healthy and if none of the rabbits shows lesions of any kind at the sites of inoculation or shows evidence of infection with B virus or with any adventitious transmissible agent attributable to the monovalent pool.

In some countries it is permissible to replace the test for B virus in rabbits by the test in rabbit kidney cell cultures.
3.4.3 Treatment before inactivation

Prior to inactivation each monovalent virus pool shall be filtered.

The importance of filtration or clarification of the crude virus suspensions as a means of improving the regularity of the inactivation process has been clearly established. Generally, filters are used in series or filtration is performed stepwise through filters of decreasing porosity. Satisfactory results have been reported with several filter types but a final filtration using a 0.22 μm filter should be used.

Filters containing asbestos should not be used.

In some countries the virus suspension is concentrated and purified before inactivation.

For vaccines produced in continuous cell lines the monovalent virus pool shall be purified before inactivation by a method approved by the national control authority (see Part D).

3.4.3.1 Purification of the monovalent pools. Each filtered monovalent pool of virus grown in a continuous cell line (see section D) shall be purified before inactivation. The purification process, which shall be shown to reduce consistently the level of cellular DNA from that of the initial virus harvest by a factor of at least $10^8$, shall be approved by the national control authority.

An acceptable method is to clarify the virus suspension by filtration, to concentrate the virus by ultrafiltration and, thereafter, collect the virus peak after passing it through a sepharose gel filtration column. Further purification is achieved by passing the virus through a DEAE ion-exchange column. Other purification procedures, such as passing the preparation through an immobilized DNA-ase column or immuno-adsorption column, may be used.

The purified pool shall be shown to contain not more that 0.1 μg of protein per D-antigen unit of poliomyelitis virus.

The examination for viral peptides by polyacrylamide gel electrophoresis and sucrose density gradient analysis have been shown to be useful. For the detection of bovine serum proteins, counter-current electrophoresis has been used. Improved methods of analysis for the purity of vaccine, however, should be developed.

3.4.4 Identity test

The virus in the monovalent pool shall be tested for identity by the neutralization of the virus with specific antiserum.
3.4.5 Sterility tests

Each pool after filtration shall be tested for sterility according to the requirements given in Part A, sections 5.1, 5.2 and 5.3, of the Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances) (3, page 49).

3.4.6 Virus titration

Before any inactivating agent is added, a sample shall be taken of each monovalent pool for titration of infective poliovirus using tissue culture methods. This titration shall be carried out in not more than 10-fold dilution steps and using 10 cultures per dilution, or any other arrangement yielding equal precision.

Each monovalent pool should show a titre of not less than $10^7$ TCID$_{50}$ per ml using a batch of tissue culture of normal sensitivity.

In several countries the use of Hep-2 cells in microtitre plates has been shown to be suitable. Other methods of virus titration, such as the enumeration of plaque-forming units, may be used. If this is done, the monovalent pool should show a titre that is on a comparable level.

The main purpose of determining the titre of virus pools destined for inactivation is to select pools that can be expected to meet potency requirements after inactivation.

3.4.7 Time of inactivation

Inactivation shall be initiated as soon as possible and not later than 72 h after filtration.

It is preferable to start inactivation within 24 h. Since the purpose of the filtration step is to remove particulate matter and other interfering substances that may diminish the effectiveness of the inactivation process, and since aggregates tend to increase on standing after filtration, efforts should be made to keep within this time limit.

3.4.8 Inactivation procedure

The virus in the monovalent pools shall be inactivated through the use of an agent or some other method that has been demonstrated to be consistently effective in the hands of the manufacturer. The method shall be approved by the national control authority.
The progress of inactivation shall be followed by suitably spaced determinations of virus titres.

Formaldehyde is used as inactivating agent in the production of vaccines. Most manufacturers have encountered some irregularities in the inactivation process that have not been fully explained. On the basis of the observation that a decrease in the concentration of free formaldehyde occurs in the course of the process, it has been recommended that tests for free formaldehyde should be performed at intervals and the concentration maintained at the desired level by intermittent readjustments. Some manufacturers use a combination of initial formaldehyde treatment with some other method of inactivation.

A second filtration during the process of inactivation shall be made.

This step is made after the virus titre has fallen below detectable levels but before the first sample for the safety test is taken.

The method of inactivation shall be shown to give consistent inactivation for the production of acceptable vaccine. A record of consistency shall be established by the production of 5 consecutive lots and if broken a further 5 monovalent lots shall be prepared and shown to be satisfactory for re-establishing this record.

3.4.9 Test for effective inactivation

Two samples of at least 500 ml or an equivalent volume of concentrated pool diluted to 500 ml from each monovalent pool shall be taken and, after removal or neutralization of the formaldehyde, shall be tested by inoculation into tissue cultures for the absence of infective poliovirus. The kinetics of inactivation shall be established by each manufacturer and approved by the national control authority. One sample shall be taken at the end of the inactivation period and the other not later than three-quarters of the way through this period. The two samples shall be inoculated into bottles of tissue cultures derived from different batches of cells. The dilution of the vaccine in the nutrient fluid shall not exceed 1:4 and the area of the cell sheet shall be at least 3 cm²/ml of vaccine. One or more bottles of each batch of cultures shall be set aside to serve as uninoculated control bottles with the same medium.

The formaldehyde in samples of vaccine for tissue culture tests is usually neutralized at the time of sampling by the addition of bisulfite. Usually, the samples are subsequently dialysed.

It is possible to conduct tissue culture tests on nondialysed material; however, this is often found to be toxic to cells, even
with a dilution of 1:4. If in such tests nonspecific degeneration of cells occurs, or if the sensitivity of the tissue culture system is reduced, the test should be repeated on dialyzed material.

In some countries this test is made using tissue cultures that are also sensitive to SV40 virus, as an additional measure for detecting this extraneous agent.

The tissue culture bottles shall be observed for at least 3 weeks. Not less than 2 subcultures shall be made from each original bottle, one at the end of the observation period and the other one week earlier. The subcultures shall be observed for at least 2 weeks.

If infectious poliovirus is isolated, the monovalent pool shall not be used. The isolation of active poliovirus from a monovalent pool must be regarded as a break in the consistency record.

If viruses other than poliovirus are present, the pool shall not be used unless it can be demonstrated that such viruses have originated from a source other than the monovalent pool being tested.

At the end of the observation period, the cell culture used for the detection of residual live virus shall be challenged with live virus of the same type as that of the monovalent pool. The number of virus particles in the challenge shall be determined by the national control authority.

The problem of detecting residual active poliovirus in a vaccine is not the same as that of measuring infective virus in untreated suspensions. Poliovirus that has been exposed to the action of formaldehyde without becoming inactivated has been shown to require a much longer time to produce cytopathogenic changes than does untreated virus. For this reason it is desirable that tissue cultures in tests for the presence of residual active virus be observed for as long a time as is technically possible. A satisfactory tissue culture system for this purpose therefore depends not only on the sensitivity of the cells used for the preparation of the cultures but also on the nutrient fluid.

The serum added to the nutrient fluid should be tested for inhibitors to poliomyelitis virus. Only serum free from inhibitors to all three types of poliovirus should be used.

Kidney cells from some monkey species, for instance those of the genera Macaca, Cercopithecus and Papio, appear to be more sensitive than others. If other tissue culture systems are used, they should have been shown to possess at least the same sensitivity as those specified above.

Maintenance of the cultures in good condition may require frequent changes of culture medium. However, it should be borne in mind that by early changes of fluid unadsorbed virus might be removed and the validity of the test thus impaired; therefore, the fluid should be changed no earlier than 5–7 days after inoculation.
3.5 Control of trivalent bulk product

Only those monovalent pools that have been shown to be satisfactory shall be blended to form a trivalent bulk.

In some countries a monovalent pool from which live virus has been isolated may be subjected to reactivation and tested again for the presence of live virus. In such cases the national control authority should approve the reactivation procedure.

3.5.1 Tests for absence of infective poliovirus

Before the addition of preservatives, a sample of at least 1500 ml or, if purified and concentrated vaccine is prepared, the equivalent of 1500 doses shall be tested in cell cultures for the absence of infective virus by the procedure described in Part A, section 3.4.9 of these requirements. If infective poliovirus is isolated, this batch of trivalent bulk product shall not be used.

Experience over the last 25 years has shown that the detection of living poliomyelitis virus is more sensitive in cell cultures than in monkeys.

If a test is also made in monkeys, however, then it is recommended that Macaca or Cercopithecus monkeys should be conditioned for at least 6 weeks before they are subjected to this test.

A pre-injection serum sample from each animal should contain no neutralizing antibody against any of the three poliovirus types in a dilution of 1:4 when tested against not more than 500 TCID₅₀ of virus.

Vaccine should be injected, under deep anaesthesia, by combined intracerebral, intraspinal and intramuscular routes into at least 10 monkeys. The intracerebral injection consists of 0.5 ml into the thalamic region of each hemisphere. The intraspinal injection consists of 0.5 ml of vaccine, or of vaccine suitably concentrated, into the lumbar spinal cord enlargement, into which it may be given in divided doses at more than one site. The intramuscular injection consists of 1.0 ml into the right leg.

In some countries the vaccine is concentrated 25-fold to 100-fold by a suitable method, e.g., by centrifugation in gelatin. It has been shown that concentration of vaccine in this way increases the sensitivity of the test.

At the time of inoculation of vaccine, an intramuscular injection of 200 mg of cortisone acetate is given, as well as an intramuscular injection of procaine penicillin. The cortisone acetate may be given in divided doses over a period of several days, starting 3 days before the inoculation of vaccine. The monkeys are observed for 17–19 days and symptoms suggestive of polio-
myelitis should be recorded. Provided that at least 60% of the animals survive the first 48 h after injection, the animals that do not survive this 48-h period may be replaced by an equal number of test animals. If fewer than 60% of the original test animals survive the first 48 h, or if the number of animals that survive the entire test period without significant weight loss is less than 80% of the initial number, the test should be repeated.

At the end of the observation period, samples of nervous tissue may be taken for virus recovery and identification. Histological sections from both spinal cord enlargements and the thalamic area are examined. Doubtful histopathological findings may necessitate (a) examination of samples of sections from several regions of the brain and spinal cord, and (b) attempts at virus recovery from the nervous tissues previously removed from the animal. At least 80% of the animals should show evidence of intraspinal and intrathalamic trauma due to injection.

The final lot passes the test if the histological and other studies show no evidence of poliomyelitis infection.

3.5.2 Sterility test

The trivalent bulk shall be tested for sterility according to the requirements given in Part A, sections 5.1 and 5.2 of the Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (3, page 49).

3.5.3 Preservatives and other substances added

Preservatives or other substances that might be added to or combined with vaccine shall have been shown to have no deleterious effect on the immunizing potency of the product.

4. FILLING AND CONTAINERS

The requirements concerning filling and containers given in Part A, section 4, of the revised Requirements for Biological Standards No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories (2, Annex 1) shall apply with the addition of the following directive:

The containers shall be of colourless and fully transparent glass so as to permit the detection of visible changes in the vaccine.

Single- and multiple-dose containers may be used.
5. CONTROL TESTS ON FINAL PRODUCT

5.1 Identity test

An identity test shall be done on at least one labelled container from each filling lot by an appropriate method.

The potency test described in Part A, section 5.4, of these requirements may serve as the identity test.

5.2 Sterility tests

Each filling lot shall be tested for sterility according to the requirements given in Part A, sections 5.1 and 5.2, of the Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (3, p. 49).

5.3 Innocuity test

Each final lot shall be tested for abnormal toxicity by appropriate tests involving injection into mice and guinea-pigs. The tests shall be approved by the national control authority.

5.4 Potency test

Each vaccine shall be tested for immunizing potency by tests approved by the national control authority. Such tests shall include an in vitro assay for antigen content and an in vivo assay for immune response. In both tests the results obtained with the test vaccine shall be compared with those obtained with a reference vaccine calibrated in international units by comparison with the WHO Reference Preparation3 (see Part A, section 1.3).

The in vitro assays that have been found to be suitable for measuring the antigen content during the stages of vaccine production are complement fixation, ELISA, gel diffusion, single

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3 The proposed WHO reference preparation under study contains 400, 40 and 160 D-antigen units per ml for types I, II and III, respectively. The antibody responses of children to this preparation have been measured.
radial dial immunodiffusion, and radioimmunoassay. It is important to use a test that is sufficiently sensitive to measure the antigen content of the final vaccine.

An in vivo assay suitable for immunogenicity is to inject by the intramuscular route groups of at least 10 rats with three dilutions of each of the test and reference vaccines. The dilutions range is chosen such that all animals may be expected to give a detectable serum antibody. Neutralization titres are determined and a geometric mean for each of the virus types determined. The regression lines for the test and reference vaccines are compared in order to calculate the potency.

The potency of the vaccines for each virus type shall be approved by the national control authority.

Vaccines made by the technique used to prepare the WHO reference preparation and that contain 40, 8 and 32 D-antigen units per dose for types I, II and III, respectively, have been found to induce a high rate of immune response following a single dose in infants in both developed and developing countries (more than 85%-95% with seroconversion and up to 100% with immunologic memory). After the second dose all individuals had post-vaccination titres of 1 IU of antibody or more for all three types.

5.5 Protein nitrogen content

Poliovirus vaccine (inactivated) shall not contain more than 10 μg of protein nitrogen per human dose.

In some countries in vitro tests for serum proteins are included. Counter-current electrophoresis is useful for such tests.

6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2, Annex 1) shall apply.

7. SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements
for Manufacturing Establishments and Control Laboratories) (2, Annex 1) shall apply.

8. LABELLING

The requirements given in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2, Annex 1) shall apply, with the addition of the following directive.

The leaflet accompanying the package shall include the following information:
(i) the nature of the cell cultures used;
(ii) the virus strain(s) used for the production of the vaccine; and
(iii) the method used for inactivating the virus.

9. DISTRIBUTION AND SHIPPING

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2, Annex 1) shall apply.

10. STORAGE AND EXPIRY DATE

10.1 Storage conditions

Poliomyelitis vaccine (inactivated) shall be stored at all times at a temperature between 2 °C and 8 °C.

10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall be not more than 18 months after the date of the last satisfactory potency test, the date of a potency test being that date on which the test animals were inoculated with the vaccine.
The expiry date shall not, however, be more than 12 months from the
date at which the vaccine was issued by the manufacturer.

In some cases the national control authority may decide, on the
basis of experimental evidence, to leave the expiry date at 18
months after the date of the last satisfactory potency test, irres-
pective of the date of issue by the manufacturer.

PART B.
NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories contained in
Part B of the revised Requirements for Biological Substances No. 1
(General Requirements for Manufacturing Establishments and Control
Laboratories) (2, Annex 1) shall apply with the addition of the follow-
ing directives:

The national control authority shall approve the strains used.
The national control authority shall approve the cell substrate used.
The national control authority shall specify potency requirements.
The national control authority shall be satisfied that the results of
all tests, including those done on monovalent pools during the process
of manufacture, are satisfactory and that consistency has been estab-
lished.

2. RELEASE AND CERTIFICATION

Poliomyelitis vaccine (inactivated) shall be released only if it fulfils
Part A of these requirements.

A statement signed by the appropriate official of the national control
laboratory shall be provided at the request of the manufacturing lab-
oratory and shall certify whether or not the lot of vaccine in question
meets all national requirements as well as the present requirements.
The certificate shall also state the date of the last satisfactory potency
test, the lot number, the number under which the lot was released,
and the number appearing on the labels of the containers. In addition,
a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of
biological substances between countries.
PART C.
REQUIREMENTS FOR HUMAN DIPLOID CELLS USED FOR VIRUS VACCINE PRODUCTION

The following requirements are applicable to the cell substrate for virus vaccine production where production is based on a cell seed system. The tests so far have been formulated for human diploid cells, but any cell bank and cell seed system shall comply with similar requirements as appropriate.

1. DEFINITIONS

1.1 Terminology

*Cell seed.* A quantity of cells derived from a single human tissue stored frozen at \(-70^\circ\text{C}\) or below in aliquots, one or more of which would be used for the production of a manufacturer’s working cell bank.

*Manufacturer’s working cell bank (MWCB).* A quantity of cells derived from a single ampoule of the cell seed and of uniform composition stored frozen at \(-70^\circ\text{C}\) or below in aliquots, one or more of which would be used for the production of each single harvest.

In normal practice such a seed culture (or ampoule) is issued to manufacturers at or near the eighth population doubling level (PDL). This is expanded by serial subculture up to a PDL selected by the manufacturer, at which point the cells are combined into one or more pools and preserved cryogenically to form the MWCB. One or more of such ampoules from a pool would be used for the production of a single harvest.

*Production cell culture.* A collection of cell cultures at the population doubling used for virus growth that have been derived from a single ampoule of the MWCB.

2. GENERAL MANUFACTURING REQUIREMENTS

The general requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2, page 11) shall apply, with the addition of the following directive:
No cell cultures other than those approved by the national control authority for the production of appropriate vaccine shall be introduced or handled in the production area.

3. PRODUCTION CONTROL

3.1 Cell seed

The use of human diploid cell cultures for vaccine manufacture shall be based on the cell seed system. Early population doubling of diploid cell cultures shall be subcultured to a population doubling that is convenient for the preparation of a cell seed.

The cell seed used for the production of virus vaccine shall be that approved by and registered with the national control authority. The accepted cell strain from which the cell seed has been derived shall have been characterized with respect to genealogy, growth characteristics, genetic markers (HLA), virus susceptibility, storage conditions, and karyology, and it shall have been shown, by tests in animals, eggs, and cell culture to be free from detectable adventitious agents.

These data shall be made available to the national control authority.

Each manufacturer shall show, to the satisfaction of the national control authority, that the cell substrate propagated from the accepted cell strain and laid down as a working cell bank conforms with the tests outlined in this section for freedom from extraneous agents, by tests in animals and eggs, for lack of tumorigenicity, for normal karyology throughout approximately the first two-thirds of its normal lifespan, and for identity.

In some countries the cells are examined also by ultra-thin sections and negative staining under the electron microscope.

3.1.1 Tests in animals and eggs for extraneous agents

The tests in animals for extraneous agents shall include the inoculation of each of the following groups of animals with cells by the intramuscular route, using a least $10^7$ viable cells divided equally between the animals in each group:

- 2 litters of suckling mice, comprising at least 10 animals, less than 24 h old;
- 10 adult mice;
- 5 guinea-pigs; and
- 5 rabbits.
At least $10^6$ viable cells shall be injected also into the allantoic cavity of each of 10 embryonated chicken eggs 9–11 days old.

The animals shall be observed for at least 4 weeks and the embryonated chicken eggs shall be examined after not less than 3 days. Any animals that are sick or show any abnormality shall be investigated to establish the cause of illness. The allantoic fluids shall be tested with guinea-pig and chick or other avian red cells for the presence of haemagglutinins.

The cells are suitable for vaccine production if at least 80% of the animals or eggs inoculated with the cells remain healthy and survive the observation period and none of the animals or eggs shows evidence of the presence in the cell cultures of any extraneous agent.

3.1.2 Tumorigenicity

The cells at the production level shall also be shown to be free from tumorigenicity by appropriate tests in animals approved by the national control authority.

Particularly for new non-continuous cell strains, some control authorities may wish to consider applying the tests for tumorigenicity as outlined in Part D, section 3.1.2. For the diploid cell strains that have been in use for many years, however, the tests suggested below have been shown to be satisfactory.

Suitable tests using immunosuppressed animals may be made as follows. Approximately $10^6$ viable cells obtained from cultures at the same passage level as those used for vaccine production are injected into (a) newborn mice or hamsters treated with antilymphocyte serum; or (b) athymic mice (nu/nu genotype); or (c) thymectomized mice irradiated and bone-marrow reconstituted (T−B+). Some of the same group of animals should be inoculated with a similar dose of HeLa or KB cells and it should be shown that tumour formation is caused by the inoculation of the neoplastic tissue, thus demonstrating the ability of the strain of animals to give rise to tumours. The animals should be observed for not less than 3 weeks. Any other test using animals treated with immunosuppressive agents and with equal sensitivity to neoplastic cells may be used.

Only those cell seeds shown not to be tumorigenic shall be used.

3.1.3 Chromosomal characterization and monitoring

3.1.3.1 Chromosomal characterization. At least 4 samples shall be examined as described in Part C, section 3.1.3.2, at approximately
equal intervals over the life-span of the cell line during serial cultivation. Each sample shall consist of 1000 metaphase cells.

It is also recommended that photographic reconstruction should be employed in the preparation of chromosome-banded karyotypes of 50 metaphase cells per 1000 cell sample using either G-banding or Q-banding techniques. This constitutes 5% of the sample and no specific limits for acceptability are yet recommended. The incidence of karyotypic abnormalities (pseudodiploidy, inversions, translocations, etc.) that are detectable with the greater resolution provided by banding should be evaluated when a larger data base than is at present available has been accumulated.

3.1.3.2 Chromosomal monitoring – preparation and testing. For the determination of the general character of each pool in the MWCDB, a minimum of 500 cells in metaphase shall be examined at the production level or at any passage thereafter for frequency of polyploidy and for exact counts of chromosomes, frequency of breaks, structural abnormalities, and other abnormalities, such as despiralization or marked attenuations of the primary or secondary constriictions.

For vaccine production, examination of the cells is usually made between the 27th and 33rd population doubling. The national control authority should determine the level of cell population doubling allowable.

For cells examined in metaphase the upper limits* of acceptability (upper fiducial limits at 95% (Poisson)) for abnormalities are, for 1000- and 500-cell samples, as follows:

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>1000 cells</th>
<th>500 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatid and chromosome breaks</td>
<td>47/1000</td>
<td>26/500</td>
</tr>
<tr>
<td>Structural abnormalities</td>
<td>17/1000</td>
<td>10/500</td>
</tr>
<tr>
<td>Hyperploidy</td>
<td>8/1000</td>
<td>5/500</td>
</tr>
<tr>
<td>Hypoploidy</td>
<td>180/1000</td>
<td>90/500</td>
</tr>
<tr>
<td>Polyploidy</td>
<td>30/1000</td>
<td>17/500</td>
</tr>
</tbody>
</table>

All cells showing abnormalities shall be subjected to detailed examination, and records shall be maintained of the detailed criteria applied to particular abnormalities evaluated in the karyotype analysis.

*These upper limits are based on extensive experience with the examination of WI-38 and MRC5 cells reported to and examined by the ad hoc Committee on Karyological Controls of Human Cell Substrates, which met in 1978 at Lake Placid, NY, USA. These values will not necessarily be applicable if another human cell strain is used.
Permanent stained slide preparations of the chromosome monitoring of the working cell bank pool, or photographs of these, shall be maintained as part of the record of the batch of vaccine for monitoring successive batches made from that cell pool.

It is desirable that a portion of the sample of pooled cell substrate removed from the culture vessels should be stored frozen so as to retain viability. This would be available for future reference for karyology or for any other purpose relating to the batch of vaccine.

Only those cell pools of the MWCB that have normal karyology shall be used for vaccine production.

3.1.4 Identity test of the cells

In some countries tests for characterizing HLA surface antigens are carried out in addition to chromosome monitoring.

3.2 Production of cell culture

A cell sample equivalent to at least 500 ml of the cell suspension of the concentration employed for seeding the vaccine production cultures shall be used to prepare control cell cultures.

In some countries in which the technology of large-scale production has been developed the national control authority should determine the size of the sample of cells to be examined, the time at which the control cells should be taken from the production cultures, and the monitoring of the control vessels.

The treatment of cells set aside as control material shall be similar to that of the production cell cultures but they shall remain uninoculated as control cultures for the detection of extraneous viruses.

These control cell cultures shall be incubated under similar conditions as the inoculated cultures for at least two weeks or until the time of the last harvest of the production cultures, whichever is the later, and shall be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures may be discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures shall be examined for degeneration caused by an infectious agent. If this examination or any of the tests required in this section show evidence
of the presence in a control culture of any adventitious agent, the virus grown in the corresponding inoculated cultures shall not be used for vaccine production.

3.2.1 Test for haemadsorbing viruses

At the end of the observation period cells comprising 25% of the control cells shall be tested for the presence of haemadsorbing viruses using guinea-pig red cells. If the guinea-pig red cells have been stored, the duration of storage shall not have exceeded 7 days and the temperature of storage shall have been in the range of 2–8 °C.

In some countries the national control authority requires that tests for haemadsorbing viruses be made in addition using other types of red cells including those from humans (blood group O), monkeys, and chickens (or other avian species). The cultures should be examined at 3–5 days and again at 12 days. All tests should be read after incubation for 30 min at 0–4 °C and again after a further incubation for 30 min at 20–25 °C. The test with monkey red cells should be read once more after yet another incubation for 30 min at 34–37 °C.

3.2.2 Tests for other extraneous agents

At the time of each harvest of the production cultures and 14 days after the day of inoculation of the production cultures with seed lot virus, a sample of the pooled fluids shall be taken at each period of collection from each group of control cultures. 10 ml of each pool shall be tested in the same cells, but not the same batch of cells, as that used for the production of virus growth, and additional 10-ml samples of each pool shall be tested in human cells and at least one other sensitive cell system.

The inoculated cultures shall be incubated at a temperature of 35–37 °C and shall be observed for a period of at least 14 days.

Some national control authorities require that at the end of this observation period a subculture should be made in the same cell culture system and observed for at least 7 days. Furthermore, some national control authorities require that these cells should be tested for the presence of haemadsorbing viruses.

For the tests to be valid, at least 80% of the culture vessels should be available and suitable for evaluation at the end of the respective test periods.

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If any cytopathogenic changes occur due to extraneous agents in any of the cultures the virus harvests produced from the batch of cells from which the control cells were taken shall be discarded.

3.2.3 Identity test

At the production level the cells shall be identified as human by tests approved by the national control authority.

Suitable tests are isoenzymes analysis, HLA, or other immunological tests or karyotyping of at least one metaphase spread of chromosomes.

PART D.
REQUIREMENTS FOR CONTINUOUS CELL LINES USED FOR INACTIVATED VIRUS VACCINE PRODUCTION

The following requirements are applicable to the cell substrate for virus vaccine production where production is based on a cell seed system from a continuous cell line.

1. DEFINITIONS

1.1 Terminology

Cell seed. A quantity of cells derived from a normal tissue and stored frozen at −70°C or below in aliquots, one or more of which would be used for the production of a manufacturer's working cell bank.

Manufacturer's working cell bank (MWCB). A quantity of cells derived from one or more ampoules of the cell seed and of uniform composition stored frozen at −70°C or below in aliquots, one or more of which would be used for the production of each single harvest.

In normal practice such a seed culture (or ampoules) is expanded by serial subculture up to a passage number selected by the manufacturer, at which point the cells are combined into one pool and preserved cryogenically to form the MWCB. One or more of the ampoules from such a pool would be used for the production of a single harvest.
Production cell culture. A collection of cell cultures at the passage number used for virus growth that have been derived from one or more ampoules of the MWCB.

2. GENERAL MANUFACTURING REQUIREMENTS

The general requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2, page 11) shall apply with the addition of the following directive:

No cell cultures other than those approved by the national control authority for the production of appropriate vaccine shall be introduced or handled in the production area.

3. PRODUCTION CONTROL

3.1 Cell seed and/or manufacturer's working cell bank

The utilization of continuous cell lines for vaccine manufacture shall be based on the cell seed system. A passage of a continuous cell line shall be subcultured to a passage number which is convenient for the preparation of a cell seed.

The cell seed used for the production of virus vaccine shall be that approved by and registered with the national control authority. The accepted continuous cell line from which the cell seed and/or the MWCB have been derived shall have been characterized with respect to genealogy, growth characteristics, immunological markers, virus susceptibility, and storage conditions, and it shall have been shown, by tests in animals, eggs and cell culture, to be free from detectable adventitious agents. In some countries karyology is also required.

These data shall be made available to the national control authority.

Each manufacturer shall show, to the satisfaction of the national control authority, that the cells intended as the virus substrate, propagated from the accepted continuous cell line and laid down as the MWCB conform with the test outlined in this section for freedom from extraneous agents by tests in animals and eggs (see Part D, section 3.1.1) and for lack of tumorigenicity. In addition, the test for tumorigenicity shall be repeated on the cells at the passage level used for vaccine production.
In some countries the cells are examined by electron microscopy to establish their ultrastructural characteristics. The cells may also be tested for the presence of retroviruses after activation with agents such as bromodeoxyuridine (BUDR), by examining electron micrographs for virus particles and/or by performing assays for viral reverse transcriptase.

3.1.1 *Tests in animals and eggs for extraneous agents*

The tests in animals for extraneous agents shall include the inoculation of each of the following groups of animals with cells by the intramuscular route, using at least $10^7$ viable cells divided equally between the animals in each group:

- 2 litters of suckling mice, comprising at least 10 animals, less than 24 h old;
- 10 adult mice;
- 5 guinea-pigs; and
- 5 rabbits.

At least $10^6$ viable cells shall be injected also into the allantoic cavity of each of 10 embryonated chicken eggs 9–11 days old.

The animals shall be observed for at least 4 weeks and the embryonated chicken eggs shall be examined after not less than 3 days' incubation. Any animals that are sick or show any abnormality shall be investigated to establish the cause of illness. The allantoic fluids shall be tested with guinea-pig and chick or other avian red cells for the presence of haemagglutinins.

The cells are suitable for vaccine production if at least 80% of the animals or eggs inoculated with the cells remain healthy and survive the observation period and none of the animals or eggs shows evidence of the presence in the cell cultures of any extraneous agent.

3.1.2 *Tests for tumorigenicity*

Cells from the MWCB at the passage used for the virus production, or up to 10 passages thereafter, shall be shown to be non-tumorigenic in a test approved by the national control authority. Such a test must show a clear difference between the continuous cell line and a reference preparation of HeLa cells.\(^5\) In an appropriate *in vivo* test, $10^6$ viable HeLa cells when given by the subcutaneous or intramuscular

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\(^5\) Information concerning the sources of suitable HeLa cells may be obtained from Chief, Biologicals, WHO, 1211 Geneva 27, Switzerland.
route shall produce progressively growing tumours in at least 9 out of 10 animals, of which one or more must show evidence of metastases, while the $10^6$ cells of an acceptable continuous cell line shall produce neither progressively growing tumours nor metastases.

The systems shown to be suitable for this test include:

(a) newborn mice, rats or hamsters that have been treated with antithymocyte serum or globulin,
(b) thymectomized and irradiated mice that have been reconstituted with bone marrow from healthy mice,
(c) chick embryo skin organ cultures.

A suitable test using newborn animals treated with antithymocyte serum is to inoculate at least 20 animals with 0.1 ml of potent serum within 24 hours of birth. The injection is given either by the intramuscular or subcutaneous route and is repeated on days 2, 7 and 14 of life. A potent antithymocyte serum or globulin is one that suppresses the immune mechanisms of the growing animals to the extent that the subsequent inoculum of $10^6$ HeLa cells regularly produces tumours and metastases.

Also on the day of birth the two groups of 10 newborn animals that have been given the antithymocyte serum are given either $10^6$ viable HeLa cells or $10^6$ viable cells of the continuous cell line by the subcutaneous route at any site at which developing tumours can be palpated (the base of the neck or the abdomen are suitable sites). The animals are observed for 21 days for evidence of nodule formation at the site of injection and measurements are made at suitable times to determine whether there has been progressive growth.

At the end of the observation period all animals from both groups are sacrificed and examined for gross evidence of tumour formation at the site of injection and in other organs, such as the lymph nodes, lungs, kidneys, and liver. All tumour-like lesions are examined histopathologically. In addition, since some cell lines may form metastases without evidence of local tumour growth, the lungs and regional lymph nodes of all animals should be examined histopathologically.

For the purposes of this requirement, a progressively growing tumour is defined as a palpable nodule that increases in diameter over the 21-day observation period and that shows viable and mitotically active inoculated cells when examined histopathologically. The presence of microscopically viable cells without gross nodule formation should not be considered a progressively growing tumour; in addition, the presence of microscopically viable cells in association with a stationary or regressing nodule should not be considered a progressively growing tumour.

In addition, some countries test the cells for tumorigenicity in animals of the homologous species from which the cells were derived. Such tests would include immunosuppression of the animals with species-specific antithymocyte serum, inoculation of
candidate cells and control tumour cells, observation for at least 3 weeks, and the histopathological examination of the inoculation sites as well as any metastatic lesions.

In some countries an in vitro test is permitted to demonstrate the freedom from tumorigenicity provided that the test has been shown to be as sensitive as a test in animals.

A suitable test using organ cultures of chick embryonic skin is to inoculate 10⁶ HeLa cells or 10⁷ cells from an acceptable continuous cell line on to organ cultures of chick embryonic skin for 3 days. At the end of this period, each culture is processed for histological evaluation and scored for cell growth and invasion. The reference HeLa cells should show extensive mitotic activity as well as extensive invasion into the chick substrate, while the continuous cell lines will show little or no invasion. In addition, secondary cell cultures derived from the same tissue as the continuous cell line may be tested in this system to provide guidance in interpreting invasive and mitotic activity.

3.1.3 Identity test of the cells

The MWCB shall be identified by a method approved by the national control authority.

The tests that may be used are karyology, isoenzymes analysis, and/or immunological markers.

3.2 Production of cell culture

A cell sample equivalent to at least 500 ml of the cell suspension at the concentration employed for seeding the vaccine production cultures shall be used to prepare control cultures.

In some countries in which the technology of large-scale production has been developed, the national control authority should determine the size of the sample of cells to be examined, the time at which the control cells should be taken from the production cultures, and the monitoring of the control vessels.

The treatment of cells set aside as control material shall be similar to that of the production cell cultures but they shall remain uninoculated as control cultures for the detection of extraneous viruses.

These control cell cultures shall be incubated under similar conditions to the inoculated cultures for at least 2 weeks or until the time of the last harvest of the production cultures, whichever is the later, and shall be examined during this period for evidence of cytopathic
changes. For the test to be valid, not more than 20% of the control cell cultures may be discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures shall be examined for degeneration caused by an infectious agent. If this examination or any of the tests required in this section shows evidence of the presence in a control culture of any adventitious agent, the virus grown in the corresponding inoculated cultures shall not be used for vaccine production.

3.2.1 Test for haemadsorbing viruses

At the end of the observation period cells comprising 25% of the control cells shall be tested for the presence of haemadsorbing viruses using guinea-pig red cells. If the guinea-pig red cells have been stored, the duration of storage shall not have exceeded 7 days and the temperature of storage shall have been in the range of 2–8°C.

3.2.2 Tests for other extraneous agents

At the end of the observation period a sample of the pooled fluids from each group of control cultures shall be tested for extraneous agents. 10 ml of each pool shall be tested in the same cells, but not the same batch of cells, as that used for the production of virus growth and additional 10-ml samples of each pool shall be tested in human cells and at least one other sensitive cell system.

The inoculated cultures shall be incubated at a temperature of 35–37°C and shall be observed for a period of at least 14 days.

For the tests to be valid, at least 80% of the culture vessels shall be available and suitable for evaluation at the end of the respective test periods.

If any cytopathogenic changes occur due to extraneous agents in any of the cultures, the virus harvests produced from the batch of cells from which the control cells were taken shall be discarded.

3.2.3 Identity test

At the production level the cells shall be identified as the species of origin by tests approved by the national control authority.

Suitable tests are isozymes analysis or other immunological tests, or karyology.
3.2.4 Purity of virus prepared on a continuous cell line

The virus grown in a continuous cell line shall be purified by a process that has been approved by the national control authority and has been shown to give consistent results.

Tests used to demonstrate the degree of purity achieved shall also be approved by the national control authority.

For polio vaccine (inactivated) the purification process shall be shown to reduce consistently the level of cellular DNA from that of the initial virus harvest by a factor of at least $10^6$ (see Part A, section 3.4.3.1).

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REFERENCES


Appendix

SUMMARY PROTOCOL OF POLIOMYELITIS VACCINE (INACTIVATED) PRODUCTION AND TESTING

(Based on Requirements for Biological Substances No 2 (Requirements for Poliomyelitis Vaccine, Inactivated) WHO Technical Report Series, No. 673, 1982, Annex 2)

Identification of Final Lot

<table>
<thead>
<tr>
<th>Name and address of manufacturer</th>
</tr>
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<tbody>
<tr>
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</table>

<table>
<thead>
<tr>
<th>Lot number of final product</th>
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</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Date of manufacture of final lot (namely, date of last potency test)</th>
</tr>
</thead>
<tbody>
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</table>

<table>
<thead>
<tr>
<th>Expiry date</th>
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</thead>
<tbody>
<tr>
<td>-------------</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total volume of final lot</th>
</tr>
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<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

Cell Cultures

Type of cell cultures used:

(Complete only the relevant part—i.e., A, B or C)

78
A. Cell cultures/monkey primary/secondary/tertiary
   - Type of cells (kidney, fetal kidney, etc.)
   - Monkey species of cells
   - Controls performed on animals from which the cells originated
   - Number of cell doublings
   - Result
   - Amount of cell culture inoculated
   - Amount of control cell culture investigated
   - Tests performed on control cultures
   - Result

B. Cell cultures/human diploid
   - Origin and short history of cell seed
   - Authority that approved cell seed
   - Amount of cell culture inoculated
   - Amount of control cell culture investigated
   - Tests performed on control cultures
   - Result

C. Cell cultures/other cells
   - Origin and short history of cell seed
   - Authority that approved cell seed
   - Amount of cell culture inoculated
   - Amount of control cell culture investigated
   - Tests performed on control cultures
   - Result
Serum For Cell Cultures

<table>
<thead>
<tr>
<th>Origin of serum used</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tests performed on serum</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
</tr>
</tbody>
</table>

Seed Lots of Virus

<table>
<thead>
<tr>
<th>Type*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain used</td>
</tr>
<tr>
<td>Origin and source of strain</td>
</tr>
<tr>
<td>Number of subcultures between primary seed lot and production</td>
</tr>
</tbody>
</table>

Information on Manufacture

1. Monovalent harvests

   **Type**

   | Date of inoculation of cells with virus |   |   |
   | Date of virus harvest               |   |   |
   | Special observations on single harvests |   |   |
   | Date of pooling                     |   |   |

Tests on pooled single harvests

Tests in Cercopithecus cell cultures

| Method used |   |
| Result of test |   |

Test in rabbits or kidney cell cultures

| Number of rabbits (or cell cultures) |   |
| Total volume injected              |   |
| Observed period                    |   |
| Result                             |   |

* Indicate: I, II or III. For each type a separate protocol should be completed.
**Monovalent product before inactivation**

Details on filtration and/or clarification and/or purification (if applied)  

Date

**Tests on monovalent pool**

**Identity test**

Method  
Result

**Sterility tests**

Method (media)  
Result

**Virus titration**

TCID₅₀ per ml  
Details on filtration and/or clarification and/or purification (if applied)  
Date(s)

**Inactivation of monovalent product**

Agent(s) and concentration  
Date of start of inactivation  
Date of taking first sample  
Date of completion of inactivation
**Test for effective inactivation**
(after removal/neutralization of inactivating agent)

- Sample size tested
- Date of first sample
- Date of second sample
- Details of testing procedure
- Period of observation of cell cultures
- Period of observation of subcultures
- Result
- Result of challenge of used culture with live virus

**2. Trivalent bulk product**
(monovalent pools incorporated)

- Date of preparation
- Preservative (if added, type and concentration)

**Tests on trivalent bulk**

**Test for absence of infective poliovirus**

- Sample size tested
- Details of testing procedure
- Period of observation of cell cultures
- Period of observation of subcultures
- Result
- Result of test in monkeys if included
Sterility tests
Method (media)

Result

3. Tests on final product
Identity test
Method used
Result

Sterility tests
Method (media)
Result

Innocuity test
Guinea-pigs Mice
Number of animals
Dose
Observation period
Result

Potency test
Method

Result

Date

Results of in vitro tests, if performed
(D-antigen)

83
Protein nitrogen

Content of protein nitrogen in µg per human dose

Serum protein tests (if done)

Result

Signature of head of laboratory

Certification by person taking overall responsibility for production of the vaccine.

I certify that lot No. ..., of trivalent poliomyelitis vaccine (inactivated) satisfied Part A of the WHO Requirements for Poliomyelitis Vaccine (inactivated).

Date

Signature

Name typed

The protocol must be accompanied by a sample of the vaccine and a copy of the leaflet.
Annex 3

A REVIEW OF TESTS ON VIRUS VACCINES


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INTRODUCTION

About 25 years ago the large-scale production of inactivated poliomyelitis vaccine initiated a new approach to the quality control of biological products. The development of a vaccine against such a
paralytic disease was heralded with enthusiasm but the release of a batch of vaccine that contained undetected living poliovirus was a stern lesson that demanded changes in the requirements for the quality control of this vaccine. At that time, the stringent tests included in the requirements for consistency of manufacture imposed the need for formal release of individual batches by national control authorities and this became the pattern for each successive new virus vaccine. Since those early days there have also been changes in the tests on the viruses used for vaccine production and there has been a decided move towards the characterization and use of cell substrates shown to be free from contaminating organisms.

The cell substrate

Originally the only freely available tissue for the production of inactivated poliomyelitis vaccine on a large scale was monkey kidney, but it was soon apparent that the tissues from these animals harboured simian RNA and DNA viruses. About one-third of the tissues prepared from the kidneys of monkeys had to be discarded for this reason. As it became known that almost all simian cell substrates from adult tissues may be infected, tests for extraneous viruses were introduced; these included the use of several species of laboratory animals, fertile eggs or cell substrates that had been shown to be sensitive for the detection of these infectious agents. It was understandable, therefore, that as new vaccines were developed, all existing tests were included, irrespective of whether the viruses for which the tests were included were likely to be present or not.

During the last 15 years there have been two major developments in the use of cell substrates. In the first, closed colonies of animals (e.g., chickens and rabbits) freed from specific pathogens have been bred under isolated conditions. Thus, the cells from such a colony have been tested extensively for the presence of contaminating viruses and to date none has been found. Such a development was important for those viruses growing on avian and rabbit cell cultures (measles, mumps, and rubella) but even for the poliomyelitis viruses, which require the cells of primates for their growth, kidneys from monkeys born in captivity or taken within a short time before expected birth have been used and have been shown to be free from contaminants. Indeed, it is now possible to expand the cell yield from monkey kidneys by the subculture of the cells obtained by the initial trypsinization. Such a technique was not possible 25 years ago because of the
high incidence of virus contaminants, many of which destroyed the cell cultures.

The second development was the establishment of a cell bank prepared by the passage of fibroblast cells growing from human fetal lung tissue. The cells from such cell banks stored in liquid nitrogen have been shown by extensive tests to be normal, to have diploid karyology, and to be free from contaminants. They have a finite life and have been used successfully over the past 15 years for the production of large quantities of virus from which millions of doses of vaccines have been prepared.

The virus strains

When a virus has been shown to be safe and effective for use in man a seed virus system is established for the preparation of vaccine. It is important, thereafter, to include tests to ensure that no changes have taken place in the character of the seed virus during its storage or during the production of the vaccine.

Although a number of tests appropriate for the characterization of the viruses have been included as “in process” controls, none has been shown to correlate completely with safety in man. The tests are a means of ensuring that the conditions of production have not changed the virus in any way. Recent technical developments, such as oligonucleotide mapping, offer a more precise means of characterizing seed viruses for vaccine production and experience should be gained in the value of including such a technique in test systems.

The need for reassessment of tests on virus vaccines

Twenty-five years ago the quality control of a virus vaccine that included all tests capable of detecting viruses was justified but the accumulated experience since then should now be taken into consideration in a reassessment of the tests for adventitious viruses. Data from manufacturers and control authorities involving 28 laboratories in 16 countries have shown that no extraneous viruses were detected in animal tests that were not detected in cell cultures. In addition, the experience indicates that the establishment of a cell bank or the continuous monitoring of a closed group of animals used as a source of tissue ensures that the substrate used for the production of each batch of vaccine is free from adventitious agents. This is in sharp contrast to the use of primary cells from wild-caught animals or from animals kept in open colonies.
Where cell banks and virus seeds shown to be free from contaminants are used, therefore, it is appropriate that the continuation of some tests on the virus harvests and final vaccines be reconsidered. Indeed, in the latest formulation and reformulation of the requirements for some virus vaccines, amendments have taken such developments into consideration.

Objectives of the group of consultants

The purpose of this report is to present to national control authorities and manufacturers the considered opinions of a group of consultants with many years’ experience in the production and testing of virus vaccines. The changes recommended are each intended to increase the meaning and purpose of quality control, which is to release only safe, potent, and stable vaccines. The Group agreed that a simple scheme for the production and testing of virus vaccines would be helpful in understanding the recommended changes to the WHO requirements and such a scheme is shown in Appendix 2. The World Health Organization¹ will be interested in discussing any test with any national control authority.

TESTS ON CELL SUBSTRATES

Cell substrates for the preparation of biological products may, for control purposes, be divided into three categories according to their provenance:

(1) tissues of wild-caught animals or from those in non-monitored open colonies;
(2) tissues from monitored, closed colonies of animals or birds; and
(3) an established cell strain or line stored as a bank.

1. Cells from wild-caught animals or from those in non-monitored open colonies

In some countries cells derived from wild-caught animals are used for the preparation of virus vaccines. Such is the case for live attenu-

¹ Correspondence should be addressed to Chief, Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

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ated and inactivated poliomyelitis vaccines prepared in the cells from the kidneys of wild-caught monkeys.

When wild-caught monkeys are used it is mandatory for the animals to be quarantined in the country of manufacture of the vaccine for not less than 6 weeks before use. Handling and housing of the monkeys and attention to their general health have been described in the WHO Technical Report Series No. 486, 1972, pages 29–31. If, during the quarantine period the overall mortality rate of a shipment reaches 5%, the batch is maintained in quarantine for a further period of at least 6 weeks. The quarantined animals are thereafter maintained in their quarantined groups until their tissue is used for the preparation of cell cultures. Monkeys from which tissue is obtained are thoroughly examined for evidence of tuberculosis and simian herpesvirus (B virus). The Group agreed that these requirements should continue to be considered as appropriate for such animals.

The Group recommended that the tests to demonstrate extraneous infectious agents in the cell cultures derived from these animals and used for vaccine production as well as those applicable to the control cell cultures should continue to be carried out in accordance with the requirements contained in WHO Technical Report Series, No. 486, 1972, pages 31–34, Part A, sections 3.2.2, 3.2.3 and 3.2.4.

These tests should be applied irrespective of the passage level of the cells when used for vaccine production.

2. Cells derived from monitored, closed colonies of animals

As mentioned above, the Group agreed that the freedom from extraneous agents of cell cultures may be achieved by the use of cells from donor animals maintained in closed colonies that have been subject to continuous and systematic veterinary and laboratory monitoring for the presence of infectious agents. Closed groups of rabbits and ducks used for cell cultures for the preparation of rubella vaccines and chickens used for measles, mumps, and live influenza vaccine production are examples of the application of this approach. The agents for which the colony should be monitored should represent those viruses and microorganisms known to be naturally occurring in the species (see WHO Technical Report Series No. 610, 1977, pages 62–64 for rubella vaccine and WHO Technical Report Series No. 638, 1979, pages 177–179 for live influenza vaccine).

It is essential for the maintenance of a closed colony that each individual animal be tested serologically before entering the colony.
and at regular intervals subsequently. The Group recommended that all animals should be bled at the time of the establishment of the colony and a random 5% sample tested at four week intervals thereafter. The sera should be screened for antibodies to the infectious agents relevant to the species; any animal that dies must be examined and attempts made to isolate an infectious agent. If there is evidence of infection by a specified pathogen then the tissue from that group of animals should not be used.

In some countries, biochemical tests for the detection of retroviruses are applied to new cell substrates. As the reliability of such tests becomes generally accepted, consideration should be given to the inclusion of the tests in national requirements.

The Group agreed that when cells are derived from monitored colonies and used for vaccine production the extent of testing of such cells for extraneous agents may be reduced compared with the testing of those cells derived from animals not subject to such monitoring procedures. In particular, the Group recommended that the requirements for the testing of these substrates in laboratory animals (suckling mice, adult mice, guinea-pigs, and rabbits) and embryonated eggs could be omitted. The control cells, however, should be tested for the presence of haemadsorbing viruses.

It is important, however, that a batch of vaccine made in such cells is not released for use until an examination of the colony subsequent to the use of the tissue as a source of cells for its production has continued to show freedom from infections.

3. The use of cell banks

The rationale for the use of a well-characterized and tested cell strain is now established by experience gained with human diploid cells. The original cell bank consists of a number of ampoules prepared from a common pool of cells of known properties in respect to their genealogy, growth characteristics, viability during storage, karyology, and lack of transplantability. Cells forming the bank shall have been shown by extensive tests in cell cultures, laboratory animals, and embryonated eggs, to be free from adventitious agents. The manufacturer is required to prepare a manufacturers working cell bank (MWCB) from a well-characterized cell strain and the Group agreed that the MWCB should continue to be tested as thoroughly as the original cell bank for freedom from extraneous agents.
The obvious advantage in the use of cells from a MWCB, as in the case of cells from a monitored colony, is the elimination of the need for extensive routine testing for adventitious agents of the production cells or virus harvests. The Group recommended that the tests on the control cells for adventitious agents in animals (suckling mice, adult mice, guinea-pigs, and rabbits) and embryonated eggs could be omitted.

TESTS ON SEED VIRUSES

The Group agreed that the general principles that have been employed for the safety testing and characterization of virus vaccines should continue to apply. These are that the seed strain shall be identified by historical records, including the origin of the strain and methods used for attenuation, where applicable, and that the strain shall be shown to be both immunogenic and safe in humans. A seed lot system shall be used, and the seed lot shall be stored at an appropriate temperature in a separate locked container set aside for this purpose only. Seed lots shall be characterized by appropriate biological and serological tests and also, where possible, by appropriate biochemical analysis.

Oligonucleotide mapping for example is suitable as a biochemical test for the characterization of virus seeds.

A range of biological and biochemical tests should be applied to the seed lots and the master seed strain from which it was derived in order to establish their identity. The Group agreed that in general the number of passages, beyond the master seed established as safe and effective in man, should be as low as possible but the decisions concerning the use of a seed lot should be based on its characteristics rather than on an arbitrary passage level.

Neurovirulence tests on seed lots for live virus vaccine should be continued where this is appropriate, such as for the three types of attenuated poliovirus and yellow fever virus. Although neurovirulence tests on the seed viruses for the preparation of measles, mumps, rubella, and influenza vaccines have been carried out in several countries and in some the individual lot of vaccine has been tested, no seed or lot has been found to be neurovirulent by these tests. The Group agreed that the continued application of such tests for new
seed viruses for live virus vaccines may be justified for the seed viruses but not for each batch of vaccine.

One country has declared that it will continue neurovirulence tests for each virus seed and each of the first five consecutive lots of vaccine.

OTHER TESTS TO PREVENT CONTAMINATION

The Group agreed that the control of materials of animal origin used in the preparation of cell cultures requires more study to devise improved methods of sterilization or avoidance of contamination by adventitious agents. The most important component of cell culture media in this regard is bovine serum, which should be shown to be free from bacteria, fungi, mycoplasma, and bovine viruses.

Some manufacturers continue to use fetal calf serum because of its growth-promoting properties but it is difficult to obtain aseptically. The Group recommended, however, that all serum should be taken aseptically from identified animals and that the use of gammad irradiation of the serum in the frozen state should be encouraged. Tests for bovine viruses should be included and a suggested test system is shown in Appendix 2.

The Group agreed that trypsin should also be sterile and free from mycoplasma and viruses and methods to ensure this should be studied and made known to all control laboratories and manufacturers.

In addition to ensuring that the substrate, seed virus, raw materials of components of media, and other ingredients are free from extraneous agents, care should be taken to avoid cross-contamination between infected and uninfected areas and contamination from operators and/or the environment.

TESTS ON PRODUCTION AND CONTROL OF CELL CULTURES

The Group confirmed that when primary kidney cell cultures of wild monkeys are used for the production of poliomyelitis vaccine, cultures prepared from 25% of the cell suspension obtained from the kidneys of each single monkey shall continue to serve as control cells and be tested for extraneous agents.
Experience over many years, however, has shown that for the cells derived from closed colonies or cell banks, the testing of 500 ml of the cell suspension at the concentration employed for seeding vaccine production cultures, irrespective of the size of the production lot, is satisfactory for the detection of adventitious agents. Therefore, the Group agreed that the continuation of the testing of 500-ml quantities of control cells prepared from tissue of animal or human origin is appropriate.

The Group saw no reason to examine the fluids from the control cultures at the time that the virus was harvested from the production cultures. They agreed, however, that the control cell cultures derived from the same batch of cells as the production cultures and that had been incubated for at least 2 weeks beyond the date of inoculation of the production cultures, or until the time of the last harvest of the production cultures, whichever is the later, shall then be examined for degeneration. At this time also cells, comprising 25% of the control cells, shall be tested for the presence of haemadsorbing viruses using guinea-pig red cells. The Group noted that some manufacturers are also using human, chicken and monkey red cells for this test. The Group agreed that there is a need for the data on the results of such additional tests to be obtained by WHO in order to assess the value of them.

In some countries fluids are collected from the control cells at the time of virus harvest and at the end of the observation period; such fluids may then be pooled before testing for extraneous viruses.

The Group confirmed that, in the tests on the pooled fluids taken from the control cultures at the time that the virus is added to the production cultures and examined for extraneous viruses, each pool shall be tested in the same cells, but not the same batch of cells, as that used for the production of virus growth, as well as in both human and simian cells. It was agreed also that the inoculated cultures shall be incubated at the appropriate temperature and shall be observed for a period of at least 14 days.

**TESTS ON VIRUS HARVEST**

As the tests applied to the control cells over the past 25 years have been shown to be successful for the detection of extraneous agents and the tests on the virus harvests have not exposed any additional con-
taminants, the Group considered that the tests on the control cells described above were alone sufficient. The virus harvests prepared for live poliovirus vaccine in primary kidney cells of wild monkey origin, however, shall continue to be tested in tissue cultures, but even with these harvests the Group agreed that the small animal tests do not add to the safety of the vaccine and should be omitted.

In some countries the virus harvests for any vaccine, irrespective of the origin of the cell substrate, will continue to be tested in cell cultures but not in animals.

It was agreed also that all virus harvests shall continue to be tested for microbial sterility, identity, and potency.

**TESTS ON LIVE VIRUS VACCINE**

The Group agreed that following the pooling of individual virus harvests and the addition of a stabilizer, the final vaccine shall continue to be tested for sterility, identity, potency, innocuity, and moisture (if freeze-dried). The Group recommended that in addition to long-term stability assays, required for the validation of the expiry date, an accelerated degradation test should be devised wherever appropriate.

In order to ensure that live virus vaccines maintain adequate potency when stored in countries with high ambient temperatures, and where the “cold chain” may be inadequate, consistency in the production of stable vaccines should be demonstrated.

**TESTS ON INACTIVATED VACCINE**

The Group confirmed that the control tests applied to the seed virus and cell substrates for adventitious agents should be similar for live and inactivated virus vaccines. When tissues from wild-caught animals or those in unmonitored groups are used for the preparation of cell cultures, tests for the presence of viruses indigenous to the animal species should always be performed. Such tests should be done on each batch of cells even though the inactivation process is generally considered to confer a greater safety to the vaccine because extraneous agents are likely to be destroyed. Some extraneous agents, however, may be relatively resistant to the inactivation process, as is the case for SV40 virus.
The Group agreed that if cells from a fully tested cell bank are used for vaccine production further tests for extraneous viruses on each batch of cells used for production could be omitted; an example is rabies vaccine prepared in a human diploid cell strain.

Recently, close and regular monitoring of animal colonies, as used in the production of living virus vaccines, has been applied to animals providing the cell substrate for inactivated vaccine production; an example is a monkey colony established for this purpose. In spite of the progress made by using closed colonies of monkeys, however, sufficient evidence has not yet been obtained by regular monitoring to establish that this system of maintaining monkeys always ensures the continuing absence of indigenous viruses in these animal species. The Group agreed, therefore, that tests for these viruses in control cultures should be retained.

As there are data available to demonstrate that the cell culture system is more sensitive than the test in monkeys for detecting the presence of small numbers of living poliovirus after completion of the inactivation process, the Group agreed that the test involving the inoculation of monkeys for this purpose could be omitted from the relevant requirements concerning inactivated polio vaccine.

The manufacturer should demonstrate to the satisfaction of the national control authority that the process of inactivation and purification is consistent in killing all virus particles and that the cell culture method used for the detection of any residual live virus is more sensitive than the test in monkeys.

THE COLLECTION AND STORAGE OF DATA ON THE TESTING OF VIRUS VACCINES

The Group considered that great benefits would accrue from a centralized systematic collection of data on the results of the testing of virus vaccines for quality control. The Group recommended that the possibility of establishing such a data bank should be explored.

IMPLICATIONS OF THE REPORT

The Group reviewed each of the WHO requirements for virus vaccines and considered the changes that would be necessary to amend
the requirements in the light of current findings. These changes are indicated in Appendix 3.

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Appendix 1

SCHEME FOR THE PRODUCTION AND TESTING OF VIRUS VACCINES
(prepared in monitored substrates or pretested cell banks and characterized virus seeds)

SUBSTRATE

- cell bank or cells from closed, regularly monitored animal colonies

Tested for freedom from adventitious agents

MATERIALS OF ANIMAL ORIGIN (SERUM AND TRYPsin)

Tests for adventitious agents

Cell Growth

VIRUS SEED

tested for adventitious agents

PRODUCTION CELLS

Tested for identity, virus titre and sterility*

VIRUS HARVEST

End of observation period—test of cells and supernatant fluid for extraneous agents

CONTROL CELLS (500 ml of cell suspension)

Incubation at suitable temperature

Incubation under similar conditions

VACCINE

Tested for safety, potency, stability, and freedom from fungi and bacteria

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* Tests in cell cultures for extraneous viruses should be included for new products and new manufacturers of an established product until consistency has been established. In some countries, however, the virus harvest of each lot will continue to be tested in cell cultures alone.
Appendix 2

TESTS FOR BOVINE VIRUSES IN SERUM

The serum to be tested is used in a growth medium to cultivate primary bovine testis cells or cells of a continuous bovine kidney cell line known to be sensitive to bovine viruses and especially the virus causing bovine viral diarrhoea (BVD).

The method employing primary bovine testis cells as a suspension in Eagles Minimum Essential Medium (MEM) containing $10^5$ cells/ml is as follows:

90 ml of the cell suspension is mixed with 10 ml of the test serum and dispensed into two 75-cm$^2$ plastic tissue culture flasks. The process is repeated with a further 90 ml to which 10 ml of control serum (pretested for freedom from bovine viruses) is added. The cultures are incubated at 36.5°C ± 1°C for a period of 28 days with frequent microscopic examination for the evidence of extraneous viruses. The growth medium may be changed as required, being replaced with fresh medium containing the relevant serum.

At least one subculture of the cells is carried out for both the test and control sera during the observation period in order to provide not less than 300 cm$^2$ (including six bottles, each of 25 cm$^2$) of cells of each group for further examination at 28 days after initiation of culture.

At the end of the observation period the following tests are each applied to at least one 25 cm$^2$ culture of cells grown on test serum and control serum. Appropriate controls are retained in each case.

(a) Challenge with a suitable cytopathogenic strain of bovine viral diarrhoea virus; the cells must be shown to be sensitive to the challenge virus.

(b) Microscopic examination for abnormalities following staining by the May Grunwald-Giemsa technique.

(c) Test for haemadsorbing agents using a mixed suspension of 0.2% chick, 0.2% guinea-pig and 0.2% human group O erythrocytes.

(d) Examination by electron microscopy of the negatively stained extract produced after disruption and clarification of the cell culture.

(e) Examination for bovine viral diarrhoea virus by the direct or indirect immunofluorescence test.

If in any of the above tests, there is evidence of the presence of commonly occurring bovine pathogens attributable to the test serum,
then the serum is unsatisfactory and may not be used for the growth of cells used in vaccine production.

Appendix 3

CHANGES SUGGESTED IN THE WHO REQUIREMENTS FOR VIRUS VACCINES

In order to take into account the deliberations of the Group of Consultants the following changes in the WHO Requirements are suggested to national control authorities for their consideration.

1. Requirements for Poliomyelitis Vaccine (Oral)
   (Requirements for Biological Substances No. 7, Revised 1971).
   Part A, section 1.4 – the definition of seed lot should be changed to virus seed lot. The reference to seed lot throughout the requirements should thereafter be changed to virus seed lot.
   Part A, section 3.1.2 – a section should be added to allow the production of vaccine in cells derived from closed and continuously monitored colonies of monkeys.
   Part A, section 3.1.4 – should be followed by the appropriate tests in sections 3.3 to 3.6 since the tests for extraneous agents shall apply to the virus seed.
   Part A, section 3.4 – the tests in animals, sections 3.3.6, 3.4.3, 3.4.4, 3.4.5, and 3.4.6, may be deleted. These tests, however, are retained for the seed virus.
   Part A, section 3.5.5.1 – it is recommended that the test in monkeys for neurovirulence should be done either by the manufacturer or by the control authority. The tissue sections should be made available for inspection. There does not appear to be any advantage in both the manufacturer and the control authority doing this test.
   Part A, a new section following section 5.4 should be added describing a test for the stability of the vaccine. An accelerated degradation test involving the time and temperature of incubation as well as the allowable decrease in virus titre under such conditions should be specified.
   Part C – should be amended to allow for the use of cells derived from monkeys in monitored colonies.

2. Requirements for Poliomyelitis Vaccines (Inactivated)  
(Requirements for Biological Substances No. 2, revised 1981)  
The Group recognized that these requirements had been revised recently (Annex 2 of WHO Technical Report Series No. 673, 1982) in order to allow the production of poliomyelitis vaccine (inactivated) in monkey kidney cells from wild caught animals, monkey kidney cells from monitored closed colonies, human diploid cells, and continuous cell lines. Furthermore, the requirements have been broadened to include the production of purified and concentrated vaccine in non-tumorigenic heteroploid cell lines.

The Group agreed that a test for stability of the final vaccine should be added when the details of such a test had been agreed by control authorities and manufacturers.

3. Requirements for Measles Vaccine (Live)  
(Requirements for Biological Substances No. 12)  
The Group did not know of any live measles vaccine produced in cells other than those derived from closed monitored flocks of birds. The amendments to these requirements, therefore, are to omit the tests in animals on the virus pools – Part A section 3.3.2., 3.3.4., 3.3.5., 3.3.6. Other sections referring specifically to the tests in dog kidney and guinea-pig kidney could be omitted as appropriate.

Part A, section 3.4.1 – the Group agreed that there was no justification for testing future batches of vaccine for neurotropic agents.

Part A, section 5 – provision should be made for allowing only the use of freeze-dried vaccine.

Part A, section 5 – a new section should be added to test for the stability of the vaccine. A test appropriate for the more stable (second generation) measles vaccine (live) in the freeze-dried form is:

The final freeze-dried vaccine shall be incubated at 37°C for 7 days. At the end of the incubation the vaccine shall retain at least 3.0 log10 of virus in each human dose and during the incubation the virus titre of the vaccine shall not have been decreased by more than 1.0 log10.

Some countries may wish to apply this test to establish the consistency of production of stable vaccines.

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4. Requirements for Measles Vaccine (Inactivated)  
(Requirements for Biological Substances No. 12)  
The Group agreed that as no measles vaccine (inactivated) was being produced there was no need to consider these requirements further.

5. Requirements for Rubella Vaccine (Live)  
(Requirements for Biological Substances No. 24)  
The Group agreed that even though the requirements for rubella vaccine had been formulated only four years ago amendments were required. These are:

Part A, section 3.1.4 – should be followed by the appropriate tests in sections 3.4 since the tests for extraneous agents shall apply to the seed virus.

Part A, section 3.2 – the monitoring of a cell bank and the tests applied to the bank and control cells in which each batch of vaccine is prepared have been described and reformulated. These requirements appear in Annex 5 of the WHO Technical Report Series, No. 658, 1981.

Part A, section 3.4 – there is no need for tests on the virus pool in animals and sections 3.4.1, 3.4.3, 3.4.4, 3.4.5, 3.4.6, 3.4.7 could be omitted.

Part A, section 5 – a test for the stability of vaccine should be added. The details of such a test should be agreed by the control authorities and manufacturers.

6. Requirements for Yellow Fever Vaccine  
(Requirements for Biological Substances No. 3)  
The Group agreed that for the special reasons outlined in the General Considerations of these requirements, revised in 1975, the requirements should not be amended.

7. Requirements for Influenza Vaccine (Inactivated)  
(Requirements for Biological Substances No. 17)  
Requirements for Influenza Vaccine (Live)  
(Requirements for Biological Substances No. 28)  
The Group agreed that as the requirements for inactivated vaccine had been revised in 1978 there was no need for amendments. The technology used in the preparation and selection of a suitable strain
for the production of live influenza vaccine was changing and amendments to these requirements should await such developments.

8. Requirements for Rabies Vaccine for Human Use
   (Requirements for Biological Substances No. 22)
   Requirements for Rabies Vaccine for Veterinary Use
   (Requirements for Biological Substances No. 29)
   The Group noted that both these sets of requirements had been revised in 1980 and no amendments were required.

9. Requirements for Hepatitis B Vaccine
   (Requirements for Biological Substances No. 31)
   The Group noted that these requirements had been formulated in 1979 and suggested no amendments.
Annex 4

PROPOSED REQUIREMENTS FOR RIFT VALLEY FEVER VACCINE (INACTIVATED) FOR HUMAN USE

(Requirements for Biological Substances No. 32)

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GENERAL CONSIDERATIONS

Specific vaccines are probably the most commonly used tools for the control of Rift Valley fever (RVF). Evidence shows that effective vaccines will protect animals and humans against illness, protect pregnant female livestock populations against abortion, and will remove the vaccinated animal from the pool of potential amplifying hosts of the virus. It is reasonable to assume that the widespread use of effective vaccines in the susceptible animal populations would eliminate the possibility of occurrence of a major RVF epizootic by reducing the number of susceptible animals. Thus, there exists a vital role for veterinary vaccines in immunizing animals, both within and adjacent to known areas of RVF endemicity. Nevertheless, in the absence of a solidly immunized livestock population, there may be a high level of virus transmission to man.

The need for immunization of humans against RVF in the classic areas of endemicity and epizootic activity is limited. Little or no exposure occurs except in persons closely associated with livestock. This would generally include herders, veterinarians, and slaughterhouse workers. However, the extension of RVF into Egypt in 1977 demonstrated the devastating public health potential of this virus when it
occurs in animal populations living in close association with man in relatively high density. Members of field epidemiological teams as well as medical staff of all hospitals may also benefit from the use of vaccine. In view of the potential need to immunize large numbers of people in such areas against this disease, requirements for inactivated RVF vaccine for use in humans have been formulated.

As with the requirements for vaccines that have been formulated or revised during the last five years, a protocol for reporting the results of all tests has been included (see Appendix). Completion of such a protocol, which is based on the present requirements, enables a control authority to check whether all the tests have been completed.

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 of their national regulations concerning RVF vaccine (inactivated), it is recommended that a clause should be included permitting modifications of manufacturing requirements on the condition that it can be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure that the degree of safety and the potency of the vaccine are at least equal to those provided by the requirements formulated below. 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 vaccine is manufactured.

PART A.
MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be "Vaccinum febris Rift Valley inactivatum (ad usum humanum)". The proper name shall be the
equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

1.2 Descriptive definition

Vaccinum febris Rift Valley inactivatum (ad usum humanum) shall consist of an aqueous suspension of Rift Valley fever virus grown in cell cultures and inactivated. The preparation shall satisfy all the requirements formulated below.

1.3 Terminology

_Virus seed lot._ A quantity of virus processed together and of uniform composition. In each manufacturing establishment a primary virus seed lot is that from which further virus seed lots are prepared. Vaccine is one passage removed from the seed lot, i.e., two passages from the primary seed lot.

_Cell seed._ A quantity of cells derived from normal tissue and stored frozen at \(-70^\circ C\) or below in aliquots, one or more of which would be used for the production of a manufacturer’s working cell bank (MWCB).

_Manufacturer’s working cell bank (MWCB)._ All of the cellular material derived by serial subculture from one or more ampoules of cell seed. After serial subculture the cells are combined into a pool and preserved cryogenically to form the MWCB. One or more ampoules from a pool would be used for the production of a single harvest.

_Single harvest._ A virus suspension harvested from cell cultures that has been derived from a single batch of cells and processed together.

_Single pool._ A virus suspension processed at the same time.

_Purified and concentrated pool._ A purified and concentrated virus suspension or vaccine processed at the same time.

_Final bulk._ The finished biological preparation present in the container from which the final containers are filled.

_Filling lot (final lot)._ A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling. A filling lot must, therefore, have been filled in one working session from a single final bulk.
Tissue culture infective dose 50% (TCID<sub>50</sub>). The smallest quantity of a virus suspension that will infect 50% of inoculated cell cultures.

Plaque-forming unit (PFU). The smallest quantity of a virus suspension that will produce a plaque in cell cultures.

2. GENERAL MANUFACTURING REQUIREMENTS

The general manufacturing requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirement for Manufacturing Establishments and Control Laboratories) (1) shall apply to establishments manufacturing Rift Valley fever (RVF) vaccine (inactivated), with the addition of the following directives:

Production areas shall be decontaminated before they are used for the manufacture of RVF vaccine.

The production of RVF vaccine shall be conducted by staff who have not handled other infectious microorganisms or animals on the same working day. The staff shall consist of persons who shall be examined medically and found to be healthy. Steps shall be taken to ensure that all such persons in the production areas are immune to RVF virus.

No culture of any microorganisms or cells other than the cultures approved by the national control authority for the production of RVF vaccine shall be introduced or handled in the production area at any time during manufacture of the vaccine.

Persons not directly concerned with the production processes shall not be permitted to enter the production area.

3. PRODUCTION CONTROL

3.1 Control of source materials

3.1.1 Virus strains and seed lot system

The strains of RVF used in the production of vaccine shall be approved by the national control authority. They shall be identified by historical records, by infectivity tests, and by immunological methods. Any strain that will yield a vaccine meeting the requirements set forth in the present document may be used. Production of vaccine shall be based on the seed lot system; the seed virus used for vaccine
production shall not have passed more than 10 subcultures, counted
from a seed lot used for the production of the vaccine on which the
original laboratory and field tests were done.

Samples of the strain used shall be deposited in the national con-
trol laboratory.

3.1.2. Cell cultures for virus propagation

3.1.2.1 Monkeys used for obtaining tissues for cell cultures. If
monkey cell cultures, such as fetal lung cultures, are used, monkeys
that are in good health and of a species approved by the national
control authority, and that have not previously been used for experi-
mental purposes, shall be used as the source of tissue for the produc-
tion of virus.

The monkeys shall be kept in well-constructed animal rooms in
adequately ventilated cages spaced as far apart as possible. Adequate
precautions shall be taken to prevent cross-infection between cages.
Not more than 2 monkeys shall be housed in each cage and cagemates
shall not be interchanged. The monkeys shall be kept in the country
of manufacture of the vaccine in quarantine groups\(^3\) for a period of
not less than 6 weeks before use. If at any time during the quarantine
period the overall death rate of a shipment consisting of one or more
groups reaches 5% (excluding deaths from accidents or causes specifi-
cally determined not to be due to infectious diseases) monkeys from
that entire shipment shall continue in quarantine from such time for
a further period of not less than six weeks.

Only monkeys that have reacted negatively to tuberculin at the
start of the quarantine period and again within 2 weeks prior to use
shall be used in the manufacture of vaccine. The groups shall be kept
continually in isolation, as in quarantine, even after completion of the
quarantine period, until the monkeys are used or discarded. After the
last monkey of a group has been taken, the room that housed the
group shall be thoroughly cleaned and decontaminated before being
used for a fresh group.

In some countries the test for sensitivity to tuberculosis in
monkeys may be omitted because the monovalent pools are sub-
jected to 2 stages of filtration through 0.22-µm filters.

\(^3\) A quarantine group is a colony of selected, healthy monkeys kept in one room,
with separate feeding and cleaning facilities, and having no contact with other monkeys
during the quarantine period.
All actions of working personnel shall be based on the premise that there is a great potential hazard at all times in the quarantine area. Personnel shall be provided with protective clothing, including gloves, footwear, and masks or visors. Street clothes shall not be permitted in the animal rooms. Personnel shall be forbidden to smoke, eat, or drink while they are in the animal rooms.

Responsibility shall be assigned to a supervisor for reporting illness among employees and ensuring that all injuries are properly treated. No worker who has cuts or abrasions on exposed areas of the body shall enter the animal area. Any unexplained febrile illness while off duty shall be considered as potentially related to the employee’s occupation.

Monkeys from which tissues are to be removed shall be thoroughly examined at necropsy, particularly for evidence of tuberculosis or simian herpesvirus (B virus) infection. Monkeys prepared for removal of tissues shall be anaesthetized.

A monkey that shows any pathological lesion relevant to the use of its tissues in the preparation of a seed lot or vaccine shall not be used, and none of the remaining monkeys of the quarantine group concerned shall be used unless it is evident that their use will not impair the safety of the product.

All operations described in this section shall be conducted outside the areas where vaccine is made.

If the monkey cells are passaged in series, the number of cell doublings permitted before the growth of the RVF virus shall be approved by the national control authority.

It has been shown that a "primary" cell culture may have undergone about 5 cell doublings, "secondary" cells about 10 cell doublings and "tertiary" cells about 15 doublings. Preferably, records of the cell doublings should be kept. It has been shown also that because of their decreased exposure to other animals, monkeys bred in captivity may have fewer extraneous agents than monkeys caught in the wild. If the cells are passaged in series, therefore, monkeys bred in captivity may be preferred.

In some countries karyology is also required. The extent of such tests should be determined by the national control authority.

3.1.2.2 Cell banks used for providing cell cultures. If human or monkey diploid cells are used for the propagation of RVF virus they shall be those approved by and registered with the national control authority. The cells shall have been characterized with respect to their genealogy, growth characteristics, viability during storage, and karyology.
They shall have been shown, by tests in animals and eggs, to be free from detectable adventitious agents and the requirements for human or monkey diploid cells used for the production of vaccine (Part C) shall apply.

If continuous cell lines are used for the propagation of RVF virus they shall be those approved by and registered with the national control authority. The requirements for the use of a non-tumorigenic continuous cell, such as VERO cells, (Part D) shall apply.

If the diploid cells are serially passaged, then the cells at the production level shall be tested for tumorigenicity (see Part C, section 3.1.2).

3.1.3 Cell culture medium

Serum used for the propagation of cells for vaccine production shall be tested to demonstrate freedom from bacteria, fungi, and mycoplasma, according to the requirements given in Part A, sections 5.2 and 5.3 of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (2) as well as freedom from pathogens of the species of the origin of the serum by methods approved by the national control authority.

In some countries sera are examined for freedom from phage.

Penicillin shall not be used at any stage in the production.

Suitable other antibiotics in minimum concentrations required for sterility may be used. Nontoxic pH indicators may be added, e.g., phenol red in a concentration of 20 mg/l.

3.1.4 Tests on virus seed lots

Each virus seed lot shall be subjected to all tests applicable to a single pool (see part A, sections 3.2, 3.4.1, 3.4.2, 3.4.4, 3.4.5, and 3.4.6). The national control authority shall approve the virus seed lots.

If the virus for vaccine production has been isolated or passaged in mice, tests for murine viruses should be included.

3.2 Control cells for vaccine prepared in monkey cells

A cell sample equivalent to at least 1% of the cell suspension, at the concentration employed for vaccine production cultures, shall be used to prepare control cell cultures.
In some countries in which the technology of large-scale production has been developed the national control authority should determine the size of the sample of cells to be examined, the time at which the control cells should be taken from the production culture, and the monitoring of the control vessels.

These control cell cultures shall be incubated for at least 2 weeks and shall be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures may be discarded for nonspecific, accidental reasons.

At this time, the control cell cultures shall be examined for any evidence of degeneration caused by an extraneous agent. If human or monkey diploid cells or continuous cell lines are used, tests on control cells shall be carried out according to the requirements described in Part C, section 3.2 or Part D, section 3.2, respectively. If primary or subcultured monkey cells are used, the control cells and the supernatant fluid from such cells shall also be tested for extraneous agents by the tests described in Part A, sections 3.2.1, 3.2.2, and 3.2.3. If this examination or any of the tests required in this section show evidence of the presence in a control culture of any adventitious agent, the RVF virus grown in the corresponding inoculated cultures shall not be used for vaccine production.

In some countries in which primary cell cultures are prepared from the tissues of monkeys caught in the wild, "foamy virus" is an inherent contaminant. Such cultures may be used for vaccine manufacture at the discretion of the national control authority.

3.2.1 Tests for haemadsorbing viruses

At the end of the observation period or at the time the virus is harvested from the production cultures, whichever is the later, 25% of the control cells shall be tested for the presence of haemadsorbing viruses using guinea-pig red cells. If the guinea-pig red cells have been stored, the duration of storage shall not have exceeded 7 days, and the temperature of storage shall have been in the range of 2–8 °C.

This test is usually made using guinea-pig red cells. In some countries the national control authority requires that tests for haemadsorbing viruses should be made in addition using other types of red cells, including those from humans (blood group O), monkeys, and chickens (or other avian species).

All tests should be read after incubation for 30 min at 0–4 °C and again after a further incubation for 30 min at 20–25 °C. The test with monkey red cells should be read once more after yet another incubation for 30 min at 34–37 °C.
3.2.2 Tests in rabbit kidney cell cultures

A sample of at least 10 ml of the pooled supernatant fluid from the control cultures shall be tested for the presence of simian herpesvirus (B virus) and other viruses in rabbit kidney cell cultures. Serum used in the nutrient medium of these cultures shall have been shown to be free from B virus inhibitors. The sample shall be inoculated into bottles of these cell cultures, in such a way that the dilution of the harvest suspension in the nutrient medium does not exceed 1 in 4. The area of the cell sheet shall be at least 3 cm²/ml of harvest suspension. At least one bottle of the cell cultures shall remain uninoculated and serve as a control.

The cultures shall be incubated at a temperature of 37°C and shall be observed for a period of at least 2 weeks.

For the test to be valid at least 80% of the culture vessels shall be available and suitable for evaluation at the end of the test period.

If the presence of B virus is demonstrated the manufacture of RVF vaccine shall be discontinued and the national control authority shall be informed. Manufacturing shall not be resumed until a thorough investigation has been completed and precautions have been taken against reappearance of the infection, and then only with the approval of the national control authority.

3.2.3 Test in Cercopithecus kidney cell cultures

A sample of at least 10 ml of the pooled supernatant fluid of the control cultures shall be tested for the presence of SV40 virus and other extraneous agents by inoculation on to cell cultures prepared from the kidneys of Cercopithecus monkeys by the method described in Part A, section 3.2.2. The cell cultures shall be incubated at 37°C and observed for a period of at least 2 weeks.

A cell culture shown to be equally sensitive to SV40 virus may be used.

For the test to be valid, at least 80% of the culture vessels shall be available and suitable for evaluation at the end of the observation period.

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2 On account of the danger of handling B virus, herpes simplex virus, has been used as the indicator for freedom from B virus inhibitors.
If there is any cyopathic effect attributable to the supernatant fluid, the virus grown on the same batch of cells shall not be used for vaccine production.

3.3 Production precautions

The general production precautions as formulated in Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (I) shall apply to the manufacture of RVF vaccine (inactivated). If animal serum is used for the growth of cell cultures, the serum concentration in the final vaccine shall be not more than 1 μl/l. The serum concentration shall be reduced to this level by rinsing the cell cultures with serum-free medium and/or purification of the virus harvests.

In some countries control tests are carried out to detect the residual animal serum content in the final vaccine (see Part A, section 5.5).

3.4 Control at the single harvests

When the tests on the control cells have shown the cells to be satisfactory, the single virus harvest from such cells may be pooled and samples taken for the following tests.

3.4.1 Test in Cercopithecus cell cultures (applied to all seeds and to all harvests grown in monkey cells except for continuous cell lines)

A sample of at least 10 ml of each single pool produced in primary or serially passaged monkey cell culture shall be tested for the presence of SV40 virus or other adventitious agents.

The single pool shall be neutralized by a high-titred antiserum against RVF.

The immunizing antigen used for the preparation of the antiserum should be shown to be free from extraneous agents and grown in cell cultures free from extraneous microbial agents that might elicit antibodies that could inhibit the growth of any adventitious agents present in the RVF virus pool.

This sample shall be tested in primary Cercopithecus kidney cell cultures or cells that have been demonstrated to be of equal sus-
ceptibility to SV40 virus. The tissue cultures shall be incubated at 37°C and observed for 14 days. At the end of this observation period at least one subculture of fluid shall be made in the same tissue culture system and both primary cultures and subcultures observed for an additional 14 days.

If necessary, serum may be added to the primary cultures at this stage, provided that the serum does not contain SV40 antibody or other inhibitors.

The virus pool passes the test if there is no evidence of the presence of SV40 virus or other adventitious agent attributable to the virus pool.

3.4.2 Test in rabbits (applied to all seeds and to all harvests grown in monkey cells except for continuous cell lines)

A sample of at least 30 ml of each single pool shall be tested as soon as possible after pooling by inoculation into three healthy rabbits, each weighing between 1.5 and 2.5 kg; proportionately larger volumes shall be used if more animals are inoculated. The inoculations shall be made at multiple sites, each rabbit being given a total of 1 ml of the single pool by intradermal injection and 9 ml by subcutaneous injection. The animals shall be observed for at least three weeks. All rabbits that die after the first 24 h of the test or that show signs of illness shall be examined by autopsy, with removal of the brain and organs for detailed inspection.

The single pool passes the test if at least 2 of the rabbits remain healthy and if none of the rabbits shows lesions of any kind at the sites of inoculation or shows evidence of infection with B virus or with any adventitious transmissible agent attributable to the single pool.

In some countries it is permissible to replace the test for B virus in rabbits by the test in rabbit kidney cell cultures.

3.4.3 Treatment before inactivation

Prior to inactivation each RVF virus pool shall be filtered.

The importance of filtration or clarification of the crude virus suspensions as a means of improving the regularity of the inactivation process has been clearly established. Generally, filters are used in series or filtration is performed stepwise through filters of decreasing porosity. Satisfactory results have been reported with several filter types but a final filtration of 0.22 μm should be used.
Filters containing asbestos should not be used. In some countries the virus suspension is concentrated and purified before inactivation.

For vaccines produced in continuous cell lines the RVF virus pool shall be purified before inactivation by a method approved by the national control authority (see Part D).

3.4.3.1 *Purification of the single pools.* Each filtered single pool of virus grown in a continuous cell line (see section D) shall be purified before inactivation. The purification process, which shall be shown to reduce consistently the level of cellular DNA from that of the initial virus harvest by a factor of at least $10^6$, shall be approved by the national control authority.

3.4.4 *Identity test*

The virus in the single pool shall be tested for identity by the neutralization of the virus with specific antiserum.

3.4.5 *Sterility tests*

Each pool after filtration shall be tested for sterility according to the requirements given in Part A, sections 5.1, 5.2 and 5.3, of the Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (2).

3.4.6 *Virus titration*

Before any inactivating agent is added, a sample shall be taken of each pool for titration of infective RVF using tissue culture methods. This titration shall be carried out in not more than 10-fold dilution steps and using 10 cultures per dilution, or any other arrangement yielding equal precision.

Each pool should show a titre of not less than $10^7$ TCID$_{50}$ per ml using a batch of tissue culture of normal sensitivity.

The main purpose of determining the titre of virus pools destined for inactivation is to select pools which can be expected to meet potency requirements after inactivation.

3.4.7 *Time of inactivation*

Inactivation shall be initiated as soon as possible and not later than 72 h after filtration.

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It is preferable to start inactivation within 24 h of filtration. Since the purpose of the filtration step is to remove particulate matter and other interfering substances that may diminish the effectiveness of the inactivation process, and since aggregates tend to increase on standing after filtration, efforts should be made to keep within this time limit.

3.4.8 **Inactivation procedure**

The virus in the single pools shall be inactivated through the use of an agent such as formaldehyde or some other method that has been demonstrated to be consistently effective in the hands of the manufacturer. The method shall be approved by the national control authority.

The time of inactivation should be at least 3 times that taken to reduce the virus titre to undetectable levels.

The progress of inactivation shall be followed by suitably spaced determinations of virus titres.

A second filtration during the process of inactivation shall be made.

This step is made after the virus titre has fallen below detectable levels but before the first sample for the safety test is taken.

The method of inactivation shall be shown to give consistent inactivation for the production of acceptable vaccine. A record of consistency shall be established by the production of 5 consecutive lots and if broken a further 5 monovalent lots shall be prepared and shown to be satisfactory to re-establish this record.

3.4.9 **Test for effective inactivation**

A sample of at least 200 ml or an equivalent volume of concentrated pool diluted to 200 ml shall be taken and, after removal or neutralization of the formaldehyde, if appropriate, shall be tested by inoculation into tissue cultures for the absence of infective RVF virus. The kinetics of inactivation shall be established by each manufacturer and approved by the national control authority. One sample shall be taken at the end of the inactivation period and the other not later than three-quarters of the way through this period. The two samples shall be inoculated into bottles of tissue cultures derived from different batches of cells. The dilution of the vaccine in the nutrient fluid shall not exceed 1:4 and the area of the cell sheet shall be at
least 3 cm$^2$/ml of vaccine. One or more bottles of each batch of cultures shall be set aside to serve as uninoculated control bottles with the same medium.

If formaldehyde is present in samples of vaccine for tissue culture tests, it is usually neutralized at the time of sampling by the addition of bisulfite. Usually, the samples are subsequently dialysed.

It is possible to conduct tissue culture tests on nondialysed material; however, this is often found to be toxic to cells even with a dilution of 1:4. If in such tests nonspecific degeneration of cells occurs, or if the sensitivity of the tissue culture system is reduced, the test should be repeated on dialysed material.

In some countries this test is made using tissue cultures that are also sensitive to SV40 virus, as an additional measure for detecting this extraneous agent.

Tissue culture bottles shall be observed for at least 2 weeks. At that time, at least 0.5 ml shall be harvested from each cell culture flask, and this may be pooled with fluids from no more than 4 other flasks. At least one-third of the individual or pooled fluid harvest shall be inoculated by intraperitoneal injection into at least 5 adult mice. The mice shall be observed daily and their condition recorded for a total of 14 days. Mice that become ill or die shall be tested for the presence of RVF virus by inoculating samples from the serum and liver into cell cultures and suckling mice.

At the end of the observation period the cell cultures used for the detection of residual live virus shall be challenged with live RVF virus. The required number of virus particles in the challenge shall be determined by the national control authority.

3.5 Control of bulk products

Only those pools that have been shown to be satisfactory shall be blended to form a bulk.

In some countries a pool from which live virus has been isolated may be subjected to reactivation and tested again for the presence of live virus. In such cases the national control authority should approve the reactivation procedure.

3.5.1 Tests for absence of RVF virus

Before the addition of preservatives, a sample of at least 200 ml or, if purified and concentrated vaccine is prepared, the equivalent of
200 doses shall be tested in cell cultures for the absence of infective virus by the procedure described in Part A, section 3.4.9 of these requirements. If infectious RVF is isolated, this batch of bulk product shall not be used.

3.5.2 Sterility test

The bulk shall be tested for sterility according to the requirements given in Part A, sections 5.1 and 5.2 of the Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (2).

3.5.3 Preservatives and other substances added

Preservatives or other substances that might be added to or combined with vaccine shall have been shown to the satisfaction of the national control authority to have no deleterious effect on the immunizing potency of the product.

4. FILLING AND CONTAINERS

The requirements concerning filling and containers given in Part A, section 4, of the revised Requirements for Biological Standards No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (1) shall apply with the addition of the following directive:

The containers shall be of colourless and fully transparent glass so as to permit the detection of visible changes in the vaccine.

Single- and multiple-dose containers may be used.

5. CONTROL TESTS ON FINAL PRODUCT

5.1 Identity test

An identity test shall be done on at least one labelled container from each filling lot by an appropriate method.

The potency test referred to in Part A, section 5.4, of these requirements may serve as the identity test.
5.2 Sterility tests

Each filling lot shall be tested for sterility according to the requirements given in Part A, sections 5.1 and 5.2, of the Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (2).

5.3 Innocuity test

Each final lot shall be tested for abnormal toxicity by appropriate tests involving injection into mice and guinea-pigs. The tests shall be approved by the national control authority.

5.4 Potency test

Each vaccine shall be tested for immunizing potency by tests approved by the national control authority. Such tests shall include an in vitro assay for antigen content and an in vivo assay for immune response. The potency of the vaccine shall be approved by the national control authority.

5.5 Protein nitrogen content

Rift Valley fever vaccine (inactivated) shall not contain more than 10 μg of protein nitrogen per human dose.

In some countries in vitro tests for serum proteins are included. Counter-current electrophoresis is useful for such tests.

6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (1) shall apply.

7. SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for
Manufacturing Establishments and Control Laboratories) \((I)\) shall apply.

8. LABELLING

The requirements given in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) \((I)\) shall apply with the addition of the following directive:

The leaflet accompanying the package shall include the following information:

(i) the nature of the cell cultures used;
(ii) the virus strain used for the production of the vaccine; and
(iii) the method used for inactivating the virus.

9. DISTRIBUTION AND SHIPPING

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) shall apply.

10. STORAGE AND EXPIRY DATE

10.1 Storage conditions

RVF vaccine (inactivated) shall be stored at all times at a temperature between 2 °C and 8 °C, if in liquid form; the freeze-dried product may alternatively be stored at -20 °C.

10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall be not more than 18 months after the date of the last satisfactory potency test, the date of a potency test being that date on which the test animals were inoculated with the vaccine. The expiry date shall not, however, be more than 12 months from the date at which the vaccine was issued by the manufacturer.
In some cases the national control authority may decide, on the basis of experimental evidence, to leave the expiry date at 18 months after the date of the last satisfactory potency test, irrespective of the date of issue by the manufacturer.

PART B.
NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (I) shall apply with the addition of the following directives:

The national control authority shall approve the strain used.
The national control authority shall approve the cell substrate used.
The national control authority shall specify potency requirements.
The national control authority shall be satisfied that the results of all tests, including those done on monovalent pools during the process of manufacture, are satisfactory and that consistency has been established.

In some countries the potency is tested by measuring the antibody response in man.

2. RELEASE AND CERTIFICATION

RVF vaccine (inactivated) shall be released only if it fulfils Part A of these requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing laboratory and shall certify whether or not the lot of vaccine in question meets all national requirements as well as the present requirements. The certificate shall also state the date of the last satisfactory potency test, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of biological substances between countries.
PART C.
REQUIREMENTS FOR HUMAN OR MONKEY DIPLOID CELLS USED FOR VIRRUS VACCINE PRODUCTION

The following requirements are applicable to the cell substrate for virus vaccine production where production is based on a cell seed system. The tests so far have been formulated for human diploid cells but any cell bank and cell seed system shall comply with similar requirements as appropriate.

1. DEFINITIONS

1.1 Terminology

*Cell seed.* A quantity of cells derived from a single human or monkey tissue stored frozen at \(-70^\circ C\) or below in aliquots, one or more of which would be used for the production of a manufacturer's working cell bank.

*Manufacturer's working cell bank (MWCB).* A quantity of cells derived from a single ampoule of the cell seed and of uniform composition stored frozen at \(-70^\circ C\) or below in aliquots, one or more of which would be used for the production of each single harvest.

In normal practice for human cells such a seed culture (or ampoule) is issued to manufacturers at or near the eighth population doubling level (PDL). This is expanded by serial sub-culture up to a PDL selected by the manufacturer, at which point the cells are combined into one or more pools and preserved in the frozen state to form the MWCB. One or more of such ampoules from a pool would be used for the production of a single harvest.

*Production cell culture.* A collection of cell cultures at the population doubling used for virus growth that have been derived from a single ampoule of the MWCB.

2. GENERAL MANUFACTURING REQUIREMENTS

The general requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufac-
turing Establishments and Control Laboratories) (I., p. 11) shall apply, with the addition of the following directive:

No cell cultures other than those approved by the national control authority for the production of appropriate vaccine shall be introduced or handled in the production area.

3. PRODUCTION CONTROL

3.1 Cell seed

The utilization of human or monkey diploid cell cultures for vaccine manufacture shall be based on the cell seed system. Early population doubling of diploid cell cultures shall be subcultured to a population doubling that is convenient for the preparation of a cell seed.

The cell seed used for the production of virus vaccine shall be that approved by and registered with the national control authority. The accepted cell strain from which the cell seed has been derived shall have been characterized with respect to genealogy, growth characteristics, genetic markers (HLA), virus susceptibility, storage conditions, and karyology, and it shall have been shown, by tests in animals, eggs, and cell culture to be free from detectable adventitious agents.

These data shall be made available to the national control authority.

Each manufacturer shall show, to the satisfaction of the national control authority, that the cell substrate propagated from the accepted cell strain and laid down as a working cell bank conforms with the test outlined in this section for freedom from extraneous agents by tests in animals and eggs, for lack of tumorigenicity, for normal karyology throughout approximately the first two-thirds of its normal life-span, and for identity.

In some countries the cells are examined also by ultrathin sections and negative staining under the electron microscope.

3.1.1 Tests in animals and eggs for extraneous agents

The tests in animals for extraneous agents shall include the inoculation of each of the following groups of animals with cells by the intramuscular route, using at least $10^7$ viable cells divided equally between the animals in each group:

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2 litters of suckling mice, comprising at least 10 animals, less than 24 h old; 10 adult mice; 5 guinea-pigs; and 5 rabbits.

At least $10^6$ viable cells shall be injected also into the allantoic cavity of each of 10 embryonated chicken eggs 9–11 days old.

The animals shall be observed for at least 4 weeks and the embryonated chicken eggs shall be examined after not less than 3 days’ incubation. Any animals that are sick or show any abnormality shall be investigated to establish the cause of illness. The allantoic fluids shall be tested with guinea-pig and chick or other avian red cells for the presence of haemagglutinins.

The cells are suitable for vaccine production if at least 80% of the animals or eggs inoculated with the cells remain healthy and survive the observation period and none of the animals or eggs shows evidence of the presence in the cell cultures of any extraneous agent.

3.1.2 Tumorigenicity

The cells at the production level shall also be shown in animals to be free from tumorigenicity by appropriate tests approved by the national control authority.

Particularly for new non-continuous cell strains some control authorities may wish to consider applying the tests for tumorigenicity as outlined in Part D, section 3.1.2. For the diploid cell strains that have been in use for many years, however, the tests suggested below have been shown to be satisfactory.

Suitable tests using immunosuppressed animals may be made as follows. Approximately $10^6$ viable cells obtained from cultures at the same passage level as those used for vaccine production are injected into (a) newborn mice or hamsters treated with antilymphocyte serum; or (b) athymic mice (nude nu/nu genotype); or (c) thymectomized mice irradiated and bone-marrow reconstituted (T-B-). Some of the same group of animals should be inoculated with a similar dose of HeLa or KB cells and it should be shown that tumour formation is caused by the inoculation of the neoplastic tissue, thus demonstrating the ability of the strain of animals to give rise to tumours. The animals should be observed for not less than three weeks. Any other test using animals treated with immunosuppressive agents and with equal sensitivity to neoplastic cells may be used.

Only those cell seeds shown not to be tumorigenic shall be used.
3.1.3 Chromosomal characterization and monitoring

3.1.3.1 Chromosomal characterization. At least four samples shall be examined as described in Part C, section 3.1.3.2, at approximately equal intervals over the life-span of the cell line during serial cultivation. Each sample shall consist of 1000 metaphase cells.

It is also recommended that photographic reconstruction should be employed in the preparation of chromosome-banded karyotypes of 50 metaphase cells per 1000 cell sample using either G-banding or Q-banding techniques. This constitutes 5% of the sample and no specific limits for acceptability are yet recommended. The incidence of karyotypic abnormalities (pseudo-diploidy, inversions, translocations, etc.) that are detectable with the greater resolution provided by banding should be evaluated when a larger data base than is at present available has been accumulated.

3.1.3.2 Chromosomal monitoring—preparation and testing. For the determination of the general character of each pool in the MWCBL, a minimum of 500 cells in metaphase shall be examined at the production level or at any passage thereafter for frequency of polyploidy and for exact counts of chromosomes, frequency of breaks, structural abnormalities, and other abnormalities such as despiralization or marked attenuations of the primary or secondary constrictions.

For vaccine production, examination of the cells is usually made between the twenty-seventh and thirty-third population doubling. The national control authority should determine the level of cell population doubling allowable.

For human cells examined in metaphase the upper limits3 of acceptability (upper fiducial limits at 95% (Poisson)) for abnormalities are, for a 1000- and 500-cell sample, as follows:

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>1000 cells</th>
<th>500 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatid and chromosome breaks</td>
<td>47/1000</td>
<td>26/500</td>
</tr>
<tr>
<td>Structural abnormalities</td>
<td>17/1000</td>
<td>10/500</td>
</tr>
<tr>
<td>Hyperploidy</td>
<td>8/1000</td>
<td>5/500</td>
</tr>
<tr>
<td>Hypoploidy</td>
<td>180/1000</td>
<td>90/500</td>
</tr>
<tr>
<td>Polyploidy</td>
<td>30/1000</td>
<td>17/500</td>
</tr>
</tbody>
</table>

3 These upper limits for human cells are based on extensive experience with the examination of W1-38 and MRC5 cells reported to and examined by the ad hoc Committee on Karyological Controls of Human Cell Substrates, which met in 1978 at Lake Placid, NY, USA. These values will not necessarily be applicable if another human cell strain or a monkey cell strain is used.

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All cells showing abnormalities shall be subjected to detailed examination, and records shall be maintained of the detailed criteria applied to particular abnormalities evaluated in the karyotype analysis.

Permanent stained slide preparations of the chromosome monitoring of the working cell bank pool, or photographs of these, shall be maintained as part of the record of the batch of vaccine for monitoring successive batches made from that cell pool.

It is desirable that a portion of the sample of pooled cell substrate removed from the culture vessels should be stored frozen so as to retain viability. This would be available for future reference for karyology or for any other purpose relating to the batch of vaccine.

Only those cell pools of the MWCB that have normal karyology shall be used for vaccine production.

3.1.4 Identity test of the cells

In some countries tests for characterizing HLA surface antigens are carried out in addition to chromosome monitoring.

3.2 Production of cell cultures

A cell sample equivalent to at least 1% of the cell suspension of the concentration employed for seeding the vaccine production cultures shall be used to prepare control cell cultures.

In some countries in which the technology of large-scale production has been developed, the national control authority should determine the size of the sample of cells to be examined, the time at which the control cells should be taken from the production cultures, and the monitoring of the control vessels.

The treatment of cells set aside as control material shall be similar to that of the production cell cultures but they shall remain uninoculated as control cultures for the detection of extraneous viruses.

These control cell cultures shall be incubated under similar conditions to the inoculated cultures for at least 2 weeks or until the time of the last harvest of the production cultures, whichever is the later, and shall be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures may be discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures shall be examined for degeneration caused by an infectious agent.
If this examination or any of the tests required in this section show
evidence of the presence in a control culture of any adventitious
agent, the virus grown in the corresponding inoculated cultures shall
not be used for vaccine production.

3.2.1 Test for haemadsorbing viruses

At the end of the observation period, cells comprising 25% of the
control cells shall be tested for the presence of haemadsorbing viruses
using guinea-pig red cells. If the guinea-pig red cells have been
stored, the duration of storage shall not have exceeded 7 days and
the temperature of storage shall have been in the range of 2–8 °C.

In some countries the national control authority requires that
tests for haemadsorbing viruses be made in addition, using other
types of red cells including those from humans (blood group O),
monkeys, and chickens (or other avian species). The cultures
should be examined at 3–5 days and again at 12 days. All tests
should be read after incubation for 30 min at 0–4 °C and again
after a further incubation for 30 min at 20–25 °C. The test
with monkey red cells should be read once more after yet
another incubation for 30 min at 34–37 °C.

3.2.2 Tests for other extraneous agents

At the time of each harvest of the production cultures and 14 days
after the day of inoculation of the production cultures with seed lot
virus, a sample of the pooled fluids shall be taken at each period of
collection from each group of control cultures. 10 ml of each pool
shall be tested in the same cells, but not the same batch of cells as
that used for the production of virus growth, and additional 10-ml
samples of each pool shall be tested in human cells and at least one
other sensitive cell system.

The inoculated cultures shall be incubated at a temperature of
35–37 °C and shall be observed for a period of at least 14 days.

Some national control authorities require that at the end of
this observation period a subculture should be made in the same
cell culture system and observed for at least seven days. Further-
more, some national control authorities require that these cells
should be tested for the presence of haemadsorbing viruses.

For the tests to be valid, at least 80% of the culture vessels should
be available and suitable for evaluation at the end of the respective
test periods.

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If any cytopathogenic changes occur due to extraneous agents in any of the cultures the virus harvests produced from the batch of cells from which the control cells were taken shall be discarded.

3.2.3 Identity test

At the production level the cells shall be identified as human or monkey by tests approved by the national control authority.

Suitable tests are isozymes, HLA, or other immunological tests or karyotyping, of at least one metaphase spread of chromosomes.

PART D.

REQUIREMENTS FOR CONTINUOUS CELL LINES USED FOR INACTIVATED VIRUS VACCINE PRODUCTION

The following requirements are applicable to the cell substrate for virus vaccine production where production is based on a cell seed system from a continuous cell line.

1. DEFINITIONS

1.1 Terminology

Cell seed. A quantity of cells derived from a normal tissue and stored frozen at −70 °C or below in aliquots, one or more of which would be used for the production of a manufacturer’s working cell bank.

Manufacturer's working cell bank (MWCB). A quantity of cells derived from one or more ampoules of the cell seed and of uniform composition stored frozen at −70 °C or below in aliquots, one or more of which would be used for the production of each single harvest.

In normal practice such a seed culture (or ampoules) is expanded by serial subculture up to a passage number selected by the manufacturer, at which point the cells are combined into one pool and preserved cryogenically to form the MWCB. One or more of the ampoules from such a pool would be used for the production of a single harvest.
Production cell culture. A collection of cell cultures at the passage number used for virus growth that have been derived from one or more ampoules of the MWCB.

2. GENERAL MANUFACTURING REQUIREMENTS

The general requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (I, p. 11) shall apply with the addition of the following directive:

No cell cultures other than those approved by the national control authority for the production of appropriate vaccine shall be introduced or handled in the production area.

3. PRODUCTION CONTROL

3.1 Cell seed and/or manufacturer's working cell bank

The utilization of continuous cell lines for vaccine manufacture shall be based on the cell seed system. A passage of a continuous cell line shall be subcultured to a passage number which is convenient for the preparation of a cell seed.

The cell seed used for the production of virus vaccine shall be that approved by and registered with the national control authority. The accepted continuous cell line from which the cell seed and/or the MWCB have been derived shall have been characterized with respect to genealogy, growth characteristics, immunological markers, virus susceptibility, and storage conditions and it shall have been shown, by tests in animals, eggs, and cell culture to be free from detectable adventitious agents.

In some countries karyology is also required.

These data shall be made available to the national control authority.

Each manufacturer shall show, to the satisfaction of the national control authority, that the cells intended as the virus substrate, propagated from the accepted continuous cell line and laid down as the MWCB conform with the test outlined in this section for freedom from extraneous agents, by tests in animals and eggs (see Part D,
section 3.1.1), and for lack of tumorigenicity. In addition, the test for tumorigenicity shall be repeated on the cells at the passage level used for vaccine production.

In some countries the cells are examined by electron microscopy to establish their ultrastructural characteristics. The cells may also be tested for the presence of retroviruses after activation with agents such as bromodeoxyuridine (BUDR), by examining electron micrographs for virus particles and/or by performing assays for viral reverse transcriptase.

3.1.1 Tests in animals and eggs for extraneous agents

The tests in animals for extraneous agents shall include the inoculation of each of the following groups of animals with cells by the intramuscular route, using at least $10^7$ viable cells divided equally between the animals in each group:

- 2 litters of suckling mice, comprising at least 10 animals, less than 24 h old;
- 10 adult mice;
- 5 guinea-pigs; and
- 5 rabbits.

At least $10^6$ viable cells shall be injected also into the allantoic cavity of each of 10 embryonated chicken eggs 9–11 days old.

The animals shall be observed for at least 4 weeks and the embryonated chicken eggs shall be examined after not less than 3 days’ incubation. Any animals that are sick or show any abnormality shall be investigated to establish the cause of illness. The allantoic fluids shall be tested with guinea-pig and chick or other avian red cells for the presence of haemagglutinins.

The cells are suitable for vaccine production if at least 80% of the animals or eggs inoculated with the cells remain healthy and survive the observation period and none of the animals or eggs shows evidence of the presence in the cell cultures of any extraneous agent.

3.1.2 Tests for tumorigenicity

Cells from the MWCB at the passage used for the virus production, or up to 10 passages thereafter, shall be shown to be non-tumorigenic in a test approved by the national control authority. Such a test must show a clear difference between the continuous cell line and a
reference preparation of HeLa cells. In an appropriate in vivo test 10⁶ viable HeLa cells when given by the subcutaneous or intramuscular route shall produce progressively growing tumours in at least 9 out of 10 animals, of which one or more must show evidence of metastases, while the 10⁶ cells of an acceptable continuous cell line shall produce neither progressively growing tumours nor metastases.

The systems shown to be suitable for this test include:
(a) newborn mice, rats or hamsters that have been treated with antithymocyte serum or globulin,
(b) thymectomized and irradiated mice that have been reconstituted with bone marrow from healthy mice,
(c) chick embryo skin organ cultures.

A suitable test using newborn animals treated with antithymocyte serum is to inoculate at least 20 animals with 0.1 ml of potent serum within 24 h of birth. The injection is given either by the intramuscular or subcutaneous route and is repeated on days 2, 7, and 14 of life. A potent antithymocyte serum or globulin is one that suppresses the immune mechanisms of the growing animals to the extent that the subsequent inoculum of 10⁶ HeLa cells regularly produces tumours and metastases.

Also on the day of birth the two groups of 10 newborn animals that have been given the antithymocyte serum are given either 10⁶ viable HeLa cells or 10⁶ viable cells of the continuous cell line by the subcutaneous route at any site at which developing tumours can be palpated (the base of the neck or the abdomen are suitable sites). The animals are observed for 21 days for the evidence of nodule formation at the site of injection and measurements are made at suitable times to determine whether there has been progressive growth.

At the end of the observation period all animals from both groups are sacrificed and examined for gross evidence of tumour formation at the site of injection and in other organs such as the lymph nodes, lungs, kidneys, and liver. All tumour-like lesions are examined histopathologically. In addition, since some cell lines may form metastases without evidence of local tumour growth, the lungs and regional lymph nodes of all animals shall be examined histopathologically.

For the purposes of this requirement, a progressively growing tumour is defined as a palpable nodule that increases in diameter over the 21-day observation period and that shows viable and mitotically active inoculated cells when examined histopathologically. The presence of microscopically viable cells without gross nodules formation shall not be considered a progressively growing tumour; in addition, the presence of microscopically viable

---

1 Information concerning the sources of suitable HeLa cells may be obtained from Chief, Biologicals, WHO, 1211 Geneva 27, Switzerland.
cells in association with a stationary or regressing nodule shall not be considered a progressively growing tumour.

In addition, some countries test the cells for tumorigenicity in animals of the homologous species from which the cells were derived. Such tests would include immunosuppression of the animals with species-specific antithymocyte serum, inoculation of candidate cells and control tumour cells, observation for at least three weeks, and the histopathological examination of the inoculation sites as well as any metastatic lesions.

In some countries an *in vitro* test is permitted to demonstrate the freedom from tumorigenicity provided that the test has been shown to be as sensitive as a test in animals.

A suitable test using *organ cultures of chick embryonic skin* is to inoculate $10^6$ HeLa cells or $10^6$ cells from an acceptable continuous cell line on to organ cultures of chick embryonic skin for 3 days. At the end of this period each culture is processed for histologic evaluation, and scored for cell growth and invasion. The reference HeLa cells should show extensive mitotic activity as well as extensive invasion into the chick substrate, while the continuous cell lines will show little or no invasion. In addition, secondary cell cultures derived from the same tissue as the continuous cell line may be tested in this system to provide guidance in interpreting invasive and mitotic activity.

3.1.3 *Identity test of the cells*

The MWCB shall be identified by a method approved by the national control authority.

The tests that may be used are karyology, isoenzymes analysis and/or immunological markers.

3.2 *Production of cell cultures*

A cell sample equivalent to at least 1% of the cell suspension at the concentration employed for seeding the vaccine production cultures shall be used to prepare control cultures.

In some countries in which the technology of large-scale production has been developed the national control authority should determine the size of the sample of cells to be examined, the time at which the control cells should be taken from the production cultures, and the monitoring of the control vessels.

The treatment of cells set aside as control material shall be similar to that of the production cell cultures but they shall remain un-inoculated as control cultures for the detection of extraneous viruses.
These control cell cultures shall be incubated under similar conditions to the inoculated cultures for at least two weeks or until the time of the last harvest of the production cultures, whichever is the later, and shall be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures may be discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures shall be examined for degeneration caused by an infectious agent. If this examination or any of the tests required in this section show evidence of the presence in a control culture of any adventitious agent, the virus grown in the corresponding inoculated cultures shall not be used for vaccine production.

3.2.1 Test for haemadsorbing viruses

At the end of the observation period cells comprising 25% of the control cells shall be tested for the presence of haemadsorbing viruses using guinea-pig red cells. If the guinea-pig red cells have been stored, the duration of storage shall not have exceeded 7 days and the temperature of storage shall have been in the range of 2–8 °C.

In some countries the national control authority requires that tests for haemadsorbing viruses be made in addition using other types of red cells including those from humans (blood group O), monkeys, and chickens (or other avian species). The cultures should be examined at 3–5 days and again at 12 days. All tests should be read after incubation for 30 min at 0–4 °C and again after a further incubation for 30 min at 20–25 °C. The test with monkey red cells should be read once more after yet another incubation for 30 min at 34–37 °C.

3.2.2 Tests for other extraneous agents

At the end of the observation period a sample of the pooled fluids from each group of control cultures shall be tested for extraneous agents. 10 ml of each pool shall be tested in the same cells, but not the same batch of cells, as that used for the production of virus growth, and additional 10-ml samples of each pool shall be tested in human cells and at least one other sensitive cell system.

The inoculated cultures shall be incubated at a temperature of 35–37 °C and shall be observed for a period of at least 14 days.

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For the tests to be valid, at least 80% of the culture vessels shall be available and suitable for evaluation at the end of the respective test periods.

If any cytopathic changes occur due to extraneous agents in any of the cultures, the virus harvests produced from the batch of cells from which the control cells were taken shall be discarded.

3.2.3 Identity test

At the production level the cells shall be identified as the species of origin by tests approved by the national control authority.

Suitable tests are isoenzymes analysis, other immunological tests, or karyology.

3.2.4 Purity of virus prepared on a continuous cell line

The virus grown in a continuous cell line shall be purified by a process that has been approved by the national control authority and that has been shown to give consistent results.

Tests used to demonstrate the degree of purity achieved shall also be approved by the national control authority.

For RVF vaccine (inactivated) the purification process shall be shown to reduce consistently the level of cellular DNA from that of the initial virus harvest by a factor of at least 10^6 (see Part A, section 3.4.3.1).

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REFERENCES


Appendix

SUMMARY PROTOCOL
OF RIFT VALLEY FEVER VACCINE (INACTIVATED)
PRODUCTION AND TESTING

(Based on Requirements for Biological Substances No. 32)
(Requirements for Rift Valley Fever Vaccine (inactivated)
for human use)

Identification of Final Lot

<table>
<thead>
<tr>
<th>Name and address of manufacturer</th>
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<table>
<thead>
<tr>
<th>Lot number of final product</th>
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<table>
<thead>
<tr>
<th>Date of manufacture of final lot (namely, date of last potency test)</th>
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<tr>
<th>Expiry date</th>
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<table>
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<tr>
<th>Total volume of final lot</th>
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</tbody>
</table>

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Cell cultures

Type of cell cultures used:
(Complete only the relevant part—e.g., A, B or C)

A. Cell cultures/monkey primary/
   secondary/tertiary
   Type of cells (kidney, fetal kidney,
   lung, etc.)
   Monkey species of cells
   Controls performed on animals from
   which the cells originated
   Number of cell doublings
   Result
   Amount of cell culture inoculated
   Amount of control cell culture
   investigated
   Tests performed on control cultures
   Result

B. Cell cultures/human or monkey diploid
   Origin and short history of cell seed
   Authority that approved cell seed
   Amount of cell culture inoculated
   Amount of control cell culture
   investigated
   Tests performed on control cultures
   Result

C. Cell cultures/other cells
   Origin and short history of cell seed
   Authority that approved cell seed
   Amount of cell culture inoculated

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### Amount of control cell culture investigated

<table>
<thead>
<tr>
<th>Tests performed on control cultures</th>
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<tbody>
<tr>
<td>Result</td>
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</table>

### Serum for Cell Cultures

<table>
<thead>
<tr>
<th>Origin of serum used</th>
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<tr>
<td>Tests performed on serum</td>
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<tr>
<td>Result</td>
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</table>

### Seed Lots of Virus

<table>
<thead>
<tr>
<th>Strain used</th>
</tr>
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<tbody>
<tr>
<td>Origin and source of strain</td>
</tr>
<tr>
<td>Number of subcultures between primary seed lot and production</td>
</tr>
</tbody>
</table>

### Information on Manufacture

#### 1. Single harvests

- Date of inoculation of cells with virus
- Date of virus harvest
- Special observations on single harvests
- Date of pooling

#### Tests on pooled harvests

##### Tests in Cercopithecus cell cultures

- Method used
- Result of test

##### Test in rabbits or kidney cell cultures

- Number of rabbits (or cell cultures)
- Total volume injected
Observation period
Result

Product before inactivation
Details on filtration and/or clarification and/or purification (if applied)

Date

Tests on pool
Identity test
Method
Result

Sterility tests
Method (media)
Result

Virus titration
TCID₅₀ per ml

Details on filtration and/or clarification and/or purification (if applied)

Date(s)

Inactivation of product
Agent(s) and concentration

Date of start of inactivation
Date of taking first sample
Date of completion of inactivation
Test for effective inactivation
(after removal/neutralization of inactivating agent)

Sample size tested
Date of first sample
Date of second sample
Details of testing procedure

Period of observation of cell cultures
Period of observation of subcultures
Result
Result of challenge of used culture with live virus

2. Bulk product
(single pools incorporated)

Date of preparation
Preservative (if added, type and concentration)

Tests on bulk

Test for absence of infective RVF virus

Sample size tested
Details of testing procedure

Period of observation of cell cultures
Period of observation of subcultures
Result

Sterility tests

Method (media)

Result

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3. Tests on final product

**Identity tests**
- Method used
- Result

**Sterility tests**
- Method (media)
- Result

**Innocuity test**
- Number of animals
- Dose
- Observation period
- Result

**Potency test**
- Method
- Result
- Date

**Protein nitrogen**
- Content of protein nitrogen in µg per human dose

**Serum protein tests (if done)**
- Result

Signature of head of laboratory
Certification by person taking overall responsibility for production of the vaccine.
I certify that lot No. ..... of Rift Valley fever vaccine (inactivated) satisfies the WHO Requirements for Rift Valley fever Vaccine (inactivated) for human use.

Date

Signature

Name typed

The protocol must be accompanied by a sample of the vaccine and a copy of the leaflet.
# Annex 5

## REQUIREMENTS FOR ANTIMICROBIC SUSCEPTIBILITY TESTS

### 1. AGAR DIFFUSION TESTS USING ANTIMICROBIC SUSCEPTIBILITY DISCS

(Requirements for Biological Substances No. 26)
(Revised 1981)

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INTRODUCTION

Since Requirements for Antibiotic Susceptibility Tests. I. Agar Diffusion Tests Using Antibiotic Susceptibility Discs (Requirements for Biological Substances No. 26) were published in 1977 (1), a number of changes have occurred in the manufacture and use of antibiotic discs. The WHO Expert Committee on Biological Standardization in its thirty-first report adopted several amendments to the Requirements to reflect, among other things, changes in the antibiotic content of amikacin discs, the addition of new antibiotic discs for cefamandole and cefoxitin, and changes in the appropriate zone sizes for judging performance of polymyxin B and for interpreting the results of susceptibility tests using carbenicillin, gentamicin, and tobramycin discs.

The WHO Secretariat has received a number of requests for information concerning possible codes for new antibiotic discs, and comments have been submitted concerning the use of codes different from those suggested in the Requirements and the use of the same code for different antibiotic discs. The WHO Secretariat therefore corresponded with a number of individuals in various countries known to have interest in and knowledge of the manufacture, marketing, and clinical use of susceptibility discs. On the basis of the replies received, the present revised Requirements for Antimicrobial Susceptibility Tests. I. Agar Diffusion Tests Using Antimicrobial Susceptibility Discs have therefore been formulated.

Since the publication of another set of amendments was not considered a satisfactory way of revising the requirements, a completely new document has been prepared. Much of it is identical with the requirements originally formulated and amended, but alterations have been made where they were considered necessary. In addition, the scope of the requirements has been broadened to cover discs containing synthetic antimicrobials as well as antibiotics.

GENERAL CONSIDERATIONS

The need for international standardization of procedures for determining the susceptibility of microorganisms to antibiotics has been recognized since 1961, when the WHO Expert Committee on Antibiotics (2) made recommendations to this end. The results of
an extensive series of international collaborative studies of different techniques of testing using broth dilution, agar dilution, and agar diffusion, organized in conjunction with WHO, were published by Ericsson & Sherris in 1971 (3). While agreement could not be reached internationally on many procedural details of experimental technique and interpretation, it was agreed that standardization of the reagents used in the testing was desirable and possible. Agar diffusion procedures are the methods most widely used internationally.

Not only antibiotic susceptibility discs but also discs containing a number of synthetic antimicrobials are used in these procedures. The latter are used concurrently in the same test procedures and for the same purpose as antibiotic susceptibility discs: to aid in the selection of effective treatment agents.

Antibiotic and antimicrobial susceptibility discs are manufactured in only a few countries, however, and international requirements defining their quality and performance would be helpful to those countries dependent on importing these biological materials or considering manufacturing them in the future. The safety of the patient and the effectiveness of treatment may well depend as much on an assurance of the quality of the antimicrobial susceptibility discs and on their method of use as on the quality and quantity of the antimicrobial preparation chosen for therapy.

The original requirements were limited to antibiotic susceptibility discs. In recognition of the wide availability and use of discs containing various synthetic antimicrobials, the present revision therefore provides requirements for both antibiotic and antimicrobial susceptibility discs. The various sections of the requirements have been revised and expanded to cover both types of disc, designated jointly as "antimicrobial susceptibility discs".

In revising these requirements it has been possible to include many of the details of the production and control of antimicrobial discs along with those of antibiotic susceptibility discs, and these details are included in Part A of the requirements. Although there is no universal agreement on the quantity of antibiotic or antimicrobial that should be contained in each disc it has been possible to indicate the quantities commonly used. Furthermore, the international code for the identification of the antibiotic discs suggested in the original requirements has been revised to provide a more flexible system of devising codes and to recognize identical codes in widespread use by a number of disc manufacturers for both antibiotic and antimicrobial discs.

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Perhaps the greatest differences between countries concern the
details of performing disc susceptibility tests because different media
and test conditions are used without a common point of reference,
e.g., reference strains. In these requirements, attempts have been
made to overcome these differences by providing guidance on the
directions necessary for the use of the discs and indicating how indi-
vidual laboratories could achieve greater uniformity by daily per-
formance testing using reference strains (see Part C).

Interpretation of the zone diameters has been a constant problem
in many countries. The aim of the test is to assess the susceptibility
or resistance of an organism to a particular antibiotic or antimicrobial
in order that the physician may successfully treat the infection. This
implies that the level of antimicrobial to which the organism is sus-
ceptible can be reached in the body at the site of infection. Although
there is no international agreement on such levels, some guidance
must be given, especially for those countries without much experience
in this technique. Therefore, a table of zone diameters has been
included indicating whether the organism is resistant, intermediate,
or susceptible. This table has been shown to be useful in one country
and is of general value if considered in relation to the recommended
use of reference strains.

Finally, because the composition of the medium plays such a
significant part in the zone sizes obtained, the requirements for a
medium (Mueller-Hinton) known to be successful and widely used
by many laboratories have been included. A performance test is
also included that would permit the use of other media (see Part D).

These requirements apply only to single discs. Multi-discs were
considered by the WHO Expert Committee on Antibiotics (2) to be
inferior, and there are no known national requirements for their
control.

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

The terms "national control authority" and "national control
laboratory", as used in these requirements, always refer to the
country in which the discs are manufactured.
PART A.

MANUFACTURING REQUIREMENTS FOR SUSCEPTIBILITY DISCS

1. DEFINITIONS

1.1 International names and proper names

The international name of each kind of disc containing a particular antimicrobial shall be the international nonproprietary name (INN) of the antibiotic or antimicrobial contained therein followed by the words “Susceptibility Disc”. The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to antimicrobial susceptibility discs that satisfy the requirements formulated below.

1.2 Descriptive definition

Antimicrobial susceptibility discs are round flat absorbent discs of uniform diameter and thickness containing an evenly distributed amount of antibiotic or antimicrobial.

Antimicrobial susceptibility discs commonly have a diameter chosen within the range 5–8 mm. Paper is the most common material from which the discs are made.

1.3 International standards, international reference preparations, and international units

The potency tests described in Part A, section 5.1, provide for the preparation of the standard discs by the laboratory performing control tests. National control authorities shall provide reference preparations of antibiotics for preparing standard discs or for determining the potency of local reference preparations of antibiotics to be used in preparing standard discs.

No international reference preparations of antimicrobial susceptibility discs are available.

International standards and reference preparations of antibiotics (\(\delta\)) are in the custody of the National Institute for Biological Standards and Control, London. Samples are distributed
1.4 Terminology

The following definitions are given for the purposes of these requirements only.

_Antimicrobial_. An antibiotic or synthetic antimicrobial.

_Disc_. An antimicrobial susceptibility (sensitivity) disc containing a specific antibiotic or antimicrobial for the determination of the susceptibility of microorganisms to the particular antibiotic or antimicrobial.

_Batch_. A quantity of discs produced during a given cycle of manufacture. The essence of a batch is its homogeneity.

_Sample disc_. A disc selected from a given batch for quality control tests.

_Standard disc_. A disc that has been prepared by a quality control laboratory, that contains a specified amount of an antimicrobial, and that is intended for use in the assay of the content of a sample disc taken from a particular batch.

_Assay organism_. A specific strain of microorganism used for the assay of particular antimicrobial susceptibility discs.

_Culture medium_. Microbiological medium for propagating assay organisms.

_Assay medium_. Microbiological medium used for preparing base layers and/or seed layers in Petri dishes used for assaying the content of discs.

_Zone of inhibition_. The clear circular area around a disc at the completion of an assay. The size of the zone is expressed as its diameter of the disc.

1.5 Contents of antimicrobial in discs

The antimicrobial content of discs shall be such that when used as described in Part C, section 3, inhibition zones are obtained with organisms against which the antimicrobial may be expected to be clinically effective. The minimum inhibitory concentration (see Part C, section 1) shall be within the range of concentrations likely to be obtained in body tissues and fluids during therapy.
Zone diameters should not exceed 40 mm with commonly tested highly susceptible microorganisms encountered in clinical practice and preferably should not exceed 30 mm.

For most antimicrobics the application of these guidelines shows that a single strength of antimicrobial disc can be employed. Some antimicrobics have such low toxicity that infections with organisms having minimum inhibitory concentrations with a range of several hundredfold may be amenable to treatment with different dosage schedules. For such antimicrobics more than one strength of disc may be required.

Within these guidelines, there is no precisely correct nominal disc content. Selection of the content to be used is arbitrary within certain limits and is based in part on the logic and convenience of having similar disc contents within a family of antimicrobics.

The following disc contents have been shown to be suitable for some families of antibiotics and for some individual antibiotics or antimicrobials:

- the penicillinase-sensitive penicillins\(^1\) (e.g., benzylpenicillin) 6 µg
- the penicillinase-resistant penicillins (e.g., meticillin) 5 µg
- the cephalosporins\(^1\) 30 µg
- the aminoglycosides\(^1\) 10–30 IU
- the tetracyclines 30 IU
- the macrolides\(^1\) 15 IU
- the lincomycins\(^1\) 2–15 IU
- the polymyxins\(^1\) 300 IU
- ampicillin 10 µg
- bacitracin 10 IU
- carbenicillin 100 µg
- chloramphenicol 30 µg
- nalidixic acid 30 µg
- nitrofurantoin 300 µg
- novobiocin 30 IU
- rifampicin 5 µg
- sulfamethoxazole/trimethoprim 23.75 µg/1.25 µg
- sulfonamides 250 µg
- trimethoprim 5 µg
- vancomycin 30 IU

\(^1\) For purposes of routine testing of clinical isolates for susceptibility to antibiotics it may be satisfactory to use one type of antibiotic susceptibility disc as a representative of its class or family. However, testing with individual members of the family may be desirable under certain circumstances.
1.6 Codes

Each disc shall be imprinted with one to three letters to identify the antimicrobial contained therein.

The purpose of the abbreviations is to identify the discs. The abbreviations should not be used in publications and in any written communication the names of antimicrobics should be given in full.

The abbreviation should consist of the first letter of the international nonproprietary name, followed by one or two other letters of the international nonproprietary name, if appropriate, except when confusion may arise by virtue of the existence of several antimicrobics with an identical initial letter or stem, or when such letters may be undesirable for other reasons.

For the sake of uniformity, the following abbreviations are suggested:

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2. GENERAL MANUFACTURING REQUIREMENTS

The general manufacturing requirements contained in the WHO Good Practices in the Manufacture and Quality Control of Drugs (5, p. 88) shall apply.

3. PRODUCTION CONTROL

3.1 Control of source material

3.1.1 Antibiotics and antimicrobials

The antibiotics and antimicrobials to be used in preparing discs shall be of the quality used in pharmaceutical manufacture and shall meet the requirements, if any, of the International Pharmacopoeia (6), except those for undue toxicity and residue on ignition.

3.1.2 Paper or other material used for preparing discs

The material used for preparing the discs and the method of manufacture shall be such that the discs are uniform and of sufficient thickness to ensure ease of handling. The material shall also permit complete and uniform adsorption of a sufficient volume of antimicrobial solution and under the conditions of use shall allow uniform release of the antimicrobial. When response lines are prepared for each antimicrobial, using discs made from the material and the assay procedures described in Part A, section 5.2, the slopes of the lines shall be comparable to those obtained with the response lines of the respective standard antimicrobial discs. The material shall be tested and shown to be free from inherent inhibitory activity and from manufacturing residues, such as sulfites, which may combine with or inactivate antimicrobics.
3.1.3 **Solvents**

Aqueous or organic solvents used to dissolve the antimicrobics or that act as a vehicle for impregnating the paper or other material shall be free from components that have inhibitory activity or may inactivate the antimicrobics or affect their diffusion properties.

3.1.4 **Inks and colours**

Dyes and inks for coding antimicrobial discs shall not prevent uniform absorption and distribution of antimicrobial in the material, nor produce inhibition zones, nor shall they interfere with, or potentiate, the activity of any antimicrobial. They shall persist for the duration of the test.

3.2 **Production methods**

3.2.1 **Preparation of antibiotic solutions**

The antimicrobial to be used for preparing solutions for impregnating paper or other material shall be weighed accurately and the activity or amount contained therein shall be calculated by multiplying the weight by the potency or purity of the antimicrobial in international units or micrograms per unit of weight, respectively. The antimicrobial shall be dissolved in an appropriate volume of one or more solvents, having regard to any change in volume that may take place during mixing.

Care should be taken to protect such solutions from factors such as excessive light and heat that are known to cause loss of antimicrobial activity.

3.2.2 **Uniformity of content**

The paper or other material used for preparing discs shall be impregnated uniformly with a solution containing a concentration of antimicrobial activity per unit volume sufficient to ensure that after drying and completion of the manufacture each disc shall contain the required quantity of antimicrobial (see Part A, section 1.5).

Disks may be made from paper through which suspensions of antimicrobial crystals have been filtered. Disks may also be made by mixing the antimicrobial substance with an inert granulate and compressing the mixture in a disc.
3.2.3 Drying of impregnated paper or other material

The impregnated paper or other material shall be dried by an appropriate process that removes the solvent without disturbing the uniformity of antimicrobial distribution in the material or causing an excessive loss of potency.

The process should be checked by determining the moisture content of representative discs from a batch shown to be satisfactory in stability studies.

4. CONTAINERS

The container shall be a tightly closed container as defined by the International Pharmacopoeia (6, p. 12).

Containers may be in the form of cartridges that are used for repetitive dispensing of discs. Dispensing devices into which a number of such cartridges are mounted may be used, provided the entire device is provided with a closure making it a tightly closed container.

Each container should contain a desiccant that indicates by a change in colour when it is no longer effective; this should be visible without opening the container.

5. CONTROL TESTS ON DISCS

5.1 Identity

The identification of the antimicrobial contained in a disc requires the application of many different analytical procedures, e.g., chromatography, electrophoresis, spectrophotometry, enzymic inactivation. Such tests are described in various pharmacopoeias but would generally require the antimicrobial to be extracted from the disc and concentrated. The character of the edge of the zone of inhibition allows differentiation between some groups of antimicrobics but is not sufficiently specific to allow positive identification. The difficulties of identification emphasize the need to ensure the application of good manufacturing practices at the time of production (see Part A, section 2).

5.2 Assay of content

5.2.1 Standard discs

Standard discs are prepared using blank discs of paper having the same diameter as the sample discs and the ability to absorb 2.5 to
3.0 times their weight of distilled water. The paper shall exhibit no inhibitory activity nor contain residual material that affects the activity or pH of the antimicrobial solution applied to it.

The national control authority should supply paper suitable for the preparation of standard discs.¹

The blank discs are supported so as to allow free circulation of air around them and a volume of 0.02 ml of an accurately prepared solution of the appropriate reference standard is added to each disc. The concentrations of the solutions are adjusted to allow the desired content of antimicrobial to be applied to each disc. At least 3 different concentrations are used to provide standard discs including (a) the quantity of antimicrobial described on the label of the sample and (b) two other quantities, one less and one greater than the labelled quantity by the same interval on a logarithmic scale.

Solutions should be unbuffered and prepared in freshly distilled water, methanol, or mixtures of these.

Examples of suitable quantities for preparing standard response lines in terms of the labelled quantity and the intervals are:

- 50%, 100%, and 200% (intervals of 2)
- 66.7%, 100%, and 150% (intervals of 1.5).

The appropriateness of the choice of the suitable log-dose interval will depend on the antimicrobial being tested and its limits of acceptance. In some countries more than 3 different standard disc contents are used.

The standard discs shall be dried rapidly in circulating air, in a vacuum, or in a desiccator. If they are stored before use such storage shall be in a desiccator under refrigeration for a period of time not exceeding that for which adequate stability data are available.

5.2.2 Assay media

A medium that has been shown to be satisfactory for the assay of the appropriate antimicrobial shall be used.

Suitable media are indicated in the International Pharmacopoeia (6, pp. 148–150), national pharmacopoeias, and the Regulations of the Food and Drug Administration of the United States of America (7, section 460.6 (a)).²

¹ Guidance to national control authorities on the choice of a suitable paper can be provided by Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.
² Information on how to purchase the Code of Federal Regulations is available from Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.
5.2.3 Assay organisms

A microorganism that has been found satisfactory for the assay of the appropriate antimicrobial shall be used.

Suitable assay organisms are described in the International Pharmacopoeia (6, pp. 148–151), national pharmacopoeias, and the Regulations of the Food and Drug Administration of the United States of America (7, section 460.6 (b)).

5.2.4 Procedure

The assay shall be carried out using flat-bottomed Petri dishes or large square plates containing a layer of seeded assay medium approximately 4 mm in depth. Replica standard discs containing the 3 different contents and replica sample discs are placed on the agar surface in a random arrangement (see Part C, section 2.1.1. for directions for the storage and handling of discs). Such replication shall be either within the same plate or on several such plates. The extent of replication necessary will vary according to the technique used but shall not be less than 5-fold. The discs are placed in position, using forceps, within as short a period as possible, and gently pressed down to ensure a uniform contact with the agar surface. The plates are incubated overnight at the temperature appropriate for the particular microorganism. After incubation, the diameters of the zones of inhibition are measured as accurately as possible. A log-dose/response line is constructed using the means of the responses, from all plates, to the three different standard disc contents. The content of each sample disc is determined from this line.

More details of assay procedures for antimicrobics are to be found in the International Pharmacopoeia (6, pp. 145–151), national pharmacopoeias, and the Regulations of the Food and Drug Administration of the United States of America (7, section 460.6 (c) to 460.6 (e) (1)). A suggested method of calculating the content from the assay data is given in Appendix 1.

5.2.5 Limits of acceptance

The content is satisfactory if the mean content of the sample discs is found to be within 75–135% of the labelled amount.

National control authorities should specify criteria for uniformity of content. A suitable measure of uniformity used in one country requires that the range of differences of zone sizes obtained in assaying replicate sample discs should not exceed 2.5 mm for 90% of the discs tested.
5.3 Sterility

Disks are not expected or required to be sterile but should be free from contamination that would interfere with their normal use, e.g., microorganisms likely to cause inactivation of the antimicrobial or to obscure the zone of inhibition.

6. RECORDS

In addition to Good practices in the manufacture and quality control of drugs (5, p. 88) the requirements given in Part A, section 6.1 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (8, p. 17) shall apply.

7. SAMPLES

Samples from each batch shall be taken in sufficient amount to satisfy the requirements for samples of the national control laboratory. Additional samples shall be retained throughout the dating period as reference material.

8. LABELLING

All products shall be clearly identified by labels. The information given on the label on the container or the label on the package shall be determined by the national control authority.

The label printed on or affixed to each container shall show at least:

— the name of the product (i.e., the international name and/or the proper name);
— the number of discs in the container;
— the nominal content of the antimicrobial in each disc;
— the name and address of the manufacturer;
— the batch number;
— the expiry date;
— the conditions of storage.

The leaflet accompanying the container shall describe directions for the use of the discs, having regard to the requirements in Part C.

Each disc shall be marked with a code that identifies the antibiotic contained in it (see Part A, section 1.6).
PART B.
NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for the quality control system given in section 10 of Good practices in the manufacture and quality control of drugs (5, p. 93) shall apply.

The national control authority shall specify the content of antimicrobics for antimicrobial susceptibility discs (see Part A, section 1.5).

The detailed test procedures for production and control shall be those approved by the national control authority. The national control authority shall provide or approve the antibiotic reference preparations for preparing standard discs or for the standardization of laboratory preparations for preparing standard discs.

The national control authority shall approve the recommended directions for use of the discs, including the zone diameter interpretative criteria to be used in reporting clinical isolates as either susceptible, intermediate, or resistant.

Criteria approved in one country are given in Appendix 2. Other categories of interpretation may be approved and the following is an example (3):

Group 1: Includes high degrees of bacterial susceptibility that make in vivo response probable when mild to moderately severe systemic infections are treated with usual dosage of antimicrobial.

Group 1 can be designated as susceptible without further qualification.

Group 2: Includes degrees of susceptibility that make in vivo response probable in systemic infections when the antimicrobial is given in high dosage or up to the limits of toxicity.

Group 3: Includes degrees of susceptibility that make in vivo response probable only in the treatment of localized infections at sites where the antimicrobial can be concentrated by physiological processes or local application.

Group 4: Includes organisms of a degree of resistance that makes in vivo response improbable. This group can be designated as resistant without further qualification.

The national control authority should seek clinical advice before approving interpretative criteria.

The national control authority shall approve the performance test and provide or approve the reference strains to be used in determining the adequacy of the procedure for testing the susceptibility of microorganisms to antimicrobics as performed in particular lab-
oratorics. The national control authority shall approve the acceptable ranges of zone diameters to be obtained when each antimicrobial susceptibility disc is tested for performance against the reference strains (see Part C, section 4).

2. RELEASE AND CERTIFICATION

A batch of antimicrobial susceptibility discs shall be released only if it fulfils Part A of the present requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify whether or not the batch of susceptibility discs in question meets all national requirements as well as Part A of the present requirements. The certificate shall further state the results of the assay for content and uniformity and the batch number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of susceptibility discs between countries.

PART C.

DIRECTIONS FOR USE OF ANTIMICROBIC SUSCEPTIBILITY DISCS

In the commonly used agar diffusion method the antimicrobial diffuses from a disc placed on a solid agar medium, the surface of which has been inoculated with the microorganism under investigation. The presence and size of a zone around the disc within which growth is inhibited is an indication of the susceptibility of the microorganism to the antimicrobial. The WHO Expert Committee on Antibiotics (2) recommended the broad principles to be followed in order to obtain satisfactory results in susceptibility testing. The Committee noted that the results of any susceptibility measurements do not yield absolute values because they are influenced, sometimes markedly, by the test conditions used. Differences in such factors as the size of the inoculum, the composition of the medium, pH, atmosphere, and incubation temperature may all affect the amount of antimicrobial required to inhibit the organism in vitro. Thus, the minimum

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inhibitory concentration of an antimicrobial for an organism will
depend on the conditions of the test.

It has not been possible to provide directions for use of anti-
microbial susceptibility discs that are internationally acceptable in all
details. Nevertheless guidelines are given that should reduce variabil-
ity in results between laboratories.

Some details of one procedure accepted on a national basis are
included as an example of a method that is being used in more than
one country. Full details of the method have been published (7, sec-
tion 460.1 (c) (2)).

1. TERMINOLOGY

*Clinical isolate.* A microorganism isolated from a clinical specimen
to be tested for its susceptibility to antimicrobics.

*Reference strains.* Stock culture(s) to be used in clinical lab-
oratories to check the performance of the method of determining the
susceptibility of microorganisms to antimicrobics. Reference strains
have well-defined characteristics and stable behaviour in the sus-
ceptibility testing procedure. They are also intended for use in con-
trolling new batches of agar medium as required in Part D and
changes in methods and materials used in manufacturing such media.

*Performance test.* Procedure for determining the validity of anti-
microbial susceptibility disc tests performed in a given clinical lab-
oratory, reference strains being used to verify that zones of inhibition
within expected ranges of sizes are obtained.

*Minimum inhibitory concentration (MIC).* The minimum con-
centration of an antimicrobial giving complete inhibition of growth of
a particular microorganism as judged by the naked eye after a given
period of incubation.

*Regression line.* The linear relationship (line of best fit) shown to
exist between the logarithm of the MIC and the diameter of the zone
of inhibition produced by a fixed disc content of a given antimicrobial
for microorganisms of comparable growth rate but of different anti-
microbial susceptibilities. Such a line shall be determined experimen-
tally using many strains of microorganisms.

A minimum of 100 strains of recently isolated and commonly
encountered pathogens has generally been used and twice tested
on two occasions in each of two or more laboratories. The
strains should be selected to cover the range of clinically im-
portant susceptibilities, and if possible there should be at least ten strains with MICs at each dilution increment.

At least 80 of the strains should have growth rates not less than that of enterococci and extending up to that of the more rapidly growing Enterobacteriaceae. A minimum of 4 genera should be included and all strains should be characterized. A separate regression line should be determined for a fixed disc content of each antimicrobial.

Examples of regression lines obtained by different laboratories, indicating the variations that may occur between laboratories, have been described (3). Details are given of the preparation of such lines, the precautions that must be taken in preparing them, and the different sources of error. At present it is not possible to define regression lines that will be universally applicable in all laboratories, although this might be possible in the future with the aid of reference microorganisms of defined susceptibility.

2. REAGENTS

2.1 Discs

Antimicrobial susceptibility discs complying with Parts A and B of these requirements shall be used.

2.1.1 Storage of discs

When received, discs shall be stored in their containers at between −20 °C and 8 °C. When needed, one container is removed from storage and before being opened allowed to stand at room temperature long enough to reach ambient temperature in order to prevent condensation of water vapour on the discs. After discs have been removed, the container shall be closed again and stored at between −20 °C and 8 °C.

For discs containing certain antibiotics (e.g., penicillins and cephalosporins) the period of storage after opening the container should not be more than 1 week.

2.2 Agar medium

Agar medium that complies with Part D of these requirements shall be used.

Mueller-Hinton agar medium has been found to be suitable and it is available commercially in a dehydrated form.
2.2.1 pH

The pH of the agar medium shall be 7.2 to 7.4 and, if necessary, shall be adjusted using sodium hydroxide or hydrochloric acid.

The exact method by which the pH is checked will depend upon the type of equipment available in a given laboratory. When the medium has cooled to room temperature its pH is checked after macerating a small amount with an equal quantity of freshly distilled water. Alternative methods are to allow a small amount of the medium to solidify around a pH electrode in a small beaker or pH cup or to use a properly calibrated surface or probe electrode.

2.3 Liquid medium

A suitable liquid medium for preparing the inoculum shall be used.

Soybean-casein digest medium (9, p. 54) and Mueller-Hinton broth are suitable.

2.4 Turbidity standard

A suitable turbidity standard may be prepared by mixing solutions of $\text{BaCl}_2$, 0.048 mol/l, and $\text{H}_2\text{SO}_4$, 0.18 mol/l. If inoculation is performed by swabbing, a turbidity standard prepared using 0.5 ml and 99.5 ml, respectively, of these solutions is suitable.

2.5 Petri dishes

Either glass or plastic dishes with a flat internal bottom surface shall be used.

Sterile dishes are commonly used.

3. PROCEDURES

3.1 Preparation of plates

The medium shall be poured into dishes on a flat horizontal surface to a uniform depth of 4 mm. This corresponds to 60 ml of medium for dishes of 14 cm internal diameter and 25 ml for dishes of 9 cm internal diameter.

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Freshly prepared plates of solidified medium may be stored at 4 °C and protected from desiccation. Plates should normally be used within 7 days, but under certain conditions they may be kept for a longer period.

Immediately before inoculation the plate shall be at room temperature and the surface of the solid medium shall be allowed to dry if necessary.

3.2 Preparation of inoculum for seeding by swabbing or flooding

Normally the inoculum shall be derived from a plate seeded with the original clinical isolate. Whenever possible, portions of 10 colonies of the organism to be tested shall be suspended in liquid medium. The density of the inoculum shall be carefully adjusted to yield a confluent growth after incubation of the plate, as described in Part C, section 3.6.

The inoculum may require a period of growth in liquid medium for several hours, possibly overnight. In some laboratories the inoculum is adjusted using a suitable turbidity standard to meet the conditions described above. In cases of clinical urgency the original clinical specimen (for instance cerebrospinal fluid) may be used as an inoculum. Preliminary and rapid results may thus be obtained if the resulting culture is pure and the density of growth on the susceptibility plate is appropriate.

3.3 Preparation of inoculum for seeding of plates by agar overlay

A suspension of just visible turbidity is prepared in 0.5 ml of liquid medium using where possible parts of 10 colonies. The suspension is incubated at 35–37 °C for at least 4 hours but not more than 8 hours and is used to inoculate melted agar at a temperature of 45–50 °C. One microlitre is used to inoculate 9.0 ml of the agar. Inoculum and agar are then mixed by gentle inversion and used immediately.

A calibrated loop may be used to measure the inoculum.

3.4 Seeding of plates

The plate shall be inoculated evenly over the entire surface of the solid medium to give the required density of confluent growth.

The density of the inoculum may be defined accurately by the count of colonies obtained per square centimetre.

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One of the methods described in the following three paragraphs shall be used.

3.4.1 Swabbing

The inoculum shall be distributed evenly by careful streaking in three directions with a cotton swab.

A bent glass rod may also be used in order to obtain even distribution. The method of inoculation may be varied but must be such that it gives uniform seeding of the plate and the density of growth required. The surface of the medium is allowed to dry for up to 15 minutes with the Petri dish lid tipped.

The details of the swabbing technique relating to the performance test evaluation (see Part C, section 4.3) and the interpretative criteria (see Appendix 2) have been described in detail (7, section 460.1 (c) (2)).

3.4.2 Flooding

The required density of confluent growth can also be obtained by a flooding technique, using 3–5 ml of the appropriate dilution of the suspension of the organism to inoculate a 14-cm plate, or 2 ml to inoculate a 9-cm plate. The inoculum is pipetted on to the plate and distributed evenly over the surface of the medium by gently rocking or tilting the plate. The plate is then tipped, the excess fluid is allowed to drain and is removed with a pipette. The surface of the medium is allowed to dry for up to 15 minutes with the Petri dish lid tipped.

3.4.3 Agar overlay

The inoculum shall be spread over the surface of a Petri dish or plate containing a 4-mm depth of agar medium. The volume of the overlay shall be sufficient to produce a uniform layer 0.6 mm thick (i.e., 9 ml for plates of 14 cm internal diameter or 3.75 ml for plates of 9 cm internal diameter). The surface of the medium is allowed to dry for up to 15 minutes with the Petri dish lid tipped.

3.5 Application of discs

The antimicrobial discs shall be applied to the surface of the seeded and dried plate and lightly pressed down immediately. Discs shall be separated by a sufficient distance to reduce the likelihood of serious
overlapping of zones and shall be at least 1.5 cm from the edge of the plate.

3.6 Incubation

The plate shall be incubated overnight (about 18 h) at 35–37 °C in an inverted position.

Incubation is normally aerobic, but anaerobic or CO₂ incubation may be necessary for some species.

If anaerobic or CO₂ incubation is used it is important to recognize that regression lines or other interpretative criteria developed from data obtained using aerobic conditions should not be used unless they have been shown experimentally to be applicable.

3.7 Reading of zone diameters

The diameter of each zone of inhibition shall be measured with sufficient accuracy to determine the proper category of susceptibility (see Appendix 2).

Some laboratories measure each zone of inhibition to the nearest whole millimetre, using templates.

When reading the zones of inhibition in the performance test (see Part C, section 4) the zone diameters shall be measured to the nearest whole millimetre.

Very small colonies within the zone seen under certain conditions of illumination should be ignored. Certain antimicrobics may produce poorly defined zone edges. In such cases, only the clear zone of inhibition should be measured and any barely visible colonies should be ignored.

Certain organisms may produce large colonies just inside the periphery of the inhibition zone given by certain antimicrobics, e.g., ampicillin and cefalotin. These colonies are regarded as defining the edge of the zone. If colonies occur throughout the zone of inhibition, the culture should be checked for purity and the test repeated. If the cell culture is found to be pure and such colonies are still present, they shall be regarded as significant, calling for special interpretation or investigation.

Strains of *Proteus* may swarm into zones of inhibition produced by certain antimicrobics (e.g., chloramphenicol). The zones are usually clearly outlined under the spreading organisms and the veil of swarming should be ignored. If difficulty in measuring is encountered, it usually indicates that an excessively heavy inoculum has been used.
3.8 Interpretation of results

There has been no international agreement on the basis of categorization of susceptibility.

One set of criteria for interpretation, based on the Bauer-Kirby procedure (10) is published in the Regulations of the United States Food and Drug Administration (7, section 460.1 (c) (2) (E)) and updated by the National Committee for Clinical Laboratory Standards (11).

This is reproduced in Appendix 2 as one example of an interpretative procedure that has been found to be satisfactory using the techniques and materials described in the requirements.

Zone diameters may be related to MIC values by the use of regression lines such as those described by Ericsson & Sherris (3). Other interpretative criteria may be approved by national control authorities (see Part B, section 1).

3.8.1 Limitations of the disc diffusion method

There are certain inherent limitations to diffusion tests. Criteria of evaluation derived from studies on rapidly growing microorganisms are not applicable to slow-growing ones unless they have been shown experimentally to be applicable, otherwise such low-growing organisms shall be tested by dilution methods. In addition, disc tests with the poorly diffusing polypeptide antibiotics—colistin and polymyxin B—are unreliable in detecting susceptibility; therefore, dilution methods shall be employed whenever these antibiotics are considered for use in treating systemic infections.

3.8.2 Problems of penicillin-resistant and meticillin-resistant staphylococci

Penicillinase-producing staphylococci are readily detected by diffusion tests. They yield smaller zone sizes with benzylpenicillin and ampicillin than do non-penicillinase-producing strains and also produce sharply defined zone edges in which colonies at the edge of the zone are of the same size or larger than those on other parts of the plate. This is in contrast to the ill-defined edges encountered with non-penicillinase producers.

The ability of diffusion methods to detect strains of staphylococci that are heteroresistant to the penicillinase-stable penicillins and to the cephalosporins is dependent on the use of a sufficiently heavy inoculum and is influenced by the medium, the antibiotic used, and
the incubation time and temperature. Special tests shall be performed at an incubation temperature of 30 °C or on a culture medium containing 5% sodium chloride; discs containing a penicillinase-resistant penicillin, such as meticillin or oxacillin, are used for such testing.

4. PERFORMANCE TEST

To check the validity of the antimicrobial susceptibility test procedure, susceptibility discs shall be tested against reference strains of microorganisms on a daily basis.

4.1 Reference strains

The reference strains used shall be approved by the national control authority.

The following have been used:

*Staphylococcus aureus* (ATCC 25923; NCTC 6571)

*Escherichia coli* (ATCC 25922; NCTC 10418)

*Pseudomonas aeruginosa* (ATCC 27853; NCTC 10662)

These strains can be obtained from the American Type Culture Collection,\(^1\) and the National Collection of Type Cultures.\(^2\)

4.2 Procedure

Inoculated plates of the reference strains shall be prepared using the procedure described in Part C, sections 3.1 to 3.4. Each reference strain shall be tested against at least one disc of each relevant antimicrobial using the procedure described in Part C, sections 3.5 to 3.7.

4.3 Evaluation of performance

The zone size obtained with each antimicrobial susceptibility disc shall be compared with the range of zone sizes approved by the national control authority as those to be expected for the appropriate organism (see Part B, section 1).

---

\(^1\) American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA.

The following ranges have been found appropriate for antimicrobial susceptibility discs 6.35 mm in diameter.

<table>
<thead>
<tr>
<th></th>
<th>Disc content</th>
<th>Expected range of zone diameters (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli (ATCC 25922)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amikacin</td>
<td>30 μg</td>
<td>19–26</td>
</tr>
<tr>
<td>ampicillin</td>
<td>10 μg</td>
<td>15–20</td>
</tr>
<tr>
<td>carbenicillin</td>
<td>100 μg</td>
<td>24–29</td>
</tr>
<tr>
<td>cefalotin</td>
<td>30 IU</td>
<td>18–23</td>
</tr>
<tr>
<td>cefamandole</td>
<td>30 μg</td>
<td>24–31</td>
</tr>
<tr>
<td>cefoxitin</td>
<td>30 μg</td>
<td>23–28</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>30 μg</td>
<td>21–27</td>
</tr>
<tr>
<td>colistin</td>
<td>300 IU</td>
<td>11–15</td>
</tr>
<tr>
<td>erythromycin</td>
<td>15 IU</td>
<td>8–14</td>
</tr>
<tr>
<td>gentamicin</td>
<td>10 IU</td>
<td>19–26</td>
</tr>
<tr>
<td>kanamycin</td>
<td>30 IU</td>
<td>17–25</td>
</tr>
<tr>
<td>nalidixic acid</td>
<td>30 μg</td>
<td>23–28</td>
</tr>
<tr>
<td>neomycin</td>
<td>30 IU</td>
<td>17–23</td>
</tr>
<tr>
<td>nitrofurantoin</td>
<td>300 μg</td>
<td>21–26</td>
</tr>
<tr>
<td>polymyxin B</td>
<td>300 IU</td>
<td>12–16</td>
</tr>
<tr>
<td>streptomycin</td>
<td>10 IU</td>
<td>12–20</td>
</tr>
<tr>
<td>sulfamethoxazole/trimethoprim</td>
<td>23.75 μg/1.25 μg</td>
<td>24–32</td>
</tr>
<tr>
<td>sulfisoxazole</td>
<td>250 μg</td>
<td>18–26</td>
</tr>
<tr>
<td>tetracycline</td>
<td>30 IU</td>
<td>18–25</td>
</tr>
<tr>
<td>trimethoprim</td>
<td>5 μg</td>
<td>21–28</td>
</tr>
<tr>
<td>tobramycin</td>
<td>10 μg</td>
<td>18–26</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus (ATCC 25923)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amikacin</td>
<td>30 μg</td>
<td>23–28</td>
</tr>
<tr>
<td>ampicillin</td>
<td>10 μg</td>
<td>24–35</td>
</tr>
<tr>
<td>bacitracin</td>
<td>10 IU</td>
<td>17–22</td>
</tr>
<tr>
<td>benzylpenicillin</td>
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</tr>
<tr>
<td>cefalotin</td>
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</tr>
<tr>
<td>cefamandole</td>
<td>30 μg</td>
<td>28–34</td>
</tr>
<tr>
<td>cefoxitin</td>
<td>30 μg</td>
<td>23–28</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>20 μg</td>
<td>19–26</td>
</tr>
<tr>
<td>clindamycin</td>
<td>2 IU</td>
<td>23–29</td>
</tr>
<tr>
<td>erythromycin</td>
<td>15 IU</td>
<td>22–30</td>
</tr>
<tr>
<td>gentamicin</td>
<td>10 IU</td>
<td>19–27</td>
</tr>
<tr>
<td>kanamycin</td>
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<td>19–26</td>
</tr>
<tr>
<td>meticillin</td>
<td>5 μg</td>
<td>17–22</td>
</tr>
<tr>
<td>neomycin</td>
<td>30 IU</td>
<td>18–26</td>
</tr>
<tr>
<td>nitrofurantoin</td>
<td>300 μg</td>
<td>20–24</td>
</tr>
<tr>
<td>novobiocin</td>
<td>30 IU</td>
<td>22–31</td>
</tr>
<tr>
<td>oleandomycin</td>
<td>15 IU</td>
<td>19–28</td>
</tr>
<tr>
<td>polymyxin B</td>
<td>300 IU</td>
<td>7–13</td>
</tr>
<tr>
<td>streptomycin</td>
<td>10 IU</td>
<td>14–22</td>
</tr>
</tbody>
</table>
Staphylococcus aureus (ATCC 25923)

<table>
<thead>
<tr>
<th>Disc content</th>
<th>Expected range of zone diameters (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sulfadiazine/trimethoprim</td>
<td>23.75 mg/1.25 μg</td>
</tr>
<tr>
<td>sulfadiazine</td>
<td>250 μg</td>
</tr>
<tr>
<td>tetracycline</td>
<td>30 IU</td>
</tr>
<tr>
<td>tobramycin</td>
<td>10 μg</td>
</tr>
<tr>
<td>trimethoprim</td>
<td>5 μg</td>
</tr>
<tr>
<td>vancomycin</td>
<td>30 IU</td>
</tr>
</tbody>
</table>

Pseudomonas aeruginosa (ATCC 27853)

<table>
<thead>
<tr>
<th>Disc content</th>
<th>Expected range of zone diameters (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>amikacin</td>
<td>30 μg</td>
</tr>
<tr>
<td>carbenicillin</td>
<td>100 μg</td>
</tr>
<tr>
<td>gentamicin</td>
<td>10 IU</td>
</tr>
<tr>
<td>tobramycin</td>
<td>10 μg</td>
</tr>
<tr>
<td>polymyxin B</td>
<td>300 IU</td>
</tr>
</tbody>
</table>

All zone diameters shall be within the expected range.

**PART D.**

**REQUIREMENTS FOR AGAR MEDIUM FOR ANTIMICROBIC SUSCEPTIBILITY TESTING USING ANTIMICROBIC SUSCEPTIBILITY DISCS**

**1. GENERAL**

In designating a suitable medium for performing antimicrobial susceptibility tests by the agar diffusion technique using antimicrobial susceptibility discs, the following characteristics are considered desirable.

(1) The composition of the medium should be defined, at least to the point of specific production details, for crude components such as peptone and agar. Ideally the medium should be fully synthetic, but no such medium is generally available.

(2) Susceptibility test results with the appropriate reference strains should be satisfactory.

(3) The medium, without enrichment, should support good growth of the majority of rapidly growing pathogens for which susceptibility tests are required.
(4) The medium should not be antagonistic to any of the antimicrobics with which susceptibility tests are made.

(5) The medium should resist marked pH changes during the growth of common pathogenic species.

(6) The medium should be approximately isotonic to blood and suitable for the addition of blood, which is necessary for the growth of fastidious organisms.

(7) The medium should be reproducible so that batches from different sources have similar performance and could be available generally.

Mueller-Hinton agar medium from different sources has been found to meet most of these criteria. A large amount of information has been collected on tests performed using this medium. It has shown good batch-to-batch reproducibility in disc diffusion antimicrobial susceptibility tests, it is low in sulfonamide and tetracycline inhibitors, and it gives satisfactory growth of most pathogens. With the addition of 5% defibrinated blood (free from antimicrobial activity) Mueller-Hinton agar can be used in performing antimicrobial susceptibility tests on certain fastidious organisms that cannot grow on the medium alone.

Different media have been proposed with "physiological" concentrations of various ions and with nitrogenous components different from those of Mueller-Hinton agar. The interpretation of zone sizes (see Part C, section 3.8) based on Mueller-Hinton agar may not be applicable to such different media, nor can it be assumed that regression lines determined using Mueller-Hinton agar can be used for such different media.

2. FORMULA

Mueller-Hinton agar medium is based on a formula for a liquid medium (12) with agar added:

- dehydrated infusion from 300 g beef acid digest of casein .............................. 17.5 g
- corn starch ........................................... 1.5 g
- agar ................................................. 17.0 g
- distilled water to make ......................... 1000 ml

pH after autoclaving ......................... 7.4

Mueller-Hinton agar medium is available in a dehydrated form, so that when it is reconstituted with distilled water to make 1000 ml and autoclaved it has a pH of 7.4.

The user should check the pH and adjust it if necessary (see Part C, section 2.2.1). Because of the influence of Mg\(^{2+}\) and Ca\(^{2+}\) on the susceptibility of *Pseudomonas aeruginosa* to aminoglycoside antibiotics, it has been recommended that Mueller-Hinton agar contain 20-
35 mg of Mg²⁺ and 50–100 mg of Ca²⁺ per litre. At this level of free divalent cations, which are known to chelate tetracyclines, the zone sizes obtained with tetracycline discs are slightly reduced in relation to the zone sizes obtained using media of lower content of Mg²⁺ and Ca²⁺.

Unless a laboratory is experienced in making media, it is recommended that dehydrated agar medium be obtained from a batch that has been shown to be suitable.

3. PERFORMANCE TESTING

Batches of agar medium should be controlled using the performance test described in Part C, section 4. The mean zone size for each antibiotic (obtained from at least four determinations on separate plates) shall be within the expected range of zone diameters listed in Part C, section 4.3.

AUTHORS

The original draft of the Requirements for Antibiotic Susceptibility Tests, I. Agar Diffusion Tests Using Antibiotic Susceptibility Discs was prepared by the following WHO consultants and staff members:

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ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice and for supplying additional data relevant to these Requirements:

Ms Anne Bolmiström, AB Biodisk, Solna, Sweden
REFERENCES

7. UNITED STATES OF AMERICA. Code of Federal Regulations, Title 21, Parts 300 to 499. Washington, DC, Office of the Federal Register (revised annually), chapter 1, part 460, subpart A.
Appendix 1

CALCULATION OF DISC CONTENT FROM ASSAY DATA

Determine the logarithm (x) of each dose of standard and the mean response (y) to these doses. Using the 3 values of x and the 3 corresponding values of y, calculate $\Sigma x$, $\Sigma x^2$, $(\Sigma x)^2$, $\Sigma y$, and $\Sigma xy$.

Calculate the slope (b), which is the regression coefficient and the γ-intercept (a) of the standard response line by the following equations:

$$b = \frac{n\Sigma xy - (\Sigma x)(\Sigma y)}{n\Sigma x^2 - (\Sigma x)^2} \quad \text{and} \quad a = \frac{\Sigma y - b\Sigma x}{n}$$

where $n$ = the number of standard doses.

Determine the response ($Y$) for each sample disc. Using the regression equation, calculate the content (X) for any observed response of sample disc as follows:

$$X = \text{antilog} \left( \frac{Y - a}{b} \right)$$

The following example illustrates the mathematical calculation of the content of a sample disc:

<table>
<thead>
<tr>
<th>Standard doses (µg or IU per disc)</th>
<th>15.0</th>
<th>30.0</th>
<th>60.0</th>
<th>$n = 3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log doses (x)</td>
<td>1.17609</td>
<td>1.47712</td>
<td>1.77815</td>
<td></td>
</tr>
<tr>
<td>$x^2$</td>
<td>1.38319</td>
<td>2.18189</td>
<td>3.16182</td>
<td></td>
</tr>
<tr>
<td>Standard zones (mm)</td>
<td>16.6</td>
<td>18.2</td>
<td>19.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.0</td>
<td>18.9</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.0</td>
<td>18.9</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.0</td>
<td>19.1</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.9</td>
<td>18.6</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.3</td>
<td>18.2</td>
<td>18.7</td>
<td></td>
</tr>
<tr>
<td>Mean responses (y)</td>
<td>16.8</td>
<td>18.7</td>
<td>19.8</td>
<td>$\Sigma y = 55.3$</td>
</tr>
<tr>
<td>$xy$</td>
<td>19.75833</td>
<td>27.62217</td>
<td>35.20739</td>
<td></td>
</tr>
</tbody>
</table>

$$b = \frac{3(19.75833) - (16.8)(55.3)}{3(35.20739) - 19.63698} = 4.98$$

$$a = \frac{55.3 - (4.98)(4.43136)}{3} = 11.07728$$

Response, Y, of sample disc = 18.9 mm

Calculated content, X, of sample disc = antilog $\left( \frac{18.9 - 11.07728}{4.98} \right) = 37$ µg or IU

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## Appendix 2

### CRITERIA FOR INTERPRETATION BASED ON THE BAUER-KIRBY PROCEDURE

*(disc diameter 6.35 mm)*

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Disc content</th>
<th>Diameter (mm) of zone of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
</tr>
<tr>
<td>amikacin</td>
<td>30 µg</td>
<td>14 or less</td>
</tr>
<tr>
<td>ampicillin¹</td>
<td>10 µg</td>
<td>11 or less</td>
</tr>
<tr>
<td>ampicillin¹</td>
<td>10 µg</td>
<td>20 or less</td>
</tr>
<tr>
<td>ampicillin¹</td>
<td>10 µg</td>
<td>19 or less</td>
</tr>
<tr>
<td>bacitracin</td>
<td>10 IU</td>
<td>8 or less</td>
</tr>
<tr>
<td>benzylpenicillin, when testing staphylococci¹⁰</td>
<td>6 µg</td>
<td>20 or less</td>
</tr>
<tr>
<td>benzylpenicillin when testing other microorganisms²⁹</td>
<td>6 µg</td>
<td>11 or less</td>
</tr>
<tr>
<td>carbenicillin¹² when testing <em>Proteus</em> species and <em>Escherichia coli</em></td>
<td>100 µg</td>
<td>17 or less</td>
</tr>
<tr>
<td>carbenicillin¹³ when testing <em>Pseudomonas aeruginosa</em></td>
<td>100 µg</td>
<td>13 or less</td>
</tr>
<tr>
<td>cefalotin when reporting susceptibility to cefalotin, cefaclor, cefadroxil, cefaloridine, ceftalexin, cefapirin, cefazolin, cefacetirile, and cefradine</td>
<td>30 IU</td>
<td>14 or less</td>
</tr>
<tr>
<td>cefalotin when reporting susceptibility to cefaloglycin</td>
<td>30 IU</td>
<td>14 or less</td>
</tr>
<tr>
<td>cefamandole¹²</td>
<td>30 µg</td>
<td>14 or less</td>
</tr>
<tr>
<td>cefoxitin¹¹</td>
<td>30 µg</td>
<td>14 or less</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>30 µg</td>
<td>12 or less</td>
</tr>
<tr>
<td>Antimicrobial</td>
<td>Disc content</td>
<td>Diameter (mm) of zone of inhibition</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
</tr>
<tr>
<td>clindamycin</td>
<td>2 IU</td>
<td>14 or less</td>
</tr>
<tr>
<td></td>
<td>reporting susceptibility to clindamycin</td>
<td></td>
</tr>
<tr>
<td>clindamycin</td>
<td>2 IU</td>
<td>16 or less</td>
</tr>
<tr>
<td></td>
<td>reporting susceptibility to lincomycin</td>
<td></td>
</tr>
<tr>
<td>colistin</td>
<td>300 IU</td>
<td>8 or less</td>
</tr>
<tr>
<td>erythromycin</td>
<td>15 IU</td>
<td>13 or less</td>
</tr>
<tr>
<td>gentamicin</td>
<td>10 IU</td>
<td>12 or less</td>
</tr>
<tr>
<td></td>
<td>reporting susceptibility to gentamicin and sisomicin</td>
<td></td>
</tr>
<tr>
<td>kanamycin</td>
<td>30 IU</td>
<td>13 or less</td>
</tr>
<tr>
<td>meticillin</td>
<td>5 ( \mu g )</td>
<td>9 or less</td>
</tr>
<tr>
<td>nalidixic acid</td>
<td>30 ( \mu g )</td>
<td>13 or less</td>
</tr>
<tr>
<td>neomycin</td>
<td>30 IU</td>
<td>12 or less</td>
</tr>
<tr>
<td>nitrofurantoin</td>
<td>300 ( \mu g )</td>
<td>14 or less</td>
</tr>
<tr>
<td>novobiocin</td>
<td>30 IU</td>
<td>17 or less</td>
</tr>
<tr>
<td>oleandomycin</td>
<td>15 IU</td>
<td>11 or less</td>
</tr>
<tr>
<td>polymyxin B</td>
<td>300 IU</td>
<td>8 or less</td>
</tr>
<tr>
<td>rifampicin</td>
<td>5 ( \mu g )</td>
<td>24 or less</td>
</tr>
<tr>
<td></td>
<td>testing <em>Neisseria meningitidis</em></td>
<td></td>
</tr>
<tr>
<td>streptomycin</td>
<td>10 IU</td>
<td>11 or less</td>
</tr>
<tr>
<td>sulfamethoxazole/trimethoprim</td>
<td>23.75 ( \mu g/1.25 ( \mu g )</td>
<td>10 or less</td>
</tr>
<tr>
<td>sulfadiazine when reporting susceptibility to sulfonamides</td>
<td>250 ( \mu g )</td>
<td>12 or less</td>
</tr>
<tr>
<td>tetracycline</td>
<td>30 IU</td>
<td>14 or less</td>
</tr>
<tr>
<td>tobramycin</td>
<td>10 ( \mu g )</td>
<td>12 or less</td>
</tr>
<tr>
<td>trimethoprim</td>
<td>5 ( \mu g )</td>
<td>10 or less</td>
</tr>
<tr>
<td>vancomycin</td>
<td>30 IU</td>
<td>9 or less</td>
</tr>
</tbody>
</table>

**Notes to Appendix 2**

1 The ampicillin disc is used for testing susceptibility to ampicillin, amoxicillin, and hetacillin. When testing *Haemophilus* species, tests should be performed using both ampicillin discs and benzylpenicillin discs, and an isolate exhibiting ampicillin susceptibility and benzylpenicillin resistance should be considered resistant to ampicillin.

2 The 30 IU cefalotin disc cannot be relied upon to detect resistance of staphilococci to cephalosporin class antibiotics. Staphylococci exhibiting resistance to meticillin should be reported as resistant to cephalosporin class antibiotics.

3 The clindamycin disc is used for testing susceptibility to both clindamycin and lincomycin.
4 Colistin and polymyxin B diffuse poorly in agar, and the accuracy of the diffusion method is thus less than is the case with other antibiotics. Resistance is always significant, but when treatment of systemic infections due to susceptible strains is considered, it is wise to confirm the results of a diffusion test with a dilution method.

4 The meticillin disc is used for testing susceptibility to all penicillinase-resistant penicillins—i.e., meticillin, cloxacillin, dicloxacillin, oxacillin, and nafcillin—and for verifying resistance to cephalosporin class antibiotics (see Part C, section 3.8.2).

5 Not applicable to medium that contains blood.

7 The oleandomycin disc is used for testing susceptibility to oleandomycin and troleandomycin.

8 The benzylpenicillin disc is used for testing susceptibility to all penicillinase-susceptible penicillins except ampicillin and carbenicillin—i.e., benzylpenicillin, phenoxymethylpenicillin, and pheneticillin.

9 This category includes some organisms such as enterococci and gram-negative bacilli that may cause systemic infections treatable with high doses of benzylpenicillin. Such organisms should only be reported susceptible to benzylpenicillin and not to phenoxymethylpenicillin or pheneticillin.

10 The tetracycline disc is used for testing susceptibility to all tetracyclines—i.e., chlortetracycline, demeclocycline, doxycycline, metacycline, oxytetracycline, rolitetracycline, minocycline, and tetracycline. Some organisms that are resistant to tetracycline may be susceptible to minocycline.

11 Cefamandole and cefoxitin have a wider spectrum of activity against gram-negative bacilli than do the cephalosporin antibiotics for which the cefalotin disc is used. Organisms resistant to cefalotin may be susceptible to cefamandole and/or cefoxitin. Therefore, the cefalotin disc cannot be used for testing susceptibility to cefamandole and cefoxitin. Further, the spectra of cefamandole and cefoxitin are dissimilar enough to justify tests using both cefamandole discs and cefoxitin discs.

12 The carbenicillin disc is used for testing susceptibility to carbenicillin and ticarcillin.

13 Susceptibility data for nalidixic acid, nitrofurantoin, and sulfonamides apply only to organisms isolated from urinary tract infections.
Annex 6

REQUIREMENTS FOR MEASLES VACCINE (LIVE)

(Requirements for Biological Substances No. 12)

Addendum 1981

The WHO Expert Committee on Biological Standardization in 1965, adopted the Requirements for Measles Vaccine (Live) and these were published in its eighteenth report (WHO Technical Report Series No. 329, 1966. Annex 2). At that time it was considered that there was no necessity for a test for the stability of the vaccine since it was freeze-dried and used in countries in which there was a good cold chain and distribution network.

Since that time and with the greater use of vaccine in developing countries it has been shown that some vaccines being marketed have inadequate stability for storage and distribution in these countries (WHO weekly epidemiological record, 56: 177, 178 (1981)). In order to ensure that only stable vaccine is available internationally it was considered advisable to include a test for stability in the WHO requirements. Such a test, based on the results obtained with many vaccines is shown below as the amendment.

Amendment

Part A, add section 5.7 Test for stability

A sample of the final freeze-dried vaccine shall be incubated at 37 °C for seven days. At the end of the incubation the sample shall retain at least 3.0 log_{10} live virus particles in each human dose. Furthermore, if during incubation the virus titre has been decreased, then it shall have done so by not more than 1.0 log_{10}.

In some countries the national control authority may wish to include this test as proof of consistency of production and may not demand a test on each filling and freeze-drying lot. Vaccines being distributed in countries with high ambient temperatures, however, should satisfy this test.

This test may be carried out in conjunction with the test for virus concentration Part A, section 5.3.
Annex 7

BIOLOGICAL SUBSTANCES:
INTERNATIONAL STANDARDS, REFERENCE PREPARATIONS, AND REFERENCE REAGENTS

A list of international biological standards, international biological reference preparations, and international biological reference reagents previously included as annexes to the reports of the WHO Expert Committee on Biological Standardization is issued as a separate publication, revised from time to time. The most recent list\(^1\) includes all changes and additions accepted by the WHO Expert Committee on Biological Standardization in 1976, 1977, 1978, 1980, and 1981 (thirty-first and thirty-second reports). Copies of the list may be obtained direct (or through booksellers) from the agents shown on the back of the cover of this report, or they may be ordered from: World Health Organization, Distribution and Sales Service, 1211 Geneva 27, Switzerland.