Annex 6

REQUIREMENTS FOR HEPATITIS B VACCINES MADE BY RECOMBINANT DNA TECHNIQUES IN YEAST

(Requirements for Biological Substances No. 39)

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GENERAL CONSIDERATIONS

Advances in molecular genetics and nucleic acid chemistry have made it possible to identify genes coding for biologically active substances, to analyse them in detail, to transfer them within and between organisms, and to obtain gene expression under controlled conditions with efficient synthesis of the encoded product. A gene that codes for a specific product can be isolated and propagated by insertion into a suitable vector with the aid of highly specific
restriction endonuclease enzymes (which cleave the vector DNA at predetermined sites) and ligases (which join the gene insert to the vector). The vector can then be introduced into host organisms, and individual clones that carry the desired gene can be selected and propagated in mass culture.

A gene is characterized by a specific nucleotide sequence in one strand of the double-stranded DNA molecule. When the strands are separated, each forms a template for the synthesis of a complementary copy, thus providing a mechanism for the faithful reproduction of genes with conservation of the linear sequence of the four types of mononucleotides. The process of decoding the genetic information and of synthesizing the gene product occurs in two phases: firstly, transcription of the DNA coding strand in the form of a messenger RNA, and secondly, translation of the information carried by the messenger RNA molecule into an amino acid sequence. The factors affecting the expression of foreign genes introduced into prokaryotic and eukaryotic cells are complex; indeed, the efficient and controlled expression of stable, cloned DNA sequences is an important field of current research.

Hepatitis B virus has several characteristics that differentiate it from the other families of DNA viruses. It has an outer coat (more substantial than a membrane or envelope) consisting of protein, lipid, and carbohydrate and bearing a unique antigen complex, hepatitis B surface antigen (HBsAg). Its nucleic acid consists of a circular DNA genome of relative molecular mass about 2 million, part of which is double-stranded and part single-stranded, which is an unusual feature among viruses. Virus recovered from the plasma of a hepatitis B carrier has been used to prepare viral DNA. That DNA has been cloned in Escherichia coli and the gene coding for the surface antigen has been isolated.

The gene coding for HBsAg has also been inserted into yeast and mammalian cells by means of appropriate expression vectors. Antigens purified from transfected cultures containing the gene for HBsAg have been shown to induce antibodies in mice and guinea-pigs and have been formulated into vaccines. Electron microscopy has revealed that the purified HBsAg used for these vaccines exists as particles 15 to 30 nm in diameter, with the morphological characteristics of free surface antigen in plasma and of the purified antigen now used in plasma-derived hepatitis B vaccines. Some of the vaccine formulations containing these materials have already been shown to be immunogenic in mice, chimpanzees and other
monkeys, and human beings, with antigenic potencies similar to those of vaccine made from plasma-derived antigen.¹

The requirements that follow apply to the control and testing of hepatitis B vaccines made by recombinant DNA methods. They have been formulated to take account of the scale-up required for commercial production. Particular emphasis is placed on introducing "in-process" control, which has been highly effective for other bacterial and viral vaccines, rather than relying entirely on tests on the end-product. General requirements, such as tests for potency, purity, toxicity, pyrogenicity, and sterility, will apply as much to hepatitis B vaccines made by recombinant DNA methods as to those derived from human plasma. Certain tests will be required on every production batch of vaccine, whereas others will be required only to establish the validity, acceptability, and consistency of a given manufacturing process.

A detailed description of how the product is made should be given. Evidence should be presented to show that any HBsAg made by recombinant DNA techniques possesses the characteristics of an immunogen that protects against hepatitis B virus.

Rigorous identification and characterization of recombinant DNA-derived vaccines will be required. The ways in which these products differ chemically, structurally, biologically, or immunologically from the naturally occurring antigen must be fully documented. Such differences could arise during processing at the genetic or post-translational level, or during purification. Variations between batches of the product may result from genetic instability during serial cultivation. Microbial contamination during fermentation may occur, and tests for contaminants must be thorough.

Special attention should be given to purity because:

— unwanted gene products may be co-expressed unexpectedly with the HBsAg, for example if transcription is initiated at several sites, or if changes occur during culture that affect transcription, initiation, or termination processes to favour the expression of other genes in the vector or the host cell;
— biologically active extraneous components such as DNA, proteins, and endogenous retroviruses derived from the host-cell system may be found in the final product;

¹ For further details, and discussion of hepatitis B vaccines produced by recombinant DNA techniques, see Annex 5.

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agents used in the purification process (column matrices, antibodies) may give rise to specific contaminants in the final product.

The product arising from the recombinant system must be shown to elicit specific antibody responses to HBsAg in laboratory animals, including, where possible, a nonhuman primate species. HBsAg of diverse subtypes should be used to characterize the specificity of the response fully.

One approach to evaluate the protective potential of recombinant DNA-derived vaccines in human beings is through chimpanzee immunization and challenge studies. The requirement for a test of vaccine efficacy in chimpanzees should be at the discretion of the national control authority but, if possible, on at least one occasion, chimpanzees given the recommended human schedule of immunization should be shown to be protected against challenge with hepatitis B virus. In some studies, more than 1000 chimpanzee infectious doses of hepatitis B virus of homotypic and heterotypic subtypes, given intravenously, have been used as a challenge.

The vaccine should reliably induce antibody responses to HBsAg in human recipients. The frequency and titre of the antibody responses should be at least equivalent to those induced by plasma-derived vaccines that fulfil WHO requirements. The aims of immunogenicity studies in human subjects should be to define the quantity of antigen and number of doses required to reliably elicit antibody responses to HBsAg. The titre, duration, and quality of the responses should be clearly defined. In vitro tests of antigenicity may be difficult to standardize, and therefore it is proposed that the content of pure HBsAg in the product should be used as the basis for comparing immunogenicity in mice and responses in human subjects. The relationship between the antigenicity of the product in in vitro tests, in mouse immunogenicity tests, and in human beings should be established. Studies in human subjects should be designed to provide information on the frequency and severity of any local and systemic adverse reactions to vaccination. Vaccine recipients in such studies should represent the intended target group for immunization in terms of age and risk.

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 hepatitis B vaccine, it is recommended that a clause should be included permitting modifications of the manufacturing requirements on the condition that it can be demonstrated, to the satisfaction of the national control authority, that such modifications ensure a degree of safety and a potency of the vaccine 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 and/or used.

PART A. MANUFACTURING REQUIREMENTS

1. Definitions

1.1 International name and proper name

The international name shall be *Vaccinum hepatitis B recombinatum*. 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 hepatitis B recombinatum* is a preparation of purified hepatitis B surface antigen (HBsAg) that has been produced by recombinant DNA techniques. The preparation shall satisfy all the requirements formulated below.

1.3 International reference materials

International standards and reference reagents are needed for the control of HBsAg to determine antigenic content and potency.

For the assay of antigenic content by techniques such as radioimmunoassay, enzyme-linked immunosorbent assay, single
radial immunodiffusion, and rocket electrophoresis, an international reference material consisting of an aqueous preparation without an adjuvant is required (see Part A, section 6.3). Such a preparation was established in 1985 as the first International Standard for Hepatitis B Surface Antigen ad Subtype (100 IU per ampoule).

For assessing the immunogenicity of vaccines, an international standard for adjuvanted vaccine is required, specifically for the measurement of immune responses of animals and the calibration of immune responses in human subjects (see Part A, section 10.6). Such a preparation is under study.

For the calibration of techniques used to measure antibody responses to hepatitis B vaccines, an international reference preparation is available. This International Reference Preparation of Hepatitis B Immunoglobulin (established in 1977) is dispensed in ampoules containing 50 IU of hepatitis B immunoglobulin (from fractionated human plasma, and freeze-dried). This preparation is in the custody of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands.

1.4 Terminology

*Hepatitis B virus:* a 42 nm double-shelled virus particle, originally known as the Dane particle, which contains the DNA genome of the virus.

*HBsAg:* hepatitis B surface antigen, comprising a complex of antigens associated with the virus envelope and subviral forms (22 nm spherical and tubular particles). Native HBsAg is encoded by envelope gene sequences (S plus pre-S) in the viral DNA. Recombinant DNA-derived hepatitis B vaccines may contain the S gene product or products of the S/pre-S combination.

*Cell seed lot:* a quantity of cells stored frozen at −70 °C or below in aliquots of uniform composition, 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, stored frozen at −70 °C or below in aliquots of uniform composition.

In normal practice a cell seed is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer, at which point the cells are combined into one pool and preserved cryogenically to form the MWCB.
Production cell culture: a collection of cell cultures being used for biological production that have been derived from one or more ampoules of the MWWC.

Single harvest: the biological material prepared from a single production run.

Final aqueous bulk: the final bulk before the addition of an adjuvant.

Final bulk: the finished biological material prepared from one or more batches of purified HBsAg, and present in the container from which the final containers are filled.

Final lot: a collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling and during the preparation of the finished vaccine. A final lot must therefore consist of finished material distributed into containers in one working session from a single final bulk.

2. General manufacturing requirements

The general manufacturing requirements contained in the revised Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories, p. 111 shall apply to establishments manufacturing hepatitis B vaccine, with the addition of the following directives.

(a) Production areas shall be decontaminated before they are used for the manufacture of hepatitis B vaccine.

(b) Hepatitis B vaccine shall be produced by staff who have not handled animals or infectious microorganisms in the same working day. The staff shall consist of persons who have been examined medically and have been found to be healthy and not to be carriers of hepatitis B.

(c) No cultures of microorganisms or eukaryotic cells other than those approved by the national control authority shall be introduced or handled in the production area at any time during manufacture of the vaccine.

(d) Persons not directly concerned with the production processes, other than official representatives of the national control authority, shall not be permitted to enter the production area.

Particular attention is drawn to the recommendations contained in Part A, section 1 of the revised Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories, p. 131 regarding the training and experience of persons in charge of production and testing and of those assigned to various positions of responsibility in the manufacturing establishment, and to the registration of such personnel with the national control authority.

The preparation of hepatitis B vaccine shall be based strictly on the cell seed lot system. A description of the system used should be provided, including the number of vials of seed available and details of their storage. Particular attention should be paid to the stability of the expression vector and to the plasmid copy number in the seed stock under conditions of storage and recovery.

Full details of the cell culture process used in manufacture should be provided to the national control authority with particular reference to tests to monitor microbial contamination in the cell culture vessels. Information on the sensitivity of methods to detect such contamination and the frequency of the tests should be provided, together with criteria for the rejection of contaminated materials.

The yield of HBsAg shall be monitored during the course of individual production runs. Criteria, based on yield, for acceptance of culture harvests for further processing into vaccine shall be defined, and consistency of production shall be established by testing at least five consecutive lots prepared by the same procedures.

3. Validation and control of manufacturing procedures

The general production precautions formulated in Part A, section 3 of the revised Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories, p. 151 shall apply to the manufacture of hepatitis B vaccine.

3.1 Strategy for cloning and expressing the gene

A full description of the biological characteristics of the host cell and expression vectors used in production should be given. This

should include details of: (a) potential retrovirus-like particles in and genetic markers of the host cell; (b) the construction, genetics, and structure of the expression vector; and (c) the origin and identification of the gene that is being cloned.

The physiological measures used to promote and control the expression of the cloned gene in the host cell should be described in detail.

Data that establish the stability of the expression system during storage of the MWCB and beyond the level used for production should be provided. Any instability of the expression system occurring in the seed culture or after a production-scale run, for example involving rearrangements, deletions, or insertions of nucleotides, must be documented. Unstable preparations must not be used until approval to continue use has been obtained from the national control authority.

3.2 Biochemical characterization of recombinant vector

The nucleotide sequence of the gene insert and of adjacent segments of the vector should be provided. Restriction enzyme mapping of the vector containing the gene insert should be given.

3.3 Purification procedures

The methods used to purify the HBsAg from culture harvests should be fully described. The capacity of each step of the purification procedure to remove and/or inactivate substances other than HBsAg derived from the host cell or culture medium, including, in particular, virus particles, proteins, and nucleic acids, shall be evaluated. If individual contaminants are difficult to monitor, the results of pilot-scale studies to follow the removal of individual, deliberately added contaminants at appropriate stages of purification will provide valuable information.

If antibodies are used in the purification procedures, their origins and characteristics should be fully described. The degree of purity of monoclonal antibodies produced from hybridoma cell lines and the criteria for freedom from cell-derived DNA and murine viruses should conform to the regulations set by the national control authority.
3.4 Characterization of the gene products (HBsAg)

3.4.1 Particle characterization

The morphological characteristics of the HBsAg particles and degree of aggregation should be established by electron microscopy and by physicochemical methods, for example gradient centrifugation. In addition, the protein, lipid, nucleic acid, and carbohydrate content should be measured.

3.4.2 Protein quantification

The protein content (composition) in comparison to a reference reagent should be established by quantitative amino acid analysis or by another accurate method.

3.4.3 Protein characterization

An ultraviolet spectrum should be recorded. The protein composition should be established by techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. The bands should be identified by sensitive staining techniques and, where possible, by specific antibodies to confirm the presence of the expected products of the hepatitis B virus envelope gene. The identity of the protein should be established by partial N-terminal and C-terminal sequence analysis.

3.4.4 Antibody responses

The recombinant DNA-derived vaccine should be shown to induce antibody responses comparable to those elicited by plasma-derived vaccines that have proved effective in the field. The antibodies induced by the vaccine in human beings should be titrated and characterized with respect to their activities against relevant determinants of the hepatitis B virus envelope, for example group and subtype determinants.

3.4.5 Consistency of yield

Data on the consistency of yield between runs and during individual production runs shall be provided, and the national control authority shall approve the criteria for an acceptable production run.

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4. Manufacturer's working cell bank (MWCB)

4.1 Origin of the cell banks

Only those cells approved by and registered with the national control authority shall be used to produce HBsAg. The national control authority shall have responsibility for approving the cell seed. A short history of the cell banks shall be provided.

4.2 Characteristics of the cell seed lot

The characteristics of the cell seed (host cell in combination with the expression vector system) shall be fully described, and information given on the absence of extraneous agents and on genetic homogeneity. The nucleotide sequence of the HBsAg gene insert and its flanking regions shall be specified. A peptide map and/or terminal amino acid sequence of the gene product shall be obtained.

4.3 Phenotypic indicators of purity and genetic consistency of the recombinant cultures

Cells must be maintained in a frozen state that allows recovery of viable cells without alteration of genotype. Recovery of the cells from the frozen state should be accomplished, if necessary, in selective media such that the genotype and phenotype consistent with the unmodified host and unmodified recombinant DNA vector are maintained and are clearly identifiable. The cell seed must be identified by appropriate tests.

5. Single harvests

5.1 Sterility

The degree and nature of any microbial contamination in the cell culture vessels shall be monitored during and at the end of the production runs by methods approved by the national control authority. The sensitivity of the test methods and criteria for the rejection of harvests shall be defined.
5.2 Consistency of yield

The yield of HBsAg from each single harvest shall be shown to be within the limits approved by the national control authority (section 3.4.5).

5.3 Plasmid retention

A sample of cells from each production fermentation must be tested to confirm the retention of the recombinant phenotype. The method used shall be approved by the national control authority.

6. Purification

The purification procedure can be applied to a single harvest, a part of a single harvest, or a pool of single harvests. The maximum number of single harvests that may be pooled shall be set by the national control authority. The antigen must be purified before adsorption onto an adjuvant. Adequate purification may require several purification steps based on different principles. This will minimize the possibility of co-purification of extraneous cellular materials. The methods used for purification of the HBsAg should be appropriately validated (see section 3.3) and approved by the national control authority.

The experience of several manufacturers has shown that it is possible to produce batches in which HBsAg is at least 95% of the total protein. One suitable method of analysing the proportion of potential contaminant proteins in the total protein of the product is separation of the proteins by polyacrylamide gel electrophoresis under both non-reducing and reducing denaturing conditions. Individual gels should be stained by Coomassie Blue and by silver stain, or a single gel may be successively stained by both methods. The silver stain is considerably more sensitive for the detection of very small quantities of proteins and is also useful for identifying any non-protein materials, such as nucleic acids, carbohydrates, and lipids, which may be present. The protein in each band can be quantified by densitometric analysis. The staining procedure shall be sensitive enough to reveal a potential contaminant protein present at a level of 1% of total protein.

High performance liquid chromatography is another very useful method for determining the purity of a protein or peptide and for investigating its molecular configuration. A widely used method is reverse-phase high performance liquid
chromatography. A protein or peptide that elutes as a single symmetrical peak in two markedly different systems, including an ion-pair system, is generally of high purity.

6.1 Protein and other components of the vaccine

The total protein content of the vaccine should be determined (see also Part A, section 3.4.2).

The total concentration of protein may be determined by measuring absorbance at 280 nm, by the micro-Kjeldahl method, by the Lowry technique, or by another suitable method.

Lipid and carbohydrate contents may also provide useful information.

6.2 Tests for agents used during purification or other phases of manufacture

At test shall be made for the presence of any potentially hazardous agent used in manufacture. The method used and the permitted concentration shall be approved by the national control authority.

6.2.1 Monoclonal antibodies

Where a monoclonal antibody is used in vaccine preparation, for example for immunological affinity chromatography to purify HBsAg, the product should be tested for residual antibody. The methods used and the permitted concentrations of antibody shall be approved by the national control authority.

Several national control authorities have drafted guidelines for the control of monoclonal antibody preparations used for the manufacture of biological products for human use.

6.3 Test for HBsAg content

The HBsAg content of the purified preparation shall be determined by an appropriate method.

Tests that have been found suitable include radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), single radial immunodiffusion, and rocket electrophoresis in which the purified preparation is compared with a known standard. Analysis of the results by the parallel-line method has been found suitable for most of these techniques.
6.4 Test for antigenic identity (molecular and immunochemical identity)

A test shall be made for the molecular characteristics of the HBsAg gene product by polyacrylamide gel electrophoresis under reducing denaturing conditions.

The gene products shall be shown to possess antigenic determinants characteristic of HBsAg by means of tests with monoclonal antibodies or other antibodies of defined specificity directed against epitopes of HBsAg known to be relevant to the protective efficacy of the vaccine. Such tests shall be approved by the national control authority.

6.5 Test for sterility of purified surface antigen

Each batch shall be tested for sterility according to the requirements given in Part A, section 5 of the revised Requirements for biological substances no. 6. General requirements for the sterility of biological substances, p. 48.1

6.6 Test for inactivating agents

If the HBsAg has been treated with formaldehyde and/or other inactivating agents, then the material shall be tested for the presence of free formaldehyde and/or the other agents. The method used and the permitted concentration shall be approved by the national control authority.

7. Final bulk before addition of adjuvant (aqueous bulk)

The final aqueous bulk consists of one or more purified HBsAg batches. Only those batches that have satisfied the requirements of sections 2–6 above shall be included in the final bulk. The national control authority shall set the maximum number of batches that may be pooled.

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7.1 Sterility tests

The final aqueous bulk shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5.2 of the revised Requirements for biological substances no. 6. General requirements for the sterility of biological substances, p. 49.¹

7.2 Test for HBsAg

The quantity of HBsAg protein compared with the total protein in the final bulk shall be determined by an adequate quantitative procedure. The quantity of protein load needs to be specified so that reasonable sensitivity of the assay can be assured. The lower limit of the ratio of HBsAg to total protein shall be approved by the national control authority. The final aqueous bulk should contain at least 90% of the total protein as HBsAg.

7.3 Test for DNA

The amount of residual cell or plasmid DNA in each batch of vaccine should be determined by sensitive methods, which must be validated, and approved by the national control authority. Suitable tests for the homologous vector used in the production of the HBsAg should be included. Maximum acceptable levels of DNA per vaccine dose shall be approved by the national control authority.

The WHO Study Group on Biologicals concluded that the probability of risk associated with heterogeneous contaminating DNA in a product derived from a continuous cell line is negligible when the amount of such DNA is 100 pg or less in a single dose given parenterally.²

8. Final bulk

8.1 Addition of adjuvant

The adjuvant and the concentration used shall be approved by the national control authority.

At this stage a preservative may need to be added.

In certain countries the aluminium adjuvant is formed in the

presence of the HBsAg, whereas in others preformed adjuvants are added to the aqueous bulk. Where preformed aluminium adjuvants are used, it may not be possible to resolubilize the aluminium compound and to test for the purity and concentration of the HBsAg in the final bulk. It must therefore be demonstrated that the correct amount of antigen is present in the final aqueous bulk before the adjuvant is added or coprecipitated with the HBsAg. Additionally, it must be shown that all the antigen is adsorbed on the adjuvant and that none is free in the aqueous phase of the preparation.

8.2 Tests on final bulk

8.2.1 Tests for sterility

The final bulk shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5.2 of the revised *Requirements for biological substances no. 6. General requirements for the sterility of biological substances*, p. 49.¹

8.2.2 Tests for preservative

The final bulk shall be tested for the presence of preservative. The method used and the permitted concentration shall be approved by the national control authority.

8.2.3 Tests for adjuvant

Each final lot shall be assayed for the content of adjuvant. The method used and permitted concentration shall be approved by the national control authority. Where aluminium compounds are used, the concentration of aluminium shall not be greater than 1.25 mg per single human dose.

9. Filling and containers

The requirements concerning filling and containers given in Part A, section 4 of the revised *Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories*, p. 16² shall apply.

Care should be taken to ensure that the material of which the container is made does not adversely affect the HBsAg under the recommended conditions of storage.
Adjuvanted HBsAg vaccine must be stored at 5–8 °C.

10. Control of the final lot

Samples shall be taken from each final lot for the tests in the following sections.

10.1 Sterility test

Each final lot shall be tested for sterility according to the requirements given in Part A, section 5 of the revised Requirements for biological substances no. 6. General requirements for the sterility of biological substances, p. 48.1

10.2 Innocuity tests

Each final lot shall be tested for innocuity by appropriate tests in mice and guinea-pigs using parenteral injections. The tests shall be approved by the national control authority.

10.3 Test for pyrogenic substances

Each final lot shall be tested for pyrogenic substances. The test shall be approved by the national control authority.

10.4 Test for preservative

Each final lot shall be tested for the presence of preservative. The test used and the permitted concentration shall be approved by the national control authority.

10.5 Assay of adjuvant

Each final lot shall be assayed for the content of adjuvant. The method used and permitted concentration shall be approved by the national control authority. Where aluminium compounds are used,

the concentration of aluminium shall not be greater than 1.25 mg per single human dose.

10.6 Potency and identity tests

The vaccine shall be identified as envelope antigens of hepatitis B virus by appropriate methods. An appropriate quantitative test for antigen content and an immunogenicity assay shall be performed on samples representative of the final filling lots. The vaccine potency shall be compared with that of a reference preparation, and the national control authority shall determine the lower limit of potency.

A suitable quantitative extinction test in mice is as follows. Several groups of at least 20 suitable mice, five weeks of age, are tested. Each mouse is vaccinated intraperitoneally with the adjuvanted hepatitis B vaccine diluted in the adjuvant used in the vaccine; a different graded dose is used for each group of mice. Similar groups of mice are inoculated with the adjuvanted reference preparation. The reactivity of positive sera toward important epitopes on HBsAg should be verified by appropriate tests. Blood samples are taken from the mice after a time period shown to permit the development of an adequate antibody response, and individual sera are assayed for antibodies to products of the envelope gene of hepatitis B virus by sensitive quantitative tests such as radioimmunoassay. The concentrations of vaccine tested should be selected to permit the calculation of 50% seroconversion to antibodies against HBsAg. The strain of mice used for this test must give a suitable dose–response curve to the reference and test antigens. In any test, a calibrated reference preparation should be included and the potency of the vaccine expressed in terms of this reference. The results of collaborative studies to establish a working reference preparation of HBsAg have been reported (WHO/BS/86.1525).

11. 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, p. 17\(^1\) shall apply.

12. 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, p. 18¹ shall apply.

13. 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, p. 18¹ shall apply, with the addition of the following directive.

The leaflet accompanying the package shall include the following information:

— the nature of the cells used to produce the antigen;
— the nature and amount of any preservative, adjuvant, or stabilizer present in the vaccine;
— the volume of one recommended human dose, the immunization schedules, and the recommended routes of administration; this information shall be given for newborn babies, children, adults, and immunosuppressed individuals, and shall be the same for a given vaccine for all regions of the world;
— the amount of total protein contained in one recommended human dose; and
— the amount of HBsAg protein contained in one recommended human dose.

14. 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, p. 18¹ shall apply.

In addition, the conditions of shipping shall ensure that the adjuvanted vaccine does not freeze.

Temperature indicators should be packaged with each vaccine shipment to show whether freezing occurs. If freezing has occurred then the vaccine should not be used.

15. Storage and expiry date

The requirements given in Part A, section 10 of the revised Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories, p. 19\(^1\) shall apply.

15.1 Storage conditions and stability

Before being distributed by the manufacturing establishment, or before being issued from a depot for the maintenance of reserves of vaccines, all vaccine in bulk form or in final containers shall be kept at 5–8 °C. The vaccine shall also be stored at this temperature after distribution or issue. The vaccine shall have been shown to maintain its potency for a period equal to that between the date of issue and the expiry date. During storage the vaccine shall not be frozen.

15.2 Expiry date

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, this being the date on which the animals were inoculated with the vaccine.

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, p. 19\(^1\) shall apply.

The national control authority shall:

—approve the methods for producing HBsAg by recombinant DNA techniques;
—approve the tests for HBsAg concentration and define its minimum value;

— approve the methods for purification;
— approve the purity of the final product;
— approve the tests for extraneous substances and total protein;
— approve the tests for preservative and the agents used for purification and other aspects of manufacture;
— approve the tests used to determine whether the final product is free from abnormal toxicity;
— approve the adjuvant assay and define the permitted concentration of adjuvant in the final product;
— approve the types of animals used in the assay of potency; and
— approve the data to establish that the vaccine produces an adequate antibody response (titre, duration, and quality) in human beings.

The national control authority shall also provide national reference preparations for defining the activity of HBsAg in a given quantity of protein.

Before releasing a vaccine, the national control authority shall be satisfied that the results of all tests, including those done on individual batches during the process of manufacture, are satisfactory and that consistency has been established.

2. Release and certification

A hepatitis B vaccine 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 establishment and shall certify whether or not the final lot of vaccine in question meets all national requirements as well as Part A of these requirements. The certificate shall state the date of the last satisfactory HBsAg 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 hepatitis B vaccine between countries.
The first draft of the Requirements for hepatitis B vaccines made by recombinant DNA techniques in yeast was prepared in 1984 by the following WHO consultants, temporary adviser, and staff members:

Dr H. Arimura, Green Cross Corporation, Osaka, Japan
Dr F. Assaad, Director, Division of Communicable Diseases, WHO, Geneva, Switzerland
Dr A.J. Beale, Scientific Director, Wellcome Biotechnology Ltd, Beckenham, Kent, England
Ms R. Bell-Madsen, Immunology, WHO, Geneva, Switzerland
Dr R. Beswick, BIOGEN, Laboratoires de Recherche Direction, Geneva, Switzerland
Dr G. Bitter, Amgen, Thousand Oaks, CA, USA
Dr W.N. Burnette, Amgen, Thousand Oaks, CA, USA
Dr F. Dorner, Immuno AG, Research Center Orth, Orth/Donau, Austria
Dr S. Drew, Research Laboratories, Merck Sharp and Dohme, Rahway, NJ, USA
Dr M. Ferguson, Division of Viral Products, National Institute for Biological Standards and Control, London, England
Dr F. Filatov, Institute of Virology, Academy of Medical Sciences, Moscow, USSR
Dr R.J. Gerety, Associate Director for Medicine and Science, Infectious Diseases Branch, Division of Blood and Blood Products, Office of Biologies Research and Review, Bethesda, MD, USA
Dr F. Hamada, Chemo-Sero-Therapeutic Research Institute, Kumamoto City, Japan
Dr M.R. Hillman, Director, Merck Institute for Therapeutic Research, Merck Sharp and Dohme Research Laboratories, West Point, PA, USA
Dr C. Husgelen, General Manager, Biological Division, Smith Kline-RIT, Rixensart, Belgium
Professor J.L. Melnick, Department of Virology and Epidemiology, Baylor College of Medicine, Houston, TX, USA,
Professor K. Murray, Department of Molecular Biology, University of Edinburgh, Edinburgh, Scotland
Dr N. Ohtomo, Director of Research and Development, The Chemo-Sero-Therapeutic Research Institute, Kumamoto City, Japan
Dr G.C. Schild, Head, Division of Viral Products, National Institute for Biological Standards and Control, London, England
Dr H. Shimojo, National Institute of Health, Tokyo, Japan (Temporary Adviser, WHO Regional Office for the Western Pacific, Manila)
Dr P. Sizaret, Biologicals, WHO, Geneva, Switzerland
Dr O. Soboslavsky, Virus Diseases, WHO, Geneva, Switzerland
Dr T. Suyama, Green Cross Corporation, Osaka, Japan
Dr T. Umemai, WHO Regional Office for the Western Pacific, Manila, Philippines
Dr M. de Wilde, Director, Molecular Genetic Department, Smith Kline-RIT, Rixensart, Belgium
Dr P. Wingfield, BIOGEN, Laboratoires de Recherche Direction, Geneva, Switzerland
Dr M.D. Winther, Wellcome Biotechnology Ltd, Beckenham, Kent, England
Professor A.J. Zuckerman, Department of MedicalMicrobiology, London School of Hygiene and Tropical Medicine, London, England

These Requirements were revised in 1985 to apply to both yeast-derived and mammalian cell-derived vaccines. The following WHO consultants and staff members
of WHO and of the International Agency for Research on Cancer prepared the revision:

Dr J. Armand, Mérieux Institute, Lyon, France
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Dr T. Bektimirov, Chief Medical Officer, Virus Diseases, WHO, Geneva, Switzerland
Dr G.A. Bitter, Amgen, Thousand Oaks, CA, USA
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Dr M. Ferguson, National Institute for Biological Standards and Control, London, England
Mr H. Fukushima, Mitsubishi Chemical Industries, Tokyo, Japan
Dr J. Gerin, Department of Microbiology, Schools of Medicine and Dentistry, Georgetown University Medical Center, Washington, DC, USA
Dr M. Girard, Pasteur Production, Marnes-la-Coquette, France
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Dr L. Johnson, Institute of Allergy and Infectious Diseases, Development and Application Branch, National Institutes of Health, Bethesda, MD, USA
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Dr K. Koike, Chief, Department of Gene Research, Cancer Institute, Tokyo, Japan
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Dr K. Mizuno, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan
Dr R. Netter, Laboratoire National de la Santé, Paris, France
Dr A.R. Neurath, New York Blood Center, New York, NY, USA
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Dr G.T. O’Connor, International Agency for Research on Cancer, Lyon, France
Dr D. Peterson, Department of Biochemistry, Medical College of Virginia, Richmond, VA, USA
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Dr G. Schild, Director, National Institute for Biological Standards and Control, London, England
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Dr O. Sobeslavsky, Medical Officer, Virus Diseases, WHO, Geneva, Switzerland
Dr H.T. Thoma, Riatai, Munich, Federal Republic of Germany

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Dr G.B. Thornton, Amgen Representative, Johnson and Johnson Biotechnology Center, San Diego, CA, USA
Professor P. Tollais, Unité de Recombinaison et Expression Génétique, Pasteur Institute, Paris, France
Dr M. de Wilde, Director, Molecular Genetic Department, Smith Kline-RIT, Rixensart, Belgium
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Dr M.D. Winther, Wellcome Biotechnology Ltd, Beckenham, Kent, England
The third draft of the Requirements was formulated in May 1986 by Dr J.C. Petricciani, Chief, and Dr V.P. Grachev, Scientist, Biologicals, WHO, Geneva, Switzerland.
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Dr G.T. O’Conor, Senior Scientist, International Agency for Research on Cancer, Lyon, France
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Dr M. Ferguson, National Institute for Biological Standards and Control, London, England
Dr M.R. Hilleman, Director, Merck Sharp and Dohme Research Laboratories, West Point, PA, USA
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Dr J. Peetemans, Technical Director, Smith Kline-RIT, Rixensart, Belgium
Professor A.J. Zuckerman, Department of Medical Microbiology, London School of Hygiene and Tropical Medicine, London, England

ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice, and for supplying additional data relevant to these Requirements: Dr J. Cameron, Director, Biological Products, Armand-Frappier Institute, Ville de Laval, Quebec, Canada; Professor F. Deinhardt, Director, Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Munich, Federal Republic of Germany; Dr M. Ferguson, Division of Viral Products, National Institute for Biological Standards and Control, London, England; Dr J. Furesz, Director, Bureau of Biologics, Drugs Directorate, Ottawa, Ontario, Canada; Dr J.L. Gerin, Director, Division of Molecular Virology and Immunology, Georgetown University, Rockville, MD, USA; Dr M. Girard, Direction Scientifique, Pasteur Vaccines, Marnes-la-Coquette, France; Dr C. Gonzalez-Rayos, Laboratory Services Division, Bureau of Animal Industry, Diliman Quezon City, Philippines; Dr A. Gray, Senior Director, Biologics, Merck Sharp & Dohme, West Point, PA, USA; Dr C. Guthrie, Production Director, Commonwealth Serum Laboratories, Parkville, Victoria, Australia; Dr M. Haase, Paul-Ehrlich Institute, Frankfurt am Main, Federal Republic of Germany; Dr N. Harford, Genetic Department, Smith Kline-RIT, Rixensart, Belgium; Dr M.R. Hilleman, Director, Merck Institute for Therapeutic Research, Merck Sharp & Dohme Research Laboratories, West Point, PA, USA; Dr R.M. Lequin, Department of Obstetrics and Gynaecology, University Hospital, Leiden, Netherlands; Dr W. Lüttmann, Paul-Ehrlich Institute, Frankfurt am Main, Federal Republic of
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Appendix

SUMMARY PROTOCOL FOR PRODUCTION AND TESTING OF HEPATITIS B VACCINES MADE BY RECOMBINANT DNA TECHNIQUES IN YEAST

Based on Requirements for biological substances no. 39,
Requirements for hepatitis B vaccines made by recombinant DNA techniques in yeast

Identification of final lot

<table>
<thead>
<tr>
<th>Name and address of manufacturer</th>
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<table>
<thead>
<tr>
<th>International name and proprietary name of vaccine</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</th>
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<table>
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<tr>
<th>Date of filling containers</th>
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<table>
<thead>
<tr>
<th>Number of containers and nature (ampoules or vials)</th>
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<table>
<thead>
<tr>
<th>Date of last potency test</th>
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<table>
<thead>
<tr>
<th>Number of doses in each container</th>
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<table>
<thead>
<tr>
<th>Volume of single dose</th>
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<table>
<thead>
<tr>
<th>Expiry date</th>
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</table>

3.1 Validation and control of manufacturing procedures

3.1 Strategy for cloning and expressing the gene²

Details of:

(a) potential retrovirus-like particles in and genetic markers of the host cell

(b) construction, genetics, and structure of the expression vector

(c) the origin and identity of the gene that was cloned

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² Numbers refer to the corresponding numbered sections in the text of the Requirements.

² Section 3.1 does not necessarily need to accompany each batch of vaccine. If necessary, it should be typed on a separate sheet.
3.2 Biochemical characterization of recombinant vector

Nucleotide sequence of the surface antigen gene insert

Restriction endonuclease mapping of the recombinant vector, where relevant

3.3 Purification procedures

Methods used to purify the HBsAg

Results

Origins and characteristics of antibodies, if used

3.4 Characterization of the gene products (HBsAg)

3.4.1 Particle characterization

Morphological characteristics of the particles

Results of examination by electron microscopy

Degree of aggregation

Method

Results

Quantity of protein

Method

Results

Quantity of lipid

Method

Results

Quantity of nucleic acid

Method

Results

Quantity of carbohydrate

Method

Results
3.4.2 Quantitative analysis of protein
Method
Results

3.4.3 Protein characterization
Ultraviolet spectrum
Protein composition
Method
Results
Identity of the protein by partial
N-terminal and C-terminal analysis
Method
Results

3.4.4 Antibodies induced by the
vaccine in human beings
Titres
Characteristics

3.4.5 Data on the consistency of yield
between runs and during individual
runs

4. Manufacturer's working cell bank (MWCB)

4.1 Origin and short history of cell banks
Date the cell banks were established
Quantity of cells stored
Passage level of the MWCB
Storage conditions

4.2. Characteristics of the cell seed lot
Purity and homogeneity of the cell seed lot
Method
Results
Genetic characteristics of the
cell seed lot
Method
Results
Purity of the recombinant DNA
vector
Method
Results

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Genetic characteristics of recombinant DNA vector
   Methods
   Results

Nucleotide sequence of the HBsAg gene insert

Peptide map of the gene product (HBsAg)

Terminal amino acid sequence of the gene product

4.3 Phenotypic indicators of purity and genetic consistency of the recombinant culture

Tests on cells after recovery from the preserved state
   Methods
   Results

Identity of cell seed
   Method
   Results

5. Single harvests

5.1 Sterility test performed at the end of the culture
   Media
   Results

5.2 Consistency of yield of HBsAg
   Method
   Results

5.3 Plasmid retention
   Method
   Results (proportion of cells still possessing the plasmid at the end of the culture)

6. Purification

Methods
   Results
6.1 Protein and other components of the vaccine

- Protein content
  - Methods
  - Results

- Lipid content
  - Methods
  - Results

- Carbohydrate content (as compared with total proteins)
  - Methods
  - Results (%)

6.2 Tests for agents used during purification or other phases of manufacture

- Methods
- Results

6.2.1 Determination of the monoclonal antibody content

- Methods
- Results

6.3 Test for HBsAg content

- Methods
- Results

6.4 Test for antigenic identity

- Methods
- Results

6.5 Test for sterility of purified surface antigen

- Media
- Results

6.6 Test for inactivating agents

- Methods
- Results

---

1 Only if immunoabsorption was used in the purification process.
7. Final bulk before addition of adjuvant (aqueous bulk)

Volume of purified bulk
Number of batches pooled
Nature and volume of diluent added

7.1 Test for sterility
Method
Results

7.2 Test for HBsAg
Quantity of HBsAg
Method
Results
Ratio of HBsAg to total protein
Method
Results

7.3 Test for DNA
Method
Results

8. Final bulk

8.1 Addition of adjuvant
Volume of bulk
Nature and volume of adjuvant added and final concentration
Nature and volume of preservative added and final concentration
Composition of final bulk (mixing of all ingredients) and identification number

8.2 Tests on final bulk

8.2.1 Tests for sterility
Method
Results

8.2.2 Tests for preservatives
Method
Results

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8.2.3 Tests for adjuvant
Method
Results

9. Filling and containers
Date of filling
Quantity of containers
Volume of vaccine per container
Control for defective containers

10. Control of the final lot

10.1 Sterility test
Method
Results

10.2 Innocuity tests
In mice
No. and weight of animals
Quantity injected
Observation period
Results

In guinea-pigs
No. and weight of animals
Quantity injected
Observation period
Results

10.3 Test for pyrogenic substances
Method
Results

10.4 Test for preservative
Method
Results

10.5 Assay of adjuvant
Method
Results
10.6 Potency and identity tests

Identification of vaccine as HBsAg
Method
Results

Antigen content
Method
Results

Immunogenicity assay
Method
Results

Vaccine potency
Method
Results

Internal certification

Certification by person taking overall responsibility for production of the vaccine

I certify that lot no. ______ of the vaccine satisfies Part A of the WHO Requirements for hepatitis B vaccines made by recombinant DNA techniques in yeast.

Signature

Name (typed)

Date

The protocol must be accompanied by a sample of the label and a copy of the leaflet.

Release certification by the national control authority

Whenever hepatitis B vaccines produced by recombinant DNA techniques are to be exported, they should be accompanied by a release certificate from the national control authority.

Sample release certificate

I hereby certify that batch no. ______ of hepatitis B vaccine produced by (name of producer) by recombinant DNA techniques meets all national requirements as well as Part A of the WHO Requirements for hepatitis B vaccines made by recombinant DNA techniques in yeast.

The date of the last satisfactory potency test carried out by the national control laboratory is ______

The final lot has been released by us under no. ______

The number appearing on the label of the containers is ______

Signature

Name (typed)

Date

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INTRODUCTION

Mumps is an acute disease of children and young adults, caused by a paramyxovirus of which there is only a single serotype. Mumps virus infection produces no symptoms in about one-third of infected persons. In those with a clinical response, glandular and nerve tissue is most often affected. The most common signs are fever and swelling of the parotid glands. Other complications that may appear simultaneously with these signs, or in any sequence, are epididymoorchitis, meningo-encephalitis, cranial nerve involvement (especially eighth cranial nerve damage leading to hearing impairment), pancreatitis, oophoritis, mastitis, and myocarditis. Frequent viruria and abnormal renal function suggest that mumps virus may infect the kidneys. In some instances, one or more of the other complications may be present in the absence of parotitis.
Mumps is generally considered to be less contagious than measles or chickenpox; for this reason some people reach adulthood without developing immunity. Outbreaks have occurred frequently among young adult males in closed communities. Potential target populations for immunization include children and susceptible adults.

The most common complication of mumps in children is meningitis, sometimes associated with encephalitis, and in young adults orchitis. Most complications due to mumps infection resolve without permanent damage. Death following mumps is rare and is mostly due to mumps encephalitis.

Several strains of attenuated mumps virus have been developed for use in vaccines. The first vaccine strain to be developed, and that most often used, is the Jeryl Lynn strain, which is grown in chick embryo cell cultures. It was licensed in the United States of America in 1967 and by 1985 had been given to nearly 50 million children and adults throughout the world. It induces seroconversion in at least 97% of children and at least 93% of adults, whether used singly or in combination with measles and rubella vaccines. When mumps vaccine is combined with other vaccines, it should be shown that the vaccine viruses do not interfere with each other's actions.

Vaccines based on the Leningrad-3 strain of attenuated mumps virus have been in use since 1974 in the USSR and subsequently in other countries. They are produced in cell cultures of Japanese quail embryo in the USSR and in chick embryo cell cultures in Yugoslavia. Approximately 20 million doses of vaccine based on this strain have so far been used, as monovalent vaccine or in combination with measles and rubella vaccines.

The Urabe strain of attenuated live mumps vaccine was first licensed in 1979 in Japan and thereafter in Belgium and France. It is produced either in the amnion of embryonated hen's eggs or in chick embryo cell cultures. By 1985, about five million persons had been immunized with the Urabe strain in Japan and other countries. Its immunogenic properties are similar to those of the Jeryl Lynn strain used as a monovalent product or in combination with measles and rubella vaccines.

Two additional strains of attenuated mumps vaccine have been licensed in Japan. These are the Hoshino and Torii strains, both of which are grown in chick embryo cell culture. These strains have been less extensively used than the Jeryl Lynn, Urabe, and Leningrad-3 strains.
Mumps vaccines have been shown to induce long-term protective immunity. In the United States of America, immunity induced by vaccines based on the Jeryl Lynn strain has apparently persisted for at least 20 years, whilst there is evidence from Japan that immunity of at least 10 years' duration is produced by vaccines containing the Urabe strain. In the United States of America widespread use of mumps vaccine since 1967 has dramatically reduced the reported incidence of mumps.

GENERAL CONSIDERATIONS

For the standardization of the infectious virus content of mumps vaccines, there is a need for an international reference preparation against which the titre can be assessed. The World Health Organization will provide such a reference preparation as soon as possible. A reference preparation of antibody to mumps virus is also required.

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 or recommendations for guidance. To facilitate the international distribution of vaccine made in accordance with these requirements, a summary protocol for recording the results of tests is included as an appendix.

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning mumps vaccine, it is recommended that a clause be included permitting modifications to the manufacturing requirements on the condition that it be demonstrated, to the satisfaction of the national control authority, that such modifications ensure a degree of safety and a potency of the vaccine at least equal to those provided by the requirements formulated below. It is desirable that the World Health Organization should then be informed of the action taken.

The terms "national control authority" and "national control laboratory" as used in these requirements always refer to the country in which the final vaccine is manufactured.
PART A. MANUFACTURING REQUIREMENTS

1. Definitions

1.1 International name and proper name

The international name shall be *Vaccinium parotitidis vivum*. The proper name in the country’s language shall be the equivalent of the international name.

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

1.2 Descriptive definition

*Vaccinium parotitidis vivum* is a preparation of live attenuated mumps virus grown in avian embryo cells or other suitable cells. The preparation shall satisfy all the requirements formulated below.

At present, live mumps vaccines are blended with an appropriate stabilizer and lyophilized. They are available as monovalent vaccine or in combination with live measles and live rubella vaccines.

1.3 International reference materials

Since no international standards or reference reagents for live mumps vaccine have yet been established, no requirements for titre based on such standards or reagents can be formulated. National control authorities should therefore provide a reference preparation of live mumps virus for validating tests to determine virus concentration (see Part A, sections 3.3.2, 3.4.1, and 5.2).

1.4 Terminology

*Primary virus seed lot*: a quantity of virus suspension that has been processed as a single lot and has a uniform composition. It is used for the preparation of secondary seed lots.

*Secondary virus seed lot*: a quantity of virus suspension that has been processed as a single lot, has a uniform composition, and is only one passage from a primary seed lot produced on the same substrate. Material is drawn from secondary seed lots for inoculating cell cultures or eggs for the production of vaccine.

*Cell substrate lot*: a number of cell cultures derived from the same pool of cells and processed and prepared together, or a number of avian embryos from an approved source.

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Single harvest: a virus suspension harvested in one continuous operation from one cell culture lot, or from one batch of embryonated eggs, all the cultures or eggs having been inoculated at the same time with the same inoculum.

Virus pool: a pool of single harvests before clarification.

Final bulk suspension: a quantity of vaccine after completion of the preparations for filling, and present in the container from which the final containers are filled.

Final lot: a collection of sealed final containers that are filled from the same final bulk and are homogeneous with respect to the risk of contamination during filling and freeze-drying. A final lot, therefore, consists of finished material distributed into containers in one working session and dried as a single lot.

2. General manufacturing requirements

The general requirements for manufacturing establishments contained in the revised Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories, Annex 1 (I) shall apply to establishments manufacturing live mumps vaccine, with the addition of the following requirements.

(a) Visitors not directly concerned with the production processes shall not be permitted to enter areas used for processing live mumps vaccine; other visitors having business in such areas shall be admitted only under supervision.

(b) Continuous cell lines shall not be introduced into areas used for the production of live mumps vaccine.

(c) Production and control shall be organized in two separate units of the manufacturing establishment that have independent responsibilities.

(d) Personnel involved in the production of the vaccine shall be shown to be immune to mumps.

Particular attention is drawn to the recommendations contained in Part A, section 1 of the revised Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories, Annex 1, p. 13 (I) regarding the training and experience of persons in charge of production and
testing and of those assigned to various positions of responsibility in
the manufacturing establishment.

3. Production control

Production shall be controlled according to the scheme
recommended in 1980 by a group of WHO consultants (see Annex 3
of 2).

3.1 Control of source materials

3.1.1 Virus strain

The strain of mumps virus used in the production of live mumps
vaccine shall be identified by historical records that include
information on the origin of the strain and its subsequent
manipulation. The virus shall at no time have been passaged in a
continuous cell line. The seed lot or five consistent lots of vaccine
derived from the seed lot shall have been shown to be non-
neuropathogenic in monkeys (see section 3.1.3) and to yield live
mumps vaccine of adequate immunogenicity and safety in human
beings. The vaccine strain shall be approved by the national control
authority.

3.1.2 Substrate for virus propagation

Mumps virus used in the production of vaccine shall be
propagated only in substrates approved by the national control
authority. If avian embryos or avian embryo cell cultures are used
for the preparation of the vaccine, the eggs shall come from a closed,
monitored healthy flock that is free from specific pathogens.
Monitoring of chickens and Japanese quail, or their embryos, shall
include tests for Mycobacterium avium, fowl or quail pox, avian
retroviruses, Newcastle disease virus, avian encephalomyelitis virus,
infectious laryngotracheitis virus, reticuloendotheliosis virus,
Marek's disease virus, infectious bursal disease virus, avian reovirus,
avian adenovirus, avian influenza virus, avian parainfluenza virus,
Haemophilus paragallinarum, Salmonella gallinarum, Salmonella
pullorum, Mycoplasma gallisepticum, and Mycoplasma synoviae.

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If human diploid cells are used in vaccine production, a manufacturer’s cell bank shall be established that meets WHO requirements for human diploid cells (3).

3.1.3 Virus seed lot system

The production of vaccine shall be based on the virus seed lot system. Seed lots shall be prepared in cells homologous to those used for production of the final vaccine.

Each seed lot shall be identified as mumps virus by appropriate serological methods (see Part A, section 5.1).

Each primary and secondary seed lot shall be shown by appropriate tests to be free from all demonstrable adventitious microbial agents, including avian leukosis virus, and shall satisfy the requirements of Part A, sections 3.3.1 to 3.3.6.

In one country the tests for adventitious agents are not required for secondary seed lots, but they are mandatory for final vaccines derived from a specified seed lot, with the exception of the test in guinea-pigs.

Each seed lot shall be shown by neurovirulence tests in monkeys to cause no unexpected histopathological changes in the central nervous system. To avoid the unnecessary use of monkeys, virus seed lots should be prepared in large quantities.

Some national control authorities require manufacturers to perform the neurovirulence test on each of five consecutive lots of vaccine derived from each virus seed lot.

In some countries the test is performed as follows. Immediately before the test, each monkey should be shown to be serologically negative for mumps. At least 10 monkeys should be used for each test. The material under test should be given to each monkey by the inoculation of 0.5 ml into the thalamic region of each hemisphere. The total amount of mumps virus inoculated into each monkey should not be less than the amount contained in the recommended single human dose of vaccine. The monkeys should be observed for 17 to 21 days for symptoms of paralysis and other evidence of neuropathological changes. Animals that die within 48 hours of injection may be replaced. The test is invalid and should be repeated if more than 20% of the monkeys die from nonspecific causes. At the end of the observation period, each monkey should be anaesthetized and killed, and at autopsy histopathological examinations should be made of appropriate areas of the brain for evidence of pathological changes. It is recommended that the areas of the central nervous system examined include periventricular areas of the brain and the choroid plexus, since these structures are
frequently affected under experimental conditions with neurotropic variants of mumps virus. At least four control animals shall be inoculated with diluent and similarly examined. *Macaca* and *Cercopithecus* monkeys are suitable for testing neurovirulence.

The seed preparation passes the neurovirulence test if there is no evidence of unexpected clinical or histopathological changes in the central nervous system that are attributable to the inoculated virus or to contaminating adventitious microbiological agents.

Histologically mild lesions of ependymal cells and inflammatory reactions in the choroid plexus of the ventricles, which are caused by some mumps vaccine strains, may be acceptable.

If freeze-dried, the seed lot shall be stored at or below — 20 °C. If not freeze-dried, the seed lot shall be stored at or below — 60 °C.

The virus in the final vaccine should be at the minimum passage level beyond that used in the preparation of a vaccine that has been shown to be immunogenic and safe in clinical studies. The passage level must be acceptable to the national control authority.

Some national control authorities require manufacturers to demonstrate consistency of the product for several consecutive (e.g., five) production lots.

**3.2 Production precautions**

The general production precautions formulated in Annex 1 of the revised *Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories* (I) shall apply to the manufacture of live mumps vaccine, with the addition of the following.

**3.2.1 Control cell cultures**

From the cells used in the preparation of the cell cultures for growing attenuated mumps virus, an amount of processed cell suspension equivalent to that used to prepare 500 ml of cell culture shall be used to prepare control cultures of uninfected cells. These control cultures shall be observed microscopically for changes attributable to the presence of adventitious agents for at least 14 days beyond the time of inoculation of the production vessels with mumps virus. After 14 days, fluids collected from the control
cultures and cell monolayers from some of the control vessels shall be tested for the presence of adventitious agents as described below.

In some countries, samples of fluid from each control vessel are collected at the same time as fluid is harvested from the corresponding production vessels. If several virus harvests are made from the same cell culture lot, the control fluid taken at each harvest is frozen and stored at or below −60°C until the last virus harvesting from that tissue culture lot is completed. The control fluids are then pooled in proportion to their amounts and submitted to the required tests.

The results of the tests on control cultures shall be considered satisfactory only if there is no evidence of adventitious agents and if at least 80% of the control vessels are available for testing at the end of the observation period.

Test for haemadsorbing or haemagglutinating viruses. The cell monolayers from one-quarter of the control vessels shall be tested at the end of the observation period for the presence of haemadsorbing viruses by the addition of guinea-pig erythrocytes that have not been stored for more than 7 days.

Test in cell cultures for adventitious agents. Samples of at least 5 ml of the fluids from each of the control cell cultures shall be inoculated into cell cultures of human and simian origin and into the cell substrate used for virus production, and shall be tested for adventitious agents by the method prescribed in Part A, section 3.3.4.

Test for avian leukosis virus. A sample of fluids pooled from the control cultures shall be tested for avian leukosis virus by a method approved by the national control authority. Suitable methods include the test for resistance-inducing factor, the complement fixation test, and the test for reverse transcriptase.

3.2.2 Control embryonated eggs

Of each batch of eggs used for producing the vaccine, 2% (or 20, whichever is the largest quantity) shall be held as uninoculated controls and incubated for the same time and at the same temperature as the inoculated eggs. At the time of harvesting the virus, amniotic fluids shall be taken from the control eggs and examined individually for haemagglutinating agents (see Annex 3, Part A, section 3.2.1, p. 179 of 4). In addition, a pool of amniotic fluid shall be tested for adventitious agents, including avian leukosis virus, by the methods specified in section 3.2.1.
3.2.3 *Cell substrate for vaccine production*

The cell cultures for vaccine production shall be grown and maintained under aseptic conditions. The maintenance medium shall contain no added proteins, except human albumin if required. Any human albumin added shall meet the *Requirements for biological substances no. 27. Requirements for the collection, processing, and quality control of human blood and blood products* (5).

If animal serum is used in the growth medium for the cell cultures, the cells shall be washed free from serum before the virus suspension is harvested, so that the final vaccine shall not contain more than 1 μl of animal serum per litre. Human serum shall not be used.

Penicillin or other β-lactams shall not be used at any stage of manufacture.

Minimal concentrations of other suitable antibiotics, such as neomycin, may be used where approved by the national control authority.

3.3 *Harvesting and testing the virus*

Virus fluid shall be harvested by a method approved by the national control authority. Each single harvest or pool of harvests shall be tested as described in the following sections.

Samples of single harvests or harvest pools shall be taken for testing at the time of harvesting and if not tested immediately shall be kept at a temperature below −60 °C.

For those tests that require prior neutralization of mumps virus, the antiserum used shall not be of human, simian, or avian origin. The immunizing antigen used for the preparation of the antiserum shall be produced in cell cultures from a species different to that used for the production of vaccine. Such cell cultures shall be free from extraneous microbial agents that might elicit antibodies inhibitory to the growth of extraneous agents present in the mumps virus pool.

3.3.1 *Sterility tests*

Each single harvest or pool of harvests shall be tested for bacterial and mycotic sterility according to Part A, sections 5.2 and 5.3 of the *Requirements for biological substances no. 6. General requirements for the sterility of biological substances* (6). Tests for mycoplasmas shall be performed by a method approved by the national control authority.

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3.3.2 Virus titration

The live virus content of each single harvest or pool of harvests may be determined by titration in cell culture against a reference preparation of live mumps virus (see Part A, section 1.3).

3.3.3 Tests for mycobacteria

Regardless of which substrate is used for producing the virus vaccine, each single harvest or pool of harvests shall be tested for the presence of Mycobacterium tuberculosis and Mycobacterium bovis. If the vaccine is produced in fibroblasts of avian origin, it shall also be tested for the presence of Mycobacterium avium. Tests for mycobacteria shall be done after centrifugation of 20 ml of the virus pool or single harvest.

3.3.4 Tests in cell cultures

A volume of each virus pool equivalent to at least 500 human doses of vaccine shall be tested for adventitious agents by inoculation into cell cultures of human and simian origin. The human cells may include those from continuous or diploid cell lines. The simian cells shall be derived from monkey kidneys. The tissue cultures shall be observed for at least 14 days.

Suitable cell lines for these tests are Vero, LLCMK₂, HeLa, and MRC5.

In one country, the virus pool is tested for the absence of adventitious agents in cell cultures derived from the same species (but not from the same group of embryos) as that used for vaccine production.

The virus pool passes the tests if at least 80% of the cell cultures remain viable and none of the tissue cultures show evidence of the presence of adventitious agents attributable to the virus pool.

3.3.5 Tests in embryonated hen's eggs

A volume of the neutralized virus pool, equivalent to at least 100 human doses of vaccine, shall be tested in a group of embryonated
hen's eggs aged between 9 and 12 days, by inoculation of 0.5 ml per egg by the allantoic and/or amniotic route.

The virus pool passes the test if, after 7 days, at least 80% of the inoculated eggs still survive, and the allantoic/amniotic fluids fail to show the presence of any haemagglutinating agent. In addition, none of the embryos or chorioallantoic membranes shall show gross pathological changes.

In some countries, it has been found that the inoculation of the yolk sac of 6-day-old to 7-day-old embryos, with incubation for 9 days, is a sensitive method for isolating nutritionally fastidious bacteria, which may then be detected by microscopic inspection of suitably stained smears.

3.3.6 Tests in small laboratory animals

Since a group of WHO consultants considered that tests for extraneous agents in small laboratory animals do not add to the safety of vaccines (see Annex 3 of 2), national control authorities may permit manufacturers to omit such tests on the virus harvest. However, these tests remain mandatory for seed lots.

Tests in adult mice. Each of at least 10 adult mice, of 15–20 g weight, shall be inoculated intracerebrally with 0.03 ml and intraperitoneally with at least 0.5 ml of the virus pool. The mice shall be observed for at least 21 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be examined after death for evidence of viral infection, both by direct macroscopical observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional mice, which shall be observed for 21 days.

The virus pool passes the test if at least 80% of the original inoculated mice survive the observation period and if no mouse shows evidence of infection with adventitious agents attributable to the virus pool.

In certain countries the national control authority requires only a 60% survival of the original animals.

Tests in suckling mice. Each of at least 20 mice, less than 24 hours old, shall be inoculated intracerebrally with 0.01 ml and intraperitoneally with at least 0.1 ml of the virus pool. The mice shall be observed daily for at least 14 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be examined after death for evidence of viral infection, both by direct
macroscopical observation and by subinoculation of appropriate
tissue suspensions by the intracerebral and intraperitoneal routes
into at least five additional suckling mice, which shall be observed
daily for 14 days.

In addition, in some countries a blind passage is made of a
suspension of the pooled emulsified tissue (minus skin and
viscera) of all mice surviving the original 14 day test.

The virus pool passes the test if at least 80% of the original
inoculated mice survive the observation period and if no mice show
evidence of infection with adventitious agents attributable to the
virus pool.

*Tests in guinea-pigs.* The virus pool is tested for adventitious
agents by the intraperitoneal inoculation of 5.0 ml of the virus pool
into each of at least five guinea-pigs of 350–450 g weight. The
animals shall be observed for at least 42 days for signs of disease. All
guinea-pigs that die after the first 24 hours of the test or that show
signs of illness shall be examined macroscopically after death, and
their tissues shall be examined both microscopically and in tissue
culture for evidence of infection. Animals that survive the
observation period without signs of illness shall be killed and
examined in a similar manner.

The virus pool passes the test if at least 80% of the guinea-pigs
survive the observation period and if none of the animals shows
evidence of infection with any adventitious agents attributable to the
virus pool.

3.4 Clarification, and control of clarified virus pool

After successful completion of appropriate testing, the virus pool
shall be clarified by a method that will ensure removal of all intact
cells and cell debris. Microscopic observation of a smear of a
concentrated sample of the virus pool is a suitable method of
checking the efficiency of the clarification process.

3.4.1 Virus titration

The live virus content of the clarified virus pool shall be
determined by titration in cell culture against a reference preparation
of live mumps virus (see Part A, section 1.3).
3.5 Control of final bulk suspension

The final bulk suspension shall be prepared from one or more clarified virus pools obtained from substrates that satisfy the requirements of Part A, section 2; the virus pools shall pass the tests of Part A, sections 3.3.1 to 3.3.5, and 3.4.

Only stabilizers, diluents, or other substances approved by the national control authority shall be added to the vaccine. Any such substances shall have been shown by appropriate tests to have, in the amounts used, no deleterious effects on the product.

In certain countries, the national control authority may approve the addition of certain antibiotics, for example neomycin at a concentration of no more than 50 µg/ml.

The final bulk suspension should be prepared under aseptic conditions, and should be stored, until it is distributed into containers and lyophilized, in conditions shown by the manufacturer to retain the activity of the vaccine.

3.5.1 Sterility tests

Each final bulk suspension shall be tested for bacterial and mycotic sterility according to Part A, sections 5.2 and 5.3 of the Requirements for biological substances no. 6. General requirements for the sterility of biological substances (6), or by a method approved by the national control authority.

4. Filling and containers

The requirements for filling and containers given in Annex 1, Part A, section 4 of the revised Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories (1) shall apply.

Single-dose containers are recommended, except for mass immunization campaigns.
5. Control tests on final product

Samples shall be taken from each final lot for the following tests.

5.1 Identity test

An identity test for mumps virus shall be performed by appropriate methods on material from two or more individual labelled containers from each final lot.

Methods such as seroneutralization in cell culture using specific antiserum are suitable.

5.2 Virus concentration

The live virus concentration of each freeze-dried final lot shall be determined by titration in a suitable cell culture system against a reference preparation of live mumps virus (see Part A, section 1.3). The minimum acceptable virus titre per human dose shall be approved by the national control authority.

It is desirable to titrate individually the contents of five or more containers.

5.3 General safety test

Each final lot shall be tested for the absence of abnormal toxicity in mice and guinea-pigs by appropriate tests approved by the national control authority.

5.4 Inspection of final containers

Each container in each final lot shall be inspected visually and those showing abnormalities shall be discarded.

5.5 Sterility tests

Final containers shall be tested for bacterial and mycotic sterility by a method approved by the national control authority.

Many countries have standard regulations for sterility testing of the final product. Where these are not available, WHO requirements should be followed (6).
5.6 Residual moisture

The residual moisture in a representative sample of each freeze-dried lot may be determined by a method approved by the national control authority. The upper limit of the moisture content may be specified by the national control authority. Generally moisture levels of less than 2% are considered satisfactory.

5.7 Test for thermostability

Samples of each final freeze-dried vaccine shall be incubated in the dry state at 37°C for 7 days. At the end of the incubation period, heated samples shall be assayed in parallel with unheated ones and with the reference preparation; the heated vaccine should meet the minimum requirement for potency established by the national control authority. The loss in titre shall be no more than one \( \log_{10} \) unit.

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 (I) 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.

The label on the carton enclosing one or more final containers, or the leaflet accompanying the container, shall contain the following additional information:
— a statement that the vaccine meets Part A of the WHO Requirements for mumps vaccine (live);
— the nature of the preparation, i.e., the designation of the strain of mumps virus contained in the vaccine and the origin of the substrate used to prepare vaccine;
— the nature and quantity of any antibiotic present in the vaccine;
— a statement concerning the photosensitivity of the vaccine, cautioning that both lyophilized and reconstituted vaccine should be protected from light;
— a statement indicating the volume and nature of the diluent to be added to reconstitute the vaccine, and specifying that such diluent should be supplied by the manufacturer;
— the statement that after the vaccine is reconstituted it should be used without delay.

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 (1) shall apply. Shipment should be at a temperature of 8 °C or below.

10. Storage and expiry date

The statements concerning storage temperature and expiry date appearing on the label and the leaflet, as required in Part A, section 10 of the revised Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories (1), shall be based on experimental evidence and shall be submitted for approval to the national control authority.

10.1 Storage conditions

Before distribution, the manufacturer shall store lyophilized vaccine at a temperature shown by the manufacturer to be compatible with a minimal titre loss. After distribution, live mumps vaccine shall be stored at all times below 8 °C.
10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall relate to the date of the last satisfactory test to determine virus concentration, this being the date on which the test system was inoculated.

In some countries, manufacturers and national control authorities have observed that mumps vaccines continuously stored in the lyophilized state at or below –20°C do not lose potency over a period of several years. In such cases, the national control authority may allow the dating period to start when the vaccine is taken out of the frozen state, provided that a satisfactory potency test has been carried out within the preceding 12 months.

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 (1) shall apply.

The national control authority shall provide a reference preparation of live mumps virus (see Part A, section 1.3) for tests to determine virus concentration (see Part A, sections 3.3.2, 3.4.1, and 5.2), and shall specify the virus content required to achieve adequate immunization of human beings with the recommended human dose.

2. Release and certification

A vaccine 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 establishment and shall certify whether the lot of vaccine in question meets all national requirements, as well as Part A of these requirements. The certificate shall state the date of the last
satisfactory determination of virus concentration, 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 live mumps vaccine between countries.

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REFERENCES

Appendix

SUMMARY PROTOCOL FOR PRODUCTION AND TESTING OF MUMPS VACCINE (LIVE)

Based on Requirements for biological substances no. 38.
Requirements for mumps vaccine (live)

Identification of final lot

Name and address of manufacturer

No. of final lot
Date of initiation of last test for determining virus concentration
Expiry date
Proprietary name of vaccine
No. of ampoules or vials in the final lot
No. of doses in each ampoule or vial

3.1 Production control

Seed virus strain
Substrate for virus propagation
For closed colonies of birds, duration of monitoring for specific pathogens
Virus seed lot system
Reference no. of seed lot
Date(s) of satisfactory test(s) for freedom from extraneous agents

Neurovirulence test
Result of blood serum test of monkeys before inoculation

Numbers refer to the corresponding numbered sections in the text of the Requirements.
### 3.2 Production precautions

**Control cell cultures or embryonated eggs**

<table>
<thead>
<tr>
<th>Substrate used for vaccine production</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Quantity of cell suspension or no. of eggs used for control</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Period of observation of uninoculated controls</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Test for haemadsorbing or haemagglutinating viruses</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
</table>

- **Test method**
- **Results**

**Cell culture tests**

<table>
<thead>
<tr>
<th>Sample size</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Cell cultures inoculated</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Results</th>
<th>Control</th>
<th>Test</th>
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<table>
<thead>
<tr>
<th>Test for avian leukosis virus</th>
<th>Control</th>
<th>Test</th>
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<table>
<thead>
<tr>
<th>Test method</th>
<th>Control</th>
<th>Test</th>
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<table>
<thead>
<tr>
<th>Results</th>
<th>Control</th>
<th>Test</th>
</tr>
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<table>
<thead>
<tr>
<th>Other tests if done (embryonated eggs)</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Type of test(s)</th>
<th>Control</th>
<th>Test</th>
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</table>

<table>
<thead>
<tr>
<th>Results</th>
<th>Control</th>
<th>Test</th>
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</table>

### Cell substrate for vaccine production

<table>
<thead>
<tr>
<th>Antibiotics added (if used)</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
</table>

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1 In terms of TCID<sub>50</sub>, the quantity of virus suspension that will infect 50% of inoculated cell cultures.
### 3.3 Harvesting and testing the virus

**Single harvests used in virus pool**

<table>
<thead>
<tr>
<th>No. of passages from the primary seed</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference no. of harvests</td>
<td></td>
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<tr>
<td>Virus concentration (optional)</td>
<td></td>
</tr>
<tr>
<td>Sterility test</td>
<td></td>
</tr>
<tr>
<td>Dates</td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>Mycoplasma test</td>
<td></td>
</tr>
<tr>
<td>Dates</td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td></td>
</tr>
</tbody>
</table>

*Tests on virus pool for absence of contamination*

If any test had to be repeated or any abnormal result was observed, this must be specified. If necessary a separate sheet of paper can be used.

| Virus concentration (optional)       |   |
| Sterility test                       |   |
| Date                                 |   |
| Media used                           |   |
| Results                              |   |
| Mycoplasma test                      |   |
| Date                                 |   |
| Media used                           |   |
| Results                              |   |
| Test for *Mycobacterium tuberculosis*|   |
| Date                                 |   |
| Media used                           |   |
| Results                              |   |

*Tests in cell culture*

| Volume tested                        |   |
| Cell cultures used                   |   |
| Observation period                   |   |
| Results                              |   |

*Tests in embryonated hen’s eggs*

| Volume tested                        |   |
| No. of eggs used                     |   |
| Route(s) of inoculation              |   |
| Results                              |   |

**Test in adult mice**\(^1\)

| No. and weight of mice               |   |

\(^1\) May be omitted if the cell cultures used for production were derived from monitored closed colonies of animals and if permission was given by the national control authority.
Date of inoculation
Route of inoculation
Quantity inoculated
Results (survival numbers, etc.)

Test in suckling mice¹
  No. and weight of mice
  Date of inoculation
  Route of inoculation
  Quantity inoculated
  Results (survival numbers, etc.)

Test in guinea-pigs¹
  No. and weight of guinea-pigs
  Date of inoculation
  Route of inoculation
  Quantity inoculated
  Results (survival numbers, etc.)

Additional tests (depending on cells used)
  Test method used
  Results of test

3.4 Clarification, and control of clarified virus pool
Reference no.
Result of test for intact cells
Virus titre²

3.5 Control of final bulk suspension
Date of preparation

Sterility test
Date
Media used
Result

¹ May be omitted if the cell cultures used for production were derived from monitored closed colonies of animals and if permission was given by the national control authority.
² In terms of TCID₅₀, the quantity of virus suspension that will infect 50% of inoculated cell cultures.
5. Control tests on final product

5.1 Identity test
Date
Method used
Result

5.2 Virus concentration
Date
Method
Result

5.3 General safety test

Test in mice
Date
No. of mice
Volume and route
Observation period
Results (give details of deaths)

Test in guinea-pigs
Date
No. of guinea-pigs
Volume and route
Observation period
Results (give details of deaths)

5.4 Inspection of final containers
Result

5.5 Sterility tests
Date
Media used
Result
Were repeat tests necessary?
(if so, give details)

5.6 Residual moisture
Size of sample
Moisture content (%)
5.7 Test for thermostability

<table>
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<tr>
<th></th>
<th>Control (unheated)</th>
<th>Samples incubated at 37° C for 7 days</th>
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<tr>
<td>No. of containers tested</td>
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<tr>
<td>Virus concentration in each container</td>
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<tr>
<td>Mean virus titre per human dose</td>
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<tr>
<td>Mean loss in titre due to heat exposure (in log_{10} units)</td>
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<td>Reference preparation</td>
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<td>Identification</td>
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<td>Theoretical titre</td>
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<tr>
<td>Actual titre</td>
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**Internal certification**

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

I certify that lot no. ____ of mumps vaccine (live) satisfies Part A of the WHO Requirements for mumps vaccine (live).

Signature: __________________________

Name (typed): __________________________

Date: __________________________

The protocol must be accompanied by a sample of the label and a copy of the leaflet. If the vaccine is to be exported, the protocol must also be accompanied by a certificate of the national control authority stating that the given product meets national as well as WHO requirements.
Annex 8

REQUIREMENTS FOR POLIOMYELITIS VACCINE (ORAL)

(Requirements for Biological Substances No. 7)

Addendum 1986

In view of several comments by the Committee, document WHO/BS/86.1509 has been replaced by this revision, and the Requirements for poliomyelitis vaccine (oral) (revised 1982) should be modified as follows. Page numbers refer to WHO Technical Report Series, No. 687, 1983.

General considerations (page 113)

Delete lines 11 to 23.

Virus titration (page 136)

Replace paragraph 5.3 by the following:

"The poliovirus titre shall be determined as described in Part A, section 3.5.4 of these requirements. If the vaccine contains more than one poliovirus type, each type shall be titrated separately by using appropriate type-specific serum for neutralizing each of the other types present. National control authorities should specify the virus titre for a human dose. It is recommended that, when Sabin strains are used, a single human dose of trivalent oral poliomyelitis vaccine should contain approximately $10^6 (10^{5.5} \text{ to } 10^{6.5})$ infectious units of type 1, $10^5 (10^{4.5} \text{ to } 10^{5.5})$ infectious units of type 2, and $10^{5.5} (10^{5.0} \text{ to } 10^{6.0})$ infectious units of type 3.

The detailed procedures for virus titration, including the use of type-specific sera, should be approved by the national control authority."
The titres of virus of each type determined by this test should be stated on the label of each container (see Part A, section 8). To facilitate the administration of oral poliomyelitis vaccine in countries where multi-dose vials are used, it is recommended that a single dose of vaccine be contained in two drops."

**Storage conditions (page 138)**

Replace section 10.1 by the following:

"Before being distributed by the manufacturing establishment, all vaccines in bulk form or in final containers shall be kept continuously in the frozen state below –20 °C.

After distribution or issue, the vaccine in the final containers shall be stored whenever possible in a frozen state.

At peripheral centres where it is not possible to maintain low temperatures specially for this purpose, the vaccine should be kept liquid at or below +8 °C.

Limited experiments have shown that several successive freezings and thawings of oral poliomyelitis vaccines have no significant effect on titres, provided that such operations are done quickly.¹"

Annex 9

REQUIREMENTS FOR RABIES VACCINE (INACTIVATED) FOR HUMAN USE PRODUCED IN CONTINUOUS CELL LINES
(Requirements for Biological Substances No. 40)

INTRODUCTION

Although the WHO Requirements for rabies vaccine for human use (1) were revised in 1980, subsequent technical developments have necessitated the preparation of these additional requirements, which complement but do not replace the existing requirements. Specifically, continuous cell lines have been introduced in the manufacture of rabies vaccine, and vaccines produced in such cell lines have been licensed by two countries. In addition, general requirements for the characterization of continuous cell lines used for the preparation of biologicals were adopted by WHO in 1985 (2).

The following international Requirements for rabies vaccine (inactivated) for human use produced in continuous cell lines have
therefore been written in parallel with the *Requirements for rabies vaccine for human use* already published by WHO (1, 3, 4 p. 17). In drafting them, account has been taken of the opinions of consultants, of the regulations and requirements for the manufacture and control of rabies vaccine that have been formulated in several countries, and of information from both published (5) and unpublished\(^1\) reports.

**GENERAL CONSIDERATIONS**

Rabies vaccines produced in neural tissue have been in worldwide use for many years and experience has indicated that they are effective (6). However, it is generally accepted that the risk of damage to the central nervous system of recipients of vaccine prepared in the brains of adult animals is about 1 in 2000 doses administered. The risk of such adverse reactions is greatly reduced when the virus is grown in the brains of newborn animals, such as rats and mice, before the development of myelin in the brain.

Recently, highly potent and safe rabies vaccines have been produced in human diploid cells, chick embryo cells, and other primary tissue culture cells. These vaccines induce high antibody levels in non-immune subjects and the risk of adverse reactions in the central nervous system is generally believed to be extremely low.

A further development has been the production of rabies vaccines in continuous cell lines. Such production methods offer great hope for improving the quality and quantity of rabies vaccines in areas of the world where they are most needed. There is, therefore, an urgent need to introduce international requirements for these vaccines.

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 rabies vaccine, it is recommended that a clause should be included permitting modifications of the manufacturing requirements on the condition

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that it can be demonstrated, to the satisfaction of the national
control authority, that such modifications ensure a degree of safety
and a potency of the vaccine 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 rabiei* (*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 and
those in (1).

1.2 Descriptive definition

*Vaccinum rabiei* (*ad usum humanum*) is a fluid or freeze-dried
preparation of rabies “fixed” virus grown in the neural tissue of
rabbits, sheep, goats, mice, or rats, or in cell cultures including
continuous cell lines, and inactivated by a suitable method. The
preparations for human use shall satisfy all the requirements
formulated below.

The use of dried vaccines should be encouraged because they
are more stable than fluid vaccines.

1.3 International standards

The International Standard for Rabies Vaccine, established in
1983 (7, p. 15), is stored and distributed in ampoules containing
freeze-dried vaccine that has been prepared in human diploid cells
and inactivated with β-propiolactone. There is an activity of 7.8 IU
per ampoule. This standard is intended for the calibration of national preparations for use in tests of potency of rabies vaccine (see Part B, section 1)). After reconstitution, the International Standard may be stored in suitable aliquots for subsequent animal immunization, provided that the storage temperature is below $-60\, ^\circ\text{C}$ and that the period of storage is not longer than one month.

An International Standard for Rabies Immunoglobulin was established in 1984 and assigned a potency of 59 IU per ampoule ($8$, p. 17).

Administration of the International Standard to human subjects is not authorized. A national reference preparation should not be considered suitable for use in human beings unless it has been approved by the national control authority.

1.4 Terminology

Primary virus seed lot: a quantity of virus that has been processed as a single lot and has a uniform composition. It is used for the preparation of secondary virus seed lots.

Secondary virus seed lot: a seed lot prepared from the primary seed lot and no more than five passages removed from it.

Cell seed lot: a quantity of cells stored frozen at $-70\, ^\circ\text{C}$ or below in aliquots of uniform composition, 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 lot and of uniform composition. The cells are stored frozen at $-70\, ^\circ\text{C}$ or below in aliquots, one or more of which would be used for production.

In normal practice a cell seed is expanded by serial subculture up to a passage number (or population doubling, as appropriate) 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 production.

Production cell culture: a collection of cell cultures derived from one or more ampoules of the MWCB and used for the production of rabies vaccine.

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The International Standards for Rabies Vaccine, Rabies Immunoglobulin, and Anti-Rabies Serum are in the custody of the International Laboratory for Biological Standards, State Serum Institute, Copenhagen, Denmark.
Adventitious agents: microorganisms contaminating the cell line, including bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses.

Single harvest: the biological material prepared from a single production run.

Bulk material: a pool of inactivated single harvests before preparation of the final bulk. The pool may be prepared from one or more single harvests and may yield one or more final bulks.

Final bulk: the finished biological material prepared from one or more purified bulks, and present in the container from which the final containers are filled.

Filling lot (final lot): a collection of sealed final containers, filled from the same final bulk, that are homogeneous with respect to the risk of contamination during filling or drying. A filling lot therefore consists of containers that are filled in one working session and (if applicable) are dried together in the same chamber at the same time.

2. General manufacturing requirements

The general requirements for manufacturing establishments contained in the revised Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories (9, p. 11) shall apply to establishments manufacturing rabies vaccine for human use.

Rabies vaccine shall be produced by staff who have not handled other infectious microorganisms, animals, or tissue cultures in the same working day. The staff shall consist of persons who have been examined medically and found to be healthy. Steps shall be taken to ensure that all such persons in the production and control areas have been immunized against rabies and maintain an antibody titre of at least 0.5 IU per ml of serum.

Only the cell cultures approved by the national control authority for the production of rabies vaccine shall be introduced or handled in the production area.

Persons not directly concerned with the production processes, other than official representatives of the national control authority, shall not be permitted to enter the production area.

Particular attention shall be given to the recommendations contained in Part A, section 1 of the revised Requirements for biological substances no. 1. General requirements for manufacturing
establishments and control laboratories (9) regarding the training and experience of the persons in charge of production and testing and of those assigned to various positions of responsibility in the manufacturing establishment.

3. Control of source materials

The general production precautions formulated in Part A, section 3 of the revised Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories (9, p. 15) shall apply to the manufacture of rabies vaccine.

3.1 Continuous cell lines

The continuous cell lines used for the propagation of rabies virus shall be approved by and registered with the national control authority. These continuous cell lines shall be characterized according to the Requirements for continuous cell lines used for biologicals production (2).

3.1.1 Cell seed and MWCB

The utilization of a continuous cell line for the manufacture of rabies vaccines shall be based on the cell seed lot system. The maximum number of passages (or population doublings) of the cell seed shall be established by the national control authority.

3.1.2 Identity test (MWCB)

The MWCB shall be identified by a method approved by the national control authority. Such methods include biochemical tests (e.g., isoenzyme analyses), immunological tests (e.g., histocompatibility antigen assays), and cytogenetic marker tests.

3.1.3 Serum used in cell culture medium

Serum used for the propagation of cells shall be shown to be free from bacteria, fungi, and mycoplasmas, 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 the sterility of biological substances (3, p. 49), and to be free from pathogens of the species of origin of the serum, by methods approved by the national control authority (10, p. 99).

In some countries, sera are also examined for bacteriophages and endotoxin.

3.2 Virus seed

3.2.1 Strain of virus

The strain of virus used in the production of all seed lots shall be a “fixed” strain and shall be identified by historical records. The virus strain shall have been shown, to the satisfaction of the national control authority, to yield safe and immunogenic vaccines when it has been inactivated (1). In addition, the vaccine strain shall be characterized by serological tests and animal inoculation.

Each time a new secondary virus seed lot is prepared, tests shall be carried out to characterize the virus strain. Such tests shall include the titration of the virus in cell cultures and in animals of various species and ages by various routes of inoculation, and serum neutralization tests. The tests shall be approved by the national control authority, and test records shall be maintained.

Monoclonal antibodies are available that can distinguish “fixed” and “street” strains of rabies virus.

The most common production strains originate from the Pasteur strain of rabies “fixed” virus, maintained in the past in rabbits, or from a derivative of this strain.¹ Such strains should be capable of producing characteristic paralysis within 5–7 days when inoculated intracerebrally into animals such as rabbits, mice, and sheep.

3.2.2 Virus seed lot system

The preparation of rabies vaccine shall be based on the use of a virus seed lot system. The secondary virus seed lot shall be not more than five passages removed from the primary virus seed lot, which shall have been thoroughly characterized. Vaccines shall be made from the secondary seed lot without further intervening passage. Virus seed lots shall be maintained either in the dried or in the frozen

¹ The Pasteur strain is available to laboratories, on request and with approval of the relevant national control authorities, from Chief, Veterinary Public Health, World Health Organization, Geneva, Switzerland.
form and each lot shall be stored separately. If frozen, the seed shall be kept continuously at a temperature below \(-60\,^{\circ}\)C.

Seed lots shall have been shown, to the satisfaction of the national control authority, to be capable of yielding vaccine that meets all the manufacturing requirements listed here.

Each virus seed lot shall be identified as rabies virus by methods approved by the national control authority.

3.2.3 *Tests for bacteria, fungi, and mycoplasmas*

Each virus seed lot shall be tested for bacterial, mycotic, and mycoplasmal contamination by appropriate tests according to Part A, section 5.2 of the revised *Requirements for biological substances no. 6. General requirements for the sterility of biological substances* (3, p. 49).

3.2.4 *Tests for adventitious agents*

Each virus seed lot shall be tested for adventitious agents in animals as described in this section, and in cell cultures as specified in Part A, sections 4.1.1 and 4.1.2. For these tests the virus shall first be neutralized by a specific anti-rabies serum.

The individual tests on the seed virus should be designed to satisfy the requirements of the national control authority. The anti-rabies serum should be free of known adventitious viruses.

*Tests in suckling mice.* A sample of the virus suspension shall be tested for the presence of adventitious agents pathogenic to mice by intracerebral inoculation of 0.01 ml and intraperitoneal inoculation of at least 0.1 ml into at least 10 suckling mice. The mice shall be less than 24 hours old and originate from more than one litter. They shall be observed daily for at least 14 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be examined for evidence of viral infection; this shall be done macroscopically and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional suckling mice, which shall be observed daily for 14 days.

In some countries, in addition, a blind passage is made of a suspension of the pooled emulsified tissue (minus skin and viscera) of all mice surviving the original 14 day test.

The virus seed passes the test if at least 80% of the mice originally inoculated remain healthy and survive the observation period, and
if none of the mice shows evidence of infection with any adventitious agent attributable to the virus seed.

Tests in adult mice. A sample of the virus suspension shall be tested for the presence of adventitious agents pathogenic to mice by intracerebral inoculation of 0.03 ml, intraperitoneal inoculation of at least 0.25 ml, and inoculation of 0.01 ml into the footpad in at least 20 adult mice, each weighing 15–20 g. The mice shall be observed for at least four weeks. All mice that die after the first 24 hours of the test or that show signs of illness shall be examined for evidence of viral infection; this shall be done macroscopically by direct observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional mice, which shall be observed for three weeks. The virus seed passes the test if at least 80% of the inoculated animals remain healthy and survive the observation period, and if none of the mice shows evidence of infection with any adventitious agent attributable to the virus seed.

Tests in guinea-pigs. A sample of the virus suspension shall be tested for the presence of Mycobacterium tuberculosis and other adventitious agents by intraperitoneal inoculation of 5.0 ml into each of at least five guinea-pigs, each weighing 350–500 g. The animals shall be observed for at least 42 days. All guinea-pigs that die after the first 24 hours of the test or that show signs of illness shall be examined macroscopically, and their tissues shall be examined both microscopically and in tissue culture for evidence of infection with Mycobacterium tuberculosis. Animals that survive the observation period shall also be examined macroscopically by autopsy for evidence of infection with Mycobacterium tuberculosis.

The virus seed passes the test if at least 80% of the inoculated guinea-pigs remain healthy and survive the observation period, and if none of the animals shows evidence of infection with Mycobacterium tuberculosis or any other adventitious agent attributable to the virus seed.

The test may be done with the pellet from 100 ml of centrifuged bulk suspension of virus resuspended in 30 ml of the supernatant. It is desirable to record the rectal temperature of the animals daily during the first three weeks. In some countries an in vitro test approved by the national control authority is used instead of the test in guinea-pigs.

Tests in cell cultures. The neutralized seed virus shall be tested for freedom from adventitious viruses in three sensitive cell culture
systems: 

(a) the continuous cell line used for production, 
(b) a different continuous cell line, and 
(c) human diploid cells.

Ten millilitres of the neutralized seed virus shall be inoculated into each cell system and the cells incubated at 35–37°C for 14 days. At day 7 and day 14 a subculture shall be made of 10% of the pooled supernatant fluids and each of the subcultures incubated for 14 days.

The cells shall be observed microscopically for cytopathic changes. At the end of the observation period, the cultures or fluids shall be tested for haemadsorbing viruses and other adventitious agents as specified in Part A, sections 4.1.1 and 4.1.2. 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 observation period. For the seed virus to be satisfactory, no cytopathic changes or adventitious agents shall be detected. Control cell cultures shall be included in the tests.

3.2.5 Virus content

A titration of the virus content of each seed lot shall be made. Such titrations may be done either in cell culture (plaque method) or by the intracerebral inoculation of mice. If mice are used, they should be inoculated with 0.03 ml quantities of suitable dilutions of the virus seed lot. Although the previously recommended end-point for this in vivo titration was death of the mice, it is reasonable instead to use clinical signs of paralysis as the end-point and to kill the animals when they reach this stage. Mice that show no signs of paralysis should be observed for 14 days. Virus activity should be such that all mice inoculated with virus suspension diluted by a factor of up to 10^4 develop clinical paralysis.

4. Control of vaccine production

4.1 Control of cell cultures

Penicillin and other β-lactams shall not be used at any stage of manufacture.

Minimal concentrations of other suitable antibiotics, such as neomycin, may be used where approved by the national control authority.

At least 5% of the cell suspension (not less than 500 ml), at the concentration employed for seeding vaccine production cultures, shall be used to prepare control cultures.
In countries in which large-scale production methods are used, the national control authority should determine the size of the cell sample to be examined and the control methods to be applied.

The control cell cultures shall be treated in a similar way to the production cell cultures, but they shall remain uninoculated so that they can be used for the detection of extraneous viruses.

The control cell cultures shall be incubated under the same conditions as the inoculated cultures for two weeks or until the last harvest of virus from 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 shall have had to be discarded because of accidental contamination or damage.

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

4.1.1 Tests for haemadsorbing viruses

At the end of the observation periods, 25% of the control cells shall be tested for the presence of haemadsorbing viruses by using guinea-pig erythrocytes. If the guinea-pig erythrocytes 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 should also be done with erythrocytes from other species, including human beings (blood group O), monkeys, and chickens (or other avian species). The results of all tests should be noted after incubation of the erythrocytes with the cultured cells for 45 minutes at 0–4°C and again after a further incubation for 30 minutes at 20–25°C. For the test with monkey erythrocytes, the results should be noted a third time, after a final incubation for 30 minutes at 34–37°C.

4.1.2 Tests for other adventitious agents

At the end of the observation period a sample of the pooled fluids from each group of control cultures shall be tested for adventitious agents. Ten millilitres of each pool shall be tested in the same cells,
but not the same batch of cells, as those used for virus production, 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 35–37°C and shall be observed for 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 test period.

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

4.1.3 Identity test (cell line)

The species of origin of the cells used for production shall be identified by tests approved by the national control authority.

Suitable tests are isoenzyme analysis, immunological tests, and karyotype analysis.

4.2 Control of single virus harvests and purified bulk material

4.2.1 Sterility tests or single virus harvests

A sample removed from each virus harvest shall be tested for bacterial and fungal contamination by appropriate methods, according to Part A, section 5.2 of the revised Requirements for biological substances no. 6. General requirements for the sterility of biological substances (3, p. 48). Any virus harvest in which contamination is detected shall be discarded.

4.2.2 Pooling of single virus harvests

Only virus harvests satisfying the requirements for sterility given in Part A, section 4.2.1 of these requirements shall be pooled.

In some countries the viral harvests are tested for the presence of extraneous viruses after neutralization of the rabies virus (see Part A, section 3.2.4). Such tests should be approved by the national control authority.

4.2.3 Purification of virus harvests

One or more single harvests grown in a continuous cell line may be purified at a time. The process shall be approved by the national control authority and shall be shown to give consistent results.
In some countries the virus is purified by continuous density-gradient centrifugation.

The purified bulk material shall be tested to determine the degree of purity achieved. The tests shall be approved by the national control authority.

Acceptable limits of cellular DNA per dose of the final product should be determined by the national control authority, taking into consideration the effect of the inactivation procedure on the biological activity of DNA as well as prior experience with DNA levels in rabies and other vaccines produced in various systems.

The WHO Study Group on Biologicals concluded that the probability of risk associated with heterogeneous contaminating DNA in a product derived from a continuous cell line is negligible when the amount of such DNA is 100 pg or less in a single dose given parenterally (11).

4.2.4 Animal serum

The concentration of animal serum in the purified bulk shall be not more than 1 μl/l.

In some countries, tests are carried out to estimate the amount of residual animal serum in the final vaccine.

4.2.5 Inactivation procedure

Methods and agents. The methods and agents used for inactivation shall be approved by the national control authority. Inactivation shall be commenced immediately after purification. The method used shall be demonstrated to be consistently effective in the hands of the manufacturer. The total inactivation time used must be double the period required to inactivate the virus completely.

When an inactivation curve is established, virus samples shall not be stored but shall be inoculated immediately into mice to test the effectiveness of the inactivation process.

Various methods for inactivating rabies virus have been used with success. The concentration of the inactivating chemical, the temperature, and the length of time necessary for inactivation must be established for the particular type of vaccine being manufactured. Satisfactory vaccines may be prepared by treating virus suspensions from tissue culture at 70°C with β-propiolactone at a dilution of 1:3500 to 1:5000 for 24 h, or until inactivation is complete, as demonstrated by the results of the test for effective inactivation specified below.
Rabies vaccines may be freeze-dried. For the best results, the time between inactivation and starting the freeze-drying cycle should be kept to a minimum.

Tests for effective inactivation. Each purified bulk material shall be tested in mice for effective inactivation of the virus before the addition of preservatives and other substances. The test shall be approved by the national control authority.

The test should be performed with undiluted purified bulk material injected intracerebrally into at least 20 mice, each weighing between 15 and 20 g. In some countries tests are also done in rabbits or guinea-pigs.

The rabies virus amplification test (5), or another test of similar sensitivity, shall be performed in the cell culture used for vaccine production, to test for the presence of live virus.

The rabies virus amplification test shall be done as follows. Not more than four days after inactivation, at least 25 ml of bulk vaccine corresponding to 25 human doses shall be inoculated on five cell cultures of the type used for vaccine production, or a type of at least equal sensitivity. At least 3 cm² of cell sheet shall be used per millilitre of vaccine. After adsorption of the inoculum for 90 minutes, medium shall be added such that the ratio of medium to vaccine is not more than 1:3. The cultures shall be observed for at least 21 days. Five millilitres of each culture fluid shall be pooled on days 14 and 21 and 0.03 ml of this pool shall be inoculated immediately intracerebrally into each of 20 weanling mice of 12–15 g. These mice shall be observed for 14 days. Any symptoms caused by rabies virus shall be confirmed by the immunofluorescence assay. At the end of the observation period, no cytopathic effects should be detected.

The bulk material passes the test if the product is shown, to the satisfaction of the national control authority, to be free from residual live virus.

4.3 Preparation and control of final bulk

4.3.1 Preservatives and other substances added

In preparing the final bulk, only preservatives and other substances approved by the national control authority shall be added. Such substances shall have been shown by appropriate tests not to impair the safety or effectiveness of the product in the amounts used.
If β-propiolactone has been used for inactivation, the procedure shall be such that this chemical is not detectable in the final bulk. The test used for β-propiolactone shall be approved by the national control authority.

No antibiotics shall be added to rabies vaccine for human use after the virus has been harvested.

4.3.2 Antigen content of the final bulk

The single radial immunodiffusion test has been shown to be useful for determining the antigen content of the final bulk.

4.3.3 Sterility tests

Each final bulk shall be tested for sterility according to Part A, section 5.2 of the revised Requirements for biological substances no. 6. General requirements for the sterility of biological substances (3, p. 48).

5. Filling and containers

The requirements concerning filling and containers given in Part A, section 4 of the revised Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories (9, p. 16) shall apply, with the addition of the following directives.

Containers of dried vaccine shall be hermetically sealed under vacuum or after filling with pure, dry, oxygen-free nitrogen or any other gas not deleterious to the vaccine. All containers shall be tested for leaks and all defective containers shall be discarded.

Generally only single-dose containers are used.

6. Control tests on final product

6.1 Identity test

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

The test for potency described in Part A, section 6.5 may serve as an identity test.
6.2 Sterility test

Each filling lot shall be tested for bacterial and mycotic sterility according to Part A, section 5.2 of the revised Requirements for biological substances no. 6. General requirements for the sterility of biological substances (3, p. 48).

6.3 Innocuity tests

Each filling lot shall be tested for abnormal toxicity by appropriate tests in mice and guinea-pigs, using parenteral injections. The test procedures shall be approved by the national control authority.

6.4 Antigen content

The single radial immunodiffusion test has been shown to be useful for determining the antigen content of the final lot.

6.5 Potency test of vaccine in final containers

The potency of each final lot shall be determined. Before being tested, dried vaccine shall be reconstituted to the form in which it is to be used in human beings.

The test shall be one in which mice are immunized and subsequently challenged with rabies virus. Each final lot shall be tested in parallel with a reference vaccine. The challenge strain, reference vaccine, and test procedure shall be approved by the national control authority (see Part B, section 1).

Reproducibility of the test depends in part on the strain of rabies virus used for challenge and its maintenance in a large homogeneous working pool kept below -60°C. The strain of mice may also affect reproducibility.

The National Institutes of Health test (12) is a suitable test. A standard preparation with an activity calibrated in international units by comparison with the International Standard for Rabies Vaccine shall be included in each test. The potency of the vaccine in international units can then be determined by comparing its activity with that of the standard.

The potency should be at least 2.5 IU per single human dose, calculated as the geometric mean of potency values found in two or more tests.

1 A suitable challenge strain, CVS, is available to laboratories, on request and with the approval of the relevant national authorities, from the Chief, Veterinary Public Health, World Health Organization, Geneva, Switzerland.
6.6 Stability test

The method of production shall yield a stable vaccine, as shown by an accelerated degradation test approved by the national control authority.

In some countries stability is ascertained by testing samples throughout the shelf-life of the vaccine. The test for potency described in Part A, section 6.5 is suitable for checking stability after the storage of samples for four weeks at 37°C. The lot passes the test if it retains minimum potency, as defined in Part A, section 6.5. In some countries each lot of vaccine must be subjected to the stability test, whereas in others the test is required only for the first lots that are licensed, to show consistency of production.

6.7 Residual moisture test on freeze-dried vaccine

The residual moisture in a representative sample of each freeze-dried lot may be determined by a method approved by the national control authority. The upper limit of moisture content shall be specified by the national control authority. Generally, moisture levels of less than 2% are considered satisfactory.

6.8 Inspection of final containers

Each container in each filling lot shall be inspected, and those showing abnormalities shall be discarded.

6.9 Test for pyrogenic substances

Each final lot shall be tested for pyrogenic substances. The test shall be approved by the national control authority.

6.10 Animal serum

The concentration of animal serum in the final product shall be not more than 1 µl/l.

7. 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 (9, p. 17) shall apply.

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8. Samples

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* (9, p. 18) shall apply.

9. Labelling

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* (9, p. 18) shall apply, with the addition of the following directive.

The leaflet accompanying the package shall include the following information:

—the continuous cell line in which the vaccine was prepared;
—the method used for inactivating the virus; and
—if the vaccine is in the dried form, a statement that, after its reconstitution, it shall be used immediately unless data are provided to show that it may be stored for a limited time without loss of potency.

10. 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* (9, p. 18) shall apply.

11. Storage and expiry date

The requirements given in Part A, section 10 of the revised *Requirements for biological substances no. 1. General requirements for manufacturing establishments and control laboratories* (9, p. 19) shall apply.
11.1 Storage conditions
Rabies vaccine shall be stored at a temperature of $5 \pm 3 \, ^{\circ}C$.

11.2 Expiry date
The expiry date shall be based on data submitted by the manufacturer and shall be determined by the national control authority.

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* (9, p. 19) shall apply.

The national control authority shall approve the strain of rabies virus used in the production of vaccine.

The national control authority shall provide or approve the strain for challenge and the standard for use in the potency test (Part A, section 6.5).

2. Release and certification
A vaccine lot 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 establishment and shall certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these 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 rabies vaccine between countries.
AUTHORS

The Requirements for rabies vaccine (inactivated) for human use produced in continuous cell lines were prepared by the following WHO staff members:

Dr V.P. Grachev, Biologicals, WHO, Geneva, Switzerland
Dr J.C. Petricciani, Chief, Biologicals, WHO, Geneva, Switzerland

ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice, and for supplying additional data relevant to these Requirements:

Dr A.J. Beale, Wellcome Research Laboratories, Beckenham, Kent, England
Mr I. Davidson, Central Veterinary Laboratory, Weybridge, Surrey, England
Dr J.G. Debbie, Veterinary Public Health, WHO, Geneva, Switzerland
Dr M. Ferguson, Division of Viral Products, National Institute for Biological Standards and Control, London, England
Dr J. Furesz, Director, Bureau of Biologics, Drugs Directorate, Ottawa, Ontario, Canada
Dr C. Guthrie, Production Director, Commonwealth Serum Laboratories, Parkville, Victoria, Australia
Mr P. Lemoine, Institute of Hygiene and Epidemiology, Brussels, Belgium
Mr J. Lyng, Head, Laboratory for Biological Standardization, State Serum Institute, Copenhagen, Denmark
Dr P. Minor, Division of Viral Products, National Institute for Biological Standards and Control, London, England
Dr B. Montagnon, Mérites Institute, Charbonnières-les-Bains, France
Dr R. Netter, Director General, Laboratoire National de la Santé, Ministère des Affaires Sociales et de la Solidarité Nationale, Paris, France
Professor E.J. Ruitenberg, Director, State Institute for Public Health, Bilthoven, Netherlands
Dr W. Schneider, Paul Ehrlich Institute, Frankfurt am Main, Federal Republic of Germany
Dr P. Sizaret, Biologicals, WHO, Geneva, Switzerland
Dr J.J. Walsh, Quality Assurance and Animal Services Director, Commonwealth Serum Laboratories, Parkville, Victoria, Australia

REFERENCES


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Appendix

SUMMARY PROTOCOL FOR PRODUCTION AND TESTING OF RABIES VACCINE (HUMAN)

Based on Requirements for biological substances no. 40.
Requirements for rabies vaccine (inactivated) for human use produced in continuous cell lines

<table>
<thead>
<tr>
<th>Identification of final lot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name and address of manufacturer</td>
</tr>
<tr>
<td>Lot number of vaccine</td>
</tr>
<tr>
<td>Date of manufacture of final lot</td>
</tr>
<tr>
<td>Expiry date</td>
</tr>
<tr>
<td>Total volume of final lot</td>
</tr>
</tbody>
</table>

3.1 Control of source materials

3.1 Continuous cell lines

<table>
<thead>
<tr>
<th>Name and identification of continuous cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell seed and MWCB</td>
</tr>
<tr>
<td>Origin and short history of cell seed</td>
</tr>
<tr>
<td>Authority that approved cell seed</td>
</tr>
<tr>
<td>Date the MWCB was established</td>
</tr>
<tr>
<td>Quantity of cell stored</td>
</tr>
<tr>
<td>The passage level of the MWCB</td>
</tr>
<tr>
<td>Storage conditions</td>
</tr>
<tr>
<td>Percentage of all MWCB ampoules tested</td>
</tr>
</tbody>
</table>

Identity test (MWCB)

<table>
<thead>
<tr>
<th>Method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results</td>
</tr>
</tbody>
</table>

¹ Numbers refer to the corresponding numbered sections in the text of the Requirements.
**Serum used in cell culture medium**

- Origin of serum used
- Tests performed on serum
- Results

### 3.2 Virus seed

#### Strain of virus

- Name and short description of history, origin, process of attenuation, and adaptation
- Date of preparation of primary virus seed lot
- Number of passages between isolation and primary seed
- Date of preparation of secondary virus seed lot
- Number of passages between primary and secondary seed

#### Virus seed lot system

- Number of subcultures between secondary virus seed lot and production
- Method for identification of the virus seed lot
- Results

#### Tests for bacteria, fungi, and mycoplasmas

- Methods used
- Results

#### Tests for adventitious agents

- Tests in suckling mice
  - No. of animals tested
  - Quantity injected
  - Observation period
  - Results (survival numbers, etc.)
- Tests in adult mice
  - No. of animals tested
  - Quantity injected
  - Observation period
  - Results (survival numbers, etc.)
Tests in guinea-pigs
  No. of animals tested
  Quantity injected
  Observation period
  Results (survival numbers, etc.)

Tests in cell cultures
  Methods
  Results

Virus content
  Method of titration
  Results

4. Control of vaccine production

4.1 Control of cell cultures

Tests for haemadsorbing viruses
  Method
  Results

Tests for other adventitious agents
  Method
  Results

Identity test (cell line)
  Method
  Results

4.2 Control of single virus harvests and purified bulk material

Sterility tests of single virus harvests
  Have all the harvests included been tested for sterility?
  Results of these tests

Pooling of single virus harvests
  No. of viral harvests included
  Date of pooling
### Purification of virus harvests

**Method**

**Degree of purity achieved**

### Animal serum in purified bulk

**Method**

**Results (concentration)**

### Inactivation procedure

**Method**

**Date**

**Temperature**

Tests for effective inactivation

- Volume and concentration of bulk material injected
- No. of mice injected
- Weight of mice
- Duration of observation
- Other animals (if used)
- Results of tests

Rabies virus amplification test

- Amount of vaccine tested (ml)
- Results

### 4.3 Preparation and control of final bulk

**Preservatives and other substances added**

- Concentration of phenol (if used)
- Other preservatives (type and concentration)
- Other substances added

**Antigen content of the final bulk**

**Method**

**Results**

**Sterility tests**

**Date of test**

**Result**

191
Other tests (chemical, biochemical)

Type of test
Result

5. Filling and containers

Date of filling
Quantity of containers
Volume of vaccine per container

Control for defective vials
Methods
Results

6. Control tests on final product

6.1 Identity test
Method
Result

6.2 Sterility test
No. of containers examined
Method
Date at start of test
Date at end of test
Result

6.3 Innocuity tests

<table>
<thead>
<tr>
<th>Mice</th>
<th>Guinea-pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td></td>
</tr>
<tr>
<td>Route of injection</td>
<td></td>
</tr>
<tr>
<td>Volume of injection</td>
<td></td>
</tr>
<tr>
<td>Date of injection</td>
<td></td>
</tr>
<tr>
<td>Date of end of test</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
</tr>
</tbody>
</table>

6.4 Antigen content
Type of test
Result

192
### 6.5 Potency test of vaccine in final containers

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of test</td>
<td></td>
</tr>
<tr>
<td>Date of immunization of mice</td>
<td></td>
</tr>
<tr>
<td>Reference vaccine (potency)</td>
<td></td>
</tr>
<tr>
<td>Challenge strain</td>
<td></td>
</tr>
<tr>
<td>Date of challenge</td>
<td></td>
</tr>
<tr>
<td>ED_{50} test vaccine(^1)</td>
<td></td>
</tr>
<tr>
<td>ED_{50} reference vaccine(^1)</td>
<td></td>
</tr>
<tr>
<td>Calculated IU/single human dose</td>
<td></td>
</tr>
<tr>
<td>Confidence limits</td>
<td></td>
</tr>
<tr>
<td>Results of other potency tests</td>
<td></td>
</tr>
</tbody>
</table>

### 6.6 Stability test

<table>
<thead>
<tr>
<th>Duration and temperature of incubation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Result</td>
<td></td>
</tr>
</tbody>
</table>

### 6.7 Residual moisture test on freeze-dried vaccine

<table>
<thead>
<tr>
<th>Method used</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Result</td>
<td></td>
</tr>
</tbody>
</table>

### 6.8 Inspection of final containers

<table>
<thead>
<tr>
<th>Result</th>
<th></th>
</tr>
</thead>
</table>

### 6.9 Test for pyrogenic substances

<table>
<thead>
<tr>
<th>Method</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Results</td>
<td></td>
</tr>
</tbody>
</table>

### 6.10 Animal serum in final product

<table>
<thead>
<tr>
<th>Method</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Results (concentration)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) \text{ED}_{50}: \text{quantity of vaccine that protects 50\% of animals against infection with the challenge strain.}
Internal certification

Certification by person taking overall responsibility for production of the vaccine

I certify that lot no. ______ of rabies vaccine satisfies Part A of the WHO Requirements for rabies vaccine (inactivated) for human use produced in continuous cell lines.

Signature
Name (typed)
Date

The protocol must be accompanied by a sample of the label and a copy of the leaflet.

Release certification by the national control authority

Whenever rabies vaccines produced in continuous cell lines are to be exported, they should be accompanied by a release certificate from the national control authority.

Sample release certificate

I hereby certify that batch no. ______ of rabies vaccine produced by (name of producer) in continuous cell lines meets all national requirements as well as Part A of the WHO Requirements for rabies vaccine (inactivated) for human use produced in continuous cell lines.

The date of the last satisfactory potency test carried out by the national control authority is ______.

The final lot has been released by us under no. ______
The number appearing on the label of the containers is ______.

Signature
Name (typed)
Date
Annex 10

BIOLOGICAL SUBSTANCES: INTERNATIONAL STANDARDS, REFERENCE PREPARATIONS, AND REFERENCE REAGENTS

The lists 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 are issued as a separate publication.1 Copies may be obtained from the agents shown on the back cover of this report, or they may be ordered from: World Health Organization, Distribution and Sales Service, 1211 Geneva 27, Switzerland.

At its thirty-seventh meeting the Expert Committee made the following changes to the previous list.

Additions

Antibiotics

Kanamycin 10 345 IU/ampoule First International Standard 1986

(This substance is held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts EN6 3QG, England.)

Antigens

Birch (Betula verrucosa) 100 000 IU/ampoule First International Standard 1986
pollen extract

Dog (Canis domesticus) 100 000 IU/ampoule First International Standard 1986
hair and dander extract

(These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts EN6 3QG, England.)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuberculin, purified protein derivative (PPD), bovine</td>
<td>58 500 IU/ampoule</td>
<td>First International Standard 1986</td>
</tr>
<tr>
<td>Blood coagulation factors II, VII, IX, and X, in human plasma</td>
<td>0.83, 0.91, 0.80, 0.81 IU/ampoule, respectively</td>
<td>First International Standards 1986</td>
</tr>
<tr>
<td>C-reactive protein, human</td>
<td>0.049 IU/ampoule</td>
<td>First International Standard 1986</td>
</tr>
<tr>
<td>Heparin, low molecular weight</td>
<td>1680 IU/ampoule</td>
<td>First International Standard 1986</td>
</tr>
</tbody>
</table>

(These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts EN6 3QG, England.)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chorionic gonadotrophin, human</td>
<td>650 IU/ampoule</td>
<td>Third International Standard 1986</td>
</tr>
<tr>
<td>Eclatron</td>
<td>15 IU/ampoule</td>
<td>First International Standard 1985</td>
</tr>
<tr>
<td>Follicle stimulating hormone, pituitary, human</td>
<td>80 IU/ampoule</td>
<td>First International Standard 1986</td>
</tr>
<tr>
<td>Insulin, bovine</td>
<td>0.03891 mg/IU</td>
<td>First International Standard 1986</td>
</tr>
<tr>
<td>Insulin, human</td>
<td>0.03846 mg/IU</td>
<td>First International Standard 1986</td>
</tr>
<tr>
<td>Insulin, porcine</td>
<td>0.03846 mg/IU</td>
<td>First International Standard 1986</td>
</tr>
<tr>
<td>Insulin, C-peptide, human</td>
<td>10 µg/ampoule</td>
<td>First International Reference Reagent 1986</td>
</tr>
<tr>
<td>Parathyroid hormone, bovine, for bioassay</td>
<td>39 IU/ampoule</td>
<td>First International Standard 1985</td>
</tr>
</tbody>
</table>
Proinsulin, bovine  25 μg/ampoule  First International Reference Reagent 1986
Proinsulin, human  6 μg/ampoule  First International Reference Reagent 1986
Proinsulin, porcine  20 μg/ampoule  First International Reference Reagent 1986

(These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts EN6 3QG, England.)

**Miscellaneous**

Endotoxin, for *Limulus* gelation tests  14 000 IU/ampoule  First International Standard 1986
Hepatitis B vaccine, plasma-derived, for immunogenicity studies  μg/ampoule not specified  First International Reference Reagent 1986

(These preparations are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts EN6 3QG, England.)

**Discontinued**

Chorionic gonadotrophin, human, for immunoassay  Not applicable  First International Reference Preparation 1975

(This preparation has become the third International Standard for Human Chorionic Gonadotrophin and is held and distributed by the National Institute for Biological Standards and Control, Potters Bar, Herts EN6 3QG, England.)
Anti-rubella virus (M-33 strain)  Not applicable  First International Reference Reagent 1983

Rubella virus (M-33 strain)  Not applicable  First International Reference Reagent 1983

(These two preparations are still distributed by the State Serum Institute, 80 Amager Boulevard, 2300 Copenhagen S, Denmark, through Microbiology and Immunology Support Services, WHO, Geneva, Switzerland.)

Insulin, bovine and porcine, for bioassay 24 IU/mg  Fourth International Standard 1958
Annex 11

REQUIREMENTS FOR BIOLOGICAL SUBSTANCES
AND OTHER SETS OF RECOMMENDATIONS

The specification of requirements to be fulfilled by preparations of biological substances is necessary in order to ensure that these products are safe, reliable, and potent prophylactic or therapeutic agents. International recommendations on requirements are intended to facilitate the exchange of biological substances between different countries and to provide guidance to workers responsible for the production of these substances as well as to others who may have to decide upon appropriate methods of assay and control.

Recommended requirements and sets of recommendations concerned with biological substances are formulated by international groups of experts and are published in the Technical Report Series of the World Health Organization, as listed here.

<table>
<thead>
<tr>
<th>No.</th>
<th>Year of publication</th>
<th>Requirements for Biological Substances:</th>
</tr>
</thead>
<tbody>
<tr>
<td>178</td>
<td>1959</td>
<td>1. General Requirements for Manufacturing Establishments and Control Laboratories</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Requirements for Poliomyelitis Vaccine (Inactivated)</td>
</tr>
<tr>
<td>179</td>
<td>1959</td>
<td>3. Requirements for Yellow Fever Vaccine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Requirements for Cholera Vaccine</td>
</tr>
<tr>
<td>180</td>
<td>1959</td>
<td>5. Requirements for Smallpox Vaccine</td>
</tr>
<tr>
<td>200</td>
<td>1960</td>
<td>Requirements for Biological Substances:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6. General Requirements for the Sterility of Biological Substances</td>
</tr>
<tr>
<td>237</td>
<td>1962</td>
<td>Requirements for Biological Substances:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7. Requirements for Poliomyelitis Vaccine (Oral)</td>
</tr>
<tr>
<td>274</td>
<td>1964</td>
<td>WHO Expert Committee on Biological Standardization:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8. Requirements for Pertussis Vaccine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate</td>
</tr>
<tr>
<td>293</td>
<td>1964</td>
<td>WHO Expert Committee on Biological Standardization:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10. Requirements for Diphtheria Toxoid and Tetanus Toxoid</td>
</tr>
<tr>
<td>323</td>
<td>1966</td>
<td>WHO Expert Group:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Requirements for Biological Substances (Revised 1965):</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1. General Requirements for Manufacturing Establishments and Control Laboratories</td>
</tr>
</tbody>
</table>

* Replaced by revised Requirements.
2. Requirements for Poliomyelitis Vaccine (Inactivated)
5. Requirements for Smallpox Vaccine
7. Requirements for Poliomyelitis Vaccine (Oral)

WHO Expert Committee on Biological Standardization:

11. Requirements for Dried BCG Vaccine
12. Requirements for Measles Vaccine (Live) and Measles Vaccine (Inactivated)

WHO Expert Committee on Biological Standardization:
9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate (Revisions adopted 1966)
13. Requirements for Anthrax Spore Vaccine (Live—for Veterinary Use)
14. Requirements for Human Immunoglobulin
15. Requirements for Typhoid Vaccine

WHO Expert Committee on Biological Standardization:
16. Requirements for Tuberculins
17. Requirements for Inactivated Influenza Vaccine

WHO Expert Committee on Biological Standardization:
4. Requirements for Cholera Vaccine (Revised 1968)
18. Requirements for Immune Sera of Animal Origin

WHO Expert Committee on Biological Standardization:
19. Requirements for Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live)
20. Requirements for Brucella abortus Strain 19 Vaccine (Live—for Veterinary Use)

WHO Expert Committee on Biological Standardization:
* Development of a National Control Laboratory for Biological Substances (A guide to the provision of technical facilities)

WHO Expert Committee on Biological Standardization:
21. Requirements for Snake Antivenins

WHO Expert Committee on Biological Standardization:
7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1971)

WHO Expert Committee on Biological Standardization:
4. Requirements for Cholera Vaccine (Revised 1968) (Addendum 1973)
6. General Requirements for the Sterility of Biological Substances (Revised 1973)
17. Requirements for Inactivated Influenza Vaccine (Addendum 1973)
22. Requirements for Rabies Vaccine for Human Use

* Replaced by revised Requirements.
† Refer also to subsequent Addendum.
WHO Expert Committee on Biological Standardization:
Recommendations for the Assessment of Binding-Assay Systems
(Including Immunoassay and Receptor Assay Systems) for Human
Hormones and their Binding Proteins (A guide to the formulation
of requirements for reagents and assay kits for the above assays and
notes on cytochemical bioassay systems)
Development of national assay services for hormones and other
substances in community health care

WHO Expert Committee on Biological Standardization:
3. Requirements for Yellow Fever Vaccine (Revised 1975)
20. Requirements for Brucella abortus Strain 19 Vaccine (Live—for
Veterinary Use) (Specification of tests used in the Requirements
(Addendum 1975)
† 23. Requirements for Meningococcal Polysaccharide Vaccine

WHO Expert Committee on Biological Standardization:
† 23. Requirements for Meningococcal Polysaccharide Vaccine
(Addendum 1976)
† 24. Requirements for Rubella Vaccine (Live)
25. Requirements for Brucella melitensis Strain Rev. 1 Vaccine (Live
—for Veterinary Use)
† 26. Requirements for Antibiotic Susceptibility Tests. I. Agar Diffusion
Tests using Antibiotic Susceptibility Discs

WHO Expert Committee on Biological Standardization:
* 17. Requirements for Inactivated Influenza Vaccine (Addendum 1977)
† 23. Requirements for Meningococcal Polysaccharide Vaccine
(Addendum 1977, incorporating Addendum 1976)
27. Requirements for the Collection, Processing, and Quality Control
of Human Blood and Blood Products

WHO Expert Committee on Biological Standardization:
* Guidelines for the Preparation and Establishment of Reference
Materials for Biological Substances

WHO Expert Committee on Biological Standardization
† 8 & 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine,
Tetanus Toxoid, and Combined Vaccines (Revised 1978)
* 11. Requirements for Dried BCG Vaccine (Revised 1978)
17. Requirements for Influenza Vaccine (Inactivated) (Revised 1978)
28. Requirements for Influenza Vaccine (Live)

WHO Expert Committee on Biological Standardization:
* 7. Requirements for Poliomyelitis Vaccine (Oral) (Addendum 1980)
† 8 & 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine,
Tetanus Toxoid, and Combined Vaccines (Addendum 1980)

* Replaced by revised Requirements.
† Refer also to subsequent Addendum.
22. Requirements for Rabies Vaccine for Human Use (Revised 1980)
23. Requirements for Meningococcal Polysaccharide Vaccine
(Addendum 1980)
29. Requirements for Rabies Vaccine for Veterinary Use
* 31. Requirements for Hepatitis B Vaccine
* 26. Requirements for Antibiotic Susceptibility Tests (Suggested
changes 1980)
24. Requirements for Rubella Vaccine (Live) (Addendum 1980)
* 30. Requirements for Thromboplastins and Plasma used to Control
Oral Anticoagulant Therapy
Guidelines for Quality Assessment of Antitumour Antibiotics
The National Control of Vaccines and Sera
Requirements for Immunoassay Kits
Procedure for Approval by WHO of Yellow Fever Vaccines in Con-
nection with the Issue of International Vaccination Certificates

673 1982 WHO Expert Committee on Biological Standardization:
† 2. Requirements for Poliomyelitis Vaccines (Inactivated) (Revised
1981)
† 8 & 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine,
Tetanus Toxoid, and Combined Vaccines (Addendum 1981)
† 26. Requirements for Antimicrobial Susceptibility Tests (Revised 1981)
32. Requirements for Rift Valley Fever Vaccine
A Review of Tests on Virus Vaccines

687 1983 WHO Expert Committee on Biological Standardization:
† 7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1982)
30. Requirements for Thromboplastins and Plasma used to Control
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33. Requirements for Louse-Borne Human Typhus Vaccine (Live)
The Standardization of Interferons
† 26. Requirements for Antimicrobial Susceptibility Tests (Addendum
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700 1984 WHO Expert Committee on Biological Standardization:
† 8 & 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine,
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35. Requirements for Rift Valley Fever Vaccine (Live, Attenuated) for
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34. Requirements for Typhoid Vaccine (Live Attenuated, Ty 21a, Oral)

725 1985 WHO Expert Committee on Biological Standardization:
† 8 & 10. Requirements for Diphtheria Toxoid, Pertussis Vaccines,
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31. Requirements for Hepatitis B Vaccine prepared from Human
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* Replaced by revised Requirements.
† Refer also to subsequent Addendum.

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36. Requirements for Varicella Vaccine (Live)
   Informal Consultation on the Standardization of Interferons

745 1987 WHO Expert Committee on Biological Standardization:
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