

## Annex 5

# REQUIREMENTS FOR MEASLES VACCINE (LIVE)

## (Requirements for Biological Substances No. 12) (Revised 1987)

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## INTRODUCTION

Measles, an almost invariable clinical experience of childhood, is in some countries a major cause of illness and of death in children. Immunization against measles has been of interest to WHO for many years, and more especially since the Expanded Programme on Immunization was launched in 1974 with measles as one of the principal diseases against which it is directed. Since the original production of measles vaccine, many years ago, the search for improved immunizing agents continues.

In support of this, reference materials such as anti-measles serum and measles vaccine virus have been established, and Requirements for Measles Vaccine (Live) and Measles Vaccine (Inactivated) were adopted by the WHO Expert Committee on Biological Standardization in 1966 (1). Although new developments may be anticipated today, only live measles vaccines are used, and it has become necessary to update the requirements for these. In drafting this revision, account has been taken of the opinions of consultants, of the regulations and requirements for the manufacture and control of measles vaccines that have been formulated in a number of countries, and of information from published and unpublished sources. In addition, opinions and data have been received from a number of experts, whose assistance is gratefully acknowledged below.

## GENERAL CONSIDERATIONS

Hundreds of millions of persons have been vaccinated with live attenuated measles virus vaccines, and there is ample evidence that measles vaccines which are safe and effective are produced throughout the world. Production of such vaccines requires the observance of certain rules, and it is thus important that up-to-date international requirements for live attenuated measles vaccines should be formulated and made available to manufacturers and national control authorities.

The antibody response in persons inoculated with live measles vaccine can be accurately measured serologically, and a number of studies have established that the presence of detectable levels of antibody is correlated with protection against the disease. Immunity following the use of live measles vaccine appears to be of long

duration; this is indicated by the persistence of neutralizing antibodies in children who, a number of years ago, received vaccines prepared from several attenuated strains derived from the Edmonston strain of measles virus. Nevertheless, it is important that studies should be made to ascertain the exact duration of immunity induced by vaccines derived from various strains.

The optimum age for the immunization of babies may differ from one country to another. If immunization is carried out too early in life, there may be no or poor protection, especially if vaccine strains have been over-attenuated. On the other hand, the pattern of incidence of measles in some developing countries is such that it may be necessary to immunize babies as early as 6 months of age. The poorer seroconversion rate when measles vaccine is administered early in life can be circumvented by a second injection later in life.

Measles vaccine can be combined with other live attenuated vaccines such as mumps and rubella vaccines, and such combinations are also highly effective.

While the basic immunogenic potential of each strain of measles vaccine can be assessed by the antibody response in man, this is not a practical method for the routine testing of the potency of batches of vaccines. Such testing is done by measuring infectivity when cell cultures are inoculated with various dilutions of vaccines. For each strain of measles vaccine, it is necessary to establish the relationship between the laboratory estimation of virus titre on the one hand and safety and efficacy for man on the other. Where measles vaccines are to be used in combination with other vaccines, dose-response curves for each component should be determined by administering it in the proposed combined form.

It is obviously important that the strains of virus used to prepare live measles vaccine should show no tendency to produce neurological complications of the type encountered in some cases of natural measles. Present experience indicates that the live vaccines so far used are, indeed, safe in this respect. In the absence of a more satisfactory test, the intracerebral inoculation of monkeys has been used as a laboratory test by which this property could be evaluated. The development of more reliable methods is desirable. The possibility exists that post-measles encephalopathy is the result of an immunopathogenic reaction; the underlying mechanism is not known.

It is essential that every precaution should be taken to exclude all adventitious agents from vaccines for use in humans. In the

requirements formulated below, tests have been described for detecting adventitious agents that might be present in cell cultures used for vaccine production.

The systematic use of cells from birds maintained in closed colonies that have been subject to continuous and systematic veterinary and laboratory monitoring for the presence of infectious agents, or of cells derived from well-characterized human diploid cell lines has resulted in the production of cell substrates of better quality than hitherto; however, in spite of the conclusion of a group convened by WHO in 1980 (2) that it was sufficient to conduct the tests for extraneous agents in cell cultures on control cells, tests for the absence of such agents are still mandatory for individual harvests and/or virus pools in these revised requirements. On the other hand, tests for extraneous agents on small laboratory animals have been abandoned, except for the testing of virus seeds and, when measles vaccines are produced in human diploid cells, for the testing of manufacturer's working cell banks.

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 used as definitive national requirements. The parts of each section that are printed in small type are comments or recommendations for guidance.

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

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

## PART A. MANUFACTURING REQUIREMENTS

### 1. Definitions

#### 1.1 International name and proper name

The international name shall be *Vaccinum morbillorum vivum*. 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 morbillorum vivum* is a preparation of live attenuated measles virus grown in a suitable cell culture. The preparation shall satisfy all the requirements formulated below.

At present, live measles vaccines are blended with an appropriate stabilizer and lyophilized. They are available for distribution only in that form, either as monovalent vaccines or in combination with live mumps and/or live rubella vaccines.

#### 1.3 International reference materials

The International Reference Preparation of Anti-Measles Serum was established in 1964. It is intended for the calibration of national standards or reference preparations for use in the manufacture and laboratory control of anti-measles serum and of human immunoglobulin. It is also intended for assessing the antibody response to measles vaccines. The International Reference Preparation of Anti-Measles Serum is in the custody of the State Serum Institute, Copenhagen, and is available on request.

An International Reference Reagent for the Assay of Measles Vaccine (Live) is available on request from the National Institute for Biological Standards and Control, Potters Bar, England, and is suitable for estimating the *in vitro* infectivity of measles vaccines.

#### 1.4 Terminology

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

*Master virus seed lot:* a quantity of virus of uniform composition, processed at one time, and distributed into a number of containers. Master virus seed lots are derived from a seed virus used in the preparation of vaccines shown to be immunogenic and safe in man, and not more passages removed from it than a number approved by the national control authority. They are used for the preparation of working virus seed lots.

*Working virus seed lot:* a quantity of virus suspension that has been processed together, is uniform with respect to composition, and is only one passage from a master seed lot produced on the same substrate. Material is drawn from working seed lots for inoculating cell cultures for the production of vaccines.

*Cell substrate lot:* a number of cell cultures derived from the same pool of cells, processed and prepared together.

*Single harvest:* a virus suspension derived from one cell substrate lot, all the cultures having been inoculated at the same time with the same inoculum. Single harvests may be derived from one cell substrate lot by repeated harvesting at intervals.

*Virus pool:* a pool of a number of single harvests before clarification.

*Final bulk suspension:* a quantity of vaccine after completion of preparations for filling and present in the container from which the final containers are filled. The final bulk may be prepared from one clarified bulk suspension, or from a blend of clarified bulk suspensions, or from a dilution thereof.

*Final lot:* a collection of sealed final containers that derive from the same final bulk and that are homogeneous with respect to the risk of contamination during filling and freeze-drying. A final lot therefore consists of finished material dispensed into containers during one working session and lyophilized together.

*Tissue culture infective dose 50% (TCID<sub>50</sub>):* the quantity of a virus suspension that will infect 50% of cell cultures.

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

## **2. Certification of the Strain of Virus for Use in Vaccine Production**

2.1 The strain of measles virus used in the production of measles vaccine shall be identified by historical records, which shall include

information on the origin of the strain as well as on the method used in the attenuation of it.

2.2 The vaccine strains of measles virus used in the production of vaccine shall have been shown to be safe and immunogenic by appropriate laboratory tests (see Part A, section 4, of these Requirements) and by tests in susceptible humans. Only strains that are approved by the national control authority shall be used.

2.3 A dose-response study should establish the minimal vaccine dose inducing seroconversion in susceptible individuals.

### **3. 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) (3) shall apply to establishments manufacturing measles vaccine, with the addition of the following directives.

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

The production of measles vaccine shall be conducted by staff who have not handled other infectious microorganisms or animals on the same working day. The staff shall consist of persons who shall be examined medically and found to be healthy. Steps shall be taken to ensure that all personnel involved in the production areas are immune to measles. Production and control shall be organized as two separate units of the manufacturing establishment with independent responsibilities.

Only the virus seed lot and cell cultures approved by the national control authority for the production of measles vaccine shall be introduced or handled in the production area.

Persons not directly concerned with the production processes, other than official inspectors, shall not be permitted to enter the production area without valid reason and specific authorization.

Particular attention shall be given to the recommendations in Part A, section 1, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3) regarding the training and experience of the persons in charge of production and testing, and of those assigned to various areas of responsibility in the manufacturing

establishment, as well as to the registration of such personnel with the national control authority.

#### 4. Production Control

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) (3) shall apply to the manufacture of measles vaccine.

##### 4.1 Substrate for virus propagation

4.1.1 Measles virus used in the production of measles vaccine shall be propagated in cell cultures approved by the national control authority. All information on the source and method of preparation of the cell culture system used shall be available to the national control authority.

4.1.2 If chick-embryo cell cultures are used for the propagation of measles vaccine virus, the eggs used as a source of cells shall be derived from a closed, specific-pathogen-free, healthy flock. Monitoring shall be performed at regular intervals for *Mycobacterium avium*, fowl 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*.

4.1.3 If human diploid cells are used for the propagation of measles virus, a manufacturer's working cell bank (MWCB) shall be established in conformity with the provisions of Part C of these Requirements. The cell seed shall be derived from an early population doubling of the approved diploid cell strain, and the MWCB shall be prepared from it by serial subculture up to an approved population doubling level. Each manufacturer shall show to the satisfaction of the national control authority that the cell substrate propagated from the accepted cell strain and laid down as a MWCB conforms with the tests in animals and eggs for freedom



from extraneous agents, for lack of tumorigenicity, for normal karyology at least up to the population doubling level at which the cells are used to propagate the measles virus (production cell cultures), and for identity, as indicated in Part C of these Requirements. The cells shall not be used beyond two-thirds of the total number of population doublings corresponding to the average finite life of the cells.

#### 4.1.4 *Serum used in cell culture medium*

Serum used for the propagation of cells for measles vaccine production shall be tested to demonstrate freedom from bacteria, fungi and mycoplasmas according to the requirements in Part A, sections 5.2 and 5.3, of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (4) and to demonstrate freedom from viruses.

Suitable tests for the detection of viruses in calf or newborn calf serum are given in Appendix 3 of the Requirements for Poliomyelitis Vaccine (Oral) (Requirements for Biological Substances No. 7) (5).

Serum shall also be shown to be free from inhibitors of measles virus. Human serum shall not be used. If human albumin is used, it shall meet the WHO Requirements for the Collection, Processing and Quality Control of Human Blood and Blood Products (Requirements for Biological Substances No. 27) (6).

In some countries sera are also examined for freedom from phage.

#### 4.1.5 *Trypsin used for preparing cell cultures*

Trypsin used for preparing cell cultures shall be bacteriologically sterile and free from mycoplasmas and viruses, especially porcine parvoviruses. The methods used to ensure this shall be approved by the national control authority.

## 4.2 **Virus seed**

The production of vaccine shall be based on the seed lot system.

It is recommended that a large working virus vaccine seed lot be set aside as the basic material that the manufacturer should use for the preparation of each batch of vaccine.

Virus seed lots shall be stored lyophilized in a special refrigerator at a temperature lower than  $-20^{\circ}\text{C}$ , or, if not lyophilized, at a temperature of  $-60^{\circ}\text{C}$  or lower.

The virus in the final vaccine shall not be more than 5 cell culture passages removed from the virus seed used in the preparation of a vaccine shown to be immunogenic and safe in man.

#### 4.2.1 *Tests on virus seed lots*

The seed lot used for the production of vaccine shall be free from detectable extraneous agents, including those which might have contaminated the original human specimen from which the virus strain was initially isolated, the cell culture used during the initial passages, or the attenuation process.

The seed lot virus shall be produced in conditions which satisfy sections 4.3 and 4.4 (with the exception of 4.4.5.1 and 4.4.5.2) of Part A of these Requirements. Tests for extraneous agents on small laboratory animals and for neurovirulence shall be done on either the master or the working seed lot as indicated in sections 4.2.1.1 and 4.2.1.2 below.

##### 4.2.1.1 *Tests in small laboratory animals*

###### 4.2.1.1.1 *Test 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 seed. 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 autopsied and examined 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 5 additional mice, which shall be observed for 21 days.

The virus seed 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 transmissible agents attributable to the virus seed.

In some countries the national control authority permits a 60% survival of the original inoculated animals.

#### 4.2.1.1.2 *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 seed. 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 autopsied and examined 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 5 additional suckling mice, which shall be observed daily for 14 days.

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

The virus seed 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 transmissible agents attributable to the virus seed.

#### 4.2.1.1.3 *Tests in guinea-pigs*

The virus seed shall be tested for adventitious agents by the intraperitoneal inoculation of 5.0 ml of the virus seed into each of at least 5 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 autopsied and examined both microscopically and by tissue culture for evidence of infection. Animals that survive the observation period shall be sacrificed and examined in a similar manner.

The virus seed passes the test if at least 80% of the guinea-pigs survive the observation period and if no guinea-pig shows evidence of infection with adventitious transmissible agents attributable to the virus seed.

#### 4.2.1.2 *Tests for neurovirulence*

Each master or working virus vaccine seed lot shall be shown to be free from neurovirulence by tests in measles-susceptible monkeys.

Such tests can be conducted as follows:  
Immediately prior to the inoculation each monkey should be shown to be serologically negative for measles. At least 10 monkeys should be employed in each test. The material under

test should be given to each monkey by inoculation of 0.5 ml into the thalamic region of each hemisphere. The total amount of measles virus inoculated into each monkey should be not less than the amount contained in the recommended single human dose of vaccine. Monkeys should be observed for 17–21 days for symptoms of paralysis and other evidence of neurological involvement. Animals that die within 48 hours after injection may be replaced. The test is invalid and should be repeated if more than 20% of the monkeys die even from nonspecific causes. At the end of the observation period each monkey is (a) bled and the serum tested for measles antibody and (b) anaesthetized, sacrificed and autopsied; histopathological examinations of appropriate areas of the brain are made for evidence of central nervous system involvement.

As a check against the inadvertent introduction of wild measles virus, at least 4 measles-susceptible uninoculated monkeys should be maintained as a control, either as cage mates of or within the same immediate area as the inoculated test animals for the entire period of observation (17–21 days) plus an additional 10 days. Serum samples should be taken from the control monkeys at the time of inoculation of the test animals and again 10 days after the test animals are killed.

The material passes the test if: (a) at least 80% of the inoculated monkeys are serologically positive for measles with an adequate level of specific antibody (0.2 IU/ml or greater) and all the serum samples from the control monkeys are shown to be free from measles antibody; and (b) there is no clinical or histopathological evidence of involvement of the central nervous system attributable to the inoculated virus.

In some countries the seed lot itself is not tested but vaccines are accepted provided each of the first 5 undiluted clarified virus pools prepared from the same seed lot satisfies the requirements of the test for neurovirulence.

#### **4.3 Control of cell cultures**

Either 5% or not less than 500 ml of the cell suspension at the concentration employed for vaccine production cultures shall be used to prepare control cultures.

Cells set aside as control material shall be treated in a similar manner to the production cell cultures, but shall remain uninoculated as control cultures for the detection of extraneous viruses; they shall be incubated under the same conditions as the inoculated cultures for at least 2 weeks, or until the time of the last harvest of the production cultures, whichever is the later, and shall be observed microscopically for changes attributable to the presence of adventitious agents. At the end of the observation period, fluids

collected from the control cultures as well as cell sheets from a proportion of the control vessels shall be tested for the presence of adventitious agents by the tests described in this section. If any such tests show evidence of the presence in a control culture of any adventitious agent, the measles vaccine virus grown in the corresponding batch of production cultures shall not be used for vaccine production.

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

#### 4.3.1 *Test for haemadsorbing viruses*

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

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

#### 4.3.2 *Tests for non-haemadsorbing extraneous agents*

At 14 days after the day of inoculation of the production cultures, or at the time of final virus harvest, if this is later, a sample of cell culture fluid shall be taken from each control culture and pooled. Ten millilitres of the pool shall be tested in the same substrate, but not the same batch as that used for the production of virus growth, and additional 10-ml samples of each pool shall be tested in both human and simian cells.

Each sample shall be inoculated into bottles of cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet shall be at least 3 cm<sup>2</sup> per ml of pooled fluid. At least one bottle of the cell cultures shall remain uninoculated as a control.

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

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

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

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

#### 4.3.3 *Additional test if chicken-cell cultures are used*

If chicken-cell cultures are used, a sample of fluids pooled from the control cultures shall be tested for adenoviruses and for avian retroviruses such as avian leukosis virus, using a method approved by the national control authority.

Satisfactory procedures for testing for avian leukosis virus include tests for detecting the resistance-inducing factor (RIF), complement-fixation tests (COFAL), and enzyme-linked immunosorbent assays (ELISA).

The control cultures pass the test if there is no evidence of the presence of virus.

A certificate of freedom from avian leukosis virus and adenovirus provided by the supplier of the fertile eggs may satisfy the licensing authority.

#### 4.3.4 *Additional tests on control cells if human diploid cells are used for production*

If human diploid cells are used for production, the cell cultures shall be identified as human by tests approved by the national control authority, as specified in Part C, section 2.2.2, of these Requirements.

## 4.4 Production and harvest of virus vaccines

### 4.4.1 *Cells used for vaccine production*

If human diploid cells are used as the substrate for the multiplication of the vaccine virus, they shall satisfy the conditions specified in Part C, section 2.2.1, of these Requirements.

On the day of inoculation with the seed lot virus, each cell culture shall be examined for degeneration caused by infective agents. If such examination shows evidence of the presence in a cell culture of any adventitious agent, the whole group of cultures concerned shall not be used for vaccine production.

After virus inoculation, cultures for vaccine production shall be incubated under controlled temperature conditions approved by the national control authority.

If animal serum is used for the maintenance of cell cultures before the harvesting of virus, the medium shall be removed and replaced with serum-free maintenance medium, the cells being rinsed before being added to the new medium.

Beta-lactam antibiotics shall not be used at any stage of manufacture.

Minimal concentrations of other suitable antibiotics may be used if approved by the national control authorities.

### 4.4.2 *Single harvests*

Harvesting of virus fluids shall be carried out by a method approved by the national control authority. A single harvest may be a combination of several consecutive harvests from one production cell lot. Single harvests are stored at a suitable temperature until pooling. No antibiotics shall be added at the time of harvesting nor at any later stage of manufacturing. Samples of single harvests shall be taken for testing for sterility and virus content; if not tested immediately, samples shall be kept at a temperature below  $-50^{\circ}\text{C}$  until testing is done.

#### 4.4.2.1 *Sterility tests*

A volume of at least 10 ml of each single harvest shall be tested for bacterial and mycotic sterility according to the requirements in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological

Substances) (4), as well as for mycoplasmas by a method approved by the national control authority.

Tests for mycoplasmas should be done using both solid and liquid media that have been shown to be capable of supporting the growth of sterol-requiring and non-sterol-requiring mycoplasmas, and using for each group of tests at least 10 ml of single harvests. Approved non-culture methods may also be used.

#### 4.4.2.2 *Virus titration*

The live virus content of each single harvest may be determined by cell culture titration, using a reference material of live measles virus, the titre of which has been determined by comparison with the International Reference Reagent (see Part A, section 1.3, of these Requirements).

#### 4.4.3 *Virus pool*

The virus pool shall be prepared from one or several single harvests and shall be submitted to the following tests, unless these tests have already been done on each single harvest; however, even in that event, sterility tests shall be done on the virus pool.

The virus pool may be clarified, stabilized and stored at  $-50^{\circ}\text{C}$  or lower before being used to prepare final bulk for freeze-drying.

In tests that require prior neutralization of measles 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 culture free from extraneous microbial agents that might elicit antibodies inhibitory to the growth of any extraneous agents that may be present in the measles virus pool.

##### 4.4.3.1 *Sterility tests*

Sterility tests shall be performed as indicated in Part A, section 4.4.2.1, of these Requirements.

##### 4.4.3.2 *Virus titration*

Virus titration shall be performed as indicated in Part A, section 6.3, of these Requirements.



#### 4.4.3.3 *Test for mycobacteria*

Regardless of which substrate is used for producing the virus vaccine, tests for detecting the presence of mycobacteria pathogenic for man shall be performed. Tests for mycobacteria shall be done on the pellet obtained after centrifugation of 20 ml of the virus pool.

#### 4.4.3.4 *Tests in cell cultures of neutralized virus pool*

A volume of each virus pool equivalent to at least 500 human doses shall be neutralized by specific antiserum and shall be tested for adventitious agents by inoculation into simian cell cultures. Similar volumes of the neutralized virus pool shall likewise be tested in human cell cultures and in cell cultures of the same type but not the same batch of cells as that used in the preparation of the virus pool. Uninoculated cell cultures shall be kept as a control. All cell cultures shall be observed for at least 14 days.

Some national control authorities require that at the end of this observation period a subculture is made in the same culture system.

The virus pool passes the tests if none of the inoculated cell cultures shows evidence of the presence of any adventitious agents.

#### 4.4.3.5 *Additional tests if chick-embryo tissue is used as substrate for production*

If chick-embryo tissue is used for vaccine production, the following additional tests shall be made.

A volume of each virus pool, equivalent to at least 100 human doses of vaccine, or 10 ml, shall be tested in a group of embryos of fertilized chicken's eggs by the allantoic route of inoculation, and a similar sample shall be tested in a separate group of eggs by the yolk sac route of inoculation, using 0.5 ml of inoculum per egg in both cases.

The virus pool passes the test if there is no evidence of the presence of any adventitious agents. If, however, an adventitious agent is detected in the uninoculated controls, the test may be repeated.

#### 4.4.4 *Clarification of the vaccine virus pool*

The vaccine virus pool suspension shall be clarified by a method that will remove cells and cell debris. Samples of the clarified bulk

suspension shall be taken immediately after clarification to ensure that no cell or cell debris is left. Samples shall also be taken to control the identity and infectious virus content of the pool. If not tested immediately, the samples shall be kept at a temperature below  $-50^{\circ}\text{C}$  until testing is done.

#### 4.4.4.1 *Test for clarification*

Microscopical observation of a smear of a concentrated sample is suitable for ensuring that no cells or cell debris are detected after clarification.

#### 4.4.4.2 *Test for virus content*

The viable virus content of samples from clarified bulk suspension shall be tested, using for comparison a reference preparation of live measles virus (see Part A, section 1.3, of these Requirements).

#### 4.4.5 *Final bulk suspension*

The final bulk suspension shall be prepared from one or more clarified virus pools that are obtained from substrates of which control cultures pass the test specified in Part A, section 4.3 of these Requirements. They shall satisfy the tests specified in Part A, sections 4.4.3 and 4.4.4.

The operations necessary for preparing the final bulk shall be conducted in such a manner as to avoid contamination of the product.

Assays of virus content may be done on the final bulk suspension.

#### 4.4.5.1 *Added substances*

In preparing the final bulk, any substance such as diluent or stabilizer that is added to the product shall have been shown to the satisfaction of the national control authority not to impair the safety and efficacy of the vaccine in the concentration used.

#### 4.4.5.2 *Residual animal serum proteins*

A sample of the final bulk shall be tested to verify that the level of contamination by heterologous serum is less than 1 part per million of the final reconstituted vaccine; that is, less than  $0.5\ \mu\text{g}$  if the human dose is 0.5 ml. Alternatively the test may be performed on the clarified bulk.

Serological tests such as ELISA are suitable for this purpose.

#### 4.4.5.3 *Storage*

Until it is distributed into containers and lyophilized, the final bulk suspension shall be stored in conditions shown by the manufacturer to retain the activity of the vaccine.

### **5. Filling and Containers**

The requirements concerning filling and containers in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3) shall apply.

Care shall be taken to ensure that the material of which the container and, if applicable, of the closure is made does not adversely affect the virus content of the vaccine under the recommended conditions of storage.

Single-dose containers are recommended, except in the case of mass immunization campaigns.

### **6. Control Tests on Final Product**

Samples shall be taken from each freeze-dried lot for the tests in the following sections.

#### **6.1 Identity tests**

The virus in 2 or more individually labelled final containers shall be identified as measles virus by appropriate methods, such as seroneutralization and then inoculation of cells.

#### **6.2 Tests for bacteria and fungi**

Reconstituted vaccine shall be tested for bacterial and mycotic sterility according to the requirements in Part A, section 5.2, of the Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances) (4), or by acceptable methods approved by the national control authority.

#### **6.3 Virus concentration**

The virus content in each of at least 3 ampoules selected at random from each drying lot shall be determined individually.

The national control authority shall determine the minimum content of the vaccine virus that should be contained in one human dose.

The minimum quantity of the vaccine virus that should be contained in one human dose is generally considered to be 1000 viral infective units. In at least one country, however, the minimum dose has been set at 5000 infective units.

An additional 3 ampoules of the final freeze-dried vaccine shall be exposed at 37°C for 7 days. The geometric mean infectious virus titre of the vials that have been exposed shall be equal to or greater than the required minimum numbers of infective units per human dose, and the geometric mean virus titre of the vaccine shall not have been decreased by more than 1.0 log<sub>10</sub> during the period of exposure. Titration of non-exposed and exposed vials shall be made in parallel and results expressed in terms of PFU and/or TCID<sub>50</sub> per human dose. A reference reagent of measles virus, the titre of which has been determined by comparison with the International Reference Reagent (see Part A, section 1.3, of these Requirements), shall be included in each assay.

The detailed procedures for carrying out this test and for interpreting the results should be those approved by the national control authority, which should specify the acceptable confidence limits.

#### **6.4 General safety tests**

Innocuity shall be tested by appropriate tests in mice and guinea-pigs, using parenteral injections. The tests shall be those approved by the national control authority.

#### **6.5 Residual moisture**

The residual moisture in a representative sample of each freeze-dried lot shall be determined by a method approved by the national control authority. The upper limit for the moisture content shall be specified by the national control authority.

Moisture levels of less than 2% are usually considered satisfactory.

## **6.6 Inspection of final containers**

Each container in each filling lot shall be inspected visually and any that show an abnormality shall be discarded.

## **7. Records**

The requirements in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3) shall apply.

## **8. Samples**

The requirements in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3) shall apply.

## **9. Labelling**

The requirements regarding labels of individual containers and labels of packages in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3) 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) a statement that the vaccine fulfils these Requirements;
- (b) a statement of the nature of the preparation, specifying the designation of the strain of measles virus contained in the live attenuated virus vaccine, the minimum number of infective units per human dose, and the origin of the substrate used in the preparation of the vaccine;
- (c) a statement of the nature and quantity of any antibiotic present in the vaccine;
- (d) a statement concerning the photosensitivity of the vaccine, cautioning that both lyophilized and reconstituted vaccine should be protected from light;

(e) a statement indicating the volume and nature of diluent<sup>1</sup> to be added in order to reconstitute the vaccine, and specifying that the diluent to be used is that supplied by the manufacturer;

(f) a statement that after the vaccine is reconstituted, it should be used without delay, or if not used immediately, stored between 0 °C and 10 °C and in the dark for a period not exceeding 8 hours.

## **10. Distribution and Shipping**

The requirements in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (Requirements for Manufacturing Establishments and Control Laboratories) (3) shall apply.

Shipments should be at temperatures of 8 °C or below and parcels should contain cold-chain monitors.

## **11. Storage and Expiry Date**

The statements concerning storage temperature and expiry date appearing on the label or the leaflet, as specified in Part A, section 9, of these Requirements, shall be based on experimental evidence and shall be submitted for approval to the national control authority.

### **11.1 Storage conditions**

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

### **11.2 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 for virus concentration, this date being that on which the test-system was inoculated. It shall be based on experimental evidence

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<sup>1</sup> No preservative or any substance that has a deleterious effect on the virus should be present in the diluent used to reconstitute the vaccine.

and shall not be less than 2 years for a temperature of storage not higher than 8 °C.

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

## **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) (3) shall apply.

The national control authority shall provide a reference material of live measles virus (see Part A, section 1.3, of the present Requirements) for tests for virus concentration (see Part A, sections 4.4.2.2, 4.4.3.2, 4.4.4.2, and 6.3) and shall specify the requirement for virus content that shall be fulfilled, in order to achieve adequate immunization of humans when the recommended human dose is used.

### **2. Release and Certification**

A vaccine shall be released only if it fulfils Part A of the present Requirements. A statement along the lines of that contained in Appendix 2 and 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 the present Requirements. The certificate shall state the date of the last satisfactory test for virus concentration by the manufacturer and the final lot number which must appear on the labels of the containers.

The purpose of the certificate is to facilitate the exchange of live measles vaccines among countries.

## **PART C. REQUIREMENTS FOR HUMAN DIPLOID CELLS TO BE USED FOR THE PRODUCTION OF MEASLES VACCINE (LIVE)<sup>1</sup>**

The following requirements concern the testing of the cell substrate for the production of measles vaccine if human diploid cells are used; they should therefore be added to or substituted for the corresponding sections in Parts A and B, as appropriate. *All the other requirements in Parts A and B remain applicable.*

### **1. Definitions**

#### **1.1 Terminology**

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

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

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

*Production cell culture:* a collection of cell cultures at the population doubling level used for virus growth that have been prepared together from one or more ampoules of the MWCB.

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<sup>1</sup> Part C is based largely on Part C of the Requirements for Poliomyelitis Vaccine (Oral) (5, pp. 140-150).



## 2. Production Control

### 2.1 Control of source materials

The cell seed and the manufacturer's working cell bank shall be those approved by and registered with the national control authority. The cells shall have been characterized with respect to their genealogy, growth characteristics, genetic markers (HLA), viability during storage, and karyology, and they shall have been shown to be free from bacteria, mycoplasma, fungi, and haemadsorbing and other viruses by the relevant tests in Part A of these Requirements. In addition the cells of the MWCB shall have been shown to be diploid and stable with respect to karyology and morphology by the tests outlined in this section.

The MWCB shall also have been shown to yield cell cultures capable of producing vaccine that is both safe and immunogenic in man.

#### 2.1.1 *Tests in animals and eggs for extraneous agents*

The cells of the MWCB are suitable if at least 80% of the animals or eggs which are inoculated with them remain healthy and survive the observation period, and none of the animals or eggs shows evidence of the presence in the cells of any extraneous agent.

##### 2.1.1.1 *Tests in animals*

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

- 2 litters of suckling mice, totalling at least 10 animals, less than 24 hours old,
- 10 adult mice of 15–20 g weight,
- 5 guinea-pigs of 350–450 g weight, and
- 5 rabbits.

The animals shall be observed for at least 4 weeks. Any animals that are sick or show any abnormality shall be investigated to establish the cause of illness.

In some countries the suckling and adult mice are also inoculated by the intracerebral route.

### 2.1.1.2 *Tests in eggs*

At least  $10^6$  viable cells shall be injected into the allantoic cavity of 10 embryonated chicken's eggs, 9–11 days old, which shall be examined after not less than 5 days. The allantoic fluids of the fertile eggs shall be tested with erythrocytes from guinea-pigs and chick or other avian species for the presence of haemagglutinins.

### 2.1.2 *Other tests for extraneous agents*

Suitable tests approved by the national control authority shall be performed in order to exclude the presence of retroviruses and the integration of nucleic acid of viral origin (hepatitis B virus and human immunodeficiency virus, HIV) in the genome of the cells.

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

### 2.1.3 *Freedom from tumorigenicity*

The cells shall also be shown to be free from potential tumorigenicity by appropriate animal tests approved by the national control authority.

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

The cells are suitable for vaccine production if at least 80% of the animals inoculated with cells remain healthy and survive the observation period, and none of the animals shows evidence of tumour formation from the cells.

#### 2.1.4 Chromosomal characterization

At least 4 samples from the cell seed shall be examined as described in Part C, section 2.1.5, of these Requirements at approximately equal intervals over the life-span of the cell line during serial cultivation. Each sample shall consist of 1000 metaphase cells.

It is also recommended that photographic reconstruction should be employed in the preparation of chromosome-banded karyotypes of 50 metaphase cells per 1000-cell sample, using either G-banding or Q-banding techniques. The incidence of karyotypic abnormalities (pseudodiploidy, inversions, translocations, etc.) that are detectable with the greater resolution provided by banding should be approved by the national control laboratory.

#### 2.1.5 Chromosomal monitoring—preparation and testing

For the determination of the general character of the manufacturer's working cell bank, a minimum of 500 cells in metaphase shall be examined at the production level or at any passage thereafter for frequency of polyploidy and for exact counts of chromosomes, frequency of breaks, structural abnormalities, and other abnormalities such as despiralization or marked attenuations of the primary or secondary constriction.

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

For WI-38 and MRC5 cells examined in metaphase, the upper limits of acceptability (upper fiducial limits at 95% (Poisson)) for abnormalities for a 1000- and 500-cell sample are as follows:<sup>1</sup>

<i>Abnormality</i>	<i>1000 cells</i>	<i>500 cells</i>
Chromatid and chromosome breaks	47/1000	26/500
Structural abnormalities	17/1000	10/500
Hyperploidy	8/1000	5/500
Hypoploidy	180/1000	90/500
Polyploidy	30/1000	17/500

<sup>1</sup> These upper limits are based on extensive experience with the examination of WI-38 and MRC5 cells reported to and examined by the *ad hoc* Committee on Karyological Controls of Human Substrates, which met in 1978 at Lake Placid, NY, USA. These values will not necessarily be applicable if other human cell strains are used.

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

Stained slide preparations of the chromosomal monitoring of the working cell bank pool, or photographs of these, shall be maintained permanently as part of the record of the MWCB.

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

## **2.2 Production precautions**

The general production precautions as formulated in Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3) shall apply to the manufacture of measles vaccine with the addition of the following.

### *2.2.1 Cell cultures used for vaccine production*

Only human diploid cell cultures derived from a MWCB approved by the national control authority shall be used for vaccine production. The production of each single harvest shall be initiated from one or more new ampoules of the cell seed. All processing of the cell seed and subsequent cell cultures shall be done in an area in which no other cells are handled during the entire period of vaccine production. The cell cultures shall be used only if no changes have occurred in their growth characteristics, and if no changes from the normal karyology have been shown to occur within the total number of population doublings that correspond to the average finite life of the cells as determined under the particular conditions of the production establishment (see Part C, section 2.1.4, of these Requirements).

It is advisable to ensure that both the trypsin and the animal serum used in the preparation or growth of the cell suspensions are free from extraneous agents.

### *3.2.2 Identity test*

An identity test shall be performed on the control cell cultures by methods approved by the national control authority.

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

The cells shall be shown to be of human origin.

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Appendix 1

**SUMMARY PROTOCOLS FOR MEASLES VACCINE  
(LIVE)**

**Based on Requirements for Biological Substances No. 12  
(Requirements for Measles Vaccine (Live))  
(Revised 1987)**

The following protocols are intended for guidance and indicate the minimum of information to be provided.

The Protocol for Final Lot must be accompanied by a sample of the label and a copy of the leaflet accompanying the vaccine container. It must also be accompanied by a certificate from the national control authority of the country in which the vaccine was produced stating that the product meets the national<sup>1</sup> as well as the WHO Requirements (see Appendix 2).

**I. PROTOCOL FOR WORKING VIRUS SEED LOT**

**A. Summary Information**

Name and address of manufacturer \_\_\_\_\_  
\_\_\_\_\_  
Virus strain \_\_\_\_\_  
Reference no. of virus seed used to  
prepare your first measles vaccine that  
was safe and immunogenic in man \_\_\_\_\_  
Reference no. of master seed virus \_\_\_\_\_  
Number of passages between the two  
above seeds \_\_\_\_\_  
Date of preparation of working seed virus \_\_\_\_\_  
No. of containers of working seed virus  
prepared \_\_\_\_\_  
Reference no. of working seed virus  
prepared \_\_\_\_\_  
Conditions of storage of seed virus  
prepared \_\_\_\_\_

<sup>1</sup> If product does not meet the national requirements, the reason why it does not should be stated.

**History of vaccine strain**

Provide a brief account indicating how the vaccine strain was acquired, history up to production of master seed virus lot, and criteria on which acceptability for virus production is based.

**B. Control of Cell Cultures (A.4.3)<sup>1</sup>**

Provide information on control cells corresponding to each single harvest, using extra pages if necessary.

Substrate used for production of virus seed lot \_\_\_\_\_  
Reference no. of control cell cultures \_\_\_\_\_  
Quantity of overall cell cultures used as control cultures \_\_\_\_\_  
Period of observation of uninoculated control cells \_\_\_\_\_

**B.1 Tests for haemadsorbing viruses (A.4.3.1)**

Type of red blood cells \_\_\_\_\_  
Date of test \_\_\_\_\_  
Result of test \_\_\_\_\_

**B.2 Test on cell cultures for non-haemadsorbing extraneous agents (A.4.3.2)**

Simian cells  
Type of cells \_\_\_\_\_  
Date of inoculation \_\_\_\_\_  
Result \_\_\_\_\_  
Human cells  
Type of cells \_\_\_\_\_  
Date of inoculation \_\_\_\_\_  
Result \_\_\_\_\_

**B.3 Other tests for non-haemadsorbing viruses**

1. Case where substrate is chicken fibroblast (A.4.3.3)  
Test for avian leukosis virus  
Method \_\_\_\_\_  
Date \_\_\_\_\_  
Results \_\_\_\_\_

<sup>1</sup> Letters and numbers in parentheses refer to the corresponding parts and sections in the text of the Requirements for Measles Vaccine (Live).



Test for avian adenovirus	
Method	_____
Date	_____
Results	_____
2. Case where cell substrate is human diploid cell (HDC) (A.4.3.4)	
Include the manufacturing protocol of the manufacturer's working cell bank (prepared along the lines indicated in Part C of the Requirements)	
HDC cultures used for testing the particular control cell cultures	
Reference no. of batch	_____
Date of inoculation	_____
Results	_____

**C. Single Harvests Used in Preparation of Seed Lot (A.4.4.2)**

Report results of tests for each single harvest, using extra pages if necessary.

Name and concentration of antibiotics used in cell culture medium	_____
No. and reference no. of single harvests	_____

**Tests on single harvests**

Date of sterility test (A.4.4.2.1)	_____
Result of sterility test	_____
Date of test for mycoplasmas	_____
Result of test for mycoplasmas	_____
Date of test on virus concentration (A.4.4.2.2)	_____
Result of test on virus concentration	_____

**D. Virus Pool (A.4.4.3)**

If any test had to be repeated or any abnormal result was observed, this must be specified.

**D.1 Tests for extraneous agents in small laboratory animals<sup>1</sup> (A.4.2.1.1)**

Report on separate pages the details of tests in suckling mice, adult mice, guinea-pigs, and chicken embryos, giving all relevant information, such as number of animals, weight (suckling mice), date and route of inoculation, quantity injected, route of inoculation, results (survival numbers).

<sup>1</sup> Not mandatory if already performed on the primary virus seed.

**D.2 Tests for neurovirulence<sup>1</sup> (A.4.2.1.2)**

No. of monkeys in test \_\_\_\_\_  
Volume injected \_\_\_\_\_  
No. of monkeys surviving without specific symptoms \_\_\_\_\_  
Result of serological tests \_\_\_\_\_  
Result of histopathological examination (specify findings) \_\_\_\_\_

**D.3 Tests for bacterial and mycotic sterility (A.4.4.3.1)**

Date of inoculation \_\_\_\_\_  
Media used \_\_\_\_\_  
Results \_\_\_\_\_

**D.4 Tests for mycoplasmas (A.4.4.3.1)**

Date of inoculation \_\_\_\_\_  
Media used \_\_\_\_\_  
Results \_\_\_\_\_

**D.5 Virus titration (A.4.4.3.2)**

Cells used for titration \_\_\_\_\_  
Date of inoculation \_\_\_\_\_  
Results \_\_\_\_\_

**D.6 Tests for mycobacteria (A.4.4.3.3)**

Date of inoculation \_\_\_\_\_  
Media used \_\_\_\_\_  
Period of observation \_\_\_\_\_  
Results \_\_\_\_\_

**D.7 Tests on cell cultures after seroneutralization (A.4.4.3.4)**

Species in which neutralizing serum was prepared and cell substrate on which immunogen was produced \_\_\_\_\_  
Human cells  
Nature of cells \_\_\_\_\_  
Date of inoculation \_\_\_\_\_  
Results \_\_\_\_\_  
Simian cells  
Nature of cells \_\_\_\_\_

<sup>1</sup> Not mandatory if already performed on the primary virus seed.

Date of inoculation \_\_\_\_\_  
Results \_\_\_\_\_

**D.8 Additional tests**

1. If chicken fibroblasts are used as substrate (A.4.4.3.5)

Tests in embryonated eggs inoculated by allantoic route

No. and age of embryonated eggs inoculated \_\_\_\_\_

Date \_\_\_\_\_

Results \_\_\_\_\_

Tests in embryonated eggs inoculated by yolk sac route

No. and age of embryonated eggs inoculated \_\_\_\_\_

Date \_\_\_\_\_

Results \_\_\_\_\_

2. If human diploid cells (HDC) are used (A.4.4.3.4)

Reference of batch no. of HDC \_\_\_\_\_

Date of inoculation \_\_\_\_\_

Results \_\_\_\_\_

**E. Certification**

Name (typed) and signature of head of production laboratory \_\_\_\_\_

Certification by the head of the control laboratory of the manufacturer taking overall responsibility for production and control of the seed:

I certify that the working virus seed lot of measles vaccine No. .... meets the requirements in Part A, sections 2 to 4.4.4.2, of the WHO Requirements for Biological Substances No. 12 (Requirements for Measles Vaccine (Live)) (Revised 1987).

Name (typed) \_\_\_\_\_

Signature \_\_\_\_\_

Date \_\_\_\_\_

## II. PROTOCOL FOR FINAL BULK SUSPENSION

### A. Summary Information

Name and address of manufacturer \_\_\_\_\_  
\_\_\_\_\_  
Virus strain \_\_\_\_\_  
Reference no. of secondary virus seed \_\_\_\_\_  
Date of completion of final bulk suspension \_\_\_\_\_

### B. Control of Cell Cultures

Give all relevant information, following as a guide section B of the Protocol for Working Virus Seed Lot. If human diploid cells were used as substrate, do not repeat information on the production of the manufacturer's working cell bank (MWCB) (Part C of the Requirements) unless a different MWCB has been used.

### C. Single Harvests

Give all relevant information as indicated in section C of the Protocol for Working Virus Seed Lot.

### D. Virus Pool

Reference no. of virus pool \_\_\_\_\_

Give all relevant information as indicated in section D of the Protocol for Working Virus Seed Lot, except that the information on tests for neurovirulence and on tests on small laboratory animals need not be repeated.

### E. Clarified Virus Pool

Date and result of test for clarification (A.4.4.4.1) \_\_\_\_\_  
Date, cell substrate used for the assay and result of the test for virus content (A.4.4.4.2) \_\_\_\_\_  
Reference preparation used in the assay \_\_\_\_\_

### F. Final Bulk Suspension

Nature of stabilizer and final concentration \_\_\_\_\_  
Reference of final bulk suspension \_\_\_\_\_  
Total volume of final bulk suspension \_\_\_\_\_  
Result of test for virus content (optional) \_\_\_\_\_

Tests for residual heterologous serum proteins

Date

Method

Result (indicate amount and nature of serum protein(s) present per human dose)

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

**G. Certification**

Name (typed) and signature of head of production laboratory

\_\_\_\_\_

Certification by the head of the control laboratory of the manufacturer taking overall responsibility for production and control:

I certify that final bulk suspension lot No. .... of measles vaccine meets the requirements in Part A, section 4.4.5, of the WHO Requirements for Biological Substances No. 12 (Requirements for Measles Vaccine (Live)) (Revised 1987).

Name (typed)

\_\_\_\_\_

Signature

\_\_\_\_\_

Date

\_\_\_\_\_

### III. PROTOCOL FOR FINAL LOT

#### A. Summary Information

Name and address of manufacturer \_\_\_\_\_  
 \_\_\_\_\_  
 Proprietary name of vaccine \_\_\_\_\_  
 Reference no. of freeze-drying lot \_\_\_\_\_  
 Expiry date \_\_\_\_\_  
 No. of containers in the lot \_\_\_\_\_  
 No. of doses per container \_\_\_\_\_

#### B. Production Details

Lot no. of final bulk suspension \_\_\_\_\_  
 Date of submission of bulk protocol \_\_\_\_\_  
 Date of filling of final lot \_\_\_\_\_

#### **Tests of final product<sup>1</sup>**

1. Identity test (A.6.1)
  - Date \_\_\_\_\_
  - Method used \_\_\_\_\_
  - Results \_\_\_\_\_
2. Tests for bacterial and mycotic sterility (A.6.2)
  - Date of inoculation \_\_\_\_\_
  - Media used \_\_\_\_\_
  - Observation period \_\_\_\_\_
  - Results \_\_\_\_\_
3. Tests for virus concentration (A.6.3)
  - Date of inoculation \_\_\_\_\_
  - Type of cell cultures \_\_\_\_\_
  - Reference preparation used \_\_\_\_\_

#### *Vaccine containers*

	<i>Not kept at 37°C</i>			<i>Kept 7 days at 37°C</i>		
	1	2	3	1	2	3
No. of containers tested						
Virus concentration found in each container (in human doses)						

<sup>1</sup> Give details of all tests and retests.

	<i>Not kept at 37°C</i>	<i>Kept 7 days at 37°C</i>
Mean of virus concentration	_____	_____
Lower 95% fiducial limit	_____	_____
<b>4. General safety tests (A.6.4)</b>		
Date of inoculation	_____	_____
No. of mice given injections	_____	_____
Volume and route of injection	_____	_____
Observation period	_____	_____
Results (give all relevant details in case of deaths)	_____	_____
No. of guinea-pigs given injections	_____	_____
Volume and route of injection	_____	_____
Observation period	_____	_____
Results (give all relevant details in case of deaths)	_____	_____
<b>5. Residual moisture (A.6.5)</b>		
Date	_____	_____
Method used	_____	_____
Size of sample	_____	_____
Moisture content (%)	_____	_____
<b>6. Inspection of final containers (A.6.6)</b>		
Date and result	_____	_____

**C. Certification**

Name (typed) and signature of head of laboratory \_\_\_\_\_  
Date \_\_\_\_\_

Certification by person taking overall responsibility for production and control of the vaccine:

I certify that lot No. .... of measles vaccine meets the requirements in Part A of the WHO Requirements for Biological Substances No. 12 (Requirements for Measles Vaccine (Live)) (Revised 1987).

Name (typed) \_\_\_\_\_  
Signature \_\_\_\_\_  
Date \_\_\_\_\_

Appendix 2

**MODEL CERTIFICATE FOR THE RELEASE  
OF MEASLES VACCINES  
BY NATIONAL CONTROL AUTHORITIES**

(to be completed by the national control authority of the country where vaccines have been manufactured, and to be provided by the vaccine manufacturer to importers)

The following final lots of measles vaccine produced by .....<sup>1</sup> in .....<sup>2</sup>, the numbers of which appear on the labels of the final containers, meet all national requirements<sup>3</sup> and Part A<sup>4</sup> of WHO Requirements for Biological Substances No. 12 (Requirements for Measles Vaccine (Live)) (Revised 1987), and WHO Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (Revised 1965).

Date of last satisfactory test for potency by producer .....

<i>Final Lot No.</i>	<i>Expiry Date</i>	<i>Final Lot No.</i>	<i>Expiry Date</i>
.....	.....	.....	.....
.....	.....	.....	.....
.....	.....	.....	.....

As a minimum, this certificate is based on examination of the manufacturing protocol.

The Director of the National Control Laboratory (or Authority as appropriate)<sup>5</sup>

Name (typed).....

Signature.....

Date.....

<sup>1</sup> Name of manufacturer.

<sup>2</sup> Country.

<sup>3</sup> If any national requirements are not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national control authority.

<sup>4</sup> With the exception of provisions on distribution and shipping, which the national control authority may not be in a position to assess.

<sup>5</sup> Or his or her representative.



## Annex 6

# REQUIREMENTS FOR JAPANESE ENCEPHALITIS VACCINE (INACTIVATED) FOR HUMAN USE

### (Requirements for Biological Substances No. 43)

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## GENERAL CONSIDERATIONS

Specific vaccines are the most commonly used means for the control of Japanese encephalitis. The causative virus is amplified in nature in a cycle involving *Culex* mosquitos and vertebrate animals,

especially pigs. Humans are susceptible at all ages unless immunized by natural infection or vaccination. Evidence shows that effective vaccines will protect animals and humans against illness and will remove the vaccinated animal from the pool of potential amplifying hosts of the virus. Although the control of mosquitos and the vaccination of pigs are effective in some circumstances in preventing Japanese encephalitis in humans, these measures have not proved effective in practice over much of the endemic area. It is also important to recognize that the infection is a zoonosis, that humans are incidental hosts, and that for protection, vaccine coverage must be maintained indefinitely in all persons exposed to the infection.

Two types of formalin-inactivated vaccines have each been used in millions of people. A vaccine produced in adult mouse brain is purified to remove myelin basic protein and is not associated with central nervous system damage in recipients. A vaccine produced in primary hamster tissue culture is also widely used.

Large outbreaks of Japanese encephalitis, sometimes involving thousands of cases, continue to occur in the classic areas of endemicity. In addition, certain special groups, such as travellers to endemic areas and laboratory workers, require immunization. In view of the need to immunize large numbers of people in such circumstances, requirements for Japanese encephalitis vaccine for human use have been formulated. In drafting them, account has been taken of the opinions of consultants, of the regulations and requirements for the manufacture and control of Japanese encephalitis vaccine that have been formulated in several countries, and of information from both published and unpublished sources.

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 Japanese encephalitis vaccine, it is recommended that a clause should be included that would permit modifications of manufacturing requirements on the condition that it can be demonstrated to the satisfaction of the national control authority that such modified requirements ensure that the degree of safety and the potency of the vaccine are at least equal to those provided by the requirements

formulated below. The World Health Organization should then be informed of the action taken.

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

## **PART A. MANUFACTURING REQUIREMENTS**

### **1. Definitions**

#### **1.1 International name and proper name**

The international name shall be *Vaccinum encephalitis japonicae*. 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 encephalitis japonicae* is a fluid or freeze-dried preparation of virus grown in neural tissue of mice or in cell cultures and inactivated by a suitable method. The preparations for human use shall satisfy all the requirements formulated below.

#### **1.3 International standards and reference reagents**

Preparations are currently under study.

#### **1.4 Terminology**

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

*Master virus seed lot*: a quantity of virus of uniform composition, processed at one time, and distributed into a number of containers. Seed lots are derived from a seed virus used in the preparation of inactivated vaccines shown to be immunogenic in man, and not more passages removed from it than a number approved by the national control authority. The master virus seed lot is used for the preparation of working virus seed lots.

*Working virus seed lot:* a quantity of virus suspension that has been processed together, is of uniform composition, and is not more passages removed from the master virus seed lot than a number approved by the national control authority. Material is drawn from working virus seed lots for inoculating cell cultures or mouse neural tissue for the production of vaccine.

*Adventitious agents:* contaminating microorganisms, including bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses.

*Single harvest:* a virus suspension derived from one cell substrate lot, all the cultures having been inoculated at the same time with the same inoculum and harvested at the same time.

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

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

*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 final lot must therefore consist of containers that have been filled in one working session and (for lyophilized products) have been freeze-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) (1) shall apply to establishments manufacturing Japanese encephalitis vaccine for human use, with the addition of the following directives.

The production of Japanese encephalitis vaccine shall be conducted 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 shall be examined medically and found to be healthy. Steps shall be taken to ensure that all such persons in the production and control areas have a serum

neutralizing antibody titre of at least 1:10 from either immunization against or natural infection with Japanese encephalitis.

Only mouse brain tissue and cell cultures approved by the national control authority for the production of Japanese encephalitis vaccine shall be introduced into or handled in the production area.

Persons not directly concerned with the production processes, other than official inspectors, shall not be permitted to enter the production area without valid reason and specific authorization.

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) (*I*, p. 13) regarding the training and experience of the persons in charge of production and testing and of those assigned to various areas of responsibility in the manufacturing establishment, as well as to the registration of such personnel with the national control authority.

### **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) (*I*, p. 15) shall apply to the manufacture of Japanese encephalitis vaccine.

#### **3.1 Animals and cell cultures used for production**

##### *3.1.1 Hamsters*

When hamster-kidney tissue is used for the propagation of Japanese encephalitis virus, only hamster stock approved by the national control authority shall be used as a source of tissue.

The animal stock should be free from infection with mycoplasmas and from microorganisms pathogenic for hamsters. The parents of animals to be used as a source of tissue should be maintained in quarantine in vermin-proof quarters for a minimum of 3 months. Neither the parent hamsters nor their progeny should previously have been used for experimental purposes involving infectious agents.

### 3.1.2 *Mice*

When mice are used for the propagation of Japanese encephalitis virus in neural tissue, only animals less than 5 weeks of age shall be used, and they shall be free from all signs of disease. Methods for intracerebral inoculation and harvesting shall be approved by the national control authority.

The animal stock should be free from microorganisms pathogenic for mice.

### 3.1.3 *Serum used in cell culture medium*

Serum used for the growth of cells shall be tested to demonstrate freedom from bacteria, fungi, and mycoplasmas according to the requirements 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) (2, pp. 49–52), as well as freedom from pathogens, such as viruses of the species of origin of the serum, by methods approved by the national control authority (3, p. 99).

In some countries sera are examined for freedom from phage and endotoxin.

### 3.1.4 *Human albumin used in cell culture medium*

If human albumin is used, it shall meet the requirements in Parts C and D of the Requirements for Biological Substances No. 27 (Requirements for the Collection, Processing and Quality Control of Human Blood and Blood Products) (4).

## 3.2 **Virus seed**

### 3.2.1 *Strain of virus*

The strains of virus used in the production of all seed lots shall be approved by the national control authority and shall yield safe and immunogenic vaccines when the virus has been inactivated. They shall be identified by historical records and by infectivity tests, serological tests, and animal inoculation.

### 3.2.2 *Virus seed lot system*

The preparation of Japanese encephalitis vaccine shall be based on the use of a virus seed lot system. The national control authority shall determine the acceptable number of passages from the master virus seed lot to produce working virus seed lots. If mice are used for the passages, suckling mice are preferred. Vaccines shall be made from a working virus seed lot without further intervening passage. Virus seed lots shall be maintained either in the dried or in the frozen form. The dried seed shall be kept at a temperature below 10 °C, while the frozen seed shall be kept at a temperature below –60 °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 present Requirements.

In some countries the national control authority distributes the master virus seed to manufacturers.

### 3.2.3 *Tests on virus seed lots*

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

#### 3.2.3.1 *Freedom from bacteria, fungi and mycoplasmas*

Each virus seed lot shall be tested for bacterial, mycotic, and mycoplasmal contamination by appropriate tests according to the requirements in Part A, sections 5.2 and 5.3, of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (2, pp. 49–52).

#### 3.2.3.2 *Tests for adventitious agents*

Each virus seed lot shall be tested for adventitious agents. For these tests the virus shall be neutralized by a specific anti-Japanese-encephalitis serum.

The individual tests on the virus seed lots should be so designed that they satisfy the requirements of the national control authority. The anti-Japanese-encephalitis serum should be free from known adventitious viruses.

#### 3.2.3.3 *Tests on working virus seed lots*

Each time a new working virus seed lot is prepared, tests shall be carried out to characterize the virus strain. Such tests shall include

the titration of virus and an identity test using standard serum provided by the national control authority.

When an international standard becomes available, the national standard serum should be calibrated against it.

## **4. Control of Vaccine Production**

### **4.1 Mouse brains**

The brains of the mice inoculated intracerebrally with the virus strain for production shall be harvested immediately before death when the animals show typical signs of encephalitis. The harvested brains shall be triturated in buffered isotonic sodium chloride solution, or any other suitable medium, and centrifuged. The supernatant shall be collected and treated by alcohol precipitation, with protamine sulfate, by ultracentrifugation, or by any other appropriate methods to serve as the virus suspension.

The virus suspension shall be subjected to the tests given in Part A. sections 4.3.1 and 4.3.2, of these Requirements.

### **4.2 Cell cultures**

Beta-lactam antibiotics shall not be used at any stage of manufacture.

Minimal concentrations of other suitable antibiotics such as kanamycin may be used when 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 some countries in which the technology of large-scale production has been developed the national control authority should determine the size of the cell sample to be examined and the control methods to be applied.

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

These control cell cultures shall be incubated under the same conditions as the inoculated cultures for at least 2 weeks or until the



time of the last harvest of the production cultures 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 for nonspecific, accidental reasons.

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

### **4.3 Control of single virus harvests**

#### *4.3.1 Sterility test of single virus harvests*

A sample removed from each virus harvest shall be tested for bacterial and fungal contamination by appropriate tests according to the requirements in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (2, p. 49). Any single virus harvest in which contamination is detected shall be discarded.

#### *4.3.2 Test of virus content*

A sample removed from each virus harvest shall be tested for virus content using the method of intracerebral inoculation of mice. The animals shall be observed for 14 days and the LD<sub>50</sub> then calculated.

4.3.2.1 The virus content shall be not less than 10<sup>7.5</sup> LD<sub>50</sub>/0.03 ml when neural tissue is used.

4.3.2.2 The virus content shall be not less than 10<sup>7.0</sup> LD<sub>50</sub>/0.03 ml when cell culture is used.

### **4.4 Control of bulk material**

#### *4.4.1 Pooling of single virus harvests*

Only virus harvests satisfying the requirements for sterility and virus content in Part A, sections 4.3.1 and 4.3.2, of these Requirements shall be pooled.

#### 4.4.2 *Animal serum*

For cell-culture-derived vaccines, the serum concentration in the bulk vaccine shall not be more than 1 part per million (1 µl/litre).

In some countries, control tests are carried out to detect the residual animal serum content in the final vaccine.

#### 4.4.3 *Inactivation of virus*

The process for the inactivation of the Japanese encephalitis virus and any adventitious agents shall be approved by the national control authority.

##### 4.4.3.1 *Treatment before inactivation*

When cell cultures are used the bulk material shall be filtered or clarified by continuous centrifugation prior to inactivation.

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

##### 4.4.3.2 *Method and agents*

The method and agents used for inactivation shall be approved by the national control authority. The method used shall be demonstrated to be consistently effective in the hands of the manufacturer. Inactivation should be commenced immediately after the preparation and sampling of single virus harvests when neural tissue is used, or immediately after filtration when cell cultures are used.

One method that has been successfully used to inactivate Japanese encephalitis virus is the treatment of the virus harvest with formalin at a final concentration of 1:2000 for 50–60 days at 4 °C.

The suspension containing inactivated virus shall serve as the bulk material when neural tissue is used, and as the final bulk when cell cultures are used.

##### 4.4.3.3 *Test for effective inactivation*

Each bulk suspension shall be tested for inactivation of virus. The test shall be approved by the national control authority. The test

shall be performed with the undiluted bulk suspension. A test sample corresponding to no less than 25 human doses of the final bulk shall be used.

In one country the test sample is dialysed at about 5 °C for not less than 24 hours against a sufficient volume of buffered isotonic sodium chloride solution—and diluted if necessary—to remove any cytopathogenic effect due to the residual inactivating agent or other substances.

The total volume of the test sample shall be inoculated into the primary culture of hamster-kidney cells, or any other cell cultures with no less susceptibility to the virus than hamster-kidney cells, and incubated at  $35 \pm 1$  °C for a period of 14 days. A cell culture sheet not less than 3 cm<sup>2</sup> shall be used for 1 ml of the test material. During the incubation period, no cytopathic change shall be detected.

At the completion of the observation, the cultured fluid shall be collected and inoculated intracerebrally at a dose of 0.03 ml into at least 10 mice of about 4 weeks of age. The animals shall be observed for 14 days. The bulk passes the test if the product has been shown to be free from residual live virus.

#### 4.4.4 *Purification of inactivated virus suspension*

4.4.4.1 The bulk suspension derived from mouse brains (see part A, section 4.1, of these Requirements) shall be purified by a process that has been approved by the national control authority and has been shown to give consistent results.

The purification process should be designed to reduce the myelin content to the lowest possible level.

4.4.4.2 The bulk suspension derived from cell culture shall be purified and concentrated by a process approved by the national control authority.

#### 4.4.5 *Potency test of bulk suspension*

The test for potency shall be made on each bulk suspension by detecting the neutralizing antibody produced in immunized mice. The method used shall be approved by the national control authority.

## **4.5 Preparation and control of final bulk**

### **4.5.1 *Preservatives and other substances added***

In the preparation of the final bulk only the preservatives or 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 formalin has been used for inactivation, the procedure shall be such that the amount of formaldehyde in the final bulk is no greater than 0.01%. The test method used shall be approved by the national control authority.

No antibiotics shall be added to Japanese encephalitis vaccine for human use after virus harvest.

### **4.5.2 *Sterility tests***

Each final bulk shall be tested for sterility according to the requirements in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (2, p. 49).

## **5. Filling and Containers**

The requirements concerning filling and containers in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (1, p. 16) shall apply, with the addition of the following directive.

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 sealed under vacuum shall be tested for leaks and all defective containers shall be discarded.

## **6. Control Tests on Final Product**

### **6.1 Identity test**

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

The test for potency, as described in Part A, section 6.5, of these Requirements may serve as an identity test.

## **6.2 Sterility tests**

Each final lot shall be tested for bacterial and mycotic sterility according to the requirements in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (2, p. 49).

## **6.3 Innocuity tests**

Each final 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 Protein content**

6.4.1 The protein content shall be no greater than 80 µg/ml when neural tissue is used.

6.4.2 The protein content shall be no higher than 200 µg/ml when human albumin is added in cell culture.

## **6.5 Potency test**

The potency test shall be determined by titration of the neutralizing antibody produced in immunized mice by the plaque-reduction method, using primary chick-embryo cells or BHK-21 cells. The test shall be made in parallel with a reference vaccine. The challenge strain and reference vaccine, as well as the test procedure used, shall be approved by the national control authority (see Part B, section 1, of these Requirements).

The test vaccine and the reference vaccine are diluted appropriately and each dilution is injected intraperitoneally in 2 doses of 0.5 ml each at 7-day intervals into at least 10 mice of 4 weeks of age. Seven days following the second injection, each animal is bled. The separated serum is pooled at each dilution of vaccine and then inactivated at 56 °C for 30 min; it may then be stored at -20 °C.

The serum is appropriately diluted and mixed with an equal volume of challenge virus, containing about 200 PFU/0.4 ml. The mixture is kept at 37 °C for 90 min for neutralization.

The virus suspension is then diluted and inoculated on to chick-embryo or BHK-21 cells. The infected cells are overlaid with 1% agar or methyl cellulose.

After incubation for an appropriate time, the cells are stained and the number of plaques formed on the cultures counted to obtain the plaque-reduction rates for the test and the reference vaccines. From the rates, the neutralizing antibody titres are calculated for each group.

The mean number of plaques of the control should be 50–150 per dish.

The potency of the test sample should be no less than that of the reference vaccine upon statistical comparison of the results.

## **6.6 Stability test**

The method of production of vaccine shall be such that stable vaccine is produced. The test used shall be 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 of liquid vaccine made after the storage of samples for 1 week at 37 °C is suitable. The test for potency of freeze-dried vaccine is made after the storage of samples for 4 weeks at 37 °C. In order to pass the test the lot should retain minimum potency, as defined in Part A, section 6.5, of these Requirements.

In some countries each lot of vaccine must be subjected to the stability test; in others the test is required only for the initial licensing lots to show consistency of production.

## **6.7 Residual moisture tests on freeze-dried vaccine**

The residual moisture in a representative sample of each freeze-dried lot shall be determined by a method approved by the national control authority. The upper limit for the moisture content shall be specified by the national control authority.

Moisture levels of less than 3% are usually considered satisfactory.

## **6.8 Inspection of final containers**

Each container in each final lot shall be inspected, and any that show any abnormality 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 serum concentration in the final product shall be not more than 1 part per million (ppm).

### **6.11 Inactivation of virus**

In some countries a test for virus inactivation is carried out by inoculating 10 mice intracerebrally with 0.03 ml of the final product.

## **7. Records**

The requirements in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (I, p. 17) shall apply.

## **8. Samples**

The requirements in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (I, p. 18) shall apply.

## **9. Labelling**

The requirements in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (I, p. 18) shall apply, with the addition of the following directive.

The leaflet accompanying the package shall include the following information.

(a) whether the vaccine was prepared by an *in vivo* or an *in vitro* method;

- (b) the method used for inactivating the virus; and
- (c) if the vaccine is in freeze-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 in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*I*, p. 18) shall apply.

## **11. Storage and Expiry Date**

The requirements in Part A, section 10, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*I*, p. 19) shall apply.

### **11.1 Storage conditions**

Japanese encephalitis vaccine shall be stored at a temperature of  $5 \pm 3^{\circ}\text{C}$  avoiding freezing if in liquid form, and at less than  $10^{\circ}\text{C}$  if in lyophilized form.

### **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 in Part B of the revised Requirements for Biological Substances No. 1 (General



Requirements for Manufacturing Establishments and Control Laboratories) (1, p. 19) shall apply.

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

The national control authority shall provide or approve the strain for challenge and the reference vaccine for use in the potency test (see Part A, section 6.5, of the present Requirements).

## 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 further 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 Japanese encephalitis vaccine between countries.

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The second draft of these Requirements was formulated in March 1987 by the following WHO staff, taking into consideration the first draft and the need for

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**Appendix 1**

**SUMMARY PROTOCOL FOR PRODUCTION AND TESTING OF JAPANESE ENCEPHALITIS VACCINE (INACTIVATED) FOR HUMAN USE**

**Identification of Final Lot**

Name and address of manufacturer \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
Lot number of vaccine \_\_\_\_\_  
Date of manufacture of final lot \_\_\_\_\_  
Expiry date \_\_\_\_\_  
Total volume of final lot \_\_\_\_\_

**Control of Source Materials**

**Serum for cell cultures**

Origin of serum used \_\_\_\_\_  
Tests performed on serum \_\_\_\_\_  
Results \_\_\_\_\_

**Virus seed**

*Strain of virus*  
Name and short description of history \_\_\_\_\_  
\_\_\_\_\_  
Date of preparation of master virus seed lot \_\_\_\_\_  
Date of preparation of working virus seed lot \_\_\_\_\_  
Number of passages between master and working virus seed lots \_\_\_\_\_  
Number of subcultures between working seed lot and production \_\_\_\_\_

*Tests for identification of the virus seed lot*

Method used \_\_\_\_\_  
Results \_\_\_\_\_

*Tests for freedom from bacteria, fungi, and mycoplasmas*

Method used \_\_\_\_\_  
Results \_\_\_\_\_

*Tests for adventitious agents*

Tests in animals:  
Methods used \_\_\_\_\_

Results \_\_\_\_\_

Tests in cell cultures:  
Methods used \_\_\_\_\_

Results \_\_\_\_\_

**Control of Vaccine Production**

**Control of single virus harvests**

*Sterility test*

Method \_\_\_\_\_  
Results \_\_\_\_\_

*Test of virus content*

Method \_\_\_\_\_  
Results \_\_\_\_\_

**Bulk material**

*Pooling of single virus harvests*

Number of single harvests pooled \_\_\_\_\_  
Volume of bulk material \_\_\_\_\_

*Animal serum test*

Method \_\_\_\_\_  
Result \_\_\_\_\_

**Inactivation**

Agent and concentration \_\_\_\_\_  
Temperature \_\_\_\_\_

Date of start of inactivation \_\_\_\_\_  
Date of completion of inactivation \_\_\_\_\_

*Test for effective inactivation*  
Method \_\_\_\_\_  
Results \_\_\_\_\_

*Purification of virus*  
Method of purification \_\_\_\_\_  
Concentration \_\_\_\_\_

*Potency test of bulk suspension*  
Method \_\_\_\_\_  
Results \_\_\_\_\_

**Final bulk**  
Preservatives and other substances \_\_\_\_\_  
Concentrations \_\_\_\_\_

*Sterility test*  
Method \_\_\_\_\_  
Results \_\_\_\_\_

**Final product**  
*Potency test*  
Method \_\_\_\_\_  
Results \_\_\_\_\_

*Identity test*  
Method \_\_\_\_\_  
Results \_\_\_\_\_

*Sterility test*  
Method \_\_\_\_\_  
Results \_\_\_\_\_

*Innocuity test*

Number of animals \_\_\_\_\_  
Route of injection \_\_\_\_\_  
Volume of injection \_\_\_\_\_  
Date of injection \_\_\_\_\_  
Date of end of test \_\_\_\_\_  
Results \_\_\_\_\_

*Protein content*

Method \_\_\_\_\_  
Results \_\_\_\_\_

*Stability test*

Method \_\_\_\_\_  
Results \_\_\_\_\_

*Pyrogenicity test*

Method \_\_\_\_\_  
Results \_\_\_\_\_

*Residual moisture test (for freeze-dried vaccine)*

Method \_\_\_\_\_  
Results \_\_\_\_\_

*Content of inactivating agent*

Method \_\_\_\_\_  
Results \_\_\_\_\_

*Content of preservatives*

\_\_\_\_\_

*Inactivation of virus*

Method \_\_\_\_\_  
Results \_\_\_\_\_

**Certification**

Name (typed) and signature of head of laboratory \_\_\_\_\_  
Date \_\_\_\_\_

Certification by person taking overall responsibility for production and control of the vaccine:

I certify that lot No. .... of Japanese encephalitis vaccine (inactivated) meets the requirements of Part A of the WHO Requirements for Biological Substances No. 43 (Requirements for Japanese Encephalitis Vaccine (Inactivated) for Human Use).

Name (typed)

\_\_\_\_\_

Signature

\_\_\_\_\_

Date

\_\_\_\_\_



## Appendix 2

### GENERAL SCHEME FOR THE PREPARATION OF JAPANESE ENCEPHALITIS VACCINES

Stage	Procedures	Tests
<b>Single harvest</b> (1 production run)		
Cell culture vaccine Mouse brain vaccine	— Inactivation	} Virus content } Sterility
<b>Bulk material</b> (1 or more pooled harvests)		
Cell culture vaccine  Mouse brain vaccine	{ Filtration or continuous centrifugation Inactivation Purification Purification	} Inactivation } Potency
<b>Final bulk</b> (1 or more pooled purified bulks)		
	Addition of preservatives and stabilizers	Sterility
<b>Final product</b>		
	Filling	Potency Identity Sterility Innocuity Protein content Stability Pyrogenicity Residual moisture Content of inactivating agent Content of preservative Inactivation of virus Animal serum Inspection of final containers

## Annex 7

# REQUIREMENTS FOR HUMAN INTERFERONS MADE BY RECOMBINANT DNA TECHNIQUES

### (Requirements for Biological Substances No. 41)

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## GENERAL CONSIDERATIONS

Advances in molecular genetics and gene engineering 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 product for which they code. A gene that codes for a specific product can be isolated and propagated by inserting its DNA 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 mononucleotides. The process of decoding this information and the synthesis of the gene product occurs in two phases: first, transcription of the DNA coding strand in the form of messenger RNA (mRNA), and, second, translation of the information carried by the mRNA 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. Currently, recombinant DNA products are produced by the following systems: bacteria, yeast, insect cells, and mammalian cells.

Interferons are proteins with antiviral, antiproliferative, and immunomodulatory properties. There are three classes of interferon: alpha-interferon ( $\alpha$ ), beta-interferon ( $\beta$ ), and gamma-interferon ( $\gamma$ ). Human alpha-interferon (HuIFN- $\alpha$ ) represents a family of more than 23 species of structurally similar proteins, many of which have been cloned. Within the HuIFN- $\alpha$  family there are two subclasses of genes, a major one encoding for interferons of 165 or 166 amino acids and a second which encodes for those of 172 amino acids. Both subclasses are found on chromosome 9. Many of the species of HuIFN- $\alpha$  are potent antiviral agents with specific activities of approximately  $2 \times 10^8$  units/mg protein and apparent relative molecular masses in the range 17–28 000. One major species of HuIFN- $\beta$  has been identified, and the gene is also located on chromosome 9. This interferon is composed of 166 amino acids, and shares 34% sequence similarity HuIFN- $\alpha_{2b}$ . It is also a potent antiviral agent with a specific activity in the range  $1-5 \times 10^8$  units/mg protein. A single gene on chromosome 12 codes for HuIFN- $\gamma$ .

The gene codes for a mature protein of 143 amino acids and has little or no sequence similarity to HuIFN- $\alpha$  or HuIFN- $\beta$ . Compared with HuIFN- $\alpha$  and HuIFN- $\beta$ , HuIFN- $\gamma$  is a more potent modulator of the immune response. Natural human beta- and gamma-interferons are glycosylated; several of the natural human alpha-interferons examined also appear to be glycosylated.

The genes for HuIFN- $\alpha_2$  have been cloned and their products isolated in pure form. HuIFN- $\alpha_{2a}$  and HuIFN- $\alpha_{2b}$  were shown to have antiviral activity *in vitro* similar to that of interferon preparations obtained from paramyxovirus-induced human leukocytes and lymphoblastoid cells.

In general, an interferon derived from the cells of a given animal species is most active when it is used to treat cells from the same species; and, being a protein, it may be antigenic when administered to an animal from another species. Therefore, although human interferons have been used in a number of animal studies, these have for the most part little relevance to their use in man. However, the antiviral and antitumour activity of human interferon preparations in man is now well established.

The Requirements which follow should be considered in the control and testing of recombinant human alpha-2-interferon made by recombinant DNA methods. They have been formulated bearing in mind the scale-up required for commercial production. Particular emphasis is placed on "in-process control" and consistency of the manufacturing process, a concept which has been highly effective in the control of other biological products, rather than on relying entirely on tests on the final product. General requirements, such as tests for potency, identity, purity, toxicity, pyrogenicity and sterility, will apply as much to interferon made by recombinant DNA methods as to those derived from lymphoblastoid cells and peripheral blood lymphocytes. Certain tests will be required on every production batch of interferon, whereas others will be required only to establish the validity, acceptability, and consistency of a given manufacturing process.

A detailed description of the strategy by which the product is manufactured should be given. Evidence should be presented to show that interferon made by recombinant DNA techniques possesses antiviral activity, plus any other biological activity expected of the product. Rigorous identification and characterization of the recombinant-DNA-derived interferon will be required since structural alterations can arise at the genetic or post-trans-

lational level during cultivation or at the protein level during purification. Therefore structural and biological characterization is necessary for each lot to ensure product consistency. In addition, microbial contamination may occur during fermentation, and testing for contaminants must therefore be thorough. Finally, whenever possible, information pertaining to the chemical, structural, biological and/or immunological properties of the naturally occurring interferon component or components should be provided and compared with the corresponding properties of the recombinant product.

Special attention should be given to the potential presence of contaminants in the final product. For instance:

(1) Unwanted gene products may be co-expressed unexpectedly with the interferon. Such gene products might arise because (a) mutations, insertions, deletions or rearrangements in the coding region of the product occur during fermentation; (b) transcription initiates at several sites; or (c) changes occur during culture that affect transcription, initiation or termination processes favouring the expression of other genes in the vector or the host cell.

(2) Biologically active extraneous components such as DNA, proteins and any adventitious agents, including retroviruses, derived from the host-cell system may be found in the final product.

(3) Agents used in the purification process (column matrices, column ligands, e.g., antibodies) may give rise to specific contaminants in the final product.

Therefore the methods used for the purification and to identify and characterize the product must be described.

The product arising from the recombinant system should have biological activity in a cell line sensitive to the given interferon in conjunction with the appropriate international standard.

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 interferon made by recombinant DNA techniques, it is recommended that a clause should be included that would permit modifications of the

manufacturing requirements on the condition that it can be demonstrated to the satisfaction of the national control authority that such modified requirements ensure that the degree of safety and the potency of the recombinant interferon product are at least equal to those provided by the requirements formulated below. The World Health Organization should then be informed of the action taken.

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

## **PART A. MANUFACTURING REQUIREMENTS**

### **1. Definitions**

#### **1.1 International name and proper name**

The international name shall be *Interferon humanum 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 interferons that satisfy the requirements formulated below.

#### **1.2 Descriptive definition**

*Interferon humanum recombinatum* is a preparation of purified interferon that has been derived through recombinant DNA techniques. The preparation shall satisfy all the requirements formulated below.

#### **1.3 International standards and reference preparations**

International standards and reference preparations shall be used for the control of interferon for use in the determination of potency.

For example, the International Standard for Interferon, Human, rDNA (HuIFN- $\alpha_2$ ( $\alpha$ A)) is intended for comparison of the sensitivity of the bioassays in different laboratories for the measurement of the antiviral activity of interferon made by recombinant DNA techniques. This preparation was established in 1984 (1).

This standard should be used for the calibration of only those national preparations of HuIFN- $\alpha_2$  that have dose-response curves parallel to the dose-response curve of this preparation.

Interferon standards and reference reagents, are held and distributed by the National Institutes of Health, Bethesda, MD, USA, and the National Institute for Biological Standards and Control, Potters Bar, England.

Samples from one or more final lots of material that has been shown to be active in clinical use, or samples directly related to such material, shall be fully characterized in ways to be specified by the national control authority and suitably stored to serve as manufacturer's reference material. For certain critical tests, such reference material shall be included in parallel with each lot of production material, which must match the specification of the reference batch with limits to be agreed by the national control authority.

#### 1.4 Terminology

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

*Cell seed*: a quantity of cells of uniform composition, stored frozen at  $-70^{\circ}\text{C}$  or below in aliquots, one or more of which would be used for the production of a manufacturer's working cell bank.

Several national control authorities have drafted documents on cell substrates used in the manufacture in their countries of biological products for human use.

*Manufacturer's working cell bank (MWCB)*: a quantity of cells derived from one or more aliquots of the cell seed, that are of uniform composition and have been dispensed in a single working session into a number of ampoules, one or more of which would be used for the production of each single harvest.

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 and preserved cryogenically to form the MWCB.

*Production cell culture*: a collection of cell cultures being used for biological production that has been derived from one or more ampoules of the MWCB.

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

*Purified interferon bulk solution*: interferon purified from one or more single harvests.

*Final bulk:* The finished biological material prepared from the purified interferon bulk solution present in the container from which the final containers are filled.

*Final lot:* a collection of sealed final containers that derive from the same final bulk and are homogeneous with respect to the risk of contamination during filling or preparation of the finished product. A final lot consists therefore of finished material dispensed into containers in one working session and processed as a single lot.

## **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) (2) shall apply to establishments manufacturing interferon, with the addition of the following directives.

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

The production of human interferon shall be conducted by staff who have not handled animals or infectious microorganisms in the same working day. The staff shall consist of persons whose state of health does not compromise the quality of the product.

No culture of any microorganism or eukaryotic cells, other than those required for the manufacturing process and approved by the national control authority, shall be introduced or handled in the production area at any time during the manufacture of the interferon including the establishment of the cell seed.

Persons not directly concerned with the production processes, other than official inspectors, shall not be permitted to enter the production area without valid reason and specific authorization.

Particular attention shall be given to the recommendation in Part A, section 1, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2) regarding the training and experience of persons in charge of production and testing and of those assigned to various areas of responsibility in the manufacturing establishment, as well as to the registration of such personnel with the national control authority.

The use of the seed lot system shall be strictly adhered to, and a description of the system used shall be provided, including the



number of vials of seed available and details of their storage. Particular attention shall 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 shall be provided to the national control authority, with particular reference to tests to monitor the presence of microbial contamination. Information on the sensitivity of methods to detect such contamination and the frequency of the tests shall be provided, together with information on the criteria for the rejection of contaminated materials. All tests shall be validated to the satisfaction of the national control authority.

The yield of interferon shall be monitored during the course of individual production runs. Criteria, based on yield, for the acceptance of single harvests for further processing into a final lot shall be defined; and consistency of production shall be established by testing a number of consecutive lots prepared by the same procedures, which shall be determined by the national control authority.

### **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) (2) shall apply to the manufacture of interferon.

#### **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 shall be given. This shall include details of: (a) phenotypic and genotypic markers of the host cell; (b) the construction, genetics, and structure of the expression vector; (c) the analysis of host cell for viral particles and viral nucleic acid, where appropriate; and (d) the origin, identification and sequencing of the gene that is being cloned, including its flanking regions.

The association of the vector and host cell may be permanent, allowing continuous expression of the product, or self-limiting

—for example, where the vector is an acceptable cytopathic virus.

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

Data that establish the stability of the expression system during storage of the MWCB and beyond the maximum level used for production shall be provided. The stability of the expression system shall be monitored at intervals to be established by the national control authority. Any instability of the expression system that occurs 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 and restriction enzyme mapping of the vector containing the gene inserts shall be provided in respect of the cell seed, the manufacturer's working cell bank, and the cells at the end of fermentation, as required by the national control authority.

### **3.3 Purification procedures**

The methods used to purify the interferon from culture harvests shall be fully described. The capacity of each step of the purification procedure to remove substances other than interferon that may be 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 in this respect.

If any substance is added during purification, it shall be reduced to an insignificant concentration during further purification or shown not to affect the safety and efficacy of the final product to the satisfaction of the national control authority.

If antibodies are used in the purification procedures, their origins and characteristics shall be fully described. The degree of purity of

monoclonal antibodies produced from hybridoma cell lines and the criteria for freedom from cell-derived or virus-derived DNA and from murine viruses shall conform to the regulations set by the national control authority.

### **3.4 Characterization of the gene product (interferon)**

#### *3.4.1 Protein quantification*

The protein content of purified interferon bulk solution shall be established by quantitative amino acid analysis or by another accurate method in comparison to a reference reagent.

#### *3.4.2 Protein characterization*

The characterization of interferons shall be established by techniques approved by the national control authority, which will specify the procedures to be applied to one or more reference batches of purified interferon bulk solution and those to be applied to each purified interferon bulk solution.

The protein composition shall be analysed by techniques such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) performed under both reducing and non-reducing conditions and/or high-performance liquid chromatography (HPLC). In the case of SDS–PAGE, the stained gels shall be analysed by an appropriate method, such as scanning densitometry, to quantify the percentage purity of the interferon preparation. If bands other than the interferon monomers are observed on the gel, Western blot analysis using antibodies shall be used to identify which species are the interferon products (e.g., oligomers, fragments) and which are non-interferon contaminants.

The following tests have also been found useful in characterizing the protein product: isoelectric focusing, size-exclusion chromatography, amino acid analysis, ultraviolet spectroscopy, affinity chromatography, circular dichroism, and neutralization by anti-interferon antibody.

Peptide mapping under reducing and non-reducing conditions shall be performed to provide confirmatory evidence that the structure of the product has not been altered.

The amino acid sequence of the amino terminal shall be analysed to confirm product identity and purity.

In addition, carboxy-terminal amino acid sequencing may be required by the national control authority.

### *3.4.3 Consistency of production*

Data on the consistency of the production process shall be provided in terms of the specific activity (units of biological activity per mg of protein) at different stages in the production process and in terms of the purity (percentage content of extraneous protein) of each lot of purified interferon bulk solution. The national control authority shall approve the criteria for an acceptable production run.

## **4. Cell Seed and Manufacturer's Working Cell Bank (MWCB)**

### **4.1 Origin of cell seed**

Only cells approved by and registered with the national control authority shall be used to produce human interferon. If continuous cell lines are used, they shall be characterized as specified in the Requirements for Biological Substances No. 37 (Requirements for Continuous Cell Lines) (3). The national control authority shall have responsibility for approving the cell seed.

### **4.2 Characterization of cell seed and MWCB**

The characteristics of the cell seed and manufacturer's working cell bank (host cell in combination with the expression vector system) shall be fully described, and information given on the absence of adventitious agents, and on genetic homogeneity. The nucleotide sequence of the human interferon gene insert and its flanking regions and the restriction mapping of the vector shall be given.

### **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 shall be accomplished, if necessary, in selective media such that the genotype and phenotype are consistent with the characteristics of the original host-vector system. The identity of the cells shall be determined by the use of appropriate tests.

## 5. Controls for Mammalian Cell Cultures

If serum is included in the medium for the production cell cultures, it shall be tested to demonstrate freedom from bacteria, fungi, and mycoplasmas according the requirements in Part A, section 5.2 and 5.3 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (4) and to demonstrate freedom from viruses. The methods used shall be approved by the national control authority.

Suitable tests for the detection of bovine viruses in serum are given in Appendix 3 of the revised Requirements for Biological Substances No. 7 (Requirements for Poliomyelitis Vaccine (Oral)) (5).

Alternatively, the serum may be processed in various ways, such as by filtration together with irradiation or chemical treatment(s), which eliminate or inactivate any bacteria, fungi, viruses, or mycoplasmas that might be present in the untreated serum. The supporting data should be presented to the national control authority, and, if accepted, it may be agreed by the national control authority that tests on each batch of serum for production are not needed.

Beta-lactam antibiotics shall not be used at any stage in the production process.

## 6. Controls for Additives

Any additives, including any inducers and enhancers, shall be defined and approved by the national control authority. If the inducer is a virus, this shall be derived according to a seed lot system under approved manufacturing conditions.

Any additives used shall be added to the production cell cultures at a concentration within a range previously shown to yield a satisfactory crude product from cultures incubated at a temperature and for a period specified. These details shall be agreed with the national control authority. The manufacturer shall demonstrate to the satisfaction of the national control authority that the presence of inducers or enhancers in the crude product does not adversely affect its stability.

## **7. Production Precautions**

### **7.1 Production cell cultures**

Only cell cultures derived from the MWCB shall be used for production. All processing of cells shall be done in an area in which no other cells or organisms are handled, other than those directly required for the process.

### **7.2 Cultural conditions for production cell cultures**

Production cell cultures shall be grown under conditions agreed with the national control authority. These conditions shall include details of the culture system used, the cell doubling time, the number of subcultures or the duration of the period of subcultivation permitted, and the incubation temperature.

Cell cultures shall be monitored for freedom from microbial contamination as required by the national control authority.

## **8. Single Harvests**

The single harvests shall have been processed to remove cells and cell debris.

### **8.1 Sterility**

The degree and nature of any microbial contamination 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 approved by the national control authority.

### **8.2 Consistency of yield**

The yield of human interferon following production shall be shown to be within the limits approved by the national control authority (see Part A, section 3.4.3, of these Requirements).

### **8.3 Stability of the expression system**

The method to assess the stability of the expression system used shall be approved by the national control authority.

#### 8.4 Cell identification

Samples from each production run shall be tested to confirm the identity of the cells by a method and at intervals specified by the national control authority.

### 9. Purification

The purification procedure to be applied at any stage of the manufacturing process shall be approved by the national control authority. Human interferon shall be purified prior to formulation. Adequate purification may require several purification steps based on different principles; this will minimize the possibility of copurification of extraneous cellular materials. The methods used for purification of the human interferon shall be appropriately validated (see Part A, section 3.3, of these Requirements) and approved by the national control authority. The purified bulk solution may be stabilized by the addition of protein or other substances of a nature and at a concentration approved by the national control authority. If the stabilizing substance is of human origin, it shall be manufactured in such a way as to ensure its freedom from adventitious agents. Any substances added shall not impair the safety and efficacy of the product.

The samples required for certain tests such as those for protein content, purity and composition analysis (e.g., SDS-PAGE), residual cellular DNA, peptide mapping, HPLC, and amino acid sequencing must be taken before any proteinaceous stabilizer is added. The test methods used shall be approved by the national control authority. Tests, to be approved by the national control authority, shall be made for any materials of animal origin (e.g., serum protein) or plant origin (e.g., lectin) used at any stage of production and purification; and the national control authority shall determine the acceptable levels of such materials in the interferon preparation.

In batches of HuIFN- $\alpha_2$  produced by manufacturers, the purity of HuIFN- $\alpha_2$  was greater than 95% as determined by photometric scanning of gels following reducing and non-reducing SDS-PAGE, the relative molecular mass was estimated to be 19 000, and the specific activity was approximately  $2 \times 10^8$  International Units per mg of protein, as determined by protection against the cytopathic effect of virus.

Cell debris and nucleic acids were flocculated and removed by centrifugation. HuIFN- $\alpha_2$  in the supernatant fluid was purified by a series of procedures involving affinity chromatography on immobilized anti-interferon monoclonal antibodies, ion-exchange chromatography, and molecular-exclusion chromatography.

### **9.1 Assay for protein content**

The total protein content of the human interferon shall be quantified (see Part A, section 3.4.1, of these Requirements).

### **9.2 Test for human interferon content**

The human interferon content of the purified preparation shall be determined by an appropriate biological method.

Tests that have been found suitable include SDS-PAGE, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), and single radial immunodiffusion with comparison to a known standard. Analysis of the data by the parallel-line method has been found suitable for most of these techniques.

### **9.3 Test for additives used during purification or other phases of manufacture**

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

#### **9.3.1 *Monoclonal antibody***

When a monoclonal antibody is used in the preparation procedure (e.g., for use in immunosorbent affinity chromatography to purify HuIFN- $\alpha_2$ ) the product shall be tested for residual antibody. The method used and the permitted concentration of antibody shall be approved by the national control authority.

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

#### **9.3.2 *Antibiotics***

A test shall be made as required by the national control authority for the presence in the interferon preparation of any antibiotics used



in the manufacturing process. The assay methods shall be described, and the permitted concentration in the final product shall be approved by the national control authority.

#### **9.4 Test for identity (molecular and immunochemical identity)**

Tests shall be made for the identity of the human interferon product by SDS-PAGE and/or neutralization of biological activity, as required by the national control authority.

#### **9.5 Protein purity**

The purity of each purified interferon bulk solution before the addition of any stabilizing solutions shall be established by methods approved by the national control authority.

Techniques that are useful include SDS-PAGE under reducing and non-reducing conditions, HPLC, Western blot analysis, isoelectric focusing, size-exclusion chromatography, peptide mapping, amino acid composition and sequence analysis, ultraviolet spectroscopy, and circular dichroism.

Procedures used for HuIFN- $\alpha_{2a}$  and HuIFN- $\alpha_{2b}$  have included SDS-PAGE, amino acid analysis and sequencing, peptide mapping, and isoelectric focusing. Purity of greater than 95% has been established for these preparations.

#### **9.6 Test for serum proteins**

If serum is used in the medium for the production cell cultures, or at any stage in the purification process, for example, as a reagent in immunosorption chromatography, tests shall be made for any residual serum in the purified interferon bulk blend by radio-immunoassay, ELISA, or another test agreed with the national control authority.

#### **9.7 Test for residual DNA**

The amount of residual DNA in each batch of product shall be determined by sensitive methods, which must be validated and approved by the national control authority. The acceptable level of DNA per human dose shall be determined by the national control authority.

A WHO Study Group on Biologicals (6) 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.

## **10. Final Bulk**

Substances such as diluent, stabilizers and/or preservatives added to the purified interferon bulk solution shall be approved by the national control authority.

### **10.1 Test for sterility**

The final bulk shall be tested for bacterial and mycotic sterility according to the requirements in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (4).

### **10.2 Test for pyrogenic substances**

The pyrogen content shall be determined by a method agreed with the national control authority.

## **11. Filling and Containers**

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

Care shall be taken to ensure that the materials of which the container and closure are made do not react with interferon.

## **12. Control of the Final Lot**

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

### **12.1 Tests for sterility**

Each final lot shall be tested for sterility according to the requirements in Part A, section 5, of the revised Requirements for

Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (4).

### **12.2 Test for identity**

Human interferon made by recombinant DNA techniques shall be identified as human interferon by appropriate methods approved by the national control authority.

Radioimmunoassay, ELISA, and neutralization assay are useful methods.

### **12.3 Test for potency**

The test for potency, which shall be based on a biological activity and approved by the national control authority, shall be performed on samples representative of the final filling lots. The essential information to be provided shall be that indicated in WHO Technical Report Series, No. 687, Annex 1 (7). An appropriate reference preparation shall be tested in parallel. Statistical analysis of the data must show that the mean potency value obtained has confidence limits within a range accepted by the national control authority.

### **12.4 Tests for innocuity**

Each final lot shall be tested for innocuity in mice and guinea-pigs by methods approved by the national control authority.

### **12.5 Test for pyrogenic substances**

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

### **12.6 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.

### **12.7 Tests for additives**

Each final lot shall be tested for the presence of additives. The tests used and the permitted concentrations shall be approved by the national control authority.

### **12.8 Moisture content**

For lyophilized products, the moisture content per vial shall not exceed a level approved by the national control authority.

### **12.9 pH and clarity**

The pH and degree of clarity of the interferon solution in the final container or in the reconstituted final container shall be within the limits approved by the national control authority.

## **13. Records**

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

## **14. Samples**

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

## **15. Labelling**

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

The leaflet accompanying the package shall include the following information:

- (a) description of the product,
- (b) clinical pharmacology,
- (c) indications and usage,
- (d) contraindications,
- (e) warnings,
- (f) precautions,
- (g) use during pregnancy,
- (h) adverse reactions,
- (i) dosage and administration,
- (j) directions for use,
- (k) how supplied,
- (l) storage conditions,
- (m) references.

## **16. Distribution and Shipping**

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

## **17. Storage and Expiry Date**

The requirements in Part A, section 10, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2) shall apply.

### **17.1 Storage conditions**

Filled containers of human interferon intended for clinical use shall be stored under conditions such that the product conforms with the specification agreed with the national control authority during the claimed shelf life.

### **17.2 Expiry date**

The expiry date shall be fixed with the approval of the national control authority on the basis of evidence for stability supplied by the manufacturer.

## **PART B. NATIONAL CONTROL REQUIREMENTS**

### **1. General**

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2) shall apply. The national control authority shall:

- (a) approve the methods for producing human interferon by recombinant DNA techniques;
- (b) approve the tests for human interferon concentration and define the minimum acceptable value of that concentration;
- (c) approve the cell seed and manufacturer's working cell bank (MWCB);
- (d) approve the methods for purification;
- (e) approve the tests for the purity of the product;
- (f) approve the tests for extraneous substances and total protein;
- (g) approve the tests for preservative and for the agents used for purification or other aspects of manufacture;
- (h) approve the tests for freedom from abnormal toxicity in the final product;
- (i) approve the types of tests used in the assay of potency; and
- (j) approve the data to establish clinical activity and safety in humans.

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**

Human interferon made by recombinant DNA techniques shall be released only if it fulfils the above requirements.

A statement signed by the appropriate official of the national control authority shall be provided at the request of the manufacturing establishment and shall certify whether or not the final lot of human interferon in question meets all national requirements as well as the above requirements. The certificate shall state the date of the last satisfactory human interferon 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 human interferon between countries.

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## Annex 8

# REQUIREMENTS FOR HEPATITIS B VACCINE PREPARED FROM PLASMA

(Requirements for Biological Substances No. 31)  
(Revised 1987)

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## INTRODUCTION

Viral hepatitis is a major public health problem occurring endemically in all parts of the world. There is substantial evidence that hepatitis B may progress to chronic liver disease, including chronic persistent hepatitis, chronic active (aggressive) hepatitis, and cirrhosis. About 80% of hepatocellular carcinomas are ascribed to chronic infection with hepatitis B virus. In some regions of the world, delta virus coinfection and superinfection have been associated with a high morbidity and mortality in hepatitis-B-positive individuals.

Hepatitis B virus (HBV) has been identified as a 42-nm particle (known as the Dane particle) containing double-stranded DNA. At least three antigenic components are produced during infection with hepatitis B virus as a result of replication of the virus in hepatocytes. These components are hepatitis B surface antigen (HBsAg), the core antigen (HBcAg), and the *e* antigen (HBeAg). Infection also produces high titres of anti-HBcIgM. The surface antigen is most frequently found as 20–22 nm spherical particles (sometimes slightly larger or smaller) and as tubular forms, and possesses common determinants *a* and generally at least two mutually exclusive subdeterminants *d* or *y* and *w* or *r*. Other subspecificities have also been recognized.

The protein moiety of hepatitis B surface antigen particles consists of a polypeptide of relative molecular mass 23 000, existing in glycosylated and non-glycosylated forms. Minor components are also present which contain amino acid sequences referred to as pre-S1 and pre-S2 as well as this polypeptide. The importance of these additional sequences in immunization is under investigation.

Hepatitis B virus has not yet been cultivated in cell culture and small laboratory animals are not susceptible to infection. The infection can be transmitted to certain of the apes, of which the chimpanzee is the only available susceptible animal model.

Transmission of hepatitis B infection from carrier mothers to their babies can occur during the perinatal period and among children in the first years of life and is an important factor determining the prevalence of the virus infection in some regions. Such transmission can be interrupted by the use of vaccine.

Because of the urgent need for a hepatitis B vaccine, particularly for groups that are at increased risk of acquiring infection (1), WHO Requirements were formulated in 1980 (2) and revised in 1984 (3). Since it has been shown that the separated viral coat proteins, containing hepatitis B surface antigen, lead to the production of protective antibody, it is possible to use purified, non-infectious 22-nm spherical hepatitis B surface antigen particles, or subunits derived from the surface antigen, as vaccines. However, the preparation of such vaccines for use in man from human viral antigens not grown in cell culture, but obtained from the plasma of infected persons—namely, from persistent carriers of hepatitis B antigens—demands special consideration in the tests applied to the production and quality control of the vaccines. Still more important, it has now been shown that human blood and plasma may harbour

a number of infectious agents in addition to hepatitis B virus. Particular attention, therefore, must be given to the selection of the donors of the plasma, the process of separation of the antigen, and the inactivation procedures to ensure that all potential infectious agents that may still be present after the purification of the antigen have been inactivated.

### **The development of vaccines**

A number of laboratories have prepared vaccines using HBsAg purified from plasma obtained from antigenaemic carriers of hepatitis B. Vaccines of varying degrees of purity and technological complexity have been prepared and some have been tested in humans.

Source plasma could contain infectious agents that possess a wide range of physico-chemical and biological characteristics and various degrees of susceptibility or resistance to different modes of inactivation. Consequently, to ensure as far as possible the inactivation of a wide range of infectious agents, it is desirable that, in addition to separation and purification, a procedure or procedures that will inactivate all infectious agents that may be present in human blood should be applied during the vaccine manufacturing process.

Whatever the procedure used, it is universally accepted that the vaccines must be safe (i.e., free from demonstrable virus and other microbial agents), potent (i.e., capable of eliciting antibody against the virus in animals and in man by the administration of a standardized dose of antigen) and efficacious (i.e., protective against the disease).

There have been a number of significant findings in the production of hepatitis B vaccine since the WHO Requirements were first formulated in 1980. In choosing methods of purification and inactivation, it is important to appreciate that hepatitis B surface antigen is a relatively stable glycoprotein that can withstand fairly harsh treatment. This permits the production of an essentially pure vaccine for which assurances as regards safety can be given. The consistency of vaccine production, including in particular uniformity of composition and potency of the final product and effectiveness of virus inactivation procedures applied during manufacture, is most readily achieved for highly purified materials. Consequently it is desirable that the manufacturing procedure

adopted enables a high degree of purification of HBsAg to be reliably achieved.

Hepatitis B vaccines prepared to date have been adjuvanted, and all are assayed for their ability to stimulate anti-HBs in small laboratory animals. An immunogenicity test performed in mice has been developed for quantification of the immunizing potency of batches of vaccine, and an International Reference Reagent has been established for this purpose.

Controlled studies in chimpanzees have shown the efficacy of several vaccines in preventing hepatitis B following challenge with human hepatitis B virus. Cross-protection studies carried out in chimpanzees have shown that subtypes are not of major importance in vaccine composition. In one country, the use of the vaccine in the staff of a renal dialysis unit has shown considerable protection against *ay* infection by *ad* vaccine. The reverse is also true because of the common *a* components.

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 be included permitting modifications of manufacturing requirements on the condition that it can be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure that the degree of safety and the potency of the vaccine are at least equal to those provided by the requirements formulated below. The World Health Organization should then be informed of the action taken.

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

# PART A

## MANUFACTURING REQUIREMENTS

### 1. Definitions

#### 1.1 International name and proper name

The international name shall be *Vaccinum hepatitis B ex plasma humanum*. The proper name shall be the equivalent of the international name in the language of the country of origin.

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

#### 1.2 Descriptive definition

*Vaccinum hepatitis B ex plasma humanum* is a preparation of purified hepatitis B surface antigen (HBsAg) that has been treated to inactivate HBV and other viruses known to be present in human blood. The preparation shall satisfy all the requirements formulated below.

#### 1.3 International reference materials

For the assessment of immunogenicity of vaccines, an international reference reagent exists in the form of an adjuvanted vaccine, for injection into animals (see Part A, section 5.6). This preparation is in the custody of the National Institute for Biological Products and Control, Potters Bar, England.

For the assay of antigenic content by techniques such as radioimmunoassay, ELISA, or single radial immunodiffusion, a purified international reference reagent without adjuvant is required (see Part A, section 3.6.2).

For the measurement of antibody to hepatitis B vaccines, an international reference preparation is available. This preparation is calibrated in terms of anti-HBs. The International Reference Preparation of Hepatitis B Immunoglobulin (established in 1977) is dispensed in ampoules containing 50 IU of hepatitis B immunoglobulin (fractionated human plasma, 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

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

*Whole blood* (sometimes referred to as blood): the blood collected in an anticoagulant solution.

*Plasma*: the liquid part of blood collected in a receptacle containing an anticoagulant.

*HBV*: hepatitis B virus. A 42-nm enveloped virus, originally known as the Dane particle.

*HBsAg*: hepatitis B surface antigen, the complex of antigens associated with the virus envelope and subviral forms (22-nm spherical and tubular particles). Native HBsAg is coded for by envelope gene sequences S plus pre-S of HBV DNA.

*HBcAg*: hepatitis B core antigen. The hepatitis B antigen found within the core of the virus.

*HBeAg*: the *e* antigen has now been identified as a cryptic HBc antigen.

*Anti-HBs*: antibody to hepatitis B surface antigen.

*Anti-HBc*: antibody to hepatitis B core antigen.

*Anti-HBe*: antibodies to the *e* antigen(s).

*Single donor plasma*: plasma obtained from a single donation of whole blood or obtained by plasmapheresis.

*Plasma pools*: pools of single-donation plasmas that have been shown to be satisfactory before pooling.

*Purified HBsAg batch*: purified HBsAg prepared from one or more plasma pools by suitable procedures that inactivate HBV and any other viruses that may be present in human blood.

*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 that have been treated to inactivate HBV and any other viruses that may be present in human blood 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 or preparation of the finished vaccine. A final lot must therefore consist of finished material dispensed 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) (4) shall apply to establishments manufacturing hepatitis B vaccine, with the addition of the following directives:

Production areas shall be decontaminated before they are used for the manufacture of hepatitis B vaccine. Completely separate areas shall be used for the separation and inactivation steps. All separation and inactivation steps shall be carried out in closed systems and closely monitored.

The production of hepatitis B vaccine shall be conducted by staff who have not handled other infectious microorganisms or animals in the same working day. The staff shall consist of persons who shall be examined medically and found to be healthy and not carriers of hepatitis B.

Steps should be taken to ensure that all such persons in the production areas are immune to hepatitis B as shown by the presence of anti-HBs, either as a result of natural infection or by immunization.

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 in Part A, section 1, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 13) regarding the training and experience of persons in charge of production and testing and of those assigned to various areas of responsibility in the manufacturing establishment, as well as to the registration of such personnel with the national control authority.

## 3. Production Control

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) (4, p. 15) shall apply to the manufacture of hepatitis B vaccine.

### 3.1 The collection of blood and plasma

#### 3.1.1 *The selection of donors*

Source materials for further processing are obtained from donations of blood or plasma. The medical criteria for accepting donors—criteria relating to the safety, purity, potency, and efficacy of the final products—must be the same for donors of whole blood (see Part A, section 3.1.2) components or blood components collected by plasmapheresis (6, Annex 1, p. 38), except that the donors must be antigenaemic and need not meet the exclusions relating to hepatitis. Only plasma from donors who are seronegative in appropriate tests for human immunodeficiency virus (HIV) shall be used (5).

In some countries separate areas or special times are set aside for the collection of plasma known to be HBsAg positive.

In some countries donors with a high HBsAg content but negative for HBeAg are selected for the donation of plasma.

Records should be kept of the identity of the donors for the identification of each batch of vaccine.

#### 3.1.2 *Donors of whole blood*

The physical fitness of a donor shall be determined by a licensed physician or a person under the direct supervision of a licensed physician. Donors shall be asymptomatic persons of either sex between the ages of 18 and 65 years, except that the findings in liver function tests may exceed normal limits provided that the values obtained are stable.

When plasma is collected from regions or populations with a high prevalence of hepatitis delta virus infection, screening of individual donors for evidence of chronic delta virus infection should be considered.

#### 3.1.3 *Medical history*

*General.* Before each donation questions shall be asked to determine that the donor is asymptomatic and has not suffered, or is not suffering, from any serious illness—e.g., malignant disease, diabetes, epilepsy, hypertension, renal disease, malaria.

Any donor who appears to be suffering from symptoms of acute or chronic disease, or who is receiving oral or parenteral medication, with the exception of vitamins or oral contraceptives, may not be



accepted for donation unless approved by a physician. The values obtained in liver function tests may exceed normal limits provided that the values obtained are stable.

Any donor who appears to be under the influence of alcohol or any drug or who does not appear to be providing reliable answers to medical history questions shall not be accepted.

National health authorities shall develop policies designed to prevent the transmission of other infectious diseases based on the prevalence of these diseases in the donor population and the susceptibility of recipients to the same diseases.

*Minor surgery.* Donors shall not have a history of tooth extraction or other minor surgery during a period of 72 hours prior to donation.

*Pregnancy.* Pregnant women shall be excluded from blood donation. In general, mothers shall also be excluded for the period of lactation and for at least 6 months after full-term delivery.

*Immunization.* Symptom-free donors who have recently been immunized may be accepted with the following exceptions:

- those receiving attenuated vaccines for measles (rubeola), mumps, yellow fever, or poliomyelitis shall be excluded until 2 weeks after the last immunization or injection;
- those receiving attenuated rubella (German measles) vaccine shall be excluded until 8 weeks after the last injection;
- those receiving rabies (therapeutic) vaccine or rabies immunoglobulin shall be excluded until 1 year after the last injection;
- those receiving passive immunization using animal serum products shall be excluded until 4 weeks after the last injection.

#### 3.1.4 *Physical examination*

Donors shall have a weight, blood pressure, pulse rate, and temperature within normal limits. Donors with any measurements outside the established normal limits of weight, blood pressure, and pulse rate may be accepted only if approved by the responsible licensed physician.

The following recommendations may be useful for guidance:

- (1) Blood pressure. Systolic blood pressure between 12 and 24 kPa (90 and 180 mmHg); diastolic blood pressure between 6.7 and 13.3 kPa (50 and 100 mmHg).
- (2) Pulse. Between 50 and 100 beats per minute and regular.
- (3) Temperature. Oral temperature not exceeding 37.5°C.

(4) Weight. Donors weighing less than 50 kg may be bled proportionately less than 450 ml per unit, provided all other donor requirements are met.

In some countries it is not required to take the body temperature, but the decision to do so or not should be made by the national control authority.

Donors shall be free from any infectious skin disease at the venepuncture site and from skin punctures or scars indicative of addiction to narcotics.

### 3.1.5 *Determination of haemoglobin or erythrocyte volume fraction*

The haemoglobin shall not be less than 125 g/l of blood for women and 135 g/l of blood for men. If erythrocyte volume fraction measurement is substituted for haemoglobin measurement the values shall be not less than 0.38 and 0.41 for men and women, respectively.

These limits are not universally accepted, and the national control authorities should raise or lower them when considered to be appropriate.

### 3.1.6 *Donors for plasmapheresis*

All phases of plasmapheresis, including explaining to donors what is involved in the process and obtaining their informed consent, shall be performed under the direct supervision of a licensed physician.

There are two groups of plasmapheresis donors: those who donate at a frequency comparable to that allowed for whole blood donations and those who donate more frequently. The former group shall be accepted on the basis of the above criteria for donors of whole blood.

In addition to these criteria, donors participating in the plasmapheresis programme shall be examined by a licensed physician on the day of the first donation, or no more than 1 week prior to the first donation. This examination shall include urine analysis and blood sampling for liver function tests, and determination of plasma proteins by electrophoresis or another suitable method.

On the day of each donation, in addition to meeting the requirements for whole blood donors, plasmapheresis donors shall be shown to have a total serum protein of no less than 60 g/l.

The medical evaluation of plasmapheresis donors shall be repeated at monthly intervals, or as specified by the national control authority.

Whenever a laboratory value other than a liver function test is found to be outside the normal limits or any important abnormalities are noted in a donor's history or on physical examination, the donor shall be removed from the programme. The donor shall not return to the programme until the abnormal finding has returned to normal and the responsible physician has given approval.

If a plasmapheresis donor donates a unit of whole blood or does not have the red blood cells returned from a unit taken during the procedure, further plasmapheresis of the donor shall be deferred for 8 weeks unless special circumstances warrant approval by the responsible physician of earlier plasmapheresis. Plasmapheresis of donors of HBsAg-positive plasma will be permitted by the responsible physician, even if the liver function test values are above normal limits in individual donors, provided that the values obtained are stable.

The upper limits of these values should be specified by the national control authority.

Appropriate guidelines to define donor changes significant to justify discontinuation of plasmapheresis should be established by the responsible physician (6, p. 43).

The maximum volume of blood or plasma that may be taken in 1 year from chronic hepatitis B carriers shall be determined by the national control authority.

No guidelines have been established for the maximum volume of plasma that can be taken in any year from a chronic hepatitis B carrier.

In some countries it is permitted to take 500 ml per week.

Where plasmapheresis is regularly performed on HBsAg-positive donors, it is advisable to have equipment set aside specifically for this purpose.

### **3.2 Tests on single-donation plasma**

Each single-donation plasma, whether obtained from whole blood or by plasmapheresis, shall be tested for HBsAg content by a method approved by the national control authority.

Several tests are suitable for this purpose (*J*). Potency should be established with reference to an appropriate potency standard included in all assays.

An international standard is available for the calibration of working reference reagents.

In some countries, unless the subtype of a particular donor has been identified, each single donation is tested for HBsAg subtype by a method approved by the national control authority.

The national control authority may require the data on the subtype composition.

In some countries the tests are done on plasma pools, when this is approved by the national control authority.

### **3.3 Pooling of single-donation plasma**

Only acceptable plasma shall be included in a plasma pool.

### **3.4 Tests on plasma pools**

#### *3.4.1 Sterility tests*

A volume of at least 10 ml of each plasma pool shall be tested for sterility according to the requirements in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (7, p. 48).

In some countries it is required that each plasma pool shall be tested for the presence of mycobacteria by culture methods approved by the national control authority.

#### *3.4.2 Tests for extraneous viruses*

##### *3.4.2.1 Tests in animals and fertile eggs*

###### *(a) Tests in adult mice*

Each plasma pool shall be tested in adult mice for adventitious agents pathogenic to mice. Each of at least 10 adult mice, of 15–20 g weight, shall be inoculated intracerebrally with 0.03 ml and at least 10 mice intraperitoneally with at least 0.5 ml of the plasma 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 autopsied and examined for evidence of viral infection, both macroscopically by direct observation and by subinoculation of

appropriate tissue suspensions by the intracerebral and intraperitoneal route into at least 5 additional mice, which shall be observed for 21 days.

The plasma 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 transmissible agents attributable to the plasma pool.

(b) *Tests in suckling mice*

Each plasma pool shall be tested in suckling mice for adventitious agents pathogenic to 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 plasma 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 autopsied and examined to determine the cause of death or illness.

In some countries a subinoculation is suggested and in others an additional 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 plasma 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 transmissible agents attributable to the plasma pool.

(c) *Tests in embryonated eggs*

A sample of at least 5 ml of each plasma pool shall be tested in a group of embryonated hens' eggs by the allantoic route of inoculation and a similar sample in a separate group of eggs by the yolk-sac route of inoculation, using at least 0.25 ml of the pool per egg for each route of inoculation. The incubation of the eggs and the observation time shall be approved by the national control authority. The embryo shall remain normal throughout the observation period.

The plasma pool passes the test if there is no evidence of the presence of any adventitious agents attributable to the plasma pool.

3.4.2.2 *Tests in cell cultures*

A sample of at least 5 ml of each plasma pool shall be tested for adventitious agents by inoculation into simian cell cultures. Similar

volumes of the plasma pool shall likewise be tested in human diploid cell cultures. The inoculated cell cultures and uninoculated control cultures shall be observed for at least 14 days. At the end of this observation period a subculture in the same cell system shall be made and cultures observed for at least 14 days.

In some countries larger volumes of the plasma pool are required to be tested.

Suitable simian cell cultures are prepared from the kidneys of *Cercopithecus* monkeys or from Vero cells. For the human cell cultures, either WI-38 or MRC-5 may be used.

The plasma pool passes the test if none of the cell cultures shows evidence of the presence of any adventitious agent attributable to the plasma pool.

#### 3.4.2.3 *Other tests*

It may be desirable to carry out tests on the plasma pools for HBV DNA by sensitive DNA hybridization assays to monitor the elimination of HBV DNA by subsequent purification steps.

### 3.5 Concentration, purification, and inactivation

Each plasma pool shall be subjected to procedures that concentrate and purify HBsAg consistently and result in the inactivation of residual HBV and any extraneous agent that may be present in human blood. The methods used shall remove the bulk of extraneous substances and inactivate infectious agents so that the resultant purified product is safe when administered to humans.

In some countries the required HBsAg content of vaccines is not less than 95% of total protein content of the finished vaccine.

The national control authority shall approve the methods used for concentration and purification of HBsAg and for inactivation of HBV and other potential contaminating agents. The national control authority shall approve the number of inactivation steps that shall be used.

The national control authority shall ensure that the production process, including purification and inactivation, is reproducible and will give rise to consecutive lots that do not differ with respect to safety.

When new processes for HBV vaccine manufactured from human plasma are introduced, the efficiency of hepatitis B DNA removal at each step during the purification process shall be validated.

Removal of HBV DNA can be monitored by DNA hybridization assays.

Chimpanzee studies may be carried out to validate the inactivation process. In such tests titrated HBV stocks containing approximately  $10^6$  chimpanzee infectious doses are added to the material prior to inactivation and residual infectivity in the inactivated material is detected by the inoculation of two chimpanzees. Titrated HBV stocks are available from The New York Blood Center.

Should proteins other than HBsAg remain in the vaccine, the national control authority shall take into consideration data which identify such proteins and which show that they do not compromise the safety of the product.

In assessing the efficiency of a procedure or procedures for inactivation the national control authority shall take into consideration data demonstrating the ability of each method to inactivate infectious agents that may be found in human blood.

Precipitation by ammonium sulfate and polyethylene glycol has been found suitable for the concentration of HBsAg.

Much experience has now been gained in the consistent production of safe batches of vaccine. Reliance is placed predominantly on different methods (chemical treatment, physical separation, and heat treatment).

A procedure that has been successfully used includes purification by zonal centrifugation, followed by three chemical treatment procedures:

- (i) pepsin, 1  $\mu\text{g}/\text{ml}$  at pH 2.0 held at  $37^\circ\text{C}$  for 18 hours;
- (ii) urea, 8 mol/litre held at  $37^\circ\text{C}$  for 4 hours; and
- (iii) formalin 1:4000 (1:10 000 formaldehyde) at  $37^\circ\text{C}$  for 3 days.

A second method involves the separation of the HBsAg, including isopyknic zonal centrifugations through cesium chloride, followed by treatment with 1:4000 formalin (1:10 000 formaldehyde) at  $30^\circ\text{C}$  for 48 hours.

A third approach includes 3 isopyknic zonal centrifugation steps with KBr and rate zonal centrifugation through sucrose followed by heat treatment of the HBsAg at  $60^\circ\text{C}$  for 10 hours and treatment with 1:2000 formalin (1:5000 formaldehyde) at  $37^\circ\text{C}$  for 4 days.

One manufacturer uses differential precipitation with polyethylene glycol and ultracentrifugation, followed by heat inactivation for 90 s at  $103^\circ\text{C}$ . After adsorption to aluminium phosphate, the product is heated for 10 hours at  $65^\circ\text{C}$ . Another

manufacturer uses differential precipitations with polyethylene glycol, selective adsorption on hydroxylapatite and isopycnic centrifugation in KBr followed by 2 heating steps under defined conditions: first at 102°C for 2 min 40 s, then at 65°C for 10 hours.

### 3.6 Tests on purified, inactivated HBsAg batches

#### 3.6.1 Sterility tests

A volume of at least 10 ml of each batch shall be tested for sterility according to the requirements in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (7, p. 48).

#### 3.6.2 Tests for HBsAg and total protein

After purification, the protein content shall be measured for both total protein and HBsAg-specific protein. The latter shall be compared with that of a suitable reference preparation.

It has been found suitable to measure the total concentration of protein by means of extinction coefficient  $E_{280}^{1\%}$  or by the micro-Kjeldahl technique, the Lowry test or another appropriate method.

The content of HBsAg shall be determined by a serological test in comparison with a suitable reference preparation. It is important that the method of production gives a reproducible content of HBsAg. The lower limit of concentration permitted shall be determined by the national control authority.

Both radioimmunoassay and ELISA methods have been shown to be suitable for this purpose. Other tests such as single radial immunodiffusion may be used.

The concentration of HBsAg shall be related to the total protein.

#### 3.6.3 Tests for extraneous substances

Tests shall be made for the presence of blood group substances and other blood proteins, including liver-specific membrane proteins, by methods approved by the national control authority.

Agglutinins and agglutinogens are tested for by haemagglutination. Immunoassays, such as immunoelectro-



phoresis, agar gel diffusion, radioimmunoassay and ELISA, and polyacrylamide gel electrophoresis have been used to test for other extraneous proteins.

The preparation shall be free from detectable blood group substances.

The permitted concentration of non-HBsAg proteins present in the vaccine shall be determined by the national control authority.

#### 3.6.4 *Test for HBV DNA*

The preparation shall be free from HBV DNA, as determined by a sensitive hybridization assay approved by the national control authority.

DNA is extracted from a volume of aqueous bulk concentrate that corresponds to 10 adult doses and tested for HBV DNA sequences using a sensitive and specific hybridization assay. The assay should be capable of detecting at least 1 pg of HBV DNA per 10 adult doses.

#### 3.6.5 *Test for antigen purity*

A test shall be made for purity of HBsAg by polyacrylamide gel electrophoresis (PAGE).

In reduced preparations there should be 2 bands shown by polyacrylamide gel electrophoresis, one at 22 000–23 000 and another at 28 000–30 000 relative molecular mass. Additional HBV-specified or non-HBV-specified bands may also be present.

The national control authority shall determine the electrophoretic pattern permitted as a demonstration of purity.

#### 3.6.6 *Tests for reagents used during manufacture*

A test shall be made for the presence of any potentially hazardous reagent, including inactivating reagents, that may have been used during the manufacture of the HBsAg.

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

### 3.7 **Final aqueous bulk**

The final aqueous bulk consists of one or more purified, concentrated HBsAg batches that have been treated to inactivate

infectious agents that may be present in human blood. Only batches that have satisfied the requirements in Part A, sections 3.5 and 3.6, shall be included in the final bulk.

### 3.7.1 *Safety test*

In view of the concern about the possibility of infectious agents being present in the plasma used for the production of hepatitis B vaccine, the national control authority shall ensure that the procedures for the collection of plasma and the production process, including purification and inactivation, are reproducible and will give rise to consecutive lots that will not differ with respect to safety.

The national control authority shall determine whether initial lots of vaccine shall be tested for the presence of infectious hepatitis B viruses in chimpanzees. The test shall be approved by the national control authority.

If a test in chimpanzees is not or cannot be carried out, alternative approaches may be considered by the national control authority. For example, clinical studies in man have been carried out that demonstrated the production of a vaccine acceptable to the national control authority.

When a chimpanzee safety test is used, the first 5 consecutive lots prepared by the same production procedures shall be tested in chimpanzees. These 5 lots shall be tested individually without pooling. If these 5 lots pass this test, safety testing of subsequent lots in chimpanzees may be discontinued. If an established manufacturing process is altered or the same process transferred under controlled conditions from one manufacturer to another after initial safety validation in chimpanzees, the national control authority may elect to reduce the number of chimpanzee safety tests required for the new production lots to less than 5.

Aqueous bulks that fail the test shall not be used to prepare vaccine for use in man. In such a case it shall be considered that the consistency has not been established and a further 5 consecutive lots must be tested. The reasons for failing the test shall be investigated and reported to the national control authority.

The chimpanzees used for testing shall have been under observation for at least 6 months before inoculation and shown to satisfy the conditions listed below. The chimpanzees shall:

(a) be free from hepatitis B virus infection, past or present, as shown by sensitive techniques (negative tests for HBsAg, anti-HBs, and anti-HBc);

(b) have normal levels of aminotransferases in at least 8 specimens taken during the 8 weeks that immediately precede the start of the study;

(c) have had at least 2 normal liver biopsies taken during the 8 weeks that precede the start of the study;

(d) be housed in adequate isolation quarters and attended by persons free from hepatitis B infection;

(e) have never received blood or blood products of human origin.

A satisfactory test involves 2 chimpanzees. One animal shall receive 1 human dose and the other shall receive 10 human doses by intravenous injection.

During the observation period of 6 months after inoculation the tests shall include:

(a) weekly determination of alanine aminotransferase (ALT), which shall remain normal for each individual chimpanzee; any abnormal finding shall be demonstrated to be unrelated to viral hepatitis;

(b) weekly determinations of the markers of HBV infection, using sensitive serological methods;

(c) antibody assays for HIV before inoculation and 4 and 6 months after inoculation;

(d) weekly weight determinations and daily checks of general health;

(e) biopsies for light microscopic examination to search for evidence of hepatitis taken monthly and at any time that the chimpanzees show any abnormality.

If after 6 months' observation the chimpanzees have shown normal alanine aminotransferase values throughout with no histological evidence of hepatitis or serological evidence of hepatitis B virus infection, the vaccine passes the chimpanzee safety test. If an animal develops only anti-HBs the test of this animal is invalidated, and an additional animal shall be added to the test.

In such circumstances consideration should be given to decreasing the amount of antigen administered.

### 3.7.2 *Sterility tests*

A volume of at least 10 ml of the final aqueous bulk shall be tested for bacterial and mycotic sterility according to the requirements in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (7, p. 49).

### 3.7.3 *Test for HBsAg*

The quantity of HBsAg compared with the total protein in the final aqueous bulk shall be determined by a quantitative serological procedure in comparison with a suitable reference reagent. The lower limit of HBsAg and the limit of total protein per human dose shall be approved by the national control authority.

The tests referred to in Part A, section 3.6.2, have been found to be suitable. The samples for this test should be taken before the addition of preservative.

### 3.7.4 *Pyrogenicity test*

Each final bulk shall be tested for pyrogenicity by a suitable test. The test shall be approved by the national control authority.

## **3.8 Final bulk**

### 3.8.1 *Addition of adjuvant*

Where the final bulk contains an adjuvant, the adjuvant and the concentration used shall be approved by the national control authority. Where aluminium salts are used, the concentration of aluminium shall not exceed 1.25 mg per single human dose.

At this stage more preservative may need to be added.

In some countries the alum used as an adjuvant is formed in the presence of the HBsAg, whereas in others preformed alum salts are added to the aqueous bulk. Where preformed aluminium adjuvants are used, it may not be possible to resolubilize the aluminium compound, and the testing for purity and concentration of the HBsAg in the final bulk may not be possible.

### 3.8.2 *Test for completeness of adsorption to adjuvant*

Tests shall be carried out to confirm that all HBsAg is adsorbed to the adjuvant. The tests shall be approved by the national control authority.

### 3.8.3 *Tests for sterility*

A volume of at least 10 ml of 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 Sterility of Biological Substances) (7, p. 49).

### 3.8.4 *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.

## **4. Filling and Containers**

The requirements concerning filling and containers in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 16) 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 can only be stored at  $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ .

## **5. Control Tests on Final Product**

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

### **5.1 Sterility tests**

The final lot shall be tested for sterility according to the requirements in Part A, section 5, of the revised Requirements for

### **5.2 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 those approved by the national control authority.

### **5.3 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.

### **5.4 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.

### **5.5 Pyrogenicity test**

Each final lot shall be tested for pyrogenicity by a suitable test. The test shall be approved by the national control authority.

### **5.6 Potency and identity test**

The vaccine shall be identified as HBsAg by appropriate methods. An appropriate quantitative potency assay shall be performed on each final lot irrespective of how many filling lots are made. The vaccine potency shall be compared with that of the international reference reagent.

A suitable quantitative extinction test in mice is as follows:

Each of a group of at least 20 suitable mice, 5 weeks of age, is vaccinated intraperitoneally with a graded dose of adjuvanted hepatitis B vaccine diluted in the adjuvant used in the vaccine. Similar groups of mice are inoculated with the adjuvanted reference preparation. The mice are bled 28 days later and the sera are kept separate. Antibody determinations are performed

by a sensitive quantitative test such as radioimmunoassay. The lower limit should be less than 25% response. The data are analysed according to seroconversion as well as according to the geometric mean titre of anti-HBs for each antigen dose. The strain of mice used for this test must give a steep dose-response curve to the reference antigen.

In some countries a quantitative extinction test in guinea-pigs has been shown to be suitable.

The potency shall be measured in terms of quantity of vaccine giving an antibody response in 50% of the animals. The national control authority shall determine the lower limit of potency.

## **6. Records**

The requirements in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 17) shall apply.

## **7. Samples**

The requirements in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 18) shall apply.

## **8. Labelling**

The requirements in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 18) shall apply, with the addition of the following directive.

The leaflet accompanying the package shall include the following information:

- the method used in the inactivation of the HBV;
- the nature and amount of any preservative, adjuvant, or stabilizer present in the vaccine;
- the volume of one recommended human dose, immunization schedules, and the recommended routes of administration; these

- shall be given for newborn babies, children, adults, and immuno-suppressed individuals and shall be the same for a given vaccine for all regions of the world;
- the amount of protein contained in one recommended human dose;
  - the amount of HBsAg contained in one recommended human dose.

## **9. Distribution and Shipping**

The requirements in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 18) shall apply.

In addition, the condition of shipping shall be such that the vaccine does not freeze.

Temperature indicators should be packaged with each vaccine shipment to show that freezing did not occur.

If freezing has occurred, the vaccine should not be used.

## **10. 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) (4, p. 19) shall apply.

In addition, the conditions of storage shall be such that the vaccine does not freeze.

### **10.1 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 vaccines in bulk form or in final containers shall be kept at  $5 \pm 3$  °C. After distribution or issue, the vaccine shall be stored at a temperature not exceeding 8 °C. The vaccine shall have been shown to maintain potency for a period equal to that between the date of issue and the expiry date. During storage the vaccine shall not be frozen.



## 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 potency test, the date of this test being that on which the test system was inoculated.

## PART B

### NATIONAL CONTROL REQUIREMENTS

#### 1. General

The general requirements for control laboratories in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 19) shall apply.

The national control authority shall:

- approve the medical evaluation of donors;
- approve the methods of sterility control;
- approve the tests for HBsAg concentration and define its minimum value;
- approve the methods for concentration, purification, and inactivation;
- approve the purity of the final product;
- approve the tests for extraneous substances and total protein;
- approve the tests for the agents used for concentration and purification, free formaldehyde, and other inactivating agents and preservatives;
- approve the test for the presence of infectious hepatitis B virus;
- approve the tests used for freedom from abnormal toxicity in the final product;
- approve the adjuvant assay and define the permitted concentration of adjuvant in the final product; and
- approve the animals used in the assay of potency.

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

Where chimpanzees are not used in the proof of safety of the production process, the national control authority must accept responsibility for the use of the vaccine in man.

## 2. Release and Certification

A hepatitis B vaccine shall be released only if it fulfils Part A of the present Requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify whether or not the final lot of vaccine in question meets all national requirements as well as Part A of the present 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.

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## REFERENCES

1. WHO Technical Report Series, No. 602, 1977.
2. WHO Technical Report Series, No. 658, 1981, Annex 4.
3. WHO Technical Report Series, No. 725, 1985, Annex 3.
4. WHO Technical Report Series, No. 323, 1966.
5. *Weekly epidemiological record*, No. 18, 1986, 138-140.
6. WHO Technical Report Series, No. 626, 1978.
7. WHO Technical Report Series, No. 530, 1973.

## Annex 9

# REQUIREMENTS FOR YELLOW FEVER VACCINE

(Requirements for Biological Substances No. 3)  
(Revised 1975)

### Addendum 1987

Since the suggestion was made 12 years ago in the Requirements for Yellow Fever Vaccine to investigate the stability of lyophilized yellow fever vaccines, much experience has been gained by manufacturers, resulting in increased stability.<sup>1</sup> The Requirements should therefore be modified as follows. Page numbers refer to WHO Technical Report Series, No. 594, 1976.

#### Control tests on final product (pages 38–41)

On page 41, after the third line (small print) *insert* the following new section:

##### **“5.6 Stability**

Many national control authorities require that 3 final containers, selected at random from the filling lot, shall be held at 37°C for 2 weeks and tested for potency as defined in section 5.5. The result of the test should comply with the requirements of section 5.5. The mean loss in titre after heating should be not more than 1.0 log<sub>10</sub>.”

*Renumber* the existing section 5.6 on page 41 to read:

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<sup>1</sup> BARME, M. & BRONNERT, C. *Journal of biological standardization*, 12: 435–442 (1984).

## **“5.7 Protein nitrogen content”**

### **Storage conditions (pages 42–43)**

In the paragraph in small print at the foot of page 42 *delete* the second sentence (“Since storage below  $-25^{\circ}\text{C}$  ... stability of the vaccine.”)

On page 42, *delete* also footnote 2.

On page 43 after the 4th line *delete* the two paragraphs in small print (“Distributed vaccines ... section 5.5 page 39.”).

## Annex 10

### LABORATORIES APPROVED BY WHO FOR THE PRODUCTION OF YELLOW FEVER VACCINE

(This list supersedes the appendix in WHO Technical Report Series, No. 658, 1981,  
Annex 1, p. 53.)

Commonwealth Serum Laboratories Parkville, Victoria Australia	Federal Laboratory Service Lagos Nigeria
Robert Koch Institute Berlin (West)	Pasteur Institute of Dakar Dakar Senegal
Oswaldo Cruz Institute Rio de Janeiro Brazil	National Institute for Virology Sandringham, Transvaal South Africa
The Wellcome Research Laboratories Beckenham, Kent England	Connaught Laboratories Inc. Swiftwater, PA USA
Pasteur Institute Paris France	Institute of Poliomyelitis and Viral Encephalitis Moscow USSR
Central Research Institute Kasauli, Himachal Pradesh India	

## Annex 11

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

(Requirements for Biological Substances No. 26)  
(Revised 1981,<sup>1</sup> 1982,<sup>2</sup> 1985<sup>3</sup>)

### Addendum 1987

At its thirty-third meeting,<sup>2</sup> the WHO Expert Committee on Biological Standardization adopted a revised list of the codes used to identify antimicrobials contained in susceptibility test discs. The list of codes was again revised at the thirty-sixth meeting of the WHO Expert Committee on Biological Standardization<sup>3</sup> in order to incorporate additions, deletions and changes in nomenclature. Since that time, further requests have been received by the WHO Secretariat for the allocation of codes for new antimicrobial substances. In order to incorporate the new entries that have been agreed, the following further additions should be made to the list of codes given in Part A, Section 1.6 of the WHO Requirements (WHO Technical Report Series, No. 687, 1983, Annex 5, pp. 175-178):

#### Add

carumonam	CAR
cefepime	FEP
cefixime	CFM
cefminox	CNX
flucytosine	FCT
ornidazole	ORN
sulbactam/ampicillin	SAM

<sup>1</sup> WHO Technical Report Series, No. 673, 1982.

<sup>2</sup> WHO Technical Report Series, No. 687, 1983.

<sup>3</sup> WHO Technical Report Series, No. 745, 1987.

## **Annex 12**

# **REQUIREMENTS FOR DRIED BCG VACCINE**

### **(Requirements for Biological Substances No. 11) (Revised 1985)**

#### **Amendment 1987**

In view of the progress that has been made in maintaining the stability of lyophilized BCG vaccines, Part A, section 10.2, of the Requirements for Dried BCG Vaccine (Revised 1985) (WHO Technical Report Series, No. 745, 1987, Annex 2, p. 81) should be replaced by the following.

#### **“10.2 Expiry date**

The date after which dried BCG vaccine should not be used shall be determined in relation to the experimental evidence referred to in Part A, section 5.6, and with the approval of the national control authority. Unless there is evidence of a greater stability, the expiry date shall be not more than 24 months after the date of distribution by the manufacturer, provided that this is not more than 36 months from the date of the last satisfactory test for viability referred to in Part A, section 5.5, and provided that the vaccine has been stored continuously at the specified storage temperature and protected from daylight. In any event, the expiry date shall be not more than 4 years after the date of harvest. Each manufacturer shall test the stability of the vaccine to ensure that it satisfies these conditions.”



### Annex 13

## BIOLOGICAL SUBSTANCES: INTERNATIONAL STANDARDS AND REFERENCE REAGENTS

A list of international biological standards, international biological reference preparations, and international biological reference reagents is issued as a separate publication.<sup>1</sup> 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.

The Expert Committee made the following changes to the previous list.

### Additions

#### Blood products and related substances

Human protein C	0.82 IU/ampoule	First International Standard 1987
Human blood coagulation factors II, IX, and X in concentrates	10.8 IU factor II/ ampoule 10.7 IU factor IX/ ampoule 9.8 IU factor X/ ampoule	First International Standards 1987
Human tissue plasminogen activator	850 IU/ampoule	Second International Standard 1987

(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.)

<sup>1</sup> *Biological substances: international standards and reference reagents, 1986.* Geneva, World Health Organization, 1987.

### Antibodies

Varicella zoster immunoglobulin	50 IU/ampoule	First International Standard 1987
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(This substance is held and distributed by the International Laboratory for Biological Standards, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, Amsterdam, Netherlands.)

### Endocrinological and related substances

Human atrial natriuretic factor	2.5 IU/ampoule	First International Standard 1987
Growth hormone, human	4.4 IU/ampoule	First International Standard 1987 <sup>1</sup>
Human interleukin-2	100 IU/ampoule	First International Standard 1987

(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

Interferon, human, recombinant, $\alpha_1(\alpha D)$	8000 IU/ampoule	First International Standard 1987
Interferon, human, recombinant, $\alpha_2(\alpha_{2b})$	17 000 IU/ampoule	First International Standard 1987

(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.)

Interferon, human fibroblast, $\beta$	15 000 IU/ampoule	Second International Standard 1987
Interferon, human, recombinant, $\beta_{\text{ser 17}}$	6000 IU/ampoule	First International Standard 1987

<sup>1</sup> Formerly established and distributed as the First International Standard for Human Growth Hormone for Bioassay; change in name only.

Interferon, murine, $\alpha$	16 000 IU/ampoule	First International Standard 1987
Interferon, murine, $\beta$	15 000 IU/ampoule	First International Standard 1987
Interferon, murine, $\gamma$	1000 IU/ampoule	First International Standard 1987

(These substances are held and distributed by the Research Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA.)

#### **Discontinued**

Blood coagulation factor IX, human	5.62 IU/ampoule	First International Standard 1976
Growth hormone, human, for immunoassay	0.35 IU/ampoule	First International Reference Preparation 1968

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

## Annex 14

# 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.

No.	Year of publication	
178	1959	Requirements for Biological Substances: * 1. General Requirements for Manufacturing Establishments and Control Laboratories * 2. Requirements for Poliomyelitis Vaccine (Inactivated)
179	1959	Requirements for Biological Substances: * 3. Requirements for Yellow Fever Vaccine * 4. Requirements for Cholera Vaccine
180	1959	Requirements for Biological Substances: * 5. Requirements for Smallpox Vaccine
200	1960	Requirements for Biological Substances: * 6. General Requirements for the Sterility of Biological Substances
237	1962	Requirements for Biological Substances: * 7. Requirements for Poliomyelitis Vaccine (Oral)
274	1964	WHO Expert Committee on Biological Standardization: * 8. Requirements for Pertussis Vaccine * 9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate

\* Replaced by revised Requirements.

- | No. | Year of<br>publication |   |
|-----|------------------------|---|
| 293 | 1964                   | WHO Expert Committee on Biological Standardization:<br>* 10. Requirements for Diphtheria Toxoid and Tetanus Toxoid  |
| 323 | 1966                   | WHO Expert Group:<br>Requirements for Biological Substances (Revised 1965):<br>1. General Requirements for Manufacturing Establishments and Control Laboratories<br>* 2. Requirements for Poliomyelitis Vaccine (Inactivated)<br>5. Requirements for Smallpox Vaccine<br>* 7. Requirements for Poliomyelitis Vaccine (Oral)   |
| 329 | 1966                   | WHO Expert Committee on Biological Standardization:<br>* 11. Requirements for Dried BCG Vaccine<br>* 12. Requirements for Measles Vaccine (Live) and Measles Vaccine (Inactivated)  |
| 361 | 1967                   | WHO Expert Committee on Biological Standardization:<br>9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate (Revisions adopted 1966)<br>13. Requirements for Anthrax Spore Vaccine (Live—for Veterinary Use)<br>14. Requirements for Human Immunoglobulin<br>15. Requirements for Typhoid Vaccine |
| 384 | 1968                   | WHO Expert Committee on Biological Standardization:<br>* 16. Requirements for Tuberculin<br>* 17. Requirements for Inactivated Influenza Vaccine  |
| 413 | 1969                   | WHO Expert Committee on Biological Standardization:<br>† 4. Requirements for Cholera Vaccine (Revised 1968)<br>18. Requirements for Immune Sera of Animal Origin  |
| 444 | 1970                   | WHO Expert Committee on Biological Standardization:<br>19. Requirements for Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live)<br>† 20. Requirements for <i>Brucella abortus</i> Strain 19 Vaccine (Live—for Veterinary Use)  |
| 444 | 1970                   | WHO Expert Committee on Biological Standardization:<br>* Development of a National Control Laboratory for Biological Substances (A guide to the provision of technical facilities)  |
| 463 | 1971                   | WHO Expert Committee on Biological Standardization:<br>21. Requirements for Snake Antivenins  |
| 486 | 1972                   | WHO Expert Committee on Biological Standardization:<br>* 7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1971)  |
| 530 | 1973                   | WHO Expert Committee on Biological Standardization:<br>4. Requirements for Cholera Vaccine (Revised 1968) (Addendum 1973)   |

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\* Replaced by revised Requirements.

† Refer also to subsequent Addendum.

- | No. | Year of publication |   |
|-----|---------------------|---|
|     |                     | 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   |
| 565 | 1975                | WHO Expert Committee on Biological Standardization:<br>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)<br>Development of national assay services for hormones and other substances in community health care |
| 594 | 1976                | WHO Expert Committee on Biological Standardization:<br>† 3. Requirements for Yellow Fever Vaccine (Revised 1975)<br>20. Requirements for <i>Brucella abortus</i> Strain 19 Vaccine (Live—for Veterinary Use) (Specification of tests used in the Requirements) (Addendum 1975)<br>† 23. Requirements for Meningococcal Polysaccharide Vaccine   |
| 610 | 1977                | WHO Expert Committee on Biological Standardization:<br>Report of a WHO Working Group on the Standardization of Human Blood Products and Related Substances  |
| 610 | 1977                | WHO Expert Committee on Biological Standardization:<br>† 23. Requirements for Meningococcal Polysaccharide Vaccine (Addendum 1976)<br>† 24. Requirements for Rubella Vaccine (Live)<br>25. Requirements for <i>Brucella melitensis</i> Strain Rev. 1 Vaccine (Live—for Veterinary Use)<br>† 26. Requirements for Antibiotic Susceptibility Tests. I. Agar Diffusion Tests using Antibiotic Susceptibility Discs   |
| 626 | 1978                | WHO Expert Committee on Biological Standardization:<br>* 17. Requirements for Inactivated Influenza Vaccine (Addendum 1977)<br>† 23. Requirements for Meningococcal Polysaccharide Vaccine (Addendum 1977, incorporating Addendum 1976)<br>27. Requirements for the Collection, Processing, and Quality Control of Human Blood and Blood Products   |
| 626 | 1978                | WHO Expert Committee on Biological Standardization:<br>* Guidelines for the Preparation and Establishment of Reference Materials for Biological Substances  |
| 638 | 1979                | WHO Expert Committee on Biological Standardization:<br>† 8 & 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Revised 1978)<br>* 11. Requirements for Dried BCG Vaccine (Revised 1978)   |

\* Replaced by revised Requirements.

† Refer also to subsequent Addendum.

- | No.        | Year of<br>publication |   |
|------------|------------------------|---|
|            |                        | 17. Requirements for Influenza Vaccine (Inactivated) (Revised 1978)   |
|            |                        | 28. Requirements for Influenza Vaccine (Live)   |
| <b>658</b> | 1981                   | 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)          |
|            |                        | 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 Connection with the Issue of International Vaccination Certificates |
| <b>673</b> | 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)          |
|            |                        | * 12. Requirements for Measles Vaccines (Live) (Addendum 1981)  |
|            |                        | † 26. Requirements for Antimicrobial Susceptibility Tests (Revised 1981)  |
|            |                        | 32. Requirements for Rift Valley Fever Vaccine  |
|            |                        | A Review of Tests on Virus Vaccines   |
| <b>687</b> | 1983                   | WHO Expert Committee on Biological Standardization:   |
|            |                        | † 7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1982)   |
|            |                        | 30. Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy (Revised 1982)                       |
|            |                        | 33. Requirements for Louse-Borne Human Typhus Vaccine (Live) The Standardization of Interferons                                 |
|            |                        | † 26. Requirements for Antimicrobial Susceptibility Tests (Addendum 1982)   |
| <b>700</b> | 1984                   | WHO Expert Committee on Biological Standardization:   |
|            |                        | † 8 & 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Addendum 1983)          |
|            |                        | 35. Requirements for Rift Valley Fever Vaccine (Live, Attenuated) for Veterinary Use  |
|            |                        | 34. Requirements for Typhoid Vaccine (Live Attenuated, Ty 21a, Oral)  |

\* Replaced by revised Requirements.

† Refer also to subsequent Addendum.

- | No. | Year of<br>publication |  |
|-----|------------------------|--|
| 725 | 1985                   | WHO Expert Committee on Biological Standardization:<br><ul style="list-style-type: none"> <li>† 8 &amp; 10. Requirements for Diphtheria Toxoid, Pertussis Vaccines, Tetanus Toxoid, and Combined Vaccines (Addendum 1984)</li> <li>* 31. Requirements for Hepatitis B Vaccine prepared from Human Plasma (Revised 1984)</li> <li>36. Requirements for Varicella Vaccine (Live)</li> </ul> Informal Consultation on the Standardization of Interferons  |
| 745 | 1987                   | WHO Expert Committee on Biological Standardization:<br><ul style="list-style-type: none"> <li>2. Requirements for Poliomyelitis Vaccine (Inactivated) (Addendum 1985)</li> <li>† 11. Requirements for Dried BCG Vaccine (Revised 1985)</li> <li>16. Requirements for Tuberculins (Revised 1985)</li> <li>† 26. Requirements for Antimicrobial Susceptibility Tests               <ul style="list-style-type: none"> <li>1. Agar Diffusion Tests Using Antimicrobial Susceptibility Discs (Addendum 1985)</li> </ul> </li> <li>37. Requirements for Continuous Cell Lines Used for Biologicals Production</li> </ul>  |
| 760 | 1987                   | WHO Expert Committee on Biological Standardization:<br>Guidelines for the Preparation and Establishment of International and other Standards and Reference Reagents for Biological Substances<br><ul style="list-style-type: none"> <li>† 7. Requirements for Poliomyelitis Vaccine (Oral) (Addendum 1986)</li> <li>8 &amp; 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Addendum 1986)</li> <li>38. Requirements for Mumps Vaccine (Live)</li> <li>39. Requirements for Hepatitis B Vaccines made by Recombinant DNA Techniques in Yeast</li> <li>40. Requirements for Rabies Vaccine (Inactivated) for Human Use Produced in Continuous Cell Lines</li> </ul> Procedure for Evaluating the Acceptability in Principle of Vaccines Proposed to United Nations Agencies for Use in Immunization Programmes<br>Model Certificate for the Release of Vaccines Acquired by United Nations Agencies<br>Report of a WHO Meeting on Hepatitis B Vaccines Produced by Recombinant DNA Techniques |
| 771 | 1988                   | WHO Expert Committee on Biological Standardization:<br><ul style="list-style-type: none"> <li>3. Requirements for Yellow Fever Vaccine (Addendum 1987)</li> <li>7. Requirements for Poliomyelitis Vaccine (Oral) (Addendum 1987)</li> <li>11. Requirements for Dried BCG Vaccine (Amendment 1987)</li> <li>12. Requirements for Measles Vaccine (Live) (Revised 1987)</li> <li>26. Requirements for Antimicrobial Susceptibility Tests               <ul style="list-style-type: none"> <li>1. Agar Diffusion Tests Using Antimicrobial Susceptibility Discs (Addendum 1987)</li> </ul> </li> </ul>  |

\* Replaced by revised Requirements.

† Refer also to subsequent Addendum.



31. Requirements for Hepatitis B Vaccine prepared from Plasma (Revised 1987)
  41. Requirements for Human Interferons made by Recombinant DNA Techniques
  43. Requirements for Japanese Encephalitis Vaccine (Inactivated) for Human Use
- Laboratories approved by WHO for the production of yellow fever vaccines
- Modification for Lyophilized BCG vaccine of the Procedure for Evaluating the Acceptability in Principle of Vaccines Proposed to United Nations Agencies for Use in Immunization Programmes
- Model Certificate for the Release of BCG Vaccines Acquired by United Nations Agencies
- Standardization of Interferons: report of a WHO informal consultation

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