



## ANNEX 2

### **RECOMMENDATIONS FOR INACTIVATED RABIES VACCINE FOR HUMAN USE PRODUCED IN CELL SUBSTRATES AND EMBRYONATED EGGS**

These Recommendations provide information and guidance to national regulatory authorities and vaccine manufacturers concerning the characterization, production and control of rabies vaccines in order to facilitate their international licensure and use. 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 national regulatory authority so desires these parts may be adopted as definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance. It is recommended that modifications to these Recommendations be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below.

In order to facilitate the international distribution of vaccine made in accordance with these recommendations, a summary protocol is given in the Appendix.

Introduction .....	
General considerations .....	
Part A. Manufacturing recommendations .....	
1. Definitions .....	
2. General manufacturing recommendations .....	
3. Control of source materials .....	
4. Control of vaccine production .....	
5. Filling and containers .....	
6. Control tests on final product .....	
7. Records .....	
8. Samples .....	
9. Labelling .....	

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10. Distribution and shipping .....  
11. Stability, storage and expiry date .....  
12. Intradermal route of administration.....

Part B. Nonclinical evaluation of rabies vaccines.....

Part C. Clinical evaluation of rabies vaccines.....

Part D. National control requirements .....  
1. General .....  
2. Release and certification .....

Authors .....  
Acknowledgements .....  
References .....  
Appendix Summary protocol .....

## INTRODUCTION

The last revision of requirements for rabies vaccines for human use was in 1980 (1). However, an additional document, WHO Requirements for rabies vaccine (inactivated) for human use produced in continuous cell lines was published in 1987 to take into consideration advances in the development of cell culture derived vaccines (2). An amendment which updated the section on the International Standard for Rabies Vaccine was published in 1994 (3,4).

The following Recommendations are for inactivated rabies vaccine for human use produced only in cell substrates and embryonated eggs. They replace all previous requirements (1,2,3,4), including those for vaccines prepared in neural tissues, which The scope of the present recommendations encompass vaccines produced in cell substrates, ranging from primary cells (hamster kidney and chick embryo fibroblasts), diploid cells, to continuous cell lines such as Vero cells. Purified vaccines produced using duck embryos are also within the scope of the document.

However, vaccines produced in mammalian neural tissues are NOT considered in this or any other document.

## GENERAL CONSIDERATIONS

Rabies is an under-reported, neglected deadly disease estimated to cause more than 50,000 human deaths annually, most of which occur in the poorest regions of the world (13). Once clinical symptoms are evident, the prognosis for survival is poor and death is almost certainly inevitable. The population at risk includes 2.5 billion people currently

living in rabies endemic regions. Unfortunately, half of the dog bite victims and subsequent rabies deaths occur in children under 15 years of age, as they are the population most at risk. (20). In light of these facts, more efforts should be made to improve the control of rabies, a zoonotic disease with the highest case fatality rate known to man.

One of the most important elements in the effective control of human rabies is the use of efficacious vaccines. Vaccines produced in mammalian neural tissues have been in use for over 100 years. However, it is the availability and use of cell culture and purified embryonated egg derived rabies vaccines that has dramatically decreased the number of human deaths throughout the world, most notably in countries where canine rabies is endemic. For example, in Thailand, the introduction of cell culture vaccines along with reduced dosage intradermal regimens decreased the incidence of human rabies by 80% in 15 years (13). Similar progress has been documented in other countries where nerve tissue vaccines have been replaced by cell culture and purified embryonated egg derived rabies vaccines.

This document is focused on the recommendations for production and control of rabies vaccines, as stated above, one of the most important elements in the effective control of human rabies. However, vaccine should always be considered as part of the complete treatment and additional information on recommendations for the treatment of the disease is available in the Report of the Expert Consultation on rabies (12).

WHO requirements for rabies vaccines were published in 1981 and 1987. The former one encompassed mammalian neural tissue derived vaccines as well as vaccines produced using embryonated eggs and variety of cell substrates, while the latter focused on vaccines produced in continuous cell lines, only. Since that time, there have been many developments in the production and quality control of vaccines as well as in their overall regulation. In particular, considerable attention has been given to safety issues.

The scientific basis for the present revision of the requirements for rabies vaccines was developed at the meetings of working group held at WHO, in May 2003 and May 2004. The issues identified for revision were: the scope of the document; the substrates for vaccine production that the revised document would cover; the inactivation process; the test for effective inactivation; potency test; the use of *in vitro* assays for determination of the antigen content as a measure of consistency of production; stability test and the value of the accelerated degradation test; and NRA requirements. Further details of these discussions and the rationale for proposed revisions are available in the meeting reports (10, 11).

Rabies vaccines produced in mammalian neural tissues (brain of adult animals such as sheep and goat; brain of suckling animals such as mouse, rat and rabbit) have been in worldwide use for many years. It is well known that their use has led to adverse reactions following immunization, such as encephalomyelitis and polyneuritis (40). Although the risk of such adverse reactions is reduced when the virus is grown in the brains of newborn animals, such as rats and mice, before the development of myelin in

the brain, the safety profile of these vaccines is still unacceptable. Moreover, there is evidence for a lack of potency of these neural tissue vaccines, leading to inadequate protection in humans, making a strong argument for the discontinuation of their production and use (37,39). The scope of the present revised recommendations is intended to improve control of rabies disease by promoting vaccines of assured quality as part of pre-exposure vaccination and post-exposure prophylaxis. In order to facilitate the international distribution of vaccine made in accordance with these recommendations, a summary protocol is given in the Appendix.

Recently developed methods for genetic sequences of rabies virus have been considered in this document. Given that genetic characteristics are part of the identity of the vaccine strains, it would be beneficial to include this information in the licensing of new vaccines and to use sequencing in monitoring subtle genetic changes of vaccine strains over time.

The approach for potency testing remains the same as previously recommended and NIH potency test, based on a mouse protection assay, is recognized as a reliable assay. A review of the data on a single dilution NIH test has led to the development of criteria for the validation of a modified NIH assay. The latter has been extensively used for lot release of veterinarian rabies vaccines while the experience in testing vaccines for human use is still at experimental stage. More data are needed to support this approach and to provide a basis for a standardized testing procedure.

Several studies conducted over the last ten years have provided useful information on the value and potential use of the *in vitro* assays for measurement of the antigen content in vaccines (NIBSC and AFSSAPS studies etc). Such assays have been successfully used by several manufacturers to control antigen concentration during production and in the final formulation of a product. However, *in vitro* data concerning antigen concentration in the final vaccine has not generally been reported and direct correlations between such determinations and evidence of protection in humans need to be evaluated. Correlations between the *in vitro* assays and the NIH test have proven challenging due primarily to the variability of the latter test. Further characterization of the reagents used for *in vitro* assays may clarify the potential of these methods to assess the quality as well as the quantity of antigen. Both manufactures and NRA/NCL staff are encouraged to further develop and use these assays, and to accumulate more data on their application to the control of rabies vaccines.

However, they also revealed some difficulties in performing the assays as well as in their analysis and the interpretation of the data. Both manufacturers and NRA/NCL staff are encouraged to develop and use these assays and to provide more data on their application to the control of a vaccine in question.

Since the intradermal route of administration has been used for some rabies vaccines initially developed for intramuscular administration, some additional considerations are provided in a separate section Intradermal route of administration, in this document. Furthermore, guidance for nonclinical and clinical testing of vaccines

intended to be used by intradermal route of administration are provided in sections B and C of this document.

Given that new rabies vaccines might be developed in the near future, a section on clinical evaluation of vaccines describes specific issues for the evaluation of data generated in clinical trials and provides some guidance on how to assess immunogenicity and safety of rabies vaccines.

The stability evaluation of vaccines is addressed in a separate section of these recommendations and emphasize the importance of real time studies under intended storage conditions.

Relevant guidance documents published since the last revision of the requirements for rabies vaccines have been considered in the present revision. The following documents are mentioned as relevant and should be consulted for further information. Updated requirements for the characterization of continuous cell lines used for the preparation of biologicals, adopted in 1998 (5), provide current recommendations for vaccines produced using cell substrates. It is important to note that the WHO recommendation for 10 ng of residual host cell DNA per single human dose for products manufactured using continuous cell lines remains the same as recommended in 1996. In addition, guidance to reduce possible risk of vaccine contamination by transmissible spongiform encephalopathies (8) and updated requirements for blood products (9) including human albumin, used in some vaccines as a stabilizer, are now available.

In recent years safety concerns have been raised over the use of thiomersal in vaccines, especially those given to infants. These concerns have been based primarily on data regarding the toxicity of a related substance, methyl mercury, and from data on chronic exposure to mercury via the food chain. Such safety concerns have, however, led to initiatives in some countries to eliminate, reduce or replace thiomersal in vaccines, both in monodose and multidose presentations. It is important to note that concerns about the toxicity of thiomersal are theoretical and there is no compelling scientific evidence of a safety problem with its use in vaccines, although public perception of risk remains in some countries. WHO policy is clear on this issue, and the Organization continues to recommend the use of vaccines containing thiomersal for global immunization programmes since the benefits of using such products far outweigh any theoretical risk of toxicity (25).

## **PART A. MANUFACTURING RECOMMENDATIONS**

### **1. Definitions**

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

The international name should be rabies vaccine for human use. The proper name should be the equivalent 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**

Rabies vaccine for human use is a freeze-dried or liquid preparation of well characterized, laboratory adapted and attenuated virus with stable biological characteristics, grown in cell substrates or embryonated eggs, and inactivated by a suitable method. The preparations for human use should satisfy all the recommendations formulated below.

## **1.3 International Standards**

The fifth International Standard for Rabies Vaccines was established by the WHO Expert Committee on Biological Standardization in 1991, with a potency of 16 International Units of Rabies Vaccine per ampoule. Recent research has indicated that the glycoprotein and ribonucleoprotein components of inactivated rabies vaccines play an important role in conferring protection. For this reason, the Committee also assigned 10 International Units of Rabies Virus PM-Glycoprotein and 135 International Units of Rabies PM-Ribonucleoprotein to the contents of each ampoule of the International Standard. It is recognized, however, that these components might differ antigenically in the different virus strains used for vaccine production; the International Standard may therefore be inappropriate for the estimation of glycoprotein and ribonucleoprotein components of vaccines not derived from the Pitman-Moore (PM) strain.

The Second International Standard for Rabies Immunoglobulin was established by the WHO in 1993. It is a preparation of human immunoglobulin and each ampoule contains 30 IU.

The fifth International Standard for Rabies Vaccine and the first International Standard for Rabies Immunoglobulin were initially held at the Statens Serum Institut in Copenhagen. Since 1997 this standard is in custody of the National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG, United Kingdom (web site: <http://www.nibsc.ac.uk>).

International reference materials mentioned above are intended for the calibration of national reference materials for use in the quality control of rabies vaccines. They are distributed free of charge, on request, to national control laboratories. The WHO catalogue of international biological standards should be consulted for the latest list of appropriate international standards and reference materials (<http://www.who.int/biologicals/IBRP/Catalogue.htm>).

## **1.4 Terminology**

The following definitions are given for the purpose of these Recommendation only.

*Virus master seed lot:* A quantity of virus, physically homogeneous, that has been prepared as a single lot . It is used for the preparation of working seed lots.

*Virus working seed lot:* A seed lot prepared from the master seed lot with no more than five passages removed from the master seed lot. Both passage level and the method of passing should be approved by the national regulatory authority.

*Cell seed:* A quantity of well-characterized cells of human or animal origin stored frozen at  $-100^{\circ}\text{C}$  or below in aliquots of uniform composition derived from a single tissue or cell, one or more of which would be used for the production of a master cell bank.

*Master cell bank:* A quantity of fully characterized cells of human or animal origin stored frozen in liquid nitrogen in aliquots of uniform composition derived from the cell seed, one or more of which may be used for the production of a manufacturer's working cell bank.

*Working cell bank (WCB):* A quantity of cells of uniform composition derived from one or more ampoules of the master cell bank, which may be used for the production cell culture. In normal practice, a cell bank is expanded by serial subculture up to passage number (or population doubling, as appropriate) selected by the manufacturer, at which point the cells are combined to give a single pool and preserved cryogenically to form the WCB. One or more of the cryotubes from such a pool may be used for the production of cell culture.

*Production cell culture:* A collection of cell cultures that have been prepared together from one or more containers from the working cell bank or in the case of primary cell cultures, from the tissues of one or more animals (5).

*Adventitious agents:* Contaminating microorganisms including bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses that have been unintentionally introduced.

*Single virus harvest:* A virus suspension of the same virus working seed lot inoculated, incubated and harvested together from either a group of embryonated eggs or a cell culture in one production run. Multiple harvests from the same production cell culture may be pooled and considered a single virus harvest.

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

*Final bulk:* The material after completion of preparations for filling, homogenous with respect to mixing of all components, and present in the container from which the final containers are filled. The final bulk may be prepared from one or more purified bulk materials.

*Final lot:* A collection of sealed, final containers of freeze-dried or liquid vaccine that is homogeneous with respect to the risk of contamination during the filling process or the preparation of the finished vaccine. A final lot must therefore have been filled or prepared in one working session from a single final bulk.

## **A.2 General manufacturing recommendations**

The general requirements for manufacturing establishments contained in good manufacturing practices for pharmaceuticals (6) and biological products (7) should apply to establishments manufacturing inactivated rabies vaccine, with the addition of the following:

Rabies has the highest case-fatality rate of any currently recognized infectious disease therefore the assignment of appropriate biosafety level for specific work with the virus at the production as well as at the control facilities is an essential precautionary measure.

The assignment of a virus to a biosafety level for production and quality control facilities must be based on a risk assessment. Such an assessment will take the risk group, as well as other factors, into consideration in establishing the appropriate biosafety level. For example, a virus assigned to risk group 2 may generally require Biosafety Level 2 facilities, equipment, practices and procedures for safe conduct of work. However, if particular phases of production require work with live virus and/or exposure to large quantities and/or high titre of virus as well as aerosol exposure, then Biosafety Level 3 may be more appropriate to provide the necessary degree of safety, since it ensures superior containment in the production and quality control facilities. The biosafety level assigned for the specific work is therefore driven by professional judgement based on a risk assessment rather than by automatic assignment of a laboratory biosafety level according to the particular risk group designation of the pathogenic agent to be used. Further guidance on the risk assessment and assignment of appropriate biosafety level are available in WHO Laboratory Biosafety Manual (22). However, countries should draw up a national classification of microorganisms, by risk group.

Personnel employed in the production and control facilities should be healthy individuals, examined medically at regular intervals. They should be adequately trained and protected against accidental infection with rabies virus. Steps should be taken to ensure that all such persons in the production and control areas have been immunized against rabies and maintain an antibody titre of at least 0.5 IU per ml of serum as measured by Rabies fluorescent focus inhibition test (RFFIT) and Fluorescent anti-virus neutralisation (FAVN). Periodic titre control and if required booster injections are recommended for people who are at continual risk of rabies exposure. Further guidance on a need for boosters are available in the Report of the Expert Consultation on Rabies (12).



The production of rabies vaccine should be conducted by dedicated staffs who do not handle other infectious microorganisms, animals, or tissue cultures in the same working day.

Steps should also be taken to minimize the risks of transmission of rabies virus from the production facility to the outside environment.

### **A.3. Control of source materials**

#### **A.3.1. Substrates for virus production**

Rabies vaccines may be produced in human diploid cells, in continuous cell lines, in primary hamster kidney cells or in primary chick embryo fibroblast cells. For human diploid and continuous cell lines section 3.1.1. should apply; for primary hamster kidney cells section 3.1.2. should apply; for primary chick embryo fibroblasts section 3.1.3 should apply; for embryonated duck eggs section 3.1.4 should apply. Section 3.1.5 applies to all types of cell substrates.

##### **A.3.1.1. Diploid cells and continuous cell lines**

The use of a diploid cell or continuous cell line for the manufacture of rabies vaccines should be based on the cell seed system. The cell seed should be approved by the national regulatory authority. The maximum number of passages (or population doublings) by which the working cell bank is derived from the cell seed should be approved by the national regulatory authority.

WHO has established a cell bank of Vero cells characterized in accordance with the requirements in the report of the WHO Expert Committee on Biological Standardization (5), which is available as a well characterized starting material to manufacturers for preparation of their own master and working cell seeds on request to the Coordinator, Quality Assurance and Safety of Biologicals, WHO, Geneva, Switzerland.

##### **A.3.1.1.1. Identity test**

Cell seed should be characterized according to the Requirements for Animal Cells Lines Used as Substrates for Biologicals Production (5), as appropriate to continuous cells or human diploid cells.

The testing performed on a replacement master cell bank (derived from the same clone or from an existing master or working cell bank) should be the same as for the establishment of the initial master cell bank, unless a justified exception is made. The WCB should be identified

by means of, for example, biochemical (e.g. isoenzyme analysis), immunological, and cytogenetic marker tests, approved by the national regulatory authority.

### **A.3.1.2 Primary hamster kidney cells for production of rabies vaccines**

#### **A.3.1.2.1. Animals**

Syrian hamsters 10-14 days old may be used as the source of kidneys for cell culture. Only hamster stock approved by the NRA should be used as the source of tissue and should be derived from a closed, healthy colony. A closed colony is a group of animals sharing a common environment and having their own caretakers who have no contact with other animal colonies. The animals are tested according to a defined programme to ensure freedom from specified pathogens, including the absence of antibodies to these pathogens. When new animals are introduced into the colony, they should be maintained in quarantine in vermin-proof quarters for a minimum of two months and shown to be free from these specified pathogens. The parents of animals to be used as a source of tissue should be maintained in vermin-proof quarters. Neither parent hamsters nor their progeny should previously have been used for experimental purposes, especially those involving infectious agents. The colony should be monitored for zoonotic viruses and markers for contamination at regular intervals.

At the time the colony is established, all founder animals should be tested to determine freedom from antibodies to the following pathogens: microorganisms pathogenic for hamsters (e.g., *Mycobacterium tuberculosis*, lymphoma virus, papilloma virus, polyomavirus, adenoviruses and retroviruses), lymphocytic choriomeningitis virus, pneumonia virus of mice, reovirus type-3, minute virus of mice, Sendai, Hantaan virus, SV-5, Toolans H-a, mouse polio, mouse hepatitis virus, virus, and Kilham rat virus. HAP, MAP and RAP tests should also be performed. A test for retroviruses using a sensitive PCR based reverse transcriptase (Rtase) assay also should be included. The results of such assays need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (23). Nucleic acid amplification tests for retrovirus may also be used. A PCR test for hamster polyoma virus should be used on a selected number of hamster tissues, especially kidneys, to qualify the colony, and at intervals thereafter.

After the colony is established, it should be monitored by testing a representative group of animals. The choice of tests and testing procedures for monitoring as well as appropriate number of animals should be approved by the national regulatory authority. In addition, the colony should be screened for the presence of pathogenic bacteria, including mycobacteria; fungi and mycoplasma. This should be performed in 100% of the animals over a defined period of time. The screening programme should be approved by the national regulatory authority.

Any animal that dies should be investigated to determine the cause of death. If the presence of an infectious agent is demonstrated in the colony, the national regulatory

authority should be informed and the manufacture of vaccine should be discontinued. In this case, manufacture should not be resumed until a thorough investigation has been completed and precautions have been taken against the infectious agent being present in the product, and only then with the approval of the national regulatory authority.

At the time of kidney harvest, the animals should be examined for the presence of any abnormalities and if kidney abnormalities or other evidence of pathology is found, those animals are not to be used for rabies vaccine production.

Each group of control cultures derived from a single group of animals used to produce a single virus harvest should remain identifiable as such until all testing, especially for adventitious agents, is completed.

#### **A.3.1.2.2. Cell cultures for virus propagation**

Kidneys derived from animals which comply with section A.3.1.2.1. should be dissected and minced under conditions approved by the NRA. A primary cell suspension is obtained after trypsin digestion and this is distributed into cell culture vessels with growth medium.

Penicillin and other Beta-lactam antibiotics should not be used at any stage of manufacture. Minimal concentrations of suitable antibiotics such as kanamycin and neomycin may be used if approved by the national regulatory authority.

#### **A.3.1.3 Chicken eggs used for primary chick embryo fibroblast preparation**

If the vaccine is to be produced in primary chick embryo fibroblasts, the eggs to be used should be from a closed, specific-pathogen-free flock. This flock should be monitored at regular intervals for agents pathogenic to birds. These include *Mycobacterium avium*, fowl pox virus, avian leukosis virus (ALV) and other avian retroviruses, Newcastle disease virus and other avian parainfluenza viruses, avian encephalomyelitis virus, infectious laryngotracheitis virus, avian reticulo-endotheliosis virus, Marek's disease virus, infectious bursal disease virus, Avian adenoviruses - group 1, Avian infectious bronchitis virus, Avian nephritis virus, Avian orthoreoviruses, Chicken anaemia virus, Egg drop syndrome virus, Influenza A virus, Turkey rhinotracheitis virus, *Haemophilus paragallinarum*, *Salmonella enterica* Gallinarum, and relevant *Mycoplasma spp.*

In some countries, all birds are bled when a colony is established, and thereafter 5% of the birds are bled each month. The resulting serum samples are screened for antibodies to the relevant pathogens. Any bird that dies should be investigated to determine the cause of death.

The flock must not have been vaccinated.

#### **A.3.1.4 Embryonated Duck Eggs**

If the vaccine is to be produced in embryonated eggs of ducks, the eggs to be used should be from a closed, specific-pathogenic-free flock. The flock should be regularly monitored for agents pathogenic to ducks. The day old ducklings come from an establishment or a hatchery where Duck Virus Enteritis, Duck viral hepatitis, Salmonella enteritidis, Salmonella anatem Salmonella aertrycke, Avian tuberculosis, Psittacosis-orinthis, fowl cholera (pasteurellosis), Egg Drop syndrome, Avian Influenza (type A) Adenovirus group III (EDS), Avian rotavirus, Avian encephalomyelitis, Avian J virus, Infectious serositus,(new duck disease), Coliform septicaemia, Spirochaetosis (Duck tick fever), Reticuloendotheliosis virus and New Castle Disease have not been reported since the last 12 months.

In some countries, all ducks are bled when a colony is established, and thereafter 5% of the ducks are bled each month. The resulting serum samples are screened for antibodies to the relevant pathogens. Any ducks that dies should be investigated to determine the cause of death.

Live vaccine of Avian influenza should never been used in the supply flocks. If any other vaccine except above is used the same has to be indicated along the name/nature of vaccine, source of vaccine and date of vaccination.

#### **A.3.1.5 Cell culture medium**

Serum used for the propagation of cells should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the requirements given in Part A, sections 5.2 and 5.3 of the revised Requirements for Biological Substances No. 6 (14), and from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the Recommendations for Production and Control of Poliomyelitis Vaccine (Oral) (15).

Validated molecular tests for bovine viruses may replace the cell culture tests of bovine sera. As an additional monitor of quality, sera may be examined for freedom from phage and for an acceptable limit of bacterial endotoxin. Irradiation may be used to inactivate potential contaminant viruses.

The acceptability of the sources(s) of any components of bovine, sheep or goat origin used in culture media should be approved by the national control authority. These components should comply with current WHO guidelines in relation to animal transmissible spongiform encephalopathies (8).

Human serum should not be used. If human albumin is used it should meet the revised Requirements for the collection, processing and quality control of blood, blood

components and plasma derivatives (Requirements for Biological Substances No.27) (14), as well as current WHO guidelines in relation to human transmissible encephalopathies (8).

The use of human albumin as a component of a cell culture medium requires careful consideration due to potential difficulties with the validity period of albumin in relation to the intended long-term storage of rabies vaccines.

Penicillin and other Beta-lactam antibiotics should not be used at any stage of manufacture. Minimal concentrations of suitable antibiotics such as kanamycin and neomycin may be used if approved by the national regulatory authority.

Nontoxic pH indicators may be added, e.g. phenol red in a concentration of 0.002%. Only substances that have been approved by the national control authority may be added.

If trypsin of animal origin is used for preparing cell cultures it should be tested and found free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially bovine or porcine parvoviruses, as appropriate. The methods used to ensure this should be approved by the national regulatory authority.

The source(s) of trypsin of bovine origin, if used, should be approved by the national regulatory authority. Bovine trypsin, if used, should comply with current WHO guidelines in relation to animal transmissible spongiform encephalopathies (8).

### **A.3.2. Virus seed**

#### **A.3.2.1. *Strains of virus***

The strains of rabies virus used in the production of all seed lots should be well characterized, laboratory adapted and attenuated\* with stable biological characteristics. The strains should be identified by historical records including the information on its origin. They should have been shown, to the satisfaction of the national regulatory authority, to yield safe and immunogenic vaccines when inactivated.

Vaccine strains used for production of vaccines derived from cell substrates and embryonated eggs known to induce protection in man against rabies include, but are not restricted to, the Pitman Moore virus; Pasteur Virus, the Vnukovo -32; the Flury LEP; and the CTN. These are the examples present in some of currently licensed vaccines and should not be interpreted as a recommendation.

\*footnote: previously used term "fixed" is based on the defined time in which clinical symptoms of the disease appear in animals when inoculated intracerebrally (e.g., rabbits, mice and sheep). This was obtained by serial passaging of the virus in rabbits. Although this characteristic reflect attenuation of virus in an appropriate animal model it is not a guarantee of a suitability of rabies virus for production of vaccines for human use. The latter should be based on the immunogenicity and safety of an inactivated virus in humans.

The choice of virus, its full characterization and adaptation to the production substrate should be justified in the overall evaluation of rabies vaccines for licensing.

Vaccine strains should be characterized by molecular and serological methods, including the use of monoclonal antibodies for the characterization of rabies virus. This should also include animal inoculation. In addition, sequencing of at least the glycoprotein and nucleoprotein genes of master or working seed should be considered.

All master and working seed lots should comply with the current guidelines to minimize the risks of transmission of animal transmissible spongiform encephalopathies (8).

#### **A.3.2.1.1. *Virus seed lot system***

Vaccine production should be based on the virus seed lot system. The working virus seed lot should be not more than five passages removed from the master virus seed lot, which should have been thoroughly characterized. Vaccines should be made from the working seed lot without further intervening passage. Virus seed lots should be maintained either in the dried or in the frozen form and each lot should be stored separately. If frozen, the seed lots should be kept continuously at a temperature below -60 °C.

#### **A.3.2.1.2. Tests on virus seed lots**

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

The virus master and working seed lots should be identified as rabies virus by methods approved by the national regulatory authority.

Monoclonal antibodies which react specifically with rabies virus nucleocapsid and glycoprotein may be used to identify the virus as rabies

All master and working seed lots should comply with the current guidelines to minimize the risks of transmission of animal transmissible spongiform encephalopathies (8).

#### **A.3.2.1.2.1. *Tests for bacteria, fungi and mycoplasmas***

Each virus seed lot should be tested for bacterial, fungal, and mycoplasmal contamination by appropriate tests according to Part A, section 5.2 of the *Requirements for biological substances no.6. General requirements for the sterility of biological substances* (14).

#### **A. 3.2.1.2.2. Tests for adventitious agents**

The virus master or working seed lots used for the production of vaccine in cell substrates or embryonated eggs should be free from detectable adventitious agents. Seed lots produced in cell substrates should comply with the recommendations in Part A . Section 3.1.1 applies to seed production in human diploid or continuous cell lines; section 3.1.2 - applies to primary hamster kidney cells; and section 3.1.3 applies to seed lots produced using primary chick embryo fibroblasts; Section 3.1.4 applies to embryonated duck eggs.

For these tests the virus should first be neutralized by a specific anti-rabies serum.

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

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

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

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

**Tests in adult mice.** A sample of the virus suspension should be tested for the presence of adventitious agents pathogenic to mice by intracerebral inoculation of 0.03 ml, intraperitoneal inoculation of at least 0.25 ml, and inoculation of 0.01 ml into the footpad in at least 20 adult mice, each weighing 15-20 g. The mice should be observed for at least four weeks. All mice that die after the first 24 hours of the test or that show signs of illness should be examined for evidence of viral infection; this should be done macroscopically by direct observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional mice, which should be observed for three weeks.

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

**Tests in cell cultures.** The neutralized seed virus should be tested for freedom from adventitious viruses in three sensitive cell culture systems: (a) the cell line used for production (b) a different cell line from a different species, and (c) human diploid cells.

Ten milliliters of the neutralized seed virus should be inoculated into each cell system and the cells incubated at 35-37°C for 14 days.

For virus seeds produced in human diploid cells, cell cultures should be held for 28 days for the detection of CMV.

The cells should be observed microscopically for cytopathic changes. At the end of the observation period, the cells or fluids should be tested for haemadsorbing viruses and other adventitious agents as specified in Part A, sections 4.1.1 and 4.1.2. For the tests to be valid, at least 80% of the culture vessels should be available and suitable for evaluation at the end of the observation period. For the seed virus to be satisfactory, no cytopathic changes or adventitious agents should be detected. Control cell cultures should be included in the tests.

#### **A.3.2.1.2.3. Additional tests if chick cell cultures are used for production of virus seed**

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

A test for retroviruses using a sensitive polymerase chain reaction (PCR)-based reverse transcriptase (Rtase) assay may be used. The results of such assays need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (23). Nucleic acid amplification tests for retrovirus may also be used.

#### **A.3.2.1.3. Virus content**

A titration of the virus content of each seed lot should be made. Such titrations may be done either in cell culture or by the inoculation of mice. If mice are used, they should be inoculated by the intra-cerebral route with 0.03 ml quantities of suitable dilutions of the virus seed lot. Although the previously recommended end-point for this *in vivo* titration was death of the mice, it is reasonable instead to use clinical signs of paralysis as the end-point and to kill the animals when they reach this stage. Mice that



show no signs of rabies infection such as ruffled fur, slow and shaky movements or paralysis should be observed for 14 days. The minimum titre of the seed should be specified by the manufacturer, according to the product, cells, virus strain etc.

#### **A.3.2.1.2.4. Additional tests if duck embryos are used for production of virus seed**

If duck embryos are used for the production of virus seed, tests for *Mycobacteria* and *Avian Viruses* should be performed.

### **4. Control of vaccine production**

#### **4.1 Control (of) cell cultures**

Penicillin and other  $\beta$ -lactams should not be used at any stage of manufacture. Minimal concentrations of other suitable antibiotics, such as kanamycin and neomycin, may be used where approved by the national control authority.

At least 5% of the cell suspension or 500 ml, whichever is greater, of the cell suspension at the concentration employed for seeding vaccine production cultures, should be used to prepare control cultures.

If bioreactor technology is used, the national regulatory authority should determine the size and treatment of the cell sample to be examined.

##### **4.1.1. Tests of control cell cultures**

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

The control cell cultures should be incubated under the same conditions as the inoculated cultures for two weeks or until the last harvest of virus from the production cultures - whichever is the later - and should 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 should have had to be discarded because of accidental contamination or damage.

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

#### **4.1.1.1. Tests for haemadsorbing viruses**

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

In tests for haemadsorbing viruses, calcium and magnesium ions should be absent from the buffer medium.

In some countries the national regulatory authority requires that tests for haemadsorbing viruses should also be done with erythrocytes from other species, including human beings (blood group 0), monkeys, and chickens (or other avian species).

The results of all tests should be noted after incubation of the erythrocytes with the cultured cells for 30 minutes at 0-4°C and again after a further incubation for 30 minutes at 20-25 °C. For the test with monkey erythrocytes, the results should be noted a third time, after a final incubation for 30 minutes at 34-37 °C.

#### **4.1.1.2. Tests for other adventitious agents in control cell fluids**

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

The inoculated cultures should be incubated at 35-37 °C and should be observed for at least 14 days.

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

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

#### **4.1.1.3. Identity test (cell line)**

At the production level, and for vaccines produced in human diploid cells or continuous cell lines only, the cells should be identified by using one of the methods specified in current requirements for the use of animal cells as in vitro substrates for production of biologicals (5). The method(s) should be approved by the national regulatory authority.

Methods for identity testing include, but are not limited to, biochemical (e.g., isoenzyme analysis), immunological (e.g., HLA assays), cytogenetic tests (e.g., for chromosomal markers), and tests for genetic markers (DNA fingerprinting).

#### **4.1.1.4 Additional tests on control cells if avian embryo cells are used for production**

A sample of the control fluid taken at the end of the observation period at the control cell cultures should be tested for avian retroviruses such as avian leucosis virus, by a method approved by the national regulatory authority.

In some countries the complement fixing test (COFAL) is used for detecting avian leucosis viruses, and liver or kidney cell cultures of embryos are used for detecting adenoviruses. A test for retroviruses using a sensitive PCR-based RTase assay may be used. The results of such assays need to be interpreted with caution because RTase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (nucleic acid amplification tests for retrovirus may also be used).

Only those cells shown to be free from contamination should be used.

#### **4.1.1.5 Additional tests on control cells if other cell cultures are used**

When other cell substrates are used for the growth of rabies virus, additional appropriate tests should be considered.

### **4.2. Control of production in embryonated duck eggs**

#### **4.2.1 Control (uninoculated) embryonated duck eggs**

A sample of 2% of, but in any case not less than 20 and not more than 50, uninoculated embryonated eggs from the batch used for vaccine production should be incubated under the same conditions as the inoculated eggs. At the time of virus harvest, the uninoculated eggs should be processed in the same manner as the inoculated eggs, and the extract from the control embryos should be shown to be free from haemagglutinating agents and from adenoviruses, avian retroviruses such as avian leukosis virus, and other extraneous agents by tests approved by the national regulatory authority.

A test for retroviruses using a sensitive PCR-based RTase assay may be used. The results of such assays need to be interpreted with caution because RTase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (23). Nucleic acid amplification tests for retrovirus may also be used.

#### **4.2.2 Single harvests from embryonated duck eggs**

After the eggs have been incubated for a suitable period they should be inoculated with seed virus. After further incubation for a suitable period, only living, typical duck embryos should be harvested with aseptic precaution. The time of harvest should be defined starting from the inoculation of the virus and should not be more than 14 days. Embryos inoculated at the same time and harvested together may be pooled and the viral harvest kept separate until completion of the sterility test (Part A, section 4.2.1.).

### **4.3. Control of single virus harvests and purified bulk material**

After inoculation of the production cells with the virus working seed lot, neither inoculated nor control cell cultures should at any time be at a temperature outside the range approved by the national regulatory authority for the defined incubation periods. The optimal range for pH, multiplicity of infection, cell density and time of incubation should be established for each manufacturer, and be approved by the national regulatory authority.

The appropriate time for harvest should be defined as number of days after virus inoculation agreed and should be approved by the national regulatory authority.

It is advisable that the inoculated cell cultures are processed in such a manner that each virus suspension harvested remains identifiable as a single harvest and is kept separate from other harvests until the results of all the tests described in part A sections 4.1 and 4.2 have been obtained.

Only virus harvests satisfying the recommendations below should be pooled and used in the preparation of the inactivated virus harvest.

#### **4.3.1. Sterility tests on single virus harvests**

A sample removed from each virus harvest should be tested for bacterial and fungal contamination by appropriate methods, according to Part A, section 5.2 of the *Requirements for biological substances no.6. General requirements for the sterility of biological substances* (14). In addition, test on mycoplasma contamination should be performed.

Any virus harvest in which contamination is detected must be discarded.

### **4.3.2 Identity**

The single harvest contains virus that should be identified as rabies virus using specific antibodies.

### **4.3.3 Virus titration for infectivity**

Each virus harvest or pool of harvests should be tested for infectivity in a sensitive assay. Both mice and cell culture of defined sensitivity are suitable for testing infectivity. Manufacturers should set an in-house specification for titre of each harvest or pool of harvests.

### **4.3.4. Determination of antigen content**

Assays for determination of glycoprotein antigen for determining the antigen content of the final bulk have been shown suitable for monitoring consistency of production. Such assays include the single radial immunodiffusion and EIA test. Selection of antibodies and other reagents is of critical importance.

### **4.3.5. Monitoring consistency of production**

Virus titre, as well as determination of the antigen content mentioned above, are appropriate parameters for monitoring consistency of production. Therefore, internal specifications should be set.

### **4.3.6. Purification and/ or concentration and of virus harvests**

One or more single harvests may be purified and/or concentrated by methods demonstrated to yield safe, potent and immunogenic vaccine. The virus harvest should be inactivated by a validated method at a defined stage of the process which may be before, during or after any concentration and purification.

The process should be approved by the national regulatory authority and should be shown to give consistent results.

### **4.3.7 Test for residual cellular DNA**

For viruses grown in continuous cell lines, purified bulk should be tested for residual cellular DNA. The purification process should be shown to consistently reduce the amount of host cell DNA. As recommended in the Requirements for the use of animal cells as *in vitro* substrates for the production of biologicals (5), the amount of residual cell DNA should be less than 10 ng per purified human dose. The assay for determination of residual cell DNA with defined sensitivity for detection of specified levels should be approved by the national regulatory authority. The specification set for the level of residual DNA should comply with current WHO guidelines for cell substrates.

#### **4.3.8 Test for Residual Animal serum**

If animal serum is used during production, the concentration of bovine serum albumin (BSA) may be used as an indicator of animal serum in the purified bulk which should not be greater than 50 ng per human dose or its equivalent.

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

#### **4.3.9 Tests for residual materials**

Each manufacturer should demonstrate, by testing each virus purified bulk or by validation of the manufacturing process, that any residual materials used in manufacture are consistently reduced to a level acceptable to the national regulatory authority.

### **4.4 Inactivation procedure**

#### **Methods and agents**

The methods and agents used for inactivation should be validated and approved by the national regulatory authority. Given that presence of virus aggregates may result in ineffective inactivation, great care should be taken to avoid this. If it is not possible, virus aggregates should be removed before starting the inactivation procedure.

Chemical or physical means, such as filtration, may be used to remove aggregates.

If clarification is performed on a crude virus suspension, it is advisable to start inactivation within 24 hours.

In the case of vaccine produced in embryonated eggs the method should also be shown to inactivate avian leucosis viruses, as demonstrated by tests in tissue culture, or, in the case of vaccine produced in the primary hamster kidney cells, any adventitious agent that may be present, as demonstrated by tests using tissue culture or by animal inoculation.

The method used should be shown to be consistently effective in the hands of the manufacturer. The kinetics of inactivation should be demonstrated by the manufacturer to be consistently effective using at least 5 consecutive batches. The total inactivation time to complete inactivation should be determined. The inactivation time used routinely should be at least double the period required to inactivate the virus completely.

As a part of validation of the inactivation process, virus samples taken at appropriate times, should be inoculated immediately into the sensitive substrate (e.g., mice, cell cultures), to determine inactivation curve. This provides information on the reproducibility of the inactivation process.

Various methods for inactivating rabies virus have been used with success. The concentration of the inactivating

chemical, the temperature, and the length of time necessary for inactivation must be defined by each manufacturer for a particular production process. Satisfactory vaccines may be prepared by treating virus suspensions from tissue culture at 2-8°C with  $\beta$ -propiolactone at a dilution of 1 : 3500 to 1 : 5000 for 24 h, or until inactivation is complete, as demonstrated by the results of the test for effective inactivation specified below.

The conditions for storage of concentrated bulks intermediates should to be validated and approved by the NRA.

Formaldehyde may also be used as an inactivating agent in the production of rabies vaccines. Tests for free formaldehyde should be performed at appropriate intervals and the concentration maintained at specified levels. Ultraviolet irradiation may be used to facilitate inactivation process.

***Tests for effective inactivation.*** Each purified bulk material should be tested in an appropriate test system for effective inactivation of the virus before the addition of preservatives and other substances. The sensitivity of the assay should be determined according to the rabies virus used for production and the most sensitive assay should be used. Test should be performed immediately after inactivation.

If samples are not tested immediately after inactivation they should be stored frozen at  $< -60^{\circ}$ . The conditions of storage should be validated to confirm no loss of virus titre. If test is performed at a later stage of production, appropriate biosafety levels should be maintained.

The rabies virus amplification test, for testing the presence of live virus, should be performed in the cell culture used for vaccine production or a type of cell line demonstrated to be of greater sensitivity. The national regulatory authority should approve the cell line and the method used. Manufacturers are encouraged to use cells such as Vero cells, BHK21 and neuroblastoma cells which are known to be highly sensitive to rabies virus.

The rabies virus amplification test should be done as follows. At least 25ml of bulk vaccine corresponding to at least 25 human doses should be inoculated on five cell cultures of the type used for vaccine production, or a type of at least equal sensitivity. At least 3 cm<sup>2</sup> of cell sheet should be used per milliliter of vaccine. After adsorption of the inoculum for an appropriate time, medium should be added such that the ratio of medium to vaccine is not more than 1:3. The cultures should be observed for at least 21 days. The cell cultures may be stained directly for the presence of rabies virus by immunofluorescence. Otherwise, five milliliters of each culture fluid should be pooled

on days 14 and 21 and 0.03 ml of this pool should be inoculated intracerebrally into each of 20 weanling mice of 12-15 g. These mice should be observed for 14 days. Any symptoms caused by rabies virus should be confirmed by the immunofluorescence assay. At the end of the observation period, no cytopathic effects should be detected.

A test involving the use of immunofluorescence for the detection of cells infected with rabies virus at day 21, which is shown to be as sensitive as mice inoculation and approved by the national regulatory authority may be used. A specification for the proportion of cells checked by immunofluorescence should be set and approved by the national regulatory authority. The inclusion of trypsinisation of the cells on day 7 should be considered as this may increase the sensitivity of the amplification assay.

For certain products such as PDEV the virus may not be adapted to growth in cell culture. Virus amplification test may, therefore, be performed in the same substrate as the one used for production. The virus is inoculated in yolk sac in pre-incubated eggs. Absence of virus can be confirmed by FAT.

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

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

### *4.5.1 Preservatives and other substances added*

In preparing the final bulk, only adjuvant, preservatives and other substances such as human albumin approved by the national regulatory authority should be added. Such substances should have been shown by appropriate tests not to impair the safety or effectiveness of the product at the concentration used.

If  $\beta$ -propiolactone has been used for inactivation, the procedure should be such that this chemical is not detectable in the final bulk. The test used for determination of  $\beta$ -propiolactone should be of defined sensitivity, performed at the time intervals appropriate for the kinetic of inactivation for the vaccine in question. The test should be approved by the national regulatory authority.

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

### *4.5.2 Antigen content of the final bulk*

Assays for determination of glycoprotein antigen of the final bulk have been shown suitable for monitoring consistency of production. Such assays include the single



radial immunodiffusion and EIA test. Selection of antibodies and other reagents is of critical importance.

Since the presence of adjuvant may affect results, it is advisable to perform the assay before adjuvant is added. Alternatively, antigen may be eluted from the adjuvant prior to assay.

Some manufacturers test glycoprotein content of the purified/ concentrated bulk to determine the dilution of the bulk to be used in the preparation of the final bulk.

#### 4.5.3 *Sterility tests*

Each final bulk should be tested for sterility according to Part A, section 5.2 of the revised Requirements for biological substances no.6. General requirements for the sterility of biological substances (14).

### **5. Filling and containers**

The requirements concerning filling and containers given in Good Manufacturing Practices for Biological Products (7) should apply to vaccine filled in the final form.

Care should be taken to ensure that the material of which the container is made does not adversely affect the virus content of the vaccine under the recommended storage condition.

### **6. Control tests on final product**

Samples should be taken from each filling lot for the tests described in the following sections.

#### **6.1 Identity test**

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

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

#### **6.2 Sterility test**

Each filling lot should be tested for bacterial and fungal sterility according to Part A, section 5.2 of the revised Requirements for biological substances no.6. General requirements for the sterility of biological substances (14).

### **6.3 General safety (innocuity) test**

Each final lot should be tested for unexpected toxicity (sometimes called abnormal toxicity) using a general safety (innocuity) test approved by the national regulatory authority.

This test may be omitted for routine lot release once consistency of production has been well established to the satisfaction of the national regulatory authority and when good manufacturing practices are in place. Each lot, if tested, should pass a test for general safety.

### **6.4 Antigen content**

If not done on the final bulk (4.3.2), antigen content should be determined and be within limits approved by the national regulatory authority.

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

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

The NIH test as described in Laboratory Techniques in Rabies (40) should be used to evaluate consistency of production of the vaccine in question. This should also be used to test product stability for the purpose of establishing shelf life as well as to calibrate reference preparations.

In this test mice are immunized and subsequently challenged with rabies virus. The test is conducted by vaccinating groups of mice, on two occasions, 7 days apart, with dilutions of an appropriate reference material calibrated against the International Standard for Rabies Vaccine and vaccine on test. Seven days after the last vaccination, the immunized animals and a control group of mice are challenged intracerebrally with the  $> 5$  LD<sub>50</sub> of Challenge Virus Standard (CVS). The titre of the challenge virus should be confirmed by inoculation of at least 3 tenfold dilutions into further groups of mice. The mice are observed for 14 days and the 50% effective dose (ED<sub>50</sub>) of the reference and test vaccine is determined on the basis of survival rate of mice. Humane end-points may also be used if validated.

The potency of the test vaccine in International Units should be determined by comparing the ED<sub>50</sub> of the test vaccine with that of the reference vaccine calibrated in International Units by comparison with the International Standard for Rabies Vaccine using appropriate statistical methods.

The assay with defined criteria for validity and test procedure, the method for statistical calculation together with the minimum number of assays to be performed for adequate interpretation of the results and the confidence limits of the assay should be approved by the national regulatory authority. In particular, calibration of reference vaccine against International Standard as well as use, storage and handling of CVS should be well defined in the approved test procedure.

The confidence limits of the assay should be in the range of 25-400%

The number of tests to be undertaken on each batch is dependent on the consistency of assays in an individual laboratory. If consistency of testing in a laboratory is well demonstrated, one assay may be sufficient. However, additional assays may increase the precision of the potency estimate.

The potency should be at least 2.5 IU per single human dose.

In some countries, more than one assay is performed. In this case, the estimated geometric mean potency is based on two valid tests and should be at least 2.5 IU/human dose.

In some countries, a modified NIH test is in use. Following licensing, and once consistency in production and quality control of the vaccine has been further confirmed on an on-going basis, of at least two years, the determination of potency in routine lot release may, with the approval of national regulatory authority, be based on the modified NIH assay, based on a single dilution. This assay will provide qualitative (or semi-quantitative) results.

Several prerequisites and conditions should be considered before designing a single dilution assay:

- The one dilution assay is advantageous when vaccine lots consistently give a lower limit for the estimated potency well in excess of 2.5 IU per single human dose. This is more likely to be consistently achieved where the antigen content of the final container vaccine is based on the assay of rabies glycoprotein content.
- This is suitable for testing large number of batches each year, in particular for testing multiple batches at the same time.
- Consistency of testing results is essential.
- Several factors such as virus strain, the homogeneity of the CVS challenge preparation and the strain and quality of mice may affect reproducibility of the results of tests.

The following criteria for validity of a single dilution assay should be taken into account:

- Full dilution assay should be well established with high percentage of valid results for defined period of time
- Reference vaccine and CVS should have a good record of values within the specified range for a laboratory in question
- Sub-potent vaccines should be included in the validation
- Acceptance/ rejection criteria must be defined.

To further confirm consistency on an ongoing basis, the potency of about 10 recent batches of vaccine should be tested using the full dilution assay. If potency expressed in International Units is within the specified range of values and if the expectations of linearity and parallelism are consistently satisfied, then fewer doses may be used and the assumptions of linearity and parallelism need not be tested in each assay.

A one-dilution assay is based on the same principles for evaluating the response as the three-dilution assays. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 2.5 IU (i.e., of the minimum potency of a single human dose), that elicits a minimum protective effect in mice, and comparing its effect with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine ( $P \leq 0.05$ ), the potency of the test vaccine is satisfactory.

One dilution assays provide assurance that the lower limit of the estimated potency is in excess of the minimum requirement. A disadvantage of such an approach is that strictly quantitative estimates of vaccine potency will not be possible.

Lot release based on a simplified approach will require periodic review to ensure that validity of all procedures is maintained. The timing of the review should be decided on a case by cases basis depending on the number of batches of vaccine produced annually and/or by time (at least every two years), as agreed by the national regulatory authority.

If a batch of vaccine fails to meet the specification set for the modified test, a full NIH mouse protection test should be performed.

Manufacturers are encouraged to support data generated by NIH potency assay by the determination of antigen content using an *in vitro* assay in order to ensure overall consistency of production.

Design of the test as well as statistical analysis of the data should be approved by the national regulatory authority.

A suitable challenge strain, CVS-11, is available upon request from the World Health Organization, Geneva, Switzerland. Such request should be approved by the relevant national regulatory authority.

### **6.6. Ovalbumin content**

For vaccines produced in embryonated eggs only, the ovalbumin content of each filling lot, if not done on the final bulk, should be determined and be within limits approved by the national regulatory authority.

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

The residual moisture in a representative sample of each freeze-dried lot may be determined by a method approved by the national control authority. The upper limit of moisture content should be specified by the national control authority. Generally, moisture levels of less than 3% are considered satisfactory.

### **6.8 Test for pyrogenic substances**

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

### **6.9 Test for residual animal serum protein**

A sample of the final lot should be tested to verify that the level of bovine serum albumin in the final reconstituted vaccine is less than 50 ng per human dose.

### **6.10 Adjuvant**

If an adjuvant has been added to the vaccine, its content should be determined by a method approved by the national regulatory authority.

Where aluminium compounds are used the concentration of aluminium should not be greater than 1.25mg per single human dose. If other substances are used as adjuvant or those with adjuvanted effect, a specification should be set.

When aluminium hydroxide is used as the adjuvant, the degree of adsorption should be determined in the final bulk. This should not be less than 95%.

### **6.11 Preservative**

If a preservative has been added to the vaccine, the content of preservative should be determined by a method approved by the national regulatory authority.

The amount of preservative in the vaccine dose should be shown not to have any deleterious effect on the antigen nor impair the safety of the product in humans. The preservative, its use at different stages of manufacturing process as well as the residual amount present in the product should be approved by the national regulatory authority.

If any modification of preservative content in already licensed vaccine is made, general principles for vaccine evaluation described in the WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines, should be followed (26).

### **6.12 Inspection of final containers**

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

### **6.13 Test for residual cellular DNA**

For viruses grown in continuous cell lines, the final product should be tested for residual cellular DNA if this test has not been carried out at final bulk stage (section 4.3.7).

## **7. Records**

The recommendations given in Good Manufacturing Practices for biological products (7) should apply.

## **8. Samples**

Vaccine samples should be retained as recommended in Good Manufacturing Practices for biological products (7, Annex 1).

## **9. Labelling**

The recommendations given in Good Manufacturing Practices for biological products (7) should apply, with the addition of the following.

The label on the container or package should include the following information:

- the designation of the strain of rabies virus contained in the vaccine;
- the minimum potency of vaccine determined by NIH test and expressed in IU per human dose;

- the substrate used for the preparation of the vaccine;
- the method used for inactivating the virus;
- the nature and amount of stabilizer, preservative or additive present in the vaccine;
- the volume and nature of diluent;
- the use of vaccines after reconstitution if the vaccine is in the dried form;
- the expiry date should be indicated at both primary and secondary package.

It is desirable that the label carry the names both of producer and of the source of the bulk material, if the producer of the final vaccine did not prepare it. The nature and residual amount of the antibiotics present in the vaccine, if any, may be included.

## **10. Distribution and shipping**

The recommendations given in Good Manufacturing Practices for biological products (7) should apply.

## **11. Stability, storage and expiry date**

### **11.1. Stability**

Stability evaluation is an important part of the quality assessment. The purpose of stability studies is to ensure that the vaccine at the end of its shelf life, storage period or period of use, still has the required characteristics supporting quality, safety and efficacy.

#### **a) for licensing**

Studies that support stability of a vaccine for the purpose of licensing have to be performed as real time real condition studies. Stability-indicating parameters should be carefully selected. They should always include, but should not be limited to, the potency test as part of real time studies under conditions recommended for storage. Tests should be conducted to determine the loss of potency at appropriate time intervals during storage. Final containers from at least three batches of vaccine derived from different bulks should be tested on the expiry date to demonstrate stability during storage.

Accelerated stability data for product stored for limited periods at temperatures that may affect stability could support preliminary data from ongoing real time stability studies but should not replace them. However, further data on stability to support shelf life of the product should be based on long-term, real conditions stability studies and should be submitted to the national regulatory authorities for approval. Any modification of the shelf life approved as part of licensing require additional stability data to support proposed modification and should be approved by relevant national regulatory authority. Following licensure, stability should be monitored throughout proposed shelf-life.

**b) for lot release**

There is no additional value in performing accelerated stability test for the purpose of lot release.

**c) at different stages of manufacturing process**

Stability testing should be performed at different stages of production, namely single harvests, final bulk and final lot. Stability indicating parameters should be selected according to the stage of production. It is advisable to assign shelf life to all materials during vaccine production, in particular intermediates such as single harvests, purified bulk and final bulk.

**d) for clinical trial approval**

For vaccines under development, stability data, such as those described under 11 a, are expected for the purpose of clinical trial approval. However, stability data generated for a more limited period are acceptable at this stage.

Appropriate documentation to support stability profile of a vaccine should be submitted to the competent national regulatory authority at all stages mentioned above.

**11.2 Storage conditions**

Recommended storage conditions and defined maximum duration of storage should be based on stability studies as described in section 11. above and approved by the national regulatory authority. For rabies vaccines, both liquid and freeze-dried, temperature of 2-8° C has been found satisfactory. This should ensure that the minimum potency specified on the label of the container or package will still be maintained after release until the end of the shelf-life, if the conditions under which the vaccine is stored are in accordance with what is stated on the label.

**11.3 Expiry date**

The expiry date should be defined on the basis of shelf life supported by the stability studies as described above (section 11.) and approved by the national regulatory authority.

**12. Intradermal route of administration**

Vaccines to be administered by the intradermal route should meet the same quality, safety and efficacy specifications as defined in WHO recommendations for production and control for vaccines for intramuscular use. This means that the potency of such vaccines, if reconstituted in the volume intended for intramuscular use, should be at least



2.5 IU per single dose. In addition, manufacturers should provide clinical evidence that vaccine is immunogenic and safe when administered intradermally.

In some countries a volume of 0.1ml per intradermal site was found appropriate due to practical aspect of vaccine administration.

Ideally, vaccines intended to be administered by intradermal route should be developed for such purpose. This includes appropriate studies where immunogenicity and safety of vaccines are demonstrated by testing vaccine, the potency of which is assigned for an intradermal dose.

For vaccines developed for intramuscular administration, intradermal use should be supported by nonclinical and clinical data (see sections B and C). In addition, potency should be assigned for the intradermal dose.

Rabies vaccines formulated with an adjuvant should not be administered intradermally.

Intradermal injections must be administered by staff trained in this technique.

Further details on immunization regimens and practices to be followed when a vaccine is to be administered by intradermal route are available in the Report of WHO Expert Consultation on Rabies (12).

## **PART B. NONCLINICAL EVALUATION OF NEW RABIES VACCINES**

Preclinical testing is a prerequisite for initiation of clinical trials in humans and includes immunogenicity studies (proof of concept) and safety testing in animals. The vaccine lots used in preclinical and nonclinical studies should be adequately representative of the formulation intended for the clinical investigation and, ideally, preclinical testing should be done on the same lots as proposed for the clinical trials. If this is not feasible, then those lots should be comparable with respect to potency, stability, and other characteristics of quality. Details on the design, conduct, analysis and evaluation of nonclinical data are available in WHO guidelines for nonclinical evaluation of vaccines (17).

If a new rabies vaccine is intended to be used intradermally, the issue of appropriate formulation for this purpose should be addressed early in its development. Dose response and minimum potency for induction of protective immune response in a relevant animal model should be demonstrated.

## **PART C. CLINICAL EVALUATION OF RABIES VACCINES**

### **C.1. EVALUATION OF NEW RABIES VACCINES FOR LICENSING**

#### **1.1 General considerations for the clinical assessment of rabies vaccines**

The clinical development program for rabies vaccines should evaluate their use for pre-exposure and post-exposure prophylaxis including different vaccination schedules and possibly routes of administration, the onset, extent and duration of protection, and the need for and timing of booster vaccination. Clinical trials should adhere to the principles described in good clinical practice (27) as well as to those formulated for design, conduct and analysis of vaccine clinical trials described in the WHO guidelines for clinical evaluation of vaccines (16). All clinical trials should be approved by the relevant National Regulatory Authority.

For ethical reasons, it is impossible to conduct placebo controlled clinical efficacy studies involving an unvaccinated group that might be or has been exposed to rabies infection. Efficacy has been demonstrated previously in well designed but uncontrolled studies for tissue culture and avian derived vaccines in individuals exposed to rabies infection. Long-term evidence has shown that vaccines that met the minimum WHO potency requirement of 2.5 IU per dose induce adequate immunogenicity and protection (29303132,333435). An antibody concentration of at least 0.5 IU per ml on days 14 and 28/30, after initial vaccination, is generally considered to be adequate. Therefore a satisfactory concentration of neutralizing antibodies could be used as a predictor of clinical efficacy. Nevertheless every effort should be made to obtain information on the protective capacity of vaccines during their actual use.

The clinical development programme must be tailored to the type of vaccine, taking into consideration the particular formulation, including any adjuvant content, the production process and intended use of a vaccine. For example, vaccines containing antigens, adjuvants or other components for which there is limited or no previous experience in humans will require more extensive clinical study than those that closely resemble licensed vaccines.

## **1.2 Characterization of vaccine lots for clinical trials**

By the beginning of the later stages of clinical development, a vaccine should have been fully characterized in terms of physico-chemical and biological properties and the final manufacturing process. Final release and end of shelf-life specifications and batch release testing procedures may not have been established since they may partly depend on the total clinical data..

Consistency of manufacturing for the vaccine lots used in clinical trials should be demonstrated and well documented. These lots should be adequately representative of the formulation intended for marketing. At a minimum, candidate vaccines for clinical trials should be prepared under conditions of Good Manufacturing Practice for clinical trial material (28). Full GMP will be required at the later stages of clinical development (6,7). All the lots to be used for clinical trials should be released by National Control Laboratories (NCL).

Any change in the manufacturing process during vaccine development should be assessed carefully regarding possible impact on the safety, immunogenicity and likely efficacy of the vaccine and the need for additional nonclinical and clinical investigations (see 1.7 below).

Similarly if the vaccine has been the subject of a transfer of production there might be a need for clinical data to assess its safety, immunogenicity and likely protective efficacy

## **1.3. Immunogenicity studies**

Dose - finding studies to identify appropriate regimens for induction of protective immune responses should be performed.

Initial immunogenicity studies should be performed in healthy adult volunteer subjects that have not been exposed to rabies and have not been previously vaccinated. After the vaccine has been proven to be immunogenic in rabies naïve healthy adults, further studies should be conducted to demonstrate immunogenicity in target populations according to the intended use:

a) pre-exposure prophylaxis - persons resident in endemic areas should be enrolled in the trial. The population should include the elderly and persons with different vaccine histories to establish the suitability of the vaccine for naïve and previously immunized

persons. If vaccine is to be licensed for pre-exposure prophylaxis of children then adequate data should be obtained in various age strata.

b) post-exposure prophylaxis - These studies can only be done in high risk areas and populations and should follow production of immunogenicity data as in a). Subjects known or thought likely to have been exposed to rabies infection should be vaccinated and followed for immunogenicity and efficacy. For initial evaluation of new vaccines, schedules recommended by WHO should be used (12). For post-exposure prophylaxis regimens, the following schedules for antibody testing are recommended as a minimum: days 0, 14, 28/30, 90, 180, 360. It is critical to include a blood sample on day 0 and 7 in order to identify and exclude previously vaccinated subjects.

For new rabies vaccines intended to be administered intradermally, the suitability of the formulation for this purpose should be tested in the target population (see 1.8).

#### **1.4. Assessment of the immune response**

When assessing pre-exposure use, immunogenicity should be assessed in terms of the time to onset, antibody titers and duration of likely protection. Variability of the immune response between subjects is an important element and should be reported wherever possible.

The ability of the vaccine to induce an immunological memory and consequently an anamnestic response after booster doses should be tested. Data generated in one study using a specific priming regimen should not be extrapolated to different regimens or routes of administration.

It would be beneficial to define the highest potency, determined by the NIH test and expressed in IU per dose, that still induces further increases in antibody levels (the so called “saturation point”).

The appropriate time intervals for taking the samples should be defined taking into account study objectives. Serum samples should be aliquotted and stored securely in the event that re-evaluation is required.

Immunogenicity should be assessed using one of the two serological assays: RFFIT or FAVN. The assay used to determine levels of neutralizing antibodies should be approved by the National Regulatory Authority. It has been demonstrated that the degree of homology between the strain of challenge virus used in the RFFIT to measure the immune response after vaccination and the strain of seed virus used for vaccine production profoundly affects reported RVNA values (36). The use of a heterologous challenge virus strain (CVS) may result in lower levels of neutralizing antibodies than those obtained with homologous CVS in the same assay.

### 1.5. Quality assurance of immunogenicity testing

It is of critical importance to perform immunogenicity testing in a laboratory with well established RFFIT/FAVN testing protocols and implemented quality assurance of testing procedures. For this purpose, following parameters should be in place:

1. Written SOP
2. Temperature monitoring and control on relevant equipment
3. All equipment should be calibrated and evaluated annually. This should include all pipettes, CO2 incubators, water baths, refrigerators/freezers, hoods, plate washers etc.
4. Cells should be monitored to determine that they are free of mycoplasma contamination
5. Passage of cells should be documented
6. Pre-batch acceptance of all reagents to verify fitness for purpose, e.g. fetal calf serum, FITC labeled anti-rabies antibody, etc.
7. Virus
  - a. Virus strain and passage history should be documented
  - b. Virus should be passaged to avoid production of defective interfering particles
  - c. Titer established for stock virus
  - d. Establish working dilution
8. Cells
  - a. Passage history
  - b. Free from contamination
  - c. Maintain same growth curve when utilized
9. Serum
  - a. Store refrigerated or frozen
  - b. Clearly labeled and destroyed adequately after usage
10. Assay
  - a. Validity criteria of assay:
    - i. All test criteria should be documented for every test
    - ii. Back titration to ensure working virus titer is within specification
    - iii. Good cell sheet is required, reasonable confluence
    - iv. A negative serum and a reference serum calibrated in IU must to be included in all assays
    - v. End point dilution of reference needs to be monitored
    - vi. Analysis of serum titers should be conducted according to documented procedures
11. Training and proficiency
  - a. All technicians need to be appropriately trained and competence demonstrated
  - b. Participation in proficiency testing schemes (PTS) is recommended

## **1.6. Analysis and interpretation of the data**

Collection, recording, analysis and interpretation of data should be conducted according to GCP guidelines. Methodological and statistical considerations described in WHO guidelines should be taken into account (16).

Data generated in clinical trials should be submitted to the National Regulatory Authority as described in the Summary protocol for vaccine evaluation (16). Data should be presented stating the batch number, vaccine presentation, potency of the vaccine used, nature and volume of a diluent where appropriate, and other relevant characteristics of tested vaccine. In addition to general statements (e.g., study sites, investigators, objectives, inclusion and exclusion criteria etc), a number of specific issues for rabies should be described: nature of exposure (WHO category 1-3); status and confirmation of rabid animal; nature of wound care; immunization schedule; details of PEP treatment including time and sites of RIG administration, nature, volume and other details of RIG as a product; care and treatment of adverse side effects; other medications given; control group using well established rabies vaccine used etc.

Immunogenicity data should include the total number and percentage of subjects with titers above and less than 0.5 IU/mL, GMT with CI, range of antibody titers. Safety data should be presented as total number, percentage and type of adverse events.

It would be beneficial for the whole scientific community to publish clinical trial data in a peer-reviewed journal.

## **1.7. Studies to support change in manufacturing processes**

Changes in production methods or scale-up before or following licensing will necessitate further product characterization to demonstrate comparability with the lots used in earlier studies of safety and immunogenicity. Changes that do not require clinical data should be defined in the national regulation and the National Regulatory Authority consulted regarding all changes prior to their implementation. However, some changes could affect the safety and immunogenicity of the vaccine and so its likely efficacy and should be supported by additional clinical data. The extent of the clinical data needed depends on the nature and extent of the changes made. The design of such studies rests on the demonstration of non-inferiority in terms of eliciting protective immune responses and safety. (16).

## **1.8. Studies to support a new route of administration**

Clinical data cannot be extrapolated between routes of administration and clinical trials should be performed. Dose finding studies should be conducted to determine the optimal intradermal dose and volume of administrations. Immunization schedules recommended by WHO are described elsewhere (12).

For vaccines already licensed for the intramuscular route of administration, the following issues should be carefully considered before proposing such a formulation for intradermal use:

- 1) presence of preservative in a vaccine: consideration should be given to the type of preservative and residual amount in the final vaccine.
- 2) potential impact of the use of opened multi-dose vials in the field. The level of immunization practice in particular area/ country and the actual risk of contamination should be taken into account by the NRA when this route of administration is submitted for approval.

Every effort should be made to reduce potential risk of contamination of the vaccine presented in the multi-dose vials (24).

The principles of the study design are as for section 1.7 above.

## **C2. CLINICAL EVALUATION AS PART OF POST MARKETING SURVEILLANCE**

### **Monitoring vaccine efficacy, effectiveness and safety**

Every effort should be made to improve current scientific understanding of the protection and safety of rabies vaccines in humans by conducting active post-marketing surveillance.

It is particularly important that data are collected on any vaccine failures including detailed data on the post-exposure prophylaxis administered. Details on the proper investigation of the treatment failures are provided elsewhere (12).

Given that limited safety data are obtained in pre-licensure studies, it is very important that safety should be monitored as part of post-marketing surveillance.

Data generated in post-marketing surveillance should be submitted to the National Regulatory Authority.

## **PART D. RECOMMENDATIONS FOR NATIONAL REGULATORY AUTHORITIES**

### **1. General**

The general recommendations for national regulatory authorities provided in Guidelines for National Authorities on Quality Assurance for Biological Products should be followed (19). These specify that no new biological substance should be licensed until consistency of production has been established.

The detailed production and control procedures as well as any change in them that may affect quality, safety and efficacy of rabies vaccine should be discussed with and approved by the national regulatory authority.

The national regulatory authority should obtain the International standard for potency testing and, where necessary, establish national working reference preparation(s) calibrated against the International standard. In addition, challenge virus standard should be obtained from a reliable source, stored and used as appropriate. The national regulatory authority should be able to provide the standard for potency testing as well as challenge virus standard on request (Part A, section 6.5).

## **2. Release and certification**

A vaccine lot should be released only if it fulfils Part A of these Recommendations. Before any vaccine lot is released from a manufacturing establishment, the recommendations for consistency of production provided in Guidelines for National Authorities on Quality Assurance for Biological products (19) should be met.

A statement signed by the appropriate official of the national control laboratory should be provided if requested by a manufacturing establishment and should certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these recommendations. The certificate should also state the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, date of the last satisfactory potency test as well as assigned expiry date on the basis of shelf life should be stated. A copy of the official national release document should be attached.

The purpose of the certificate is to facilitate the exchange of rabies vaccine between countries.



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## Appendix

### SUMMARY PROTOCOL FOR PRODUCTION AND TESTING OF INACTIVATED RABIES VACCINE PRODUCED IN CELL SUBSTRATES AND EMBRYONATED EGGS FOR HUMAN USE (to be revised after text is adopted by ECBS)

#### Identification of final lot

Name and address of manufacturer -----  
-----  
Lot number of vaccine -----  
Date of manufacture of final lot -----  
Date of start of the potency test -----  
Expiry date -----  
Total volume of final lot -----  
Temperature of storage -----

#### 3. Control of source materials

##### 3.1 Substrate for virus production (complete only relevant part, i.e. A, B, C)

Name and identification of cell substrate -----

##### A. Continuous cell lines / Diploid cells

##### Cell seed and WCB

Origin and short history of master and working cell

bank (date of approval by NRA) -----

Authority that approved cell seed -----

Date the MWCB was established and approved by NRA .....

Quantity of cell stored -----

The passage level of the WCB -----

Storage conditions -----

Percentage of all WCB ampoules tested -----

##### Identity test (WCB)

Method used -----

Results -----

Amount of cell culture inoculated -----

Test performed on control cultures -----

Result -----

**Trypsin used for preparation of cell cultures**

Origin of trypsin used -----

Tests performed on trypsin -----

Results -----

**B. Primary cell cultures**

Type of cells (e.g., kidney) -----

**B1. Animal species of cells** -----

Controls performed on animals from which the  
cells originated -----

Result -----

**B2. Chicken eggs (for primary chick embryo fibroblasts)**

Controls performed on eggs from which the  
cells originated -----

Result -----

**C. Embryonated duck eggs**

Controls performed on flock -----

Result -----

Control performed on eggs -----

Result -----

**3.1.5. Serum used in cell culture medium**

Origin of serum used -----

Tests performed on serum -----  
Results -----

**3.2 Virus seed**

**Strain of virus**

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

**Virus seed lot system**

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

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

Method used -----  
Results -----

**Tests for adventitious agents**

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



Observation period -----  
 Results (survival numbers, etc.,) -----  
 Tests in guinea-pigs  
 No. of animals tested -----  
 Quantity injected -----  
 Observation period -----  
 Results (survival numbers, etc.) -----  
 Tests in cell cultures  
 Methods -----  
 Results -----  
**Virus content**  
 Method of titration -----  
 Results -----

#### 4. Control of vaccine production

##### 4.1 Control of cell cultures

###### Tests for haemadsorbing viruses

Method -----  
 Results -----

###### Tests for other adventitious agents

Method -----  
 Results -----

###### Identity test (cell line)

**Method** -----  
 Results -----

##### 4.2 Control of single virus harvests and purified bulk material

###### Sterility tests of single virus harvests

Have all the harvests included been tested for sterility? -----  
 Results of these tests -----

###### Pooling of single virus harvests

No. of viral harvests included -----

Date of pooling -----

**Purification of virus harvests**

Method -----

Degree of purity achieved -----

**Animal serum in purified bulk**

Method -----

Results (concentration) -----

**Inactivation procedure**

Method -----

Date -----

Temperature -----

Tests for effective inactivation

Volume and concentration of bulk material injected -----

No. of mice injected -----

Weight of mice -----

Duration of observation -----

Other animals (if used) -----

Results of tests -----

**Rabies virus amplification test**

Substrate used for amplification -----

Amount of vaccine tested (ml) -----

Results -----

**4.3 Preparation and control of final bulk**

**Preservatives and other substances added**

Concentration of phenol (if used) -----

Other preservatives (type and concentration) -----

Other substances added -----

**Antigen content of the final bulk**

Method -----

Results -----

**Sterility tests**

Date of test -----

Result -----

**Residual DNA**

Test performed -----

Result -----

**Other tests (chemical, biochemical)**

Type of test -----

Results -----

**5. Filling and containers**

Date of filling -----

Quantity of containers -----

Volume of vaccine per container -----

**Control for defective vials**

Methods -----

Results -----

**6. Control tests on final product**

**6.1 Identity test**

Method -----

Result -----

**6.2 Sterility test**

No. of containers examined -----

Method -----

Date at start of test -----

Date at end of test -----

Result -----

**6.3 Innocuity tests (if required by the NRA)**

	Mice	Guinea-pigs
No. of animals	-----	-----
Route of injection	-----	-----
Volume of injection	-----	-----
Date of injection	-----	-----
Date of end of test	-----	-----
Result	-----	-----

**6.4 Antigen content**

Type of test	-----
Result	-----

**6.5 Potency test of vaccine in final containers**

Type of test	-----
Date of immunization of mice	-----
Reference vaccine (potency)	-----
Challenge strain	-----
Date of challenge	-----
ED <sub>50</sub> test vaccine <sup>1</sup>	-----

ED <sub>50</sub> reference vaccine <sup>1</sup>	-----
Calculated IU/single human dose	-----
Confidence limits	-----
Results of other potency tests	-----

**6.6 Stability test**

Duration and temperature of incubation	-----
Result	-----

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

Method	-----
Result	-----

**6.8 Inspection of final containers**

Result	-----
--------	-------

---

<sup>1</sup> ED<sub>50</sub>: quantity of vaccine that protects 50% of animals against infection with the challenge strain.

**6.9 Test for pyrogenic substances**

Method -----  
Results -----

**6.10 Test for adjuvant**

Date of test -----

Nature and concentration of adjuvant per  
single human dose -----

Degree of adsorption -----

**Internal certification**

*Certification by person taking overall responsibility for production of the vaccine*  
I certify that lot no.----- of rabies vaccine satisfies Part A of the WHO  
*Recommendations for inactivated rabies vaccine produced in cell substrates and  
embryonated eggs for human use.*

Signature -----  
Name (typed) -----  
Date -----

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

**Release certification by the national control authority**

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

*Sample release certificate*

I hereby certify that batch no.----- of rabies vaccine produced by (*name of producer*) in continuous cell lines meets all national requirements as well as Part A of the WHO *Recommendations for inactivated rabies vaccine produced in cell substrates and embryonated eggs for human use.*

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

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

The number appearing on the label of the containers is-----

Signature

Name (typed)

Date

-----

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## Appendix

### SUMMARY PROTOCOL FOR PRODUCTION AND TESTING OF INACTIVATED RABIES VACCINE FOR HUMAN USE PRODUCED IN CELL SUBSTRATES AND EMBRYONATED EGGS

#### Identification of final lot

Name and address of manufacturer	----- -----
Lot number of vaccine	-----
Date of manufacture of final lot	-----
Date of start of the potency test	-----
Expiry date	-----
Total volume of final lot	-----
Temperature of storage	-----

#### 3. Control of source materials

##### 3.1 Substrate for virus production

Name and identification of cell substrate	-----
<i>Cell seed and WCB</i>	
Origin and short history of master and working cell bank	-----
(date of approval by NRA)	
Authority that approved cell seed	-----
Date the MWCB was established and approved by NRA	.....
Quantity of cell stored	-----
The passage level of the WCB	-----
Storage conditions	-----
Percentage of all WCB ampoules tested	-----
<i>Identity test (WCB)</i>	

Method used -----  
Results -----

*Serum used in cell culture medium*

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

*Trypsin used for preparation of cell cultures*

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

**3.2 Virus seed**

*Strain of virus*

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

*Virus seed lot system*

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

*Tests for bacteria, fungi, and mycoplasmas*

Method used -----  
Results -----

*Tests for adventitious agents*



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

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

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

Tests in cell cultures  
 Methods -----  
 Results -----

*Virus content*  
 Method of titration -----  
 Results -----

**4. Control of vaccine production**

**4.1 Control of cell cultures**

*Tests for haemadsorbing viruses*

Method -----  
 Results -----

*Tests for other adventitious agents*

Method -----  
 Results -----

*Identity test (cell line)*

Method -----

Results -----

**4.2 Control of single virus harvests and purified bulk material**

*Sterility tests of single virus harvests*

Have all the harvests included been tested for sterility? -----

Results of these tests -----

*Pooling of single virus harvests*

No. of viral harvests included -----

Date of pooling -----

*Purification of virus harvests*

Method -----

Degree of purity achieved -----

*Animal serum in purified bulk*

Method -----

Results (concentration) -----

*Inactivation procedure*

Method -----

Date -----

Temperature -----

Tests for effective inactivation

Volume and concentration of bulk material injected -----

No. of mice injected -----

Weight of mice -----

Duration of observation -----

Other animals (if used) -----

Results of tests -----

Rabies virus amplification test

Amount of vaccine tested (ml) -----

Results -----

**4.3 Preparation and control of final bulk**

*Preservatives and other substances added*

Concentration of phenol (if used) -----

Other preservatives (type and concentration) -----

Other substances added -----

*Antigen content of the final bulk*

Method -----

Results -----

*Sterility tests*

Date of test -----

Result -----

*Other tests (chemical, biochemical)*

Type of test -----

Results -----

**5. Filling and containers**

Date of filling -----

Quantity of containers -----

Volume of vaccine per container -----

*Control for defective vials*

Methods -----

Results -----

**6. Control tests on final product**

**6.1 Identity test**

Method -----

Result -----

**6.2 Sterility test**

No. of containers examined -----

Method -----

Date at start of test -----  
Date at end of test -----  
Result -----

**6.3 Innocuity tests**

	Mice	Guinea-pigs
No. of animals	-----	-----
Route of injection	-----	-----
Volume of injection	-----	-----
Date of injection	-----	-----
Date of end of test	-----	-----
Result	-----	-----

**6.4 Antigen content**

Type of test -----  
Result -----

**6.5 Potency test of vaccine in final containers**

Type of test -----  
Date of immunization of mice -----  
Reference vaccine (potency) -----  
Challenge strain -----  
Date of challenge -----  
ED<sub>50</sub> test vaccine<sup>1</sup> -----

ED<sub>50</sub> reference vaccine<sup>1</sup> -----  
Calculated IU/single human dose -----  
Confidence limits -----  
Results of other potency tests -----

**6.6 Stability test**

Duration and temperature of incubation -----  
Result -----

---

<sup>1</sup> ED<sub>50</sub>: quantity of vaccine that protects 50% of animals against infection with the challenge strain.

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

Method -----  
 Result -----

**6.8 Inspection of final containers**

Result -----

**6.9 Test for pyrogenic substances**

Method -----  
 Results -----

**6.10 Test for adjuvant**

Date of test -----  
 Nature and concentration of adjuvant per  
     single human dose -----  
 Degree of adsorption -----

**Internal certification**

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

I certify that lot no.----- of rabies vaccine satisfies Part A of the WHO

*Recommendations for inactivated rabies vaccine for human use produced in cell  
 substrates and embryonated eggs.*

Signature -----  
 Name (typed) -----  
 Date -----

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

**Release certification by the national control authority**

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

*Sample release certificate*

I hereby certify that batch no.----- of rabies vaccine produced by (*name of producer*) in continuous cell lines meets all national requirements as well as Part A of the WHO *Recommendations for inactivated rabies vaccine produced in cell substrates and embryonated eggs for human use.*

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

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

The number appearing on the label of the containers is-----

Signature

Name (typed)

Date

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