
Final

ANNEX 3

RECOMMENDATIONS FOR PRODUCTION AND CONTROL OF INFLUENZA VACCINE (INACTIVATED)

Introduction
General considerations

Part A
Manufacturing recommendations
A1 Definitions
A2 General manufacturing recommendations
A3 Production control
A4 Filling and containers
A5 Control tests on final lot
A6 Records
A7 Retained samples
A8 Labelling
A9 Distribution and transport
A10 Stability testing, storage and expiry date

Part B
Recommendations for National Control Authorities
B1 General
B2 Release and certification
B3 Clinical evaluation of influenza vaccines

Authors
Acknowledgements
Introduction

Influenza is a significant cause of morbidity and mortality and has a major social and economic impact throughout the world. During major epidemics many people require medical treatment or hospitalization. Excess mortality often accompanies influenza epidemics, the vast majority affected being the elderly. Since they constitute the most rapidly increasing sector of the populations of many countries, the epidemiology of influenza can be expected to change accordingly, especially in the developed countries. The only means of influenza prophylaxis generally available at present is vaccination.

In 1967, a group of experts formulated requirements for inactivated influenza vaccine and these were published as an annex to the twentieth report of the Expert Committee on Biological Standardization (1). During the following five years, technical developments in the purification of the virus suspensions from which vaccines were made, as well as in the measurement of the virus content, were such that the potency of whole virus vaccines could be expressed in international units. Accordingly, an addendum to the requirements was annexed to the twenty-fifth report of the Expert Committee on Biological Standardization (2, pp. 15-17). In its twenty-ninth report (3), the Committee recognized that technical developments had completely altered the method of
measurement of the haemagglutinin content of the vaccines and that the International Reference Preparation of Influenza Virus Haemagglutinin (Type A) established in 1967 was no longer appropriate for controlling the haemagglutinin content of inactivated influenza vaccines since it no longer represented the haemagglutinin of the prevalent strains. Accordingly, the International Reference Preparation was withdrawn, and the Committee recommended that the requirements for inactivated Influenza vaccine should be revised. Revised requirements were approved by the WHO Expert Committee on Biological Standardization in 1978 published in 1979(4) and later modified in 1990 (5).

Since 1990, there have been significant new developments in methods of influenza vaccine production: increased development of mammalian cell lines for vaccine production; experience in use of adjuvants; rapid development of reverse genetics technologies for generation of vaccine viruses. There has also been considerable effort in pandemic planning to ensure that safe, effective vaccines can be quickly produced in response to a pandemic emergency. Consequently it is necessary to revise these requirements in order to reflect the new developments. In accordance with current WHO policy, the revised document is renamed as 'Recommendations'.

**General considerations**

Inactivated influenza vaccines have been in widespread use for nearly 60 years. The efficacy of immunization has varied according to circumstances, but protection rates of 75-90% have been reported. Differences in protective efficacy may result from continuing antigenic variation in the prevalent epidemic strains. Because of this variation, the composition of inactivated influenza virus vaccine, unlike that of most viral vaccines, must be kept constantly
under review. Accordingly, WHO twice annually publishes recommendations concerning the strains to be included in the vaccine.

Influenza vaccines usually contain one or more influenza A viruses. However, because influenza A viruses undergo frequent and progressive antigenic drift in their haemagglutinin and neuraminidase antigens, vaccines containing formerly prevalent viruses are expected to be less protective against virus variants showing antigenic drift than against the homologous virus. When a new subtype of influenza A virus bearing new haemagglutinin (and neuraminidase) antigen(s) appears, it is likely that vaccine containing the antigen(s) of the influenza A subtype(s) formerly prevalent will be ineffective, so that a vaccine containing the new pandemic virus will be required.

Changes in the structure of the haemagglutinin and neuraminidase molecules, which result in changes in antigenicity as new epidemic strains appear, involve surface residues in the region of the molecule furthest from the viral envelope. The information available does not permit prediction of future variations because the mechanism of selection of antigenic variants, which is termed antigenic drift, is not known and multiple evolutionary pathways appear possible. Antigenic shift (i.e. the appearance of epidemic strains with a new haemagglutinin subtype) is also unpredictable.

Influenza B virus antigenic drift is less frequent than that in the A strains and antigenic shift is unknown. Although distinct lineages of influenza B may occasionally co-circulate it is usual for influenza vaccines to contain only one influenza B strain.
In addition to antigenic drift and shift, there is another type of variation among influenza viruses, namely the preferential growth of certain virus subpopulations in different host cells in which the virus is cultivated. Influenza viruses grown in embryonated eggs often exhibit antigenic and biological differences as compared with those isolated and maintained in mammalian cells. Sequence analysis of the haemagglutinin gene of such variants has shown that, typically, virus grown in mammalian cells differs from virus from the same source cultivated in eggs only by the substitution of a single amino acid in the haemagglutinin molecule.

The WHO Expert Committee on Biological Standardization recommended in its twenty-ninth report (3) that the potency of influenza vaccines should be expressed in mcg of haemagglutinin per ml (or dose), as determined by suitable immunodiffusion methods. In order to standardize these methods, reference antigen (calibrated in mcg of the haemagglutinin per ml) and specific anti-haemagglutinin serum, suitable for use in the assay of the haemagglutinin content of each component of inactivated vaccines, are prepared and distributed by reference laboratories (Appendix 1). A new reference antigen and antiserum is prepared each time it is necessary to introduce a new virus strain into the vaccines, and these are standardized by international collaborative study.

Over the past twenty years there have been many clinical trials of whole virus, split and subunit influenza vaccines. As a result of this experience, it is generally accepted that one dose of vaccine containing 15mcg of haemagglutinin per strain per dose, will stimulate haemagglutination-inhibition antibody levels consistent with immunity in most primed individuals (7).
There has been much progress in developing adjuvanted influenza vaccines and some vaccines are now licensed. The main issues for vaccine quality are: demonstration of compatibility of the adjuvant with the antigenic components of the vaccine; proof of consistent association with vaccine antigens (if appropriate) at time of production and throughout shelf life; effect of adjuvant on vaccine potency assays; biochemical purity of adjuvant.

There is a long history of safety for egg grown vaccines. However it is known that influenza viruses cultivated in eggs can be contaminated with other viral agents and there has been a recent example of avian adenovirus contamination of a candidate pandemic vaccine virus. These recommendations have therefore been revised in view of the findings with egg grown viruses, the increasing use of mammalian cells for virus isolation and vaccine production and improved methods for detecting extraneous agents.

Influenza pandemic alerts occurred in 1997 (H5N1 virus), 1999 (H9N2 virus) and 2003 (H5N1 and H7N7 viruses), where avian influenza viruses caused serious illness and, on occasions, death in man. Experience gained from these events illustrate that different strategies for production and clinical use of vaccine may be needed in response to a pandemic: it may be necessary to generate a vaccine virus from a highly pathogenic virus by use of reverse genetics; initially reference reagents for vaccine potency testing may not be available; an adjuvanted vaccine may be desirable; monovalent vaccine may be preferred to conventional trivalent vaccine; whole virus vaccine may be preferred to split or subunit; a different dosing strategy may be needed (ie two doses). It is important to develop WHO vaccine recommendations for production and quality control that reflect the special needs of a pandemic.
Although the technology is still under development, use of reverse genetics for vaccine virus development is likely to affect interpandemic and pandemic vaccines alike. This technology involves transfecting mammalian cells with plasmids coding for influenza virus genes in order to produce a virus reassortant. Production of reassortants by reverse genetics is in concept similar to traditional methods, but there are some quality issues which should be taken into account: the influenza virus haemagglutinin and neuraminidase genes may be derived from a variety of sources (an egg isolate, an isolate in cells approved or not approved for human vaccine production, virus present in clinical specimens); the reassortant virus will have been generated in mammalian cells; in some countries, reassortants produced by reverse genetics may be classified as Genetically Modified Organisms and vaccine production should comply with national Contained Use regulations (although the final inactivated product will not be a Genetically Modified Organism).

**Recommendations published by WHO**

Recommendations published by WHO are intended to be scientific and advisory in nature. Thus, each of the following sections consitutes a recommendation. The parts of each section printed in type of normal size have been written in a form, so that, should a national control authority so desire, they may be adopted as they stand as definitive national requirements or used as the basis of such requirements. Those parts of each section printed in small type are comments and recommendations for guidance for those manufacturers and national control authorities who may benefit from additional information.
It is recommended that modifications 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 formulated below.

In order to facilitate the international distribution of vaccine made in accordance with these recommendations, a summary protocol for the recording of results of the tests is given in appendix 2.

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

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 Proper name

The proper name shall be "influenza vaccine (whole virion, inactivated)" or "influenza vaccine (split virion, inactivated)", "influenza vaccine (surface antigens, inactivated)" or “influenza vaccine (inactivated, adjuvanted)” translated into the language of the country of use.

The use of the proper name should be limited to vaccines that satisfy the recommendations specified below.
A.1.2 Descriptive definition

Influenza vaccine is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of these two types, which have been grown individually in embryonated hens’ eggs or in mammalian cells. Four types of influenza vaccine are available, as follows: (i) is a suspension of whole virus particles inactivated by a suitable method; (ii) is a suspension treated so that the virus particles have been partially or completely disrupted by physicochemical means (split vaccine); (iii) is a suspension treated so that the preparation consists predominantly of haemagglutinin and neuraminidase antigens (subunit vaccine); is a suspension of inactivated influenza virus particles, split or subunit components formulated with an adjuvant.

The preparation shall satisfy all the requirements formulated below.

A.1.3 Choice of vaccine strain

The World Health Organization reviews the world epidemiological situation twice annually and if necessary recommends new strains in accordance with the available epidemiological evidence.

Such strains, or those antigenically related to them, should be used in accordance with the regulations in force in the country concerned.
It is now common practice to use reassortant strains giving high yields of the appropriate surface antigens. Reassortant strains for vaccine production have the surface glycoproteins (haemagglutinin and neuraminidase) of the recommended reference virus and internal proteins of a high growth donor virus.

These recommendations shall also apply to the subsequent production and quality control of reassortant vaccine viruses produced by reverse genetics.

The passage history of the parent and reassortant virus strains shall be approved by the national control authority.

A.1. 4 Reference reagents

WHO influenza reference antigens for strain characterization are preparations antigenically representative of viruses isolated throughout the world. They may be obtained from one of the WHO Collaborating Centres for Reference and Research on Influenza (see Appendix 1)

Candidate influenza vaccine viruses are preparations antigenically representative of virus strain likely to be included in current vaccine. They may be wild type viruses or reassortant viruses with surface antigens appropriate for the current vaccine strain recommendations. They are distributed on demand when a new virus appears and the likelihood of its spread throughout
the world makes its inclusion in a vaccine desirable. They may be obtained from one of the custodian laboratories listed in Appendix 1.

Antigen reagents for vaccine potency standardization contain a calibrated quantity of haemagglutinin antigen of influenza virus measured in mcg/ml. The calibrations are performed by international collaborative study using single radial immunodiffusion tests (8) with purified virus of known haemagglutinin antigen concentration. The reference antigen and antiserum reagents are used to calibrate the haemagglutinin content of inactivated influenza vaccines by an in vitro immunodiffusion test. These reference haemagglutinin antigens, together with the specific antihaemagglutinin sera, may be obtained for the purpose of such tests from one of the custodian laboratories listed in Appendix 1.

A.1.5 Terminology

Master seed lot: A quantity of virus, antigenically representative of a WHO-recommended strain, that has been processed at one time to assure a uniform composition and fully characterized. It is used for the preparation of working seed lots. The master seed lot and its passage level are approved by the national authority.

Working seed lot: A quantity of virus of uniform composition and fully characterized that is derived from a master seed lot by a number of passages that does not exceed the maximum approved by the national authority.
*Cell seed:* A quantity of well-characterized cells of human or animal origin stored frozen in liquid nitrogen 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. The testing performed on a replacement master cell bank (derived from the same clone or from an existing master or working cell bank) is the same for the initial master cell bank, unless a justified exception is made.

*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 a 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 MWCB. One or more of the ampoules from such a pool may be used for the production cell culture.

*Production cell culture:* A cell culture derived from one or more ampoules of the MWCB and used for production of the live influenza virus.

*Single harvest:* A quantity of virus suspension derived from either a group of embryonated eggs or a culture of mammalian cells that were inoculated with the same virus working seed lot, incubated together and harvested together in one session.
Monovalent virus pool: A pool of a number of single harvests of a single virus strain processed at the same time.

Final bulk: The finished vaccine prepared from one or more monovalent pools present in the container from which the final containers are filled. It may contain one or more virus strains.

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

A.2 General manufacturing requirements

The general requirements for manufacturing establishments contained in good manufacturing practices for pharmaceuticals (9) and biological products (10) shall apply to establishments manufacturing inactivated influenza vaccine, with the addition of the following:

Details of standard operating procedures for the preparation and testing of influenza vaccines adopted by a manufacturer together with evidence of appropriate validation of the production process shall be submitted for approval to the national control authority. Proposals for modification of the manufacturing/control methods shall also be submitted for approval before they are implemented, on the request of the national control authority.
Personnel employed in the production and control facilities shall be adequately trained and protected against accidental infection with influenza virus.

High levels of biological containment are likely to be required for the production of pandemic or potential pandemic vaccines. The WHO will provide advice where appropriate (14) and national or regional safety guidelines must be followed.

Standard operating procedures need to be developed for dealing with emergencies involved with accidental spillage, leakage or other dissemination of influenza virus.

The areas where processing of inactivated influenza vaccine takes place shall be separate from those where work with live influenza virus is performed.

A.3 Production control

A.3.1 Control of source materials

A.3.1.1 Eggs used for seed virus growth

If the vaccine is to be produced in embryonated eggs, the eggs to be used should be from closed, specific-pathogen-free, healthy flocks (19).

This flock shall be monitored at regular intervals for specific agents:

Agents monitored may include *Mycobacterium avium*, fowl pox virus, ALV and other avian retroviruses,

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.

**A.3.1.2 Eggs used for vaccine production**

If the vaccine is to be produced in embryonated eggs, the eggs should be from a healthy flocks, which are monitored by methods approved by local animal health authorities.

As large numbers of eggs are needed for vaccine production, it is not feasible to use eggs from specific pathogen free flocks.

Monitoring of flocks for avian influenza viruses is performed in some countries.
In both situations (production of vaccine seed and production of vaccine), the flock must not have been vaccinated with live Newcastle disease virus vaccine. It is also recommended that eggs should be obtained from young birds.

In some countries, where use of live Newcastle disease vaccine is mandatory, vaccination should take place during the first few weeks of the chickens’ life and well before the use of flocks for supply of eggs.

A.3.1.3 Master cell bank and manufacturer’s working cell bank

If a cell line is used for the manufacture of influenza vaccines it should be based on the cell bank system. The national control authority should approve the master cell bank and should establish the maximum number of passages (or population doublings) by which the MWCB is derived from the master cell bank and the maximum number of passages of the production cultures.

WHO has established a reference cell bank of Vero cells characterized in accordance with the requirements in the report of the WHO Expert Committee on Biological Standardization (17). This should not be considered as a master cell bank for direct use in vaccine production, but may be used to develop a master cell bank by thorough requalification.

A.3.1.3.1 Identity test

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1 Available to manufacturers on application to Quality Assurance and Safety of Biologicals: Vaccines and Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.
The master cell bank should be characterized according to the WHO Requirements for the Use of Animal Cells as In Vitro Substrates for the Production of Biologicals (17) as they relate to continuous cell lines, or to human diploid cells, as appropriate.

The MWCB should be identified by means, inter alia, of biochemical (e.g. isoenzyme analysis), immunological, and cytogenetic marker tests, DNA fingerprinting, approved by the national control authority.

A.3.1.4 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 sections A.5.2 and A.5.3 of the revised Requirements for Biological Substances No. 6 (16) and from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the Recommendations for Poliomyelitis Vaccine (Oral) (18).

Where approved by the national control authority, alternative tests for bovine viruses may be used.

As an additional monitor of quality, sera may be examined for freedom from phage and endotoxin.

Irradiation may be used to inactivate potential contaminant viruses.
The acceptability of the sources(s) of serum of bovine origin should be approved by the national control authority. The serum should comply with current guidelines in relation to animal transmissible spongiform encephalopathies (19).

Human serum should not be used. If human albumin is used, it should meet the revised Requirements for Biological Substances No. 27 (Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives) (20), as well as current guidelines in relation to human transmissible encephalopathies (19).

Manufacturers are encouraged to explore the possibilities of using serum-free media for production of inactivated influenza vaccine.

Penicillin and other beta-lactams should not be used at any stage of the manufacture.

Other antibiotics may be used at any stage in the manufacture, provided that the quantity present in the final product is acceptable to the national control authority. Non-toxic 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.

Trypsin used for preparing cell cultures should be tested and found free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially parvoviruses appropriate to the
species of animals used. The methods used to ensure this should be approved by the national control authority.

The source(s) of trypsin of bovine origin, if used, should be approved by the national control authority. Bovine trypsin, if used, should comply with current guidelines in relation to animal transmissible spongiform encephalopathies (19).

A.3.1.5 Virus

Strains of influenza virus used in the production of inactivated influenza vaccine shall be identified by historical records, which shall include information on the origin of the strains and their subsequent manipulation,

Only strains that have been isolated in embryonated hens’ eggs, in cells derived from eggs, or in mammalian cells approved for human vaccine production under validated laboratory conditions shall be used. The national control laboratory shall approve the virus strain. It is now common practice to use reassortant strains giving high yields of the appropriate surface antigens. However, it has been noted that antigenic changes may occur during the development of high-yielding reassortants, and the absence of such changes should be shown by haemagglutination-inhibition tests using antibodies to the haemagglutinin of the reassortant and wild type viruses.
Where reassortant strains are used, the parent high-yield strain and the method of preparing the reassortant should be approved by the national control authority.

Where reverse genetics is used to generate the reassortant vaccine virus, the influenza haemagglutinin and neuraminidase genes may be derived from a variety of sources (egg isolate, mammalian cell isolate, virus in clinical specimen). The haemagglutinin and neuraminidase genes are expected to be free of extraneous agents associated with the wild type virus by virtue of the recombinant DNA technology employed. The cell substrate used for transfection to generate the reassortant virus shall be approved for human vaccine production. The derivation of the reassortant virus shall be approved by the national control authority.

Strains of virus suitable for manufacture of a pandemic or potential pandemic vaccine shall be supplied by procedures agreed by the WHO Collaborating Centres for Reference and Research on Influenza and the custodian laboratories for supply of candidate strains (Appendix 1) and shall be approved by the national control authority.
If any materials of animal (non-avian) origin are used in production, they shall comply with the guidance given in the report of a WHO consultation on medicinal and other products in relation to human and animal transmissible spongiform encephalitis (12) and shall be approved by the national control authority.

Reference strains for antigenic analysis may be obtained from the WHO Collaborating Centres for Reference and Research on Influenza, or other custodian laboratories (Appendix 1).

A.3.1.5.1 Seed lot system

The production of vaccine shall be based on a seed lot system. Each seed lot shall be identified as influenza virus of the appropriate strain by methods acceptable to the national control authority (section A.1.3). The maximum number of passages between a master seed lot and a working seed lot shall be approved by the national control authority. The vaccine shall be not more than one passage from the working seed lot.

Each manufacturer shall identify the haemagglutinin and neuraminidase antigens of the vaccine virus strains by suitable tests capable of detecting biologically significant variation, as well as cross-contamination during manipulation.

A.3.1.5.2 Tests on seed lots
Either the master or working seed virus shall be shown to be free from relevant extraneous agents by tests or procedures approved by the national control authority in accordance with the requirements of Part A, sections 5.2 and 5.3, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (2, pp. 49-52).

Strategies to ensure freedom from extraneous agents in the final vaccine may involve a combination of testing the seed virus and validation of the production process, depending on the substrate used for production. For egg derived vaccines, the emphasis should be on a validation process, whereas for cell derived vaccines, the emphasis may be on a testing strategy. The national control authority should approve the strategy chosen.

**Validation strategy**

The production process should be validated to remove and/or inactivate likely potential contaminating agents. Validation may be performed using appropriate model agents.
If removal/inactivation cannot be demonstrated for a potential contaminant, a testing strategy should be implemented.

Testing strategy

Cell derived vaccine

The susceptibility of mammalian cells to various human pathogens should be considered and this information should be used in considering a list of potential human pathogens to be included in testing for extraneous agents in seed virus. Pathogens to be considered could include adenovirus, parainfluenza virus, respiratory syncytial virus, coronavirus, rhinovirus, enterovirus, Epstein Barr virus, herpes simplex virus, cytomegalovirus and mycoplasmas.

It is recognized that whenever a vaccine strain changes, there may be time constraints that make testing seed viruses for extraneous agents problematic and results of such testing may not be completely available before further processing. Use of rapid assays (e.g. multiplex pcr) which could be applied within these time constraints are encouraged.
If an agent is detected in a seed virus and the mammalian cells used for production are shown to be susceptible to this agent, the seed virus should not be used for vaccine production.

If an agent is detected in a seed virus and the mammalian cells are not susceptible to the agent, steps should be in place to ensure that the contaminating agent is removed and/or inactivated by the production process. If removal/inactivation cannot be demonstrated for the agent, appropriate and specific downstream testing at the level of each inactivated monovalent bulk should be implemented to demonstrate that any contaminant which may have been identified in the seed virus, is absent from the vaccine.

Egg derived vaccine

A testing strategy for specific potential contaminating agents may be needed, if removal/inactivation of the agent by the production process cannot be demonstrated. The use of rapid tests (eg pcr) is encouraged. If the agent is detected, the seed virus should not be used for vaccine production.
The seed lot should be stored at a temperature lower than –60°C, unless it is in the lyophilized form, in which case it should be stored at a temperature lower than –20°C.

A. 3.2 Production precautions

The general production precautions formulated in Part A, section 3, of the revised Requirements for Biological Substances No. I (General Requirements for Manufacturing Establishments and Control Laboratories), (8, p. 15) shall apply to the manufacture of inactivated influenza vaccines, with the addition of the following:

- for egg derived vaccines, only allantoic and amniotic fluids may be harvested;

- beta-lactam antibiotics shall not be used at any stage in the manufacture of the vaccine.

Minimal concentrations of other suitable antibiotics may be used.

If vaccines are produced by the splitting of the virus by chemical means, the splitting conditions and the concentration of the chemicals used shall be approved by the national control authority. If an adjuvant is used, the concentration of adjuvant shall be approved by the national control authority.
A. 3.3 Production of monovalent virus pools

A.3.3.1 Single harvests

For egg derived vaccine, the virus of each strain shall be grown in the allantoic cavity of embryonated hens' eggs derived from healthy flocks. After incubation at a controlled temperature, both the allantoic and amniotic fluids may be harvested. For mammalian cell derived vaccine, the virus of each strain shall be grown in cells approved for human vaccine production.

For both cell and egg derived vaccines, a number of single harvests of the same strain of virus may be combined to give a monovalent virus pool. Cell derived monovalent virus pools should not be mixed with egg derived monovalent virus pools.

A.3.3.2 Inactivation of monovalent virus pools

Time of inactivation. In order to limit the possibility of contamination, monovalent virus pools shall be inactivated as soon as possible after their preparation. However, if delay is unavoidable, the temperature and duration of the storage should be validated with respect to bioburden and quality of the haemagglutinin and neuraminidase antigens.

Validation with respect to bioburden may be omitted for cell culture derived monovalent virus
pools, with the agreement of the national regulatory authority.

Before monovalent virus pools are inactivated, samples shall be taken and tested for bacterial and fungal contamination. Limits for bioburden should be approved by the national control authority.

**Inactivation procedure.** The virus in the monovalent virus pools shall be inactivated by a method that has been demonstrated to be consistently effective in the hands of the manufacturer and has been approved by the national control authority. For egg derived vaccine, the inactivation process shall also have been shown, to the satisfaction of the national control authority, to be capable of inactivating avian leukosis viruses and mycoplasmas. If the virus pool is stored after inactivation, the temperature and duration of the storage should be validated. The inactivation procedure should have been shown to be capable of inactivating influenza viruses without destroying their antigenicity.

The usual temperature of storage is $5 \, ^\circ \text{C} \pm 3 \, ^\circ \text{C}$

Consideration should be given to investigating whether influenza virus inactivation also inactivates human or avian pathogens capable of becoming extraneous agents. These investigations may be performed using appropriate model agents eg *egg derived vaccines*: avian leukosis virus, mycoplasma, avian adenovirus; *cell derived vaccines*:...
poliovirus, human immunodeficiency virus, human adenovirus, parainfluenza virus, minute virus of mice. If formalin (40% formaldehyde) or beta-propiolactone (2-oxetanone) is used, the concentration by volume should not exceed 0.1% at any time during inactivation. Other suitable inactivating agents can also be used.

Consideration should be given to strategies to limit the entry into the manufacturing process of potential adventitious agents that may not be inactivated by the influenza inactivation conditions.

A.3.3.3 Testing of control cells

A cell sample equivalent to at least 500 ml of the cell suspension, at the concentration employed for vaccine production cultures, should be used to prepare control cell cultures. In some countries in which the technology of large-scale production has been developed, the national control authority should determine the size of the sample of cells to be examined, the time at which the control cells should be taken from the production culture, and the monitoring of the control vessels.

These control cell cultures should be incubated for at least 2 weeks 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 may be discarded for nonspecific, accidental reasons.
If this examination or any of the tests required in this section show evidence of the presence in a control culture of any adventitious agent, the influenza virus grown in the corresponding inoculated cultures should not be used for vaccine production.

Samples not tested immediately should be stored at –60 °C or below.

A.3.3.3.1 Tests for haemadsorbing viruses

At the end of the observation period or at the time the virus is harvested from the production cultures, whichever is the later, at least 25% of the control cells should be tested for the presence of haemadsorbing viruses using guinea-pig red cells. If the guinea-pig red cells have been stored, the duration of storage 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 medium.

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

In some countries the sensitivity of each new batch of red blood cells is demonstrated by titration against a haemagglutin antigen before use in the haemadsorption test.

A.3.3.3.2 Tests on supernatent fluids

A sample of at least 10 ml of the pooled supernatent fluid from the control cultures collected at the end of the observation period should be tested in the same cell substrate, but not the same batch, as that used for production. Additional samples of at least 10 ml should be tested in both human and simian cells. The samples should be inoculated into bottles of these cell cultures, in such a way that the dilution of the supernatent fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm²/ml of supernatent fluid. At least one bottle of the cell cultures should remain uninoculated and serve as a control.

The cultures should be incubated at a temperature of 35 - 37 °C and should be observed for a period of at least 2 weeks.

Use of rapid assays (e.g. multiplex pcr), which could be applied within the time constraints are encouraged.
A.3.3.3 Identity test

For vaccines produced in continuous cells the control cells should be identified by means, inter alia, of biochemical (e.g. isoenzyme analysis), immunological and cytogenetic marker tests, approved by the national control authority.

A.3.3.4 Concentration and purification

The monovalent material shall be concentrated and purified by high-speed centrifugation or other suitable methods approved by the national control authority, either before or after the inactivation procedure.

The aim is to separate the virus or viral components from other constituents in either the allantoic and amniotic fluids or the mammalian cell culture fluids as efficiently as possible. It is advisable to concentrate and purify the virus under optimum conditions to preserve its antigenic properties.
Consideration should be given to investigating whether the concentration and purification steps, remove potential extraneous agents.

A.3.4 Control of monovalent virus pools

A.3.4.1 Effective inactivation

The inactivated and purified monovalent virus pool shall be shown not to contain viable influenza virus when tested by a method approved by the national control authority. Tests for viable virus shall be conducted in eggs for egg derived vaccine and in the mammalian cells used for vaccine production for cell derived vaccine.

A suitable method for egg derived vaccine consists of inoculating 0.2 ml of undiluted monovalent pool and 1 : 10 and 1 : 100 dilutions of the monovalent pool into the allantoic cavities of groups of fertile eggs (ten eggs in each group), and incubating the eggs at 33-37 °C for three days. At least eight of the ten eggs should survive at each dosage level.

A volume of 0.5 ml of allantoic fluid is harvested from each surviving egg. The fluid harvested from each group is pooled and 0.2 ml of each of the three pools is inoculated, undiluted, into a further group of ten fertile eggs.
Haemagglutinin activity should not be detected in these new groups of eggs.

In some countries, alternative methods are used.

In some countries, the requirement that 80% of the eggs should survive during incubation may be impossible to satisfy. The national control authority should then specify the requirement to be satisfied.

For mammalian cell derived vaccine, methods could be modeled on those used for egg derived vaccines with the exception that a mammalian cell substrate with validated sensitivity should be used. The national control authority should approve the method used and specify the requirement to be satisfied.

As testing of residual virus infectivity after inactivation may be problematic due to aggregation, validation of test sensitivity should be performed.
A.3.4.2 Haemagglutinin content

The content of haemagglutinin in the monovalent virus pool shall be determined by a suitable and approved technique, such as single radial immunodiffusion. In the test, an influenza reference haemagglutinin antigen reagent or a national preparation calibrated against it shall be used for purposes of comparison (see section A.1.4).

For adjuvanted vaccines it should be established whether the adjuvant is compatible with the antigenic components of the vaccine and whether the presence of adjuvant interferes with the test for haemagglutinin content. If there is likely to be interference, this test may be performed before the addition of adjuvant. This practice should be fully validated.

There is evidence to suggest that when cell derived vaccines are produced from viruses isolated in eggs, the conventional reference antigen reagents (egg derived) are suitable for measurement of haemagglutinin content. However it should be considered that the use of conventional antigen reagents may not be suitable to measure the haemagglutinin content of mammalian cell derived vaccines, when viruses isolated in cells are used for production. As information accumulates, advice will be provided by WHO.
During early stages of pandemic vaccine production, there may be no reference reagents to measure vaccine haemagglutinin content by conventional methods. It may be necessary to use alternative estimates of antigen content as advised by WHO and national control authorities.

A.3.4.3 Presence of neuraminidase

Vaccine should be prepared under conditions that allow retention of detectable levels of viral neuraminidase for each strain.

In some countries, a test is included for the presence of neuraminidase enzymatic or antigenic activity. The ratio of haemagglutinin to neuraminidase should be consistent for the particular virus strain and method of vaccine production used, but the neuraminidases of different strains vary markedly in their stability during processing.
A.3.4.4 Virus disruption (split vaccines)

Monovalent pools in which the virus has been split by chemical means shall be shown by procedures approved by the national control authority to consist predominantly of disrupted virus particles.

This test need be performed on only three samples of monovalent pool for each vaccine strain provided that the test result is satisfactory.

A.3.4.5 Surface antigens (subunit vaccines)

The purity of monovalent pools intended for the preparation of subunit vaccine shall be determined by polyacrylamide gel electrophoresis or by other suitable techniques approved by the national control authority. Mainly haemagglutinin and neuraminidase antigens for each strain should be present.

This test need be performed on only three samples of monovalent pool for each vaccine provided that the test result is satisfactory.
A.3.4.6 Identity

Antigenic specificity may be confirmed by an immunodiffusion or haemagglutination-inhibition technique using appropriate specific immune sera. The tests for haemagglutinin content (A.3.4.2) and presence of neuraminidase (A.3.4.3) also serve as identity tests. Reference viruses for identity tests may be obtained from reference laboratories (Appendix 1).

Alternatively antigenic identity may be confirmed by:

- injection of vaccine into mice, chickens or other suitable animals and demonstration of the production of antibodies to the haemagglutinin of the influenza virus used to produce the vaccine. In addition, demonstration of production of antibody to neuraminidase may also be performed
- suitable genetic tests.

With split and subunit vaccines, the identity test may be performed before virus disruption.
A.3.4.7 Extraneous agents

Cell derived vaccines

If a contaminating agent is found in the working seed, mammalian cells are not susceptible to infection by the agent (A.3.1.3.2) and removal and/or inactivation of the agent by the production process cannot be demonstrated, monovalent bulks shall be tested to ensure freedom from the agent. The data shall be approved by the national control authority.

Egg derived vaccines

If removal/inactivation of a potential contaminating agent by the production process cannot be demonstrated, monovalent bulks shall be tested to ensure freedom from the agent. The data shall be approved by the national control authority.

A.3.4.8 Purity of cell derived vaccines

To monitor consistency in purity, monovalent virus pools derived from mammalian cell cultures should be tested for the ratio haemagglutinin content:total protein. This ratio should be within the limits approved by the national control authority.
For viruses grown in continuous cells the purified monovalent pool should be tested for residual cellular DNA. The purification process should be shown to reduce consistently the level of cellular DNA to less than 10 ng per human dose. This test may be omitted, with the agreement of the national control authority, if the manufacturing process is validated to achieve this specification.

**A.3.4.9 Tests for chemicals used in production**

The concentration of each detergent, organic solvent and inactivating agent remaining in the final vaccine shall be determined by methods approved by the national control authority. These concentrations shall not exceed upper limits specified by the national control authority. For preservatives, both the method of testing and the concentration shall be approved by the national control authority.

Alternatively, tests for chemicals may be performed on final bulk.

**A.3.5 Control of final bulk**

Final bulks are prepared by mixing and diluting monovalent pools of the relevant strains. In the preparation of the final bulk, only preservatives or other substances, including diluents, approved by the national control authority shall be added. Such substances shall have been shown by appropriate tests not to impair the safety or effectiveness of the product in the
concentrations used, and shall not be added before samples have been taken for any tests that would be affected by their presence.

Pandemic vaccines are likely to contain only one strain.

**A.3.5.1 Test for content of haemagglutinin antigen**

The haemagglutinin concentration in the final bulk shall be determined as described in section A.3.4.2.

This test may be omitted if such a test is performed on each final lot.

**A. 3.5.2 Sterility tests**

Each bulk shall be tested for sterility by a method approved by the national control authority.

Many countries have regulations governing sterility testing. Where these do not exist, the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (2) shall be satisfied. If a preservative has been added to the vaccine, appropriate measures shall be taken to prevent it from interfering with the sterility test.
A.3.5.3 Total protein

The total protein content shall be not more than six times the total haemagglutinin content of the vaccine, as determined in the test for haemagglutinin content, but in any case not more than 100 mcg of protein per virus strain per human dose and not more than a total of 300 mcg of protein per human dose.

In some countries, protein stabilisers are added to vaccine. The total protein content should reflect such additions. For subunit vaccines, a lower protein content is achievable i.e. not more than 40 mcg of protein per virus strain per human dose and not more than a total of 120 mcg of protein per human dose.

A.3.5.4 Ovalbumin (egg derived vaccines)

The ovalbumin content shall be not more than 5 mcg per human dose. The amount of ovalbumin shall be determined by a suitable technique using a suitable reference preparation of ovalbumin.

Values of less than 1 mcg of ovalbumin per human dose are attainable and lower limits may be set.
A.3.5.5 Adjuvant content

If an adjuvant has been added to the vaccine, its content shall be determined by a method approved by the national control authority. The amount and nature of the adjuvant shall be within the range shown to be clinically effective and shall be approved by the national control authority.

The formulation of adjuvant and antigen shall be stable and consistent. The purity of the adjuvant should be demonstrated to be within the range found for vaccine lots shown to be clinically effective.

A.4 Filling and containers

The requirements concerning filling and containers given in good manufacturing practices for biological products (10, annex 1, section 4) shall apply. Single- and multiple-dose containers may be used. If the latter are used, a suitable preservative, approved by the national control authority, shall be incorporated.
A.5 Control tests on final lot

A.5.1 Identity test

An identity test shall be performed by a method approved by the national control authority on at least one container from each final lot.

The identity of the haemagglutinins in the vaccine shall be determined by an immunological technique, such as immunodiffusion or haemagglutinin inhibition, using the appropriate specific immune serum.

In some countries, a test to identity the specific neuraminidase antigens is also included.

A.5.2 Sterility test

Final containers shall be tested for sterility as described in section A.3.5.2.
A.5.3 Haemagglutinin content

The test for haemagglutinin antigen concentration is performed as described in section A.3.4.2.

The vaccine shall contain in each human dose at least 15 mcg of haemagglutinin of each strain used in the preparation.

In some countries, lower limits may be set, based on clinical experience.

Expression of haemagglutinin antigen content can also reflect uncertainty of measurement by stipulating that the lower confidence interval (p = 0.95) of the assay should be not less than 12mcg of haemagglutinin of each strain per dose.

It may be necessary to formulate a pandemic vaccine to contain a different haemagglutinin antigen concentration. Advice will be provided by WHO and the national control authority.
A. 5.4 General Safety (Innocuity) tests

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

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

A. 5.5 Endotoxin

A test for endotoxin shall be included, e.g., the Limulus amoebocyte lysate test.

The permissible level of endotoxin is determined by the national control authority. It is likely that the permissible level of endotoxin for mammalian cell derived vaccine will be lower than that for egg derived vaccine.
A. 5.6 Inspection of final containers

Each container in each final lot shall be inspected visually, and those showing abnormalities such as improper sealing, lack of integrity shall be discarded.

A.6 Records

The requirements given in section 8 of *Good Manufacturing Practices for Biological Products* (10, annex 1) shall apply.

A.7 Retained Samples

The requirements given in section 9 of *Good Manufacturing Practices for Biological Products* (10, annex 1) shall apply.

A.8 Labelling

The requirements given in section 7 of *Good Manufacturing Practices for Biological Products* (10, annex 1) shall apply, with the addition of the following information:

The label on the carton, the container or the leaflet accompanying the container shall state:
that the vaccine has been prepared from virus propagated in embryonated hens’ eggs or in
mammalian cells;
- the type of cell line ie monkey, dog etc (if appropriate);
- the strain or strains of influenza virus present in the preparation;
- the haemagglutinin content in mcg per virus strain, expressed as mcg of haemagglutinin
per dose;
- the number of doses, if the product is issued in a multiple-dose container.
- the influenza season for which the vaccine is intended.
- the method used for inactivating the virus;
- the name and maximum quantity of any antibiotic present in the vaccine;
- the name and concentration of any preservative added.
- the name and concentration of any adjuvant added
- the temperature recommended during storage and transport.
- the expiry date

For a pandemic vaccine – special dosing schedules (eg two doses)

A.9 Distribution and transport

The requirements given in section 8 of Good Manufacturing Practices for Biological
Products (10, annex 1) shall apply.
A.10 Stability testing, storage and expiry date

A.10.1 Stability testing

Adequate stability studies form an essential part of vaccine development. The stability of the vaccine in its final form and at the recommended storage temperatures shall be demonstrated to the satisfaction of the national control authority on final containers from at least three lots of final product.

Stability data may be presented to the National Regulatory Authority after use of vaccine. Accelerated stability studies may be used.

In some countries vaccine haemagglutinin content should comply with final product specifications (see A5.3) at the end of its expiry date.

The formulation of vaccine antigens and adjuvant (if used) must be stable throughout its shelf life. Acceptable limits for stability shall be agreed with national authorities.

When any changes are made in the production process, that may affect stability of the products the vaccine produced by the new method shall be shown to be stable.
A.10.2 Storage conditions

Inactivated influenza vaccine shall be stored at a temperature of 2-8°C.

If other storage conditions are used, they should be fully validated and approved by the national control authority.

A.10.3 Expiry date

The expiry date shall be fixed with the approval of the national control authority, taking account of the experimental data on stability of the vaccine.

In general, the expiry date should not exceed one year from the date of issue by the manufacturer since one year's viral strains may not be appropriate the next year.

Part B. Requirements for National Control Authorities

B.1 General
The general requirements for control laboratories, contained in *Guidelines for National Authorities on Quality Assurance of Biological Products* shall apply.

The national control authority shall give directions to manufacturers concerning the Influenza virus strains to be used, the haemagglutinin content, whether or not neuraminidase is present, and the recommended human dose.

**B.2 Release and certification**

A vaccine lot shall be released only if it fulfils national requirements and/or Part A of these Requirements. A statement signed by the appropriate official of the national control authority shall be provided at the request of the manufacturing establishment and shall certify that the lot of vaccine in question satisfies all national requirements as well as Part A of these Requirements. The certificate shall state the number under which the lot was released by the national controller, and the number appearing on the labels of containers. Importers of influenza vaccine (inactivated) shall be given a copy of the official national release document. The purpose of the certificate is to facilitate the exchange of inactivated influenza vaccine between countries.

An example of a suitable certificate is given in Appendix 3.

**B.3 Clinical evaluation of influenza vaccines**

In the case of a new manufacturer, the national control authority shall assess the safety and immunogenicity of the vaccine by arranging for studies in human volunteers of one or
more of the lots of vaccine that have satisfied the above-mentioned requirements. Such studies shall include the assessment of the immune responses and adverse reactions in various age groups.

In the case of a significant change in the manufacturing process, clinical studies may also be required by the national control authority.

Some national authorities require a limited clinical evaluation for licensing purposes whenever a new vaccine strain is introduced.

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References


Appendix 1  Reference Laboratories

WHO Collaborating Centres for Reference and Research on Influenza

Centers for Disease Control, Atlanta;
National Institute for Medical Research, Mill Hill;
National Institute for Infectious Disease, Tokyo;
WHO Collaborating Center for Reference and Research on Influenza, Melbourne.

Custodian laboratories for candidate influenza vaccine viruses and antigen reagents for vaccine potency

National Institute for Biological Standards and Control, Potters Bar;
Center for Biologics Evaluation and Research, Bethesda;
Therapeutic Goods Administration, Canberra;
National Institute for Infectious Diseases, Tokyo,
Centers for Disease Control, Atlanta;
National Institute for Medical Research, Mill Hill;
WHO Collaborating Center for Reference and Research on Influenza, Melbourne.

Laboratories performing calibration of haemagglutination content

National Institute for Biological Standards and Control, Potters Bar,
Center for Biologics Evaluation and Research, Bethesda,
Therapeutic Goods Administration, Canberra
National Institute for Infectious Diseases, Tokyo
Appendix 2  Summary protocol for influenza vaccine (inactivated) (master/working seed lot)

Type A or Type B) The model summary protocol that follows is provided as general guidance to manufacturers. It is not intended to constrain them in the presentation of data relevant to the complete review of the quality-control tests performed on the vaccine. It is important to note that satisfactory test results do not necessarily imply that the vaccine is safe and effective, since many other factors must be taken into account, including the characteristics of the manufacturing facility.

Name and address of manufacturer

Laboratory reference no. of lot

Date when the processing was completed

Information on manufacture

Virus used to inoculate eggs or cells for the manufacture of the lot:

(a) strain and substrain

(a) passage level

(b) source and reference no.

(c) remarks

Results of sterility test
Results of tests for extraneous agents
Results of tests on adjuvant (if any)
Conditions of storage

**Monovalent virus pool Type A or Type B**

Name and address of manufacturer

Laboratory reference no. of virus pool

Virus used to inoculate eggs/cells:

(a) master seed strain and source
(b) passage level of master seed
(c) working seed lot, reference no. and source

Date of inoculation

Date of harvesting allantoic/amniotic fluids or cell culture fluids

Storage conditions before inactivation

Date of inactivation

Time of inactivation

Method of inactivation

Concentration of inactivating agent

Storage conditions after inactivation

Concentration/purification procedure

Antibiotics used during preparation, if any

Identification of adjuvant added, if any

**Tests on monovalent pool**

**Test for absence of viable influenza virus**

No. of eggs/cell culture vessels inoculated

Incubation time and temperature

Date of test

Results

_________________

1 If there are more than four virus pools in the monovalent pool, the relevant data should be given on a separate sheet.
Determination of haemagglutinin content
Method
Date of determination
Results

Tests for presence of neuraminidase (if performed)
Method
Date of test
Results

Virus disruption (for split vaccine)
Method
Date
Results

Purity (for subunit vaccine)
Method
Date
Results

Purity (for cell derived vaccine)
Method
Identity tests
Method
Date of test
Results

Test for extraneous agents (if performed)
Method
Date
Results

Final bulk
Name and address of manufacturer
Identification of final bulk
Identification of monovalent virus pool used to prepare final bulk
Date of manufacture

Control of final bulk
Preservative(s) added and concentration
Any other substances added and concentration

Determination of haemagglutinin content
Method
Date of determination
Results

Sterility
Date of test
Results

*Total protein content*
Method
Date of test
Results

*Ovalbumin content (egg derived vaccines)*
Method
Date of test
Results

*Test for residual DNA, if performed*
Method
Date
Results

*Test for adjuvant (if performed)*
Method
Date
Results
Tests for chemicals used

Date of tests
Results

Final lot

Identity test
Method
Date of test
Results

Sterility
Method
Date of test
Results

Determination of haemagglutinin content
Method
Date of determination
Results

Innocuity (if performed)
No. and species of animals
Doses injected
Period of observation
Date of test
Results

*Endotoxin content*
Method
Date of test
Results

*Inspection of final container*
Results

*Other tests*

*Additional comments (if any)*
A sample of a completed final container label and package insert shall be attached.
Certification by producer

Name of head of production of the final vaccine

Certification by head of the quality assurance department taking overall responsibility for production and control of the final vaccine:

I certify that lot no ... of influenza vaccine (inactivated), whose number appears on the label of the final container, meets all national requirements\(^1\) and satisfies Part A of the Requirements for Biological Substances No. 17, revised 1990.

Signature _____________________________________________

Name (typed) _____________________________________________

Date: _____________________

Certification by the national controller

If the vaccine is to be exported, provide a copy of the certificate from the national control authority as described in section B. 2, a label of a final container, and a leaflet of instructions to users.

_________________
If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot has nevertheless been authorized.

Appendix 3 Model certificate for the release of influenza vaccine (inactivated)¹

The following lots of influenza vaccine (inactivated) produced by in -,² whose numbers appear on the labels of the final containers, meet all national requirements,³,⁴ Part A of the Requirements for Biological Substances No. 17 (Requirements for Influenza Vaccine (Inactivated) [revised 1990; if applicable, addenda 19 _ ]),⁵ and the Requirements for Biological Substances No. I (General Requirements for Manufacturing Establishments and Control Laboratories [revised 1965]).⁶

<table>
<thead>
<tr>
<th>Lot Number</th>
<th>Date of last potency test by manufacturer</th>
<th>Expiry Lot number</th>
<th>Date of last potency test by manufacturer</th>
<th>Expiry Date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</table>

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

The number of this certificate is

The Director of the National Control Laboratory (or Authority as appropriate):'

Name (typed)

______________________________________________

Signature

______________________________________________

Date: __________________________
To be provided by the national control authority of the country where the vaccines have been manufactured, on request by the manufacturer.

Name of manufacturer.

Country.

If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national control authority.

With the exception of the provisions on shipping, which the national control authority may not be in a position to control.


Or his or her representative.