# Annex 4

## Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (adsorbed)

### Replacement of Annex 2 of WHO Technical Report Series, No. 800, and Annex 5 of WHO Technical Report Series, No. 927

Recommendations published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes recommendations for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Recommendations be made only on condition that modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. The parts of each section printed in small type are comments or examples intended to provide additional guidance to manufacturers and NRAs.

## Introduction

Diphtheria toxoid, produced by the chemical detoxification of diphtheria toxin, was one of the earliest vaccines available for protection against a bacterial disease, and it remains the basis for diphtheria vaccines today. The widespread use of diphtheria vaccines in routine immunization programmes has significantly reduced the incidence of the disease and its related mortality both in developed and developing countries (*1*).

Diphtheria–tetanus–pertussis vaccine has been part of the WHO Expanded Programme on Immunization since the inception of the programme in 1974, and during 1980–2000, the reported number of diphtheria cases was reduced by more than 90% (*2*). However, diphtheria is still a significant health concern in countries with poor vaccination coverage. In addition, a large proportion of the adult population in countries with good vaccination coverage may be susceptible to diphtheria due to the waning of immunity and the absence of natural boosting. The potential for severe outbreaks of the disease is enhanced in populations where there are large numbers of susceptible adults and unimmunized children. This was evident during the epidemic affecting countries in the former Soviet Union during the 1990s; however, the epidemic also highlighted the protective efficacy of the diphtheria vaccines used to control it (*3*, *4*). Such outbreaks highlight the need to maintain good coverage of childhood immunizations and appropriate booster immunizations, which are given beyond infancy and early school age, in order to provide sustained protective immunity against diphtheria.

Single-antigen diphtheria toxoid vaccine (i.e. containing only diphtheria toxoid) is rarely used for immunization, and the antigen is most commonly used in combination with tetanus toxoid alone, or with tetanus toxoid and whole-cell pertussis ("DTP"), or with tetanus toxoid and acellular pertussis antigens ("DTaP"). Diphtheria toxoid is also used in other combination vaccines that may contain, in addition to tetanus and pertussis antigens, inactivated poliovirus, hepatitis B surface antigen or *Haemophilus influenzae* type b capsular polysaccharide conjugates, or some combination of these. All diphtheria vaccines that are currently in use contain a mineral carrier, such as aluminium hydroxide or aluminium phosphate, as an adjuvant. Diphtheria vaccines intended for booster immunizations in older children, adolescents and adults are formulated with a lower amount of diphtheria toxoid when compared with vaccines intended for primary immunization; these lower doses for booster immunizations are designated with a lower-case "d", while those intended for primary immunization are designated with an upper-case "D".

New diphtheria vaccines are evaluated in populations with a low disease burden and, as such, are not evaluated according to their efficacy. Rather, evaluations assess their ability to induce levels of diphtheria antitoxin that

are considered to offer protection against the disease. Further considerations surrounding the clinical evaluation of diphtheria vaccines are included in Part C of these Recommendations.

## History of WHO Requirements and Recommendations, and standardization

The early developments leading to the formulation of the first Requirements for diphtheria toxoid are described in detail in Requirements for diphtheria toxoid and tetanus toxoid (Requirements for biological substances No. 10), published in 1964 (*5*). The development of diphtheria toxoid vaccines, and the publication of Requirements for their manufacture and for quality control, was helped considerably by the availability of international standards and international reference preparations for diphtheria toxoid and antitoxin. The International Standard for diphtheria antitoxin, equine (established in 1934) enabled toxoid to be assessed in terms of its ability to produce diphtheria antitoxin in humans and animals; results were expressed in IUs. In addition, the International Standard for Diphtheria Antitoxin for Flocculation Test (established in 1956) enabled antigen concentrations to be expressed in limit for flocculation units (Lf), and led to requirements for antigen content and purity being included in the 1964 publication (*5*). International standards for diphtheria toxoid, plain (established in 1951) and adsorbed (established in 1955), for use in biological potency assays, had been available for a number of years, but there was no general agreement on how they should be used in assaying different types of preparations, and they were not widely included in potency assays. Prior to 1964 the requirements for potency were specified by national regulatory authorities (NRAs) on the basis of the results of laboratory and field studies, and there was little uniformity among countries. As a result, the 1964 Requirements included a recommendation that the international standard for toxoid should be more widely used in biological assays in order to permit the formulation of more satisfactory potency tests based on the use of the international standard, which would be incorporated into future revisions of the Requirements (*5*).

The subsequent formulation of requirements for the assay of diphtheria vaccine potency was a significant milestone in the history of diphtheria vaccine production and quality control. The 1978 revision of the Requirements, which covered tetanus and pertussis vaccines as well as diphtheria, included a requirement for a potency assay that involved immunizing guinea-pigs, and following this with a challenge from a lethal dose of toxin (given subcutaneously) or a challenge with a series of toxin doses given intradermally (*6*). The requirement to compare immunizing potency against a reference material calibrated against the international standard was also included so that vaccine potency could be expressed in IUs. A minimum requirement

for diphtheria potency (for vaccines intended for the primary immunization of children) of 30 IU per single human dose (SHD) was also included in the revised Requirements, together with a requirement that sufficient animals should be used to achieve a 95% confidence interval that is less than 50–200% (*6*). It has been recognized that there are difficulties in providing evidence of a direct correlation between the estimated potency of a vaccine in a biological assay and the level and duration of protective immunity in humans. Despite this lack of direct evidence, the minimum requirement for diphtheria potency of 30 IU/SHD has helped to ensure the production and release of safe and effective diphtheria vaccines as assessed by the satisfactory performance of the vaccines in clinical studies and the low incidence of diphtheria in populations with good immunization coverage. The recommendation of 30 IU/SHD as a minimum requirement for diphtheria potency for primary immunization is therefore retained in this latest revision of the Recommendations. Following the publication in 1978 of revised Requirements, it became apparent that the large numbers of guinea-pigs required for the potency test made conformity difficult to achieve in many countries, and in 1986 an addendum to the Requirements specified that 95% confidence intervals greater than 50–200% were acceptable provided that the lower limit of the 95% confidence interval was still above the minimum potency required in each SHD (*7*).

Subsequent activities were undertaken aimed at providing greater flexibility in procedures, reducing the number of animals used and refining end-points without prejudice to the principle of expressing vaccine potency in IUs to demonstrate whether the product being tested meets the minimum requirement for potency. In 1988, WHO held a scientific consultation in Geneva during which special emphasis was placed on methods of determining the potency of diphtheria (and tetanus) toxoid vaccines that would require fewer animals. It was acknowledged that measurement of toxoid antigen content by in vitro methods would not necessarily indicate whether a vaccine was of acceptable potency, and that immunogenicity tests in animals remained necessary for assessing potency (*8*). When the Requirements were revised again in 1989, they included the option to refine the end-point of the potency assay by using toxin neutralization tests (TNTs) in vivo or in vitro after bleeding instead of a toxin challenge, which would in turn allow mice (which are not sensitive to challenge with the toxin) to be used instead of guinea-pigs (*8*). In addition, although multiple-dilution assays were still recommended for the demonstration of production consistency, product stability and the calibration of reference materials, the option to perform the routine potency test using a single dilution of the test and reference vaccines was included, with the provision that consistency in production and quality control had been demonstrated previously for that product (*8*). Further extensive international consultation highlighted a need to clarify the recommendations on the use of simplified potency assays for routine lot release, and an amendment was added

to the Requirements to include a division of the section on potency testing to distinguish clearly the recommendations for licensing from the recommendations for routine lot release (*9*). This latest revision of the Recommendations includes a new section on the nonclinical evaluation of diphtheria vaccines (see Part B). As a result, the procedures for potency testing included in Part A refer to routine lot-release testing, while procedures for potency testing before licensure are included in Part B.

Developments in biological standardization continue to play a crucial part in the formulation of requirements and recommendations for the production and quality control of diphtheria vaccines. For potency testing of diphtheria vaccines, the approach taken by the European Pharmacopoeia (*10*), like that of WHO, relies on the use of a reference preparation calibrated against the international standard as well as the expression of vaccine potency in IUs. In some countries (including the United States), the potency test is based on the United States National Institutes of Health assay for diphtheria toxoid (*11*). In this test, the vaccine is assessed according to its ability to induce an antibody response in guinea-pigs that reaches a minimum threshold of 2 units per ml (as measured by an in vivo TNT against a standard antitoxin preparation) without comparison to a reference vaccine. Although data have demonstrated that vaccines meeting such requirements can induce significant levels of antitoxin response in humans, the use of quantitative assays is recommended by WHO, and the expression of diphtheria vaccine potency in IUs remains the approach recommended by WHO. Nevertheless, there are no universally accepted methods for potency testing for diphtheria vaccines, and the global harmonization of procedures and requirements remains a challenge. The lack of harmonization leads to problems with the international exchange of vaccines due to difficulties in the mutual recognition of the results of testing.

During the revision of these Recommendations, WHO held a scientific consultation in Beijing, China, in November 2011. At that meeting, the option of harmonizing the minimum potency requirements for diphtheria vaccine with those recommended in the European Pharmacopoeia was discussed. It was acknowledged that amending the WHO minimum requirement for potency could improve harmonization and the international exchange of vaccines. As a result, the minimum requirement for the potency of diphtheria vaccine, tested according to the methods described in these Recommendations, was amended so that the specification of 30 IU/SHD for vaccines intended for primary immunization now applies to the lower 95% confidence limit, thus demonstrating that the vaccine potency significantly exceeds 30 IU/SHD. Because the minimum potency requirement now applies to the lower limit of the 95% confidence interval, there is no requirement to achieve a 95% confidence interval narrower than 50–200%. However, the revised section on potency testing in Part A includes information on criteria that should be met in order for the potency estimate to

be statistically valid. This latest revision of the Recommendations also includes a recommendation that the use of product-specific minimum requirements for potency is acceptable, provided they are based on the results of clinical and laboratory studies, and have been approved by the NRA.

The main changes in this latest revision include:

- a change of title from Requirements to Recommendations;
- an update of the section on international standards and reference preparations, which has been moved to the General considerations section;
- an update of the section on general manufacturing recommendations and control tests;
- amendment of the minimum requirements for the potency of diphtheria vaccines to clarify the value that applies to the lower limit of the 95% confidence interval;
- inclusion of new sections to provide guidance on the clinical and nonclinical evaluations of diphtheria vaccines to assess safety, quality and efficacy.

In order to facilitate the release process of vaccines made in accordance with these Recommendations, a model protocol is provided in Appendix 1.

## Scope of the Recommendations

These Recommendations apply to the production and quality control of adsorbed diphtheria vaccines, and have been updated from the 1989 revision of the Requirements for diphtheria, tetanus, pertussis and combined vaccines (*8*) and the amendments made in 2003 (*9*). These current Recommendations highlight advances in the production and testing of diphtheria vaccines and their related intermediates. The recommendations for the testing and quality control of diphtheria vaccines included in this document are based on currently licensed vaccines. Other products (such as those containing a new type of antigen or produced using novel technology) may require additional considerations. Other issues, such as guidelines for lot release (*12*), are covered in more detail by other documents.

Although these recommendations apply to the production and quality control of diphtheria vaccines, most diphtheria vaccines are presented in their final formulation with at least one other vaccine. Therefore, in addition to monovalent diphtheria vaccine, these recommendations also apply to diphtheria vaccines used in combination vaccines. The tests recommended for the final bulk or final fill also apply to combined vaccines where appropriate.

## General considerations

The supply of effective diphtheria vaccines depends on the use of well characterized and standardized production processes, together with extensive in-process quality control tests and monitoring of the product and its related intermediates. A detailed, written description of clearly defined standard operating procedures used for the production and testing of diphtheria vaccines (or combined vaccines containing diphtheria vaccine), together with evidence of appropriate validation for each critical production step and relevant control tests, should be submitted by the vaccine manufacturer to the NRA for approval as part of the licensing application. Proposals for any variations to manufacturing or quality control methods should be submitted to the NRA for approval before implementation and should conform to national regulatory requirements.

For the production of diphtheria toxoid, the Park Williams 8 strain of *Corynebacterium diphtheriae* has been successfully used as the source of diphtheria toxin owing to its low infectivity and high capacity for toxin production in vitro, and this strain continues to be recommended for use. The approach adopted for diphtheria vaccine production is to obtain the greatest possible quantity of toxin during the growth phase of the microorganisms, and thereafter to convert the toxin into stable toxoid by the most effective method. Formaldehyde is most commonly used for detoxifying the toxin to produce toxoid.

The demonstration of safety and the confirmation of vaccine potency are fundamental requirements for the production of diphtheria toxoid vaccine. The requirement for the product to be purified (either before or after detoxification) is retained, since diphtheria toxoid in unpurified form is liable to cause severe vaccination reactions in humans. In view of the risk of reversion to toxicity, especially when a toxin is detoxified after purification, the present recommendations have been formulated to address this risk by retaining the recommended 6-week incubation period for diluted, purified toxoid stored at elevated temperatures during the irreversibility test. The assay to detect diphtheria toxin as part of in-process safety testing can be performed using guinea-pigs or using an in vitro cell culture system. The purpose of the potency test is to demonstrate, using a suitable animal model, the capacity of the product being tested to induce an immune response analogous to that of toxoid shown to be efficacious in humans. Although there is no direct correlation between the potency result obtained in a biological assay and the level and duration of immunity induced in humans after immunization, diphtheria vaccines that have been released based on the minimum requirement of 30 IU/SHD, introduced in the 1978 revision to the previous Requirements, have been

shown to be clinically effective. Clinical studies should also be performed to support the licensure of a new diphtheria vaccine. Long-term studies to monitor antibody persistence and to determine the need for booster doses should also be considered, although these are not necessarily a prelicensure requirement. More information on clinical evaluation is included in Part C of these Recommendations.

## Terminology

Definitions for some common terms used throughout this document are given below. They may have different meanings in other contexts.

**Bulk purified toxoid:** the processed, purified material that has been prepared from either a single harvest or a pool of single harvests. It is the parent material from which the final bulk is prepared.

**Final bulk:** the homogeneous final vaccine present in a single container from which the final containers are filled either directly or through one or more intermediate containers.

**Final lot:** a collection of sealed final containers that is homogeneous in all respects. In principle, a final lot must have been filled from a single final bulk container and processed further (e.g. freeze-dried) in one continuous working session. Different final lots may be filled or processed from the same final bulk in different working sessions. These related final lots (or batches) are sometimes referred to as sub-batches, sublots, filling lots or freeze-drying lots, and should be identifiable by a distinctive final lot number.

**Master seed lot:** a quantity of bacterial suspension that has been derived from a single strain, has been processed as a single lot, and has a uniform composition. It is used to inoculate media for preparation of the working seed lot. The master seed lot should be stored as frozen stock in liquid glycerol (usually at or below −80 °C) or as lyophilized stock at a temperature known to ensure stability.

**Seed lot:** a quantity of bacterial suspension that has been derived from one strain, has been processed as a single lot, and has a uniform composition. It is used to prepare the inoculum for the production medium.

**Single harvest:** the toxic filtrate or toxoid obtained from one batch of cultures that have been inoculated, harvested and processed together.

**Working seed lot:** a bacterial culture consisting of a single substrain derived from the master seed lot. Working seed lots are stored in aliquots under the conditions described above for master seed lots. The working seed lot should be prepared from the master seed lot using as few cultural passages as possible; it should have the same characteristics as the master seed lot. It is used to inoculate media for the preparation of single harvests.

## International reference materials

Subsequent sections of this document refer to WHO reference materials that may be used in laboratory or clinical evaluations. Key standards used in the control of diphtheria vaccines include the following.

- The Second International Standard of Diphtheria Toxoid for Flocculation Test – this material (NIBSC code 02/176) was established in 2007 (*13*), with an assigned unitage of 1100 Lf/ampoule, replacing the First International Reference Reagent of Diphtheria Toxoid for Flocculation Test. This standard is intended for use in flocculation tests to determine the antigen content of diphtheria toxoid.
- The Fourth WHO International Standard for Diphtheria Toxoid Adsorbed – this material (NIBSC code 07/216) was established in 2009 (*14*), and has an assigned potency of 213 IU/ampoule based on calibration against the Third WHO International Standard for Diphtheria Toxoid Adsorbed (NIBSC code 98/560) in guinea-pig challenge assays. This standard replaces the previous standard and is intended for use as a reference vaccine in diphtheria vaccine potency assays.
- The WHO International Standard Diphtheria Antitoxin Equine – this dried hyperimmune equine serum was established in 1934. The material is used to prepare a liquid fill containing 10 IU/ml in 66% glycerol in normal saline approximately every two years. The current fill has the NIBSC code number 11/200, and is intended for use as a reference preparation in TNTs in vivo or in vitro to determine the potency of diphtheria antitoxin.
- The First WHO International Standard for Diphtheria Antitoxin Human – this material (NIBSC code 10/262) was established in 2012, and has an assigned unitage of 2 IU/ampoule. This material is intended for use as a reference preparation in assays used to measure diphtheria antibody levels in human serum.

The above-mentioned international standards and reference materials listed are held by the National Institute for Biological Standards and Control, Medicines and Healthcare Products Regulatory Agency, Potters Bar, Hertfordshire, EN6 3QG, England.[1] As reference materials mentioned may be superseded by replacement standards, the WHO catalogue of international reference preparations should be consulted for the latest list of established standards.[2]

[1] See: http://www.nibsc.org/

[2] See: http://www.who.int/bloodproducts/catalogue/en/index.html

International reference materials are intended for use in the calibration of national, regional or other secondary standards that are used for the production and quality control of diphtheria vaccines. They may also be suitable for use as a primary reference preparation for some assays.

# Part A. Manufacturing recommendations

## A.1 Definitions

### A.1.1 International name and proper name

The international name should be diphtheria vaccine (adsorbed). The proper name should be the equivalent of the international name in the language of the country of origin.

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

### A.1.2 Descriptive definition

Diphtheria vaccine (adsorbed) is a preparation of diphtheria toxoid prepared by treating diphtheria toxin using chemical means to render it nontoxic without destroying its immunogenic potency. The toxoid is adsorbed onto a suitable adjuvant. The preparation should satisfy the recommendations formulated below.

The most common method of preparing toxoid from toxin is by using formaldehyde.

In some countries, the adsorbent is precipitated in the presence of the toxoid.

## A.2 General manufacturing recommendations

The general manufacturing recommendations contained in WHO good manufacturing practices for pharmaceutical products: main principles (*15*) and Good manufacturing practices for biological products (*16*) apply to the production of diphtheria vaccines. These practices include demonstrating the purity and quality of the production strain and seed lots, implementing in-process quality control testing, testing for process additives and process intermediates, and developing and establishing lot-release tests.

A written description of the procedures used in the preparation and testing of the diphtheria vaccine, together with appropriate evidence that each production step has been validated, should be submitted to the NRA for approval. Proposals for modifying the manufacturing process or quality control methods should also be submitted to the NRA for approval before such modifications are implemented.

## A.3 Production, processing and control

### A.3.1 Production precautions

The general production precautions, as formulated in Good manufacturing practices for biological products (*16*), apply to the manufacture of diphtheria vaccine.

> Suitable methods for the production of diphtheria vaccine are given in the WHO *Manual for the production and control of vaccines: Diphtheria toxoid* (*17*).

Personnel employed in production and quality control should be adequately trained, should have completed a course of immunization against diphtheria, and should have received appropriate booster immunization(s). Appropriate health surveillance should also be carried out.

### A.3.2 Production strain and seed lots

#### A.3.2.1 Strains of *C. diphtheriae*

Strains of *C. diphtheriae* used in preparing diphtheria toxoid should be identified using a record of their history and of all tests made periodically to verify the strain's characteristics. The strain should be approved by the NRA and should be maintained as a freeze-dried culture or as frozen stock in liquid glycerol.

> A highly toxigenic strain of *C. diphtheriae* should be used. A strain that has proved satisfactory in many laboratories is the Park Williams 8 strain.

#### A.3.2.2 Seed-lot system

The preparation of seed lots should comply with the recommendations in Part A, section A.3.1, of this document. The production of diphtheria toxin should be based on a well defined seed lot system in which toxigenicity is conserved. Cultures of the working seed should have the same characteristics as those of the strain from which the master seed lot was derived. Detailed records of the origin, passage history, purification and characterization procedures, and storage conditions should be provided to the NRA when new master seeds or working seeds are introduced. Working seeds that are in use should be characterized at defined intervals that have been approved by the NRA on the basis of prior production history and experience. The maximum number of passages of each seed lot used for production should be specified based on the number shown to produce a safe and effective product.

> When possible, a combination of validated biochemical, molecular and genetic tests should be used for identification and characterization of seed lots. Suitable methods include multilocus enzyme electrophoresis (MEE),

matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and restriction fragment length polymorphism (RFLP) analysis.

#### A.3.2.3 Culture medium for production of toxin

*C. diphtheriae* should be cultured in media that are suitable to support growth and to ensure a good yield of diphtheria toxin. Examples of suitable growth media that support the production of diphtheria toxin are given in the WHO *Manual for the production and control of vaccines: Diphtheria toxoid* (*17*).

Semisynthetic culture media consisting of enzymatic digests of protein (such as casein) have been shown to be suitable to support the growth of *C. diphtheriae*, but toxin yield is highly dependent on the level of available iron in the growth medium (*17–19*), and the tox gene is regulated at the transcriptional level by iron (*19*).

The culture media should be free from adventitious agents, and components that are known to cause allergic reactions in humans should be avoided. Human blood or blood products should not be used. If the medium is prepared from a protein digest (e.g. casein hydrolysate or digested muscle), precautions should be taken to ensure that digestion has proceeded sufficiently. Materials or components of animal origin should be identified and approved by the NRA, and their use should comply with the WHO Guidelines on transmissible spongiform encephalopathies (*20*). The methods for detecting these substances should be approved by the NRA.

Any change in the media used should be submitted to the NRA for approval.

### A.3.3 Single harvests

The consistency of production should be demonstrated. This process may include using measurements of culture purity, growth rate, pH and rate of toxin production. Acceptance specifications with defined limits should be approved by the NRA.

Any culture showing anomalous growth characteristics should be investigated and should be shown to be satisfactory before being accepted as a single harvest. Contaminated cultures must be discarded.

Suitable methods for the production of diphtheria toxin are given in the WHO *Manual for the production and control of vaccines: Diphtheria toxoid* (*17*).

Single harvests that meet the acceptance criteria may be pooled to prepare the bulk purified toxoid. Storage times should be supported by data obtained from appropriate stability studies, and should be approved by the NRA.

#### A.3.3.1 Control of bacterial purity

Samples of cultures used for preparing single harvests should be tested for bacterial purity by microscopic examination of stained smears, and by inoculation into appropriate culture media. Single harvests should be discarded if contamination has occurred at any stage during their production.

#### A.3.3.2 Filtration

After the culture medium has been sampled to control for purity, filtration should be used to separate the medium aseptically from the bacterial mass as soon as possible. A preservative may be added, but phenol should not be used for this purpose.

> To facilitate filtration, cultures may be centrifuged, provided that suitable precautions have been taken to avoid the formation of potentially hazardous aerosols. A filter aid may be added beforehand. A filter that does not shed fibres should be used.

#### A.3.3.3 Determination of crude toxin concentration

Prior to inactivation, the toxin content of the culture supernatant should be determined using a method approved by the NRA.

> The flocculation test is suitable for the measurement of toxin content, and is described in the WHO *Manual for the production and control of vaccines: Diphtheria toxoid* (*17*) and the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*21*). A reference material calibrated against the International Standard for diphtheria toxoid for flocculation test should be included, and the results should be expressed in Lf.

The measurement of toxin content is a good indicator of the consistency of production, and acceptance limits should be defined for monitoring purposes.

> It is preferable that culture filtrates used to prepare purified toxoid contain at least 50 Lf/ml.

#### A.3.3.4 Detoxification and purification

Detoxification of diphtheria toxin may be performed using crude toxin (culture filtrate) or purified toxin. Detoxification of purified toxin results in a purer product, although particular care must be taken to avoid a reversion to toxicity; reversion may also occur when crude toxin is used for detoxification. The method of purification should be such that no substance is incorporated into the final product that is likely to cause adverse reactions in humans.

The method of purification and the agent used for detoxification should be suitably validated, and should be approved by the NRA. The rate of

detoxification may vary, and in-process monitoring of the detoxifying process should be performed.

> Formaldehyde is most commonly used as the detoxifying reagent, and amino acids such as lysine or glycine may be added during detoxification to facilitate cross-linking of toxin molecules, and to help prevent reversion. The detoxification conditions should be well defined and controlled with respect to temperature, time, concentration of the detoxifying reagent, toxin concentration and any other critical parameters.

The method used for purification should be approved by the NRA.

> Crude toxoid can be concentrated using ultrafiltration prior to purification by fractionation with ammonium sulfate, dialysis, gel filtration, ion-exchange chromatography, or a combination of these methods.
>
> Bioburden testing may also be performed after purification to ensure that potential levels of contamination have been minimized for subsequent steps that are not performed aseptically.

When measured in the final bulk vaccine, the amount of residual free detoxifying agent remaining after detoxification and purification have been completed should not exceed the limit stated in section A.3.5.2.7.

Harvests should be treated as potentially toxic, and subject to the appropriate safety restrictions until the detoxification has been shown to be complete by performance of a specific toxicity test (as detailed in section A.3.4.4) or any other suitably validated in vivo or in vitro method.

> Detoxification can be confirmed by subcutaneous inoculation of the toxin into guinea-pigs, or by intradermal injection into guinea-pigs or rabbits. A cell culture assay, such as the Vero cell assay, is also suitable.

Storage times should be supported by data obtained from appropriate stability studies, and should be approved by the NRA.

## A.3.4 Bulk purified toxoid

### A.3.4.1 Preparation

The bulk purified toxoid should be prepared from either a single harvest or a pool of single harvests, and should be sterile. If the NRA approves, a preservative may be added, provided that the preservative has been shown not to adversely affect the safety and immunogenicity of the toxoid. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity of diphtheria vaccines.

> It is advisable to sterilize the bulk purified toxoid by filtration.

### A.3.4.2 Sterility

Each bulk purified toxoid should be tested for bacterial and mycotic sterility in accordance with the requirements in Part A, section 5, of General requirements for the sterility of biological substances (*22*) or by a method approved by the NRA. The sterility test is performed using at least 10 ml of each bulk purified toxoid. If a preservative has been added to the purified bulk, appropriate measures should be taken to prevent it from causing any interference in the sterility test.

### A.3.4.3 Antigenic purity

Each bulk purified toxoid should be tested for antigenic purity by determining the antigen concentration in Lf and the concentration of protein (nondialysable) nitrogen. The antigen concentration should be determined by comparing it with a reference material calibrated against the International Standard for diphtheria toxoid for flocculation test or against an equivalent reference preparation approved by the NRA. The method of testing should be approved by the NRA. The bulk purified toxoid passes the test if it contains at least 1500 Lf/mg of protein (nondialysable) nitrogen.

> The flocculation (Ramon) assay is suitable for measuring antigen content, and is described in the WHO *Manual for the production and control of vaccines: Diphtheria toxoid* (*17*) and in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*21*).
>
> Physicochemical analysis, using methods such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC) may be used to monitor antigenic purity and to provide additional information on antigen integrity and the extent of aggregation and proteolysis. These additional characterization tests should be performed whenever a new working seed is introduced.

### A.3.4.4 Specific toxicity

Each bulk purified toxoid should be tested for the presence of diphtheria toxin. The test may be performed in vivo using guinea-pigs or in vitro using a suitable cell culture assay, such as the Vero cell assay.

A suitable in vivo test consists of injecting the toxoid into at least five guinea-pigs, each weighing 250–350 g. The guinea-pigs should not have been used previously for experimental purposes. Each guinea-pig should be given a subcutaneous injection of 1 ml of a dilution of purified toxoid containing at least 500 Lf of toxoid. The diluted toxoid is prepared in such a way that the chemical environment is comparable to that found in the final vaccine except for the absence of adjuvant. Animals are observed for 42 days, and any animals that die should undergo necropsy and be examined for symptoms of diphtheria

intoxication (e.g. red adrenal glands). The bulk purified toxoid passes the test if no guinea-pig shows symptoms of specific toxicity and if at least 80% (i.e. four fifths) of the animals survive the test period. If more than one animal dies from nonspecific causes, the test should be repeated. If more than one animal dies during the retest, then the bulk purified toxoid does not comply with the test.

> Some manufacturers carry out an alternative test for determining whether diphtheria toxin is present: they inject intradermally into rabbits or guinea-pigs at least 20 Lf of purified toxoid and observe the injection sites for specific erythema. Erythema with a diameter greater than 5 mm is typically considered to be positive.

Alternatively, an in vitro cell culture test, such as the Vero cell assay, may be used, provided that the sensitivity of the test has been demonstrated to be not less than that of the guinea-pig test. For the Vero cell assay, a dilution of bulk purified toxoid is prepared so that the chemical environment is comparable to that present in the final bulk vaccine except for the absence of adjuvant, preservative and other excipients, which may cause nonspecific toxicity in Vero cells. A duplicate titration of toxoid is performed in the presence of diphtheria antitoxin to confirm that any signs of cytotoxicity are specific and due to the presence of diphtheria toxin. So that the sensitivity of the assay can be confirmed, a purified preparation of diphtheria toxin should be included in the test, diluted in a purified bulk diphtheria toxoid that has previously been shown to be nontoxic to Vero cells. The test procedure and the interpretation of results should be approved by the NRA. An example of the Vero cell method is included in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*21*).

#### A.3.4.5 Reversion to toxicity

Each bulk purified toxoid should be tested to ensure that reversion to toxicity does not take place during storage. The test may be performed in vivo using guinea-pigs or in vitro using a suitable cell culture assay, such as the Vero cell assay. The test employed should be approved by the NRA, and should be sufficiently sensitive to detect very small amounts of toxin. For the in vivo assay, the bulk purified toxoid should be diluted in order to obtain the same concentration and chemical environment as present in the final bulk vaccine, except for the absence of adjuvant.

> For bulk toxoid that will be used in the preparation of more than one final-bulk formulation, the test should be performed using dilutions of the bulk toxoid that represent the lowest and highest concentrations of toxoid that will be present in the final formulations.

To determine whether reversion has occurred, diluted toxoid that has been stored at 34–37 °C for six weeks should be tested. At the end of the incubation period,

groups of five guinea-pigs are each given a subcutaneous injection of the diluted toxoid sample. A total injection volume of 5 ml should be used (using multiple injection sites where necessary (such as two injections of 2.5 ml each), which is the equivalent of 10 SHDs. The animals are observed for 42 days for signs of ill health. No toxicity should be detected.

> Similar dilutions of toxoid held at 2–8 °C during the same period of time as those held at 34–37 °C may be tested as controls.
>
> Intradermal tests in guinea-pigs are considered to be suitable provided that the dose has been adjusted accordingly, and the sensitivity of the test has been shown to be not less than that of the subcutaneous test.

Alternatively, an in vitro cell culture test, such as the Vero cell assay, may be used, provided that the sensitivity of the test has been demonstrated to be not less than that of the guinea-pig test. For the Vero cell assay, a dilution of bulk purified toxoid is prepared in such a way that the chemical environment is comparable to that present in the final bulk vaccine, except for the absence of adjuvant, preservative and other excipients, which may cause nonspecific toxicity in Vero cells.

> For bulk toxoid that will be used in the preparation of more than one final bulk formulation, the test should be performed using dilutions of the bulk toxoid that represent the lowest and highest concentrations of toxoid that will be present in the final formulations.

The diluted toxoid is stored at 34–37 °C for six weeks, and a duplicate sample is stored at 2–8 °C for the same period. So that the sensitivity of the assay can be confirmed, a purified preparation of diphtheria toxin should be included in the test, diluted in a purified bulk diphtheria toxoid that has previously been shown to be nontoxic to Vero cells. The test procedure and the interpretation of the results should be approved by the NRA. An example of the Vero cell method is included in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*21*).

#### A.3.4.6 Storage of bulk purified toxoid

Storage times for the bulk purified toxoid should be supported by data from appropriate stability studies, and should be approved by the NRA (*23*).

### A.3.5 Final bulk

#### A.3.5.1 Preparation

The final bulk is prepared from bulk purified toxoid adsorbed onto a suitable adjuvant. The final formulation of the vaccine should be based on formulations

that have been shown to be safe and effective in clinical use. The number of Lf per SHD should be approved by the NRA.

> It is recommended that the diphtheria antigen content in vaccines intended for primary immunization should not exceed 30 Lf/SHD.

In vaccines intended for use as booster vaccines, the quantity of diphtheria toxoid in the vaccine should be approved by the NRA. It should be shown that the vaccine does not cause adverse reactions in people from the age groups for which the vaccine is intended.

> In some countries it is recommended that the diphtheria antigen content of diphtheria vaccines intended for boosting should not exceed 2.5 Lf/SHD.

### A.3.5.2 Control tests

#### A.3.5.2.1 *Preservative*

If the vaccine is to be dispensed into multidose containers, a suitable antimicrobial preservative should be added. The amount of preservative in the final bulk should be shown to have no deleterious effect on the toxoid or on other vaccine components with which the toxoid may be combined; the preservative should also be shown to cause no unexpected adverse reactions in humans. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity of diphtheria vaccines. The preservative and its concentration should be shown to be effective, and should be approved by the NRA. The WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines should be followed (*24*).

> Determine the amount of preservative by using a suitable chemical method. The amount should be at least 85% and not more than 115% of the intended amount.

#### A.3.5.2.2 *Adjuvants*

The nature, purity and concentration of the adjuvant used in the formulation should be determined by methods approved by the NRA. When aluminium compounds are used as adjuvants the concentration of aluminium should not exceed 1.25 mg/SHD. If other adjuvants are used, specifications should be established by the manufacturer and approved by the NRA.

> In some countries these recommended limits for adjuvant concentrations are considered too high, and lower limits have been approved and shown to be safe and effective.

A.3.5.2.3 ***Degree of adsorption***

The degree of adsorption should be measured and should be shown to be comparable to that measured in vaccine lots used in clinical studies to support licensing. The measurement of antigen content and the degree of adsorption to adjuvant are good indicators of the consistency of production; in-house acceptance limits can be established once a suitable number of lots have been tested.

Suitable methods for determining the degree of adsorption in diphtheria vaccines are described in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*21*).

These tests may be omitted provided they are performed on the final lot.

A.3.5.2.4 ***Sterility***

Each final bulk should be tested for bacterial and mycotic sterility in accordance with the recommendations in Part A, section 5, of the revised General requirements for the sterility of biological substances (*22*) or by a method approved by the NRA. The sterility test should be performed using at least 10 ml of each final bulk. If a preservative has been added to the final bulk, appropriate measures should be taken to prevent it from causing any interference in the sterility test.

A.3.5.2.5 ***Specific toxicity***

Each final bulk should be tested for specific toxicity in at least five guinea-pigs; each guinea-pig should weigh 250–350 g and not have been used previously for experimental purposes. Each guinea-pig is given a subcutaneous injection of a quantity equivalent to at least 5 SHDs, and is observed for 42 days. Animals that die should undergo necropsy and be examined for symptoms of diphtheria intoxication (e.g. red adrenal glands). The final bulk passes the test if no guinea-pig shows symptoms of specific intoxication within six weeks of injection, and if at least 80% (i.e. four fifths) of the animals survive the test. If more than one animal dies from nonspecific causes, the test should be repeated. If more than one animal dies during the retest, then the final bulk does not comply with the test.

> If the NRA approves, the specific toxicity test used on the final bulk may be omitted from routine lot-release procedures once consistency in production has been demonstrated.

A.3.5.2.6 ***Potency***

The potency of each final bulk (or final lot) should be determined by comparison with a suitable reference preparation that has been calibrated in IUs against the Fourth WHO International Standard for Diphtheria Toxoid Adsorbed. Appropriate statistical methods should be used to calculate the potency of the final bulk (*21*). The NRA should approve the assay method and the method used for calculating the results. Details on methods to be used for the potency testing

of diphtheria vaccines can be found in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*21*).

The minimum potency specifications introduced in the 1978 WHO Recommendations have helped to ensure the production and use of safe and effective diphtheria vaccines, as evidenced by the satisfactory performance of these products in clinical studies and the low incidence of diphtheria in populations with good immunization coverage. Therefore, it is recommended that the potency of a diphtheria vaccine used for the primary immunization of children should significantly exceed 30 IU/SHD (based on data showing that the lower 95% confidence limit of the estimated potency is at least 30 IU/SHD).

The minimum potency specification for diphtheria vaccine intended for booster immunization in older children, adolescents and adults should be approved by the NRA.

> In some cases it is recommended that the lower 95% confidence limit of the estimated potency of diphtheria vaccines intended for boosting should be not less than 2 IU/SHD.

Product-specific minimum requirements for diphtheria potency are acceptable, provided that they have been justified and are based on potency values obtained for the vaccine in question. A suitable number of lots should be tested in order to define the minimum requirement for potency. Vaccine lots used for the establishment of the potency specification should include lots that have been shown to be safe and effective in clinical studies. Product-specific minimum requirements should be approved by the NRA. Once defined and approved, the potency of the vaccine should be shown to exceed the minimum requirement significantly (based on data showing that the lower 95% confidence limit of the estimated potency is at least that of the minimum requirement).

The following criteria should be met in order for the potency estimate to be statistically valid:

- the statistical analysis should show a significant regression ($P < 0.05$) of the log dose–response lines without significant deviation from linearity and parallelism ($P > 0.05$);
- for subcutaneous challenge assays, the 50% protective dose should lie between the smallest and largest vaccine doses – for intradermal challenge assays, the mean score obtained for the smallest vaccine dose should be less than 3, and the mean score obtained for the largest vaccine dose should be more than 3.

When more than one assay is performed, the results of all statistically valid tests should be combined into a geometric mean estimate, and the confidence limits calculated.

Manufacturers are also encouraged to monitor the potency of different vaccine bulks and lots by setting minimum and maximum alert criteria once a suitable number of lots have been tested.

#### *Calibration of reference preparations*

Secondary reference preparations (regional, national, working or product-specific standards) should be calibrated using a multiple-dilution assay to immunize guinea-pigs with appropriate dilutions of both the international standard and the proposed reference preparation; immunization should be followed by challenge with diphtheria toxin (via the subcutaneous or intradermal route) or titration of immune serum samples using an in vivo TNT (in guinea-pigs) or an in vitro TNT (i.e. a Vero cell assay). Adequate controls should be in place to ensure and monitor the stability of all secondary standards; where possible, replacement lots should be calibrated against the international standard (*25*).

#### *Potency test for routine lot release*

For routine testing, the potency of diphtheria vaccine may be determined using guinea-pigs or mice. When potency tests are carried out in mice instead of guinea-pigs, transferability should be demonstrated for the product being tested (*21*).

To determine the potency of a diphtheria vaccine, guinea-pigs or mice are immunized with appropriate dilutions of the calibrated reference preparation and the product being tested. Care should be taken to ensure that the diluents are inert (e.g. phosphates might interfere with the adsorption of toxoid) and not pyrogenic. Guinea-pigs may be challenged with diphtheria toxin or bled for titration of immune serum. Mice should be bled for titration of immune serum. Titration of immune serum samples may be performed using an in vivo or in vitro TNT – such as a Vero cell assay – or using another in vitro method, such as the enzyme-linked immunosorbent assay (ELISA), subject to validation. If in vitro serological assays are used, they should show that the product induces an appropriate antibody response in animals when compared with the reference preparation.

> The ELISA assay or another suitable in vitro method may be used to measure the antibody response to diphtheria toxoid, provided that these assays have been validated against the challenge assay or the TNT using the particular product in question. A minimum of three assays with a suitable dose–response range is likely to be required for validation of a particular product (*26*). These methods require precise definition of the characteristics of reagents (such as the antigen, and positive and negative control serum samples) that are critical for the successful performance of the testing method.

***Potency assay modifications: reduced dilution schemes***

Consistency limits for diphtheria potency should be established once a suitable number of lots has been tested using a multiple-dilution assay. Once consistency in production has been demonstrated for the vaccine, the potency assay (using the challenge or serological model) may, with the approval of the NRA, be performed using a reduced number of animals or doses, or both. Production consistency should be demonstrated using vaccine potency expressed in IUs and obtained for at least 10 consecutive vaccine lots derived from different toxoid bulks; the expectations of linearity and parallelism must be consistently satisfied, and the potency must be consistently higher than the minimum requirement. Once approved, fewer doses of the test and reference vaccines may be used, and the assumptions of linearity and parallelism need not be tested for each assay. When vaccine lots consistently give the lower limit of the 95% confidence interval for the estimated potency values (i.e. well in excess of the minimum requirement), one-dilution tests may offer advantages. If one-dilution assays are not advantageous, a reduction in animal usage may nevertheless be achieved by using two-dilution assays or another suitable design modification.

> A one-dilution assay is based on the same principles for evaluating the response as three-dilution assays. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 30 IU (or the minimum requirement for the product expressed as an SHD), that elicits a minimum protective effect (or antibody response) in immunized animals; the effect of the reference vaccine is compared 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 assurances that the potency significantly exceeds the minimum requirement. A disadvantage of this approach is that it is not possible to obtain strictly quantitative estimates of vaccine potency. Therefore, in order to ensure the overall consistency of production, there is a need to support the data generated by a simplified potency assay with data from physicochemical methods or other in vitro assays. When a one-dilution assay is used with serological analysis, measurement of the geometric mean antibody response in a group immunized with the test vaccine can provide some information about production consistency on a continual basis, provided that the in vitro assay used to measure antibody titres contains suitable internal controls.

Lot release based upon the use of a simplified approach requires periodic review to ensure that the validity of all procedures (including assumptions of linearity and parallelism) is maintained. The timing of the review should be decided on a case by case basis, depending on the number of lots of vaccine

produced annually, or by time schedule (at least every two years), and should be approved by the NRA. It should be noted that if there is a significant change in the production process, testing should revert to the full multiple-dilution assay, and production consistency should be reconfirmed before the reduction scheme is reintroduced.

#### A.3.5.2.7 *Amount of residual free detoxifying agent*

The amount of residual free detoxifying agent in each final bulk should be determined. The method used and the acceptable limits should be approved by the NRA.

> If formaldehyde has been used, the residual content should not exceed 0.2 g/l. The colorimetric determination of the reaction product of formaldehyde and fuchsin–sulfurous acid is a suitable method for detecting residual free formaldehyde.
>
> Where applicable, appropriate tests should be performed for the quantification of other detoxifying agents. The tests used and the maximum residual content of such chemicals should be approved by the NRA.

#### A.3.5.2.8 *pH*

The pH of the final bulk should be measured and should be within the range of values measured in vaccine lots shown to be safe and effective in clinical use.

### A.3.5.3 Storage of final bulk

The final bulk may be stored in a single container or in multiple containers. When multiple storage containers are used, the contents must be pooled into a single container for filling into the final containers. Storage times for the final bulk should be supported by stability studies, and approved by the NRA.

## A.4 Filling and containers

The requirements concerning filling and containers given in WHO good manufacturing practices for pharmaceutical products: main principles (*15*) and Good manufacturing practices for biological products (*16*) apply.

Single-dose or multiple-dose containers may be used. Vaccine in multidose containers should contain a suitable antimicrobial preservative (see section A.3.5.2.1).

The filling process should be suitably validated by comparing key parameters measured in the final bulk and in the final lot. Such studies should include measurement of the degree of adsorption.

## A.5 Control of final product

Quality-control procedures and tests should be validated and approved by the NRA to ensure that the final containers hold the antigen and formulation appropriate for the intended use of the final product.

Unless otherwise justified and authorized, the following tests should be performed on labelled containers from each final lot by means of validated methods approved by the NRA.

### A.5.1 Identity

An identity test should be performed on at least one container from each final lot using a validated method approved by the NRA.

> The method used should be based on the specific interaction between the diphtheria antigen in the vaccine and diphtheria antitoxin. Suitable detection methods include flocculation (Ramon and light-scattering methods), immunoprecipitation assays and ELISA (*17*, *21*). Tests on toxoid adsorbed on to an aluminium carrier should be performed after the carrier has been dissolved or the adsorbed toxoid has been wholly or partially eluted by sodium citrate or ethylenediaminetetraacetic acid (EDTA) (*17*, *21*).

### A.5.2 Sterility

Final containers should be tested for bacterial and mycotic sterility using a method approved by the NRA.

Many countries have regulations governing the sterility testing of the final product. Where these regulations do not exist, the requirements published by WHO should be met (*22*). If a preservative has been added to the purified bulk, appropriate measures should be taken to prevent it from causing any interference in the sterility test.

### A.5.3 Potency

A potency test should be carried out on each final lot as described in Part A, section A.3.5.2.6, if such a test has not been performed on the final bulk.

### A.5.4 Innocuity

Each final lot should be tested for innocuity by intraperitoneal injection of 1 human dose (but not more than 1 ml) into each of five mice (weighing 17–22 g) and by intraperitoneal injection of at least 1 SHD (but not more than 1 ml) into each of two guinea-pigs (weighing 250–350 g). The tests should be approved by the NRA. The final product is considered to be innocuous if the animals survive

for at least seven days without showing significant signs of toxicity. This test is also referred to as the abnormal toxicity test or the general safety test.

If the NRA approves, the innocuity test on the final lot may be omitted from routine lot release once the consistency of production has been demonstrated.

### A.5.5 Adjuvant content

The adjuvant content of each final lot should be determined using a method approved by the NRA (see Part A, section A.3.5.2.2).

The formulation should be such that after shaking, the vaccine remains suspended as a homogeneous solution for a defined period (to allow sufficient time for administration).

### A.5.6 Degree of adsorption

A test for the degree of adsorption should be carried out on each final lot as indicated in Part A, section A.3.5.2.3.

### A.5.7 Preservative content

The preservative content of each final lot should be determined as described in section A.3.5.2.1. The method used should be approved by the NRA.

If the NRA approves, this test may be performed only on the final bulk.

### A.5.8 pH

The pH of the final lot should be measured and should be within the range of values measured in vaccine lots shown to be safe and effective in clinical use.

In some cases, determination of osmolality may also be required.

### A.5.9 Extractable volume

For vaccines filled into single-dose containers, the extractable content should be checked and shown to be not less than the intended dose.

For vaccines filled into multidose containers, the extractable content should be checked and should be shown to be sufficient for the intended number of doses.

### A.5.10 Inspection of final containers

Each container in each final lot should be inspected visually or mechanically, and those containers showing abnormalities (e.g. improper sealing, clumping or the presence of particles) should be discarded.

## A.6 Records

The requirements given in WHO good manufacturing practices for pharmaceutical products: main principles (*15*) and Good manufacturing practices for biological products (*16*) apply. Written records should be kept of all tests, irrespective of their results. The records should be of a type from which annual trends can be determined.

A model of a suitable summary protocol for diphtheria vaccines (adsorbed) is given in Appendix 1.

## A.7 Retained samples

Vaccine samples should be retained, as recommended in WHO good manufacturing practices for pharmaceutical products: main principles (*15*) and Good manufacturing practices for biological products (*16*).

## A.8 Labelling

The label printed on or affixed to each container, and the label on the carton enclosing one or more containers, should be approved by the NRA. The labels should be easily readable and should show as a minimum:

- the words "diphtheria vaccine (adsorbed)" or the proper name of the product, or both
- the licence number of the product
- the name of the manufacturer
- the number of the final lot
- the identity of any preservative or adjuvant
- the amount of antigen in Lf or the minimum potency in IU/SHD, or both
- the recommended storage temperature and the expiry date if kept at that temperature
- the recommended SHD and route of administration.

In addition, the label printed on or affixed to the container, or the label on the cartons, or the leaflet accompanying the container should contain the following:

- a statement that the vaccine satisfies the recommendations of this document;
- the address of the manufacturer;
- the recommended temperature for transport;

- a warning that the adsorbed vaccine should not be frozen;
- a warning that the adsorbed vaccine should be shaken before use;
- instructions for the use of the vaccine, and information on contraindications and the reactions that may follow vaccination.

## A.9 Distribution and transport

The requirements given in WHO good manufacturing practices for pharmaceutical products: main principles (*15*) and Good manufacturing practices for biological products (*16*) apply.

## A.10 Stability, storage and expiry date

### A.10.1 Stability

Stability evaluation is a critical part of quality assessment, and the general principles of stability evaluation are described in the WHO Guidelines on stability evaluation of vaccines (*23*). The purpose of stability studies is to confirm that at the end of its shelf-life (or other defined storage period) the vaccine has the required characteristics to ensure its quality, safety and efficacy. The stability of the vaccine in final containers maintained at the recommended storage temperature should be demonstrated to the satisfaction of the NRA. Containers from at least three consecutive final lots (each derived from unique toxoid bulks) should be tested. The vaccine should be tested up until its expiry date to demonstrate its stability during storage.

The vaccine should be manufactured in such a way that reversion to toxicity does not occur during the defined shelf-life, provided that the vaccine is stored under the conditions recommended on the label. To confirm that the vaccine does not revert to toxicity during storage, the specific toxicity test described in Part A, section A.3.5.2.5, should be scheduled up until the expiry date as part of the stability studies. In addition, at the time of the expiry date, the vaccine should meet the requirements or acceptance limits for the final product in terms of sterility, potency, adjuvant content, degree of adsorption, preservative content and pH (see Part A, sections A.5.2, A.5.3 and A.5.5–A.5.8), provided that it has been stored at the recommended temperature. The frequency of testing should be approved by the NRA.

When any changes that may affect the stability of the product are made in the production process, the vaccine produced by the new method should be shown to be stable.

> Stability studies performed at temperatures other than those recommended for storage may be useful in providing information about transporting the vaccine at different temperatures for a limited time.

### A.10.2 Storage conditions

The recommended storage conditions and the defined maximum duration of storage should be based on stability studies, as described in section A.10.1, and should be approved by the NRA. For diphtheria vaccines, a temperature of 2–8 °C is considered to be satisfactory and should ensure that the minimum potency specified on the label of the container or package will be maintained after release and until the end of the shelf-life, provided that the vaccine is stored under the recommended conditions.

Adsorbed vaccines must not be frozen.

### A.10.3 Expiry date

The statement concerning the expiry date that appears on the label, as required in Part A, section A.8, should be based on experimental evidence, and approved by the NRA on the basis of data obtained during the stability studies referred to in section A.10.1. The date of manufacture (i.e. blending or filling) or the start date of the last satisfactory potency determination (i.e. the date on which the test animals were immunized with the vaccine) performed in accordance with Part A, section A.5.3 (or section A.3.5.2.6), is taken as the start date for the shelf-life.

In some cases, the date of the first satisfactory potency determination is used as the start date for the shelf-life.

## Part B. Nonclinical evaluation of diphtheria vaccines

### B.1 Introduction

The nonclinical testing of vaccines and their related intermediates is an essential part of the development of candidate vaccines, and is a prerequisite for the initiation of clinical trials in humans. Within the scope of this document, nonclinical evaluation means all in vivo and in vitro testing performed before and during the clinical development of the vaccine. Studies are aimed at defining the in vitro and in vivo characteristics of candidate vaccines, and such studies include evaluations of safety and immunogenicity. The recommendations included in this document are intended for new manufacturers of diphtheria vaccine, and should also be referred to if a significant change to the production process or product formulation is made by a manufacturer already producing diphtheria vaccine.

These recommendations refer only to products based on those that are currently licensed and in clinical use – i.e. vaccines based on the use of chemically detoxified diphtheria toxin as the antigen adsorbed onto an aluminium-based or calcium-based adjuvant. Diphtheria vaccines based on novel antigens or formulations that have not previously been evaluated for safety and efficacy in

clinical trials are likely to require more extensive nonclinical characterization, which is beyond the scope of this document.

The nonclinical evaluation of diphtheria vaccines should be based on WHO guidelines on nonclinical evaluation of vaccines (*27*) which incorporate definitions for commonly used terms related to nonclinical evaluations. Nonclinical evaluations of vaccine intermediates and the final product should be performed in accordance with the principles of good laboratory practice (GLP). Adhering to the principles of GLP promotes the development of high-quality test data, and provides a tool to ensure that a sound approach is taken to the management of laboratory studies, including how they are conducted, and how their data are reported and archived (*28*).

The nonclinical characterization of vaccine intermediates and in-process materials should be based on the use of adequately characterized, homogenous starting materials of defined origin and acceptable quality, including the bacterial strain and production seed lots. Demonstrating consistency in production may not be applicable during the early stages of nonclinical evaluation, but adequate validation of the production process is required to demonstrate that the manufacturing conditions are reproducible.

## B.2 Nonclinical testing and characterization of intermediates and in-process materials

Intermediates and in-process materials must be tested and characterized to confirm that they meet the recommendations in Part A of this document. The source and quality of all starting materials should be documented and should include detailed descriptions of the characterization of the strain, master seed lot and working seed lot. Defined procedures should also be shown for the preparation of new working seeds from the master seed. Seed lots should be shown to retain the characteristics of the parent strain throughout seed lot production, and should be characterized whenever a new master seed or working seed is introduced. Seed lots should be identified and characterized using a combination of validated biochemical, molecular and genetic tests. Methods such as MEE, MALDI-TOF mass spectrometry, PFGE, MLST and RFLP analysis should be considered. The maximum number of passages of each seed lot used for production should be specified and based on the number shown to result in the production of a safe and effective product; the maximum number of passages should be approved by the NRA.

The toxigenicity of the *C. diphtheriae* strain used for production should be confirmed by titration of crude toxin harvested from the culture supernatant using an appropriate in vivo or in vitro method. The culture medium used for toxin production should be well defined, and any animal components present in the medium should be identified and documented. Protein contaminants derived from the bacterium or from components of the culture medium may increase

the potential for adverse reactions to immunization with the toxoid, and the detoxification and purification processes used should minimize the presence of any substances likely to cause adverse reactions in humans. The methods used for the detoxification and purification of crude toxin should be adequately described and should be supported by appropriate validation data.

### B.2.1 Safety evaluation

The detoxification step of the production process should be validated to confirm that the detoxification of diphtheria toxin is complete and irreversible. Both the specific toxicity test (section A.3.4.4) and the reversion-to-toxicity test (A.3.4.5) should be performed on the bulk purified toxoid. Where possible, in vivo methods should be performed during nonclinical evaluations of the vaccine, but in vitro alternatives may be included as part of the validation studies.

### B.2.2 Immunogenicity and/or potency

The adsorbed bulk vaccine should be tested for immunogenicity and/or potency during the nonclinical evaluation as described in section B.3.3.

### B.2.3 Stability

Stability studies should be based on the WHO Guidelines on stability evaluation of vaccines (*23*). The stability of all intermediates not used within a short period of time should be evaluated and demonstrated using suitable methods. The choice of stability-indicating parameters as well as the frequency of testing should be justified to and approved by the NRA. Storage periods proposed for intermediates produced during the manufacturing process should be based on data obtained from the stability studies.

### B.2.4 Adjuvants

Where appropriate, adjuvants should be characterized in terms of chemical composition, physical form and adsorption capacity, purity, endotoxin content and sterility. The interaction between the adjuvant and antigen should also be evaluated; this evaluation should include measurement of the degree of adsorption. This should be shown to be consistent from lot to lot and throughout the intended storage period, and quality specifications should be established once a sufficient number of lots have been produced.

## B.3 Nonclinical characterization of formulated vaccine

Lots of the final formulated vaccine used in nonclinical studies should be adequately representative of those intended for clinical investigation, and, ideally, should be the same lots as those intended for clinical use. Manufacturers should

make every effort to keep some of this characterized material for future reference. As a minimum, candidate vaccines should be prepared under conditions of good manufacturing practice (GMP) for clinical trial material (*29*), and full implementation of the principles of GMP will be required during the later stages of clinical development (*15*, *16*).

The final formulated vaccine should be evaluated using a combination of immunological and physicochemical approaches to determine key product characteristics including sterility, pH, antigen content and degree of adsorption, immunogenicity/potency, and safety, as described in Part A of this document. Particular attention should be paid to the assessment of safety, toxicology, immunogenicity and stability. In some cases, comparability testing should be performed (e.g. after a significant change in the manufacturing process or at the time of scale-up following licensure). Comparator studies may also be required when a new manufacturer produces a diphtheria vaccine. The requirement for and extent of comparative studies, and the choice of the comparator vaccine, should be approved by the NRA.

### B.3.1 Safety

The vaccine should be tested to confirm the absence of specific toxicity and general toxicity using the in vivo methods described in Part A (the specific toxicity test and innocuity test). In vitro methods are not suitable for toxicity testing of the final vaccine formulation due to the presence of adjuvant.

### B.3.2 Toxicology

Nonclinical toxicology studies should be such that reasonable assurance is obtained that it is safe to proceed to clinical evaluation. The potential toxic effects of the vaccine should be evaluated in at least one animal species; this evaluation should include histopathology of important organs. The study should investigate the potential for local inflammatory reactions, systemic toxicity and effects on the immune system. The animal species used should be sensitive to the biological effects of the vaccine and to the toxin. Where feasible, the highest dose to be used in the proposed clinical trial should be evaluated in an animal model. Further information on considerations related to dose, route of administration, controls, and parameters to be monitored can be found in the WHO guidelines on nonclinical evaluation of vaccines (*27*). A full toxicology assessment may not be required in all cases (e.g. when a manufacturer already producing the vaccine changes the production process), although any decision not to perform toxicology studies should be approved by the NRA. Diphtheria vaccines produced using a novel antigen or adjuvant, or both, are likely to require a full toxicology assessment, which is described elsewhere (*27*).

For diphtheria vaccines intended to be used in adolescents and adults (e.g. as booster vaccines or to manage diphtheria outbreaks), the need to perform developmental toxicology studies should be considered unless scientific and clinically sound arguments can be made that such studies are not necessary. Further information about developmental toxicity studies can be found in the WHO guidelines on nonclinical evaluation of vaccines (*27*).

### B.3.3 Immunogenicity and/or potency

Immunization studies in appropriate animal models can provide valuable proof-of-concept information during the preclinical development stages. For diphtheria vaccines, immunogenicity studies should include measurement of toxin neutralizing antibody responses in serum samples from vaccinated animals.

The potency of the vaccine should be determined, and those lots that have been shown to meet the recommendations described in Part A, section A.3.5.2.6, are likely to induce adequate immune responses in clinical trials. The measurement of vaccine potency by comparison with a suitable reference vaccine calibrated in IUs is useful for assessing production consistency. During nonclinical evaluations, the potency test should consist of a multiple-dilution assay (with at least three dilutions of each test vaccine and the reference preparation), should be performed using guinea-pigs or mice and should have a functional end-point (i.e. a challenge with diphtheria toxin when guinea-pigs are used, or titration of immune serum samples by TNT when guinea-pigs or mice are used). More details on the methods used for the potency testing of diphtheria vaccines can be found in the WHO *Manual for quality control of diphtheria, tetanus and pertussis vaccines* (*21*).

### B.3.4 Stability

Stability testing should be seen as a continual process occurring from the development of the vaccine through licensing and on to post-licensure monitoring. Stability studies should be based on the WHO Guidelines on stability evaluation of vaccines (*23*). During the early stages of clinical trials, the amount of real-time stability data may be limited, but sufficient data should be generated to support the stability of the vaccine for the intended duration of the trial. For licensure, however, studies should be carried out under the proposed storage conditions, and should be performed in real time. Accelerated stability studies of products stored for limited periods at temperatures that may affect stability could support preliminary data from continuing real-time stability studies but should not replace them. Following licensure, continuing assessments of stability are recommended to support the shelf-life specifications. The cumulative nature of the actual age of the antigen at the end of the shelf-life of the final vaccine product

should be considered, and data covering the cumulative age of the antigen should be collected and reported to the NRA.

Stability studies should confirm that the production process results in a final product that does not revert to toxicity during long-term storage. As a result, safety testing, using both the specific toxicity test and the innocuity test, should be performed on the expiry date of the product. Additional tests that may be used to demonstrate stability include the potency test, and physical and chemical characterization; as a minimum, tests for potency, sterility, adjuvant content, degree of adsorption, preservative content and pH should be performed. Final containers from at least three vaccine lots, each of which has been derived from different bulks should be tested on their expiry date to demonstrate that stability has been maintained during storage at the recommended temperature. The time points selected for testing should be appropriate for the vaccine being evaluated, should be supported by validation data and should be approved by the NRA.

# Part C. Clinical evaluation of diphtheria vaccines

## C.1 Introduction

This section addresses issues that are relevant during the clinical development of diphtheria vaccines. Progression through the phases of clinical development should follow the principles outlined in WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (*30*) which contains definitions for commonly used terms during clinical evaluations. Only those vaccines assessed by the NRA as having an adequate nonclinical evaluation should be considered for clinical evaluation.

Clinical evaluation is required for new diphtheria vaccines, and may also be required for existing vaccines if a significant change to the manufacturing process has been proposed. The content and extent of the clinical programme will vary according to each possible scenario. It is strongly recommended that early dialogue is established between the vaccine manufacturer and the NRA to clarify requirements for clinical studies as well as for marketing approval.

### C.1.1 General considerations for clinical studies

All clinical trials on pharmaceutical products should adhere to the standards of good clinical practice set out by WHO (*31*). Vaccines have special aspects that demand careful consideration during clinical evaluation because they are given to healthy people, mostly in the paediatric population, and are given to prevent disease rather than to cure it, which limits the tolerance to adverse events.

It is expected that at least some clinical studies, including those in the primary target population, will be conducted with different lots of vaccine manufactured using the same process as the vaccine intended for marketing.

Consistency in the manufacture of the vaccine lots used in clinical trials should be demonstrated and well documented. Although a formal clinical trial to evaluate lot consistency may not always be needed, in some instances clinical data may be required to provide evidence to validate manufacturing consistency (e.g. if there is a particular concern about consistency). Vaccine lots used in clinical studies should ideally be the same lots that have been evaluated in nonclinical studies, and should be adequately representative of the formulation intended for marketing. Where this is not feasible, the lots used clinically should be comparable to those used in the nonclinical studies with respect to the manufacturing process, immunogenicity and potency, safety, stability and other relevant characteristics of quality. The number of different vaccine lots evaluated as part of the clinical studies should be approved by the NRA but should be more than one. It is important to note that clinical data used to provide evidence of production consistency do not replace the need to demonstrate consistency in the manufacturing process during nonclinical evaluations.

#### C.1.1.1 Scope of the studies

The size and design of the studies, and the selection of end-points for evaluation, require justification; they should provide reasonable assurance of the clinical benefit and safety of the candidate vaccine. Studies should include evaluations of the performance of the investigational vaccine when co-administered with other vaccines routinely recommended for the target population. Of particular interest in the evaluation of diphtheria vaccines are any effects on safety and immunogenicity found during co-administration with polysaccharide conjugate vaccines containing CRM197 or diphtheria toxoid as the carrier protein.

Where vaccines containing the same antigen(s) are already in common use, or the incidence of disease is very low – as is the case for diphtheria vaccines – it may not be feasible to perform a study based on protective efficacy. In the case of diphtheria, for which there are generally accepted serological correlates of protection, studies are based on the evaluation of antidiphtheria antibody responses in addition to a thorough assessment of vaccine safety. The primary immunogenicity end-point(s) should be those most relevant to the target population, and these will differ for evaluations of priming and booster doses of diphtheria toxoid.

#### C.1.1.2 Comparator vaccine

Immunological correlates of protection are well established for diphtheria vaccines (see section C.2.2), and it should be acknowledged that comparison studies, where used, do not bridge to efficacy but to serological correlates. In some cases, it may be decided to perform immunogenicity analyses that are not comparative, although in most cases comparative studies are recommended. The inclusion of a

comparator aids interpretation of the results of the trial, particularly if the expected seroprotection rate in the investigational group is not observed (e.g. if the study is conducted in a population where high levels of maternal antibody suppress the response to immunization in infants). Decisions about whether to include a comparator vaccine, and the selection of a comparator, should be justified by the manufacturer, and approved by the NRA. In studies performed to support major changes to the manufacturing process for a licensed vaccine, including a change in formulation or antigen dose, the candidate vaccine should be compared with the existing product (i.e. one manufactured according to the licensed production process). In this case, a comparative study is particularly useful for directly evaluating the effect of the change on the process or formulation. In studies of a new vaccine, the comparator is typically a licensed vaccine.

In some comparative studies, safety end-points may also be considered primary end-points (e.g. where the antigen content of a vaccine has been reduced with the intention of lowering the frequency of local reactions).

## C.2 Assessment of immunogenicity in humans

### C.2.1 Assays to assess antibody responses

Assays to measure the antibody response to diphtheria vaccine can be divided into functional assays (which demonstrate the capacity of diphtheria antibody in a serum sample to prevent the toxic effects of diphtheria toxin) and nonfunctional binding assays (which demonstrate the capacity of diphtheria antibody in a serum sample to bind to diphtheria toxin or toxoid).

The Vero cell assay is an in vitro toxin neutralization (or microneutralization) test that can be used to measure neutralizing antibodies in serum (*32*); it is considered to be the gold standard for measuring responses to diphtheria vaccines. This method can be used to confirm the relevance and performance of other in vitro serological assays as part of validation studies. However, the Vero cell assay is not commercially available, and it requires cell culture facilities and a relatively large volume of serum compared with other in vitro serological assays. Therefore, other in vitro serological assays may be preferred if they use a lower sample volume, are faster, and can be automated, all of which make it easier to screen large numbers of samples. These nonfunctional binding assays include ELISA, double-antigen ELISAs (DAEs), the dual double-antigen time-resolved fluorescence immunoassay (dDA-DELFIA), the passive haemagglutination assay (PHA) and the toxin-binding inhibition (ToBI) test. Nonfunctional in vitro serological assays show variable degrees of correlation with the Vero cell assay, particularly when the levels of functional antibody are low (*33*, *34*); the nonfunctional in vitro assays should be validated against the Vero cell TNT.

The method chosen to measure antibody response should be approved by the NRA. Where feasible, an assay that measures functional antibody responses should be used at some stage during the clinical evaluation of the vaccine (e.g. to analyse a subset of the clinical trial samples).

The International Standard for diphtheria antitoxin human, calibrated in IUs of diphtheria antitoxin, can be used in the TNT and in vitro immunoassays. Secondary reference materials intended for use with ELISA and other in vitro serological assays should be calibrated against the international standard using a TNT, but the results of all assays must be expressed in IU per ml.

### C.2.2 Criteria for evaluation of immune responses

The end-points and criteria used to evaluate immune responses require justification, and must take into account the assay used to measure diphtheria antibody responses, the intended use of the vaccine (i.e. for primary or booster immunization) and established immunological correlates of protection.

Immunological correlates of protection are well established for diphtheria vaccines, and are recommended for use as primary or secondary end-points, depending on the scenario. When measured using a TNT, antidiphtheria antibody levels of less than 0.01 IU/ml are considered to indicate that a person is highly susceptible to the disease; an antibody level of 0.01 IU/ml is considered to be the minimum required for some degree of protection; levels of 0.1 IU/ml or higher are considered to confer full protection against the disease; and levels 1.0 IU/ml or higher are associated with long-term protection against diphtheria (*1*). However, it should be acknowledged that there is no sharply defined level of antitoxin above which all persons can be considered to be fully protected. When an ELISA is used, the minimum level of antibody considered to confer some degree of protection is usually 0.1 IU/ml, and at this level of response there is a good correlation with the Vero cell assay (*34*). The end-points and evaluation criteria proposed by the manufacturer should be approved by the NRA.

#### C.2.2.1 Primary immunization of infants

The proportion of subjects with a diphtheria antibody concentration above a prespecified threshold (indicating seroprotection) should be determined approximately one month following the last priming dose. When the Vero cell assay is used to measure antidiphtheria antibodies, the proportion of subjects with a post-vaccination level of 0.01 IU/ml or higher may be acceptable as the primary immunogenicity end-point. In countries where a booster dose of diphtheria toxoid is not routinely administered during the second year of life, a level of 0.1 IU/ml or higher may be recommended as the primary end-point (even when the Vero cell assay is used). When a nonfunctional assay such as an ELISA is used to measure antidiphtheria antibodies, the proportion of subjects

with a post-vaccination level of 0.1 IU/ml or higher is typically used as the primary end-point.

The noninferiority of the investigational vaccine relative to a comparator vaccine should be evaluated. Noninferiority is demonstrated if the upper limit of the 95% confidence interval for the difference in the seroprotection rates (i.e. the comparator vaccine minus the investigational vaccine) is less than the prespecified margin (usually 10%, although a margin of 5% may be recommended if the expected rates of seroprotection are very high). The specified threshold antibody concentration, noninferiority margin and bleeding time should be approved by the NRA.

In studies performed without a comparator vaccine, an acceptance criterion should be used for the proportion of subjects (usually 90% or 95%) that reaches the prespecified threshold antibody concentration as measured approximately 1 month following the last priming dose. The acceptance criterion should be based on the 95% confidence interval for the proportion of subjects achieving the prespecified antibody concentration (e.g. the lower limit of the 95% confidence interval for the observed proportion should be greater than 90% or 95%). The NRA should approve the specified antibody threshold, acceptance criterion and bleeding time.

In addition to the level of antibody specified as the primary end-point, it is recommended that secondary analyses of the proportion of subjects achieving other clinically relevant thresholds of diphtheria antibody (see section C.2.2) are also performed for the investigational vaccine and, where used, the comparator vaccine. The geometric mean titre (GMT) of the antidiphtheria antibody response should also be evaluated, and presented as a secondary end-point. In comparative studies, the GMT ratio of the investigational vaccine to the comparator vaccine may be evaluated using a predefined margin of noninferiority (e.g. the lower limit of the 95% confidence interval of the observed ratio of the investigational vaccine to the comparator vaccine should be greater than 0.67). The presentation of reverse cumulative distribution (RCD) curves, which show the accumulated proportion of subjects with an antibody concentration greater than or equal to a given level, may also provide useful information for comparison.

#### C.2.2.2 Primary immunization of adolescents or adults

In some countries it may be desirable to evaluate a diphtheria vaccine to be used for primary immunization in adolescents or adults. In these instances, it may be necessary to conduct pre-enrolment screening to identify previously unvaccinated (i.e. naive) subjects. Criteria to identify naive subjects for enrolment might include a diphtheria antibody level less than 0.01 IU/ml as measured by Vero cell assay prior to and seven days after receipt of the first dose of diphtheria vaccine. In some cases, it may be difficult to identify sufficient numbers of naive individuals, and the choice of study design (i.e. comparative or noncomparative)

may therefore be dependent on the number of naive subjects that can be identified in the target population. If sufficient numbers of naive subjects in older age groups cannot be identified, consideration may be given to extrapolating the effectiveness of primary immunization from infants to older age groups.

The end-points for the evaluation of the primary immune response in adolescents or adults are the same as those recommended for the primary immunization of infants (see section C.2.2.1). As with studies of primary immunization in infants (see section C.2.2.1), noninferiority criteria should be specified for comparative studies; acceptability criteria should be specified for noncomparative studies.

### C.2.2.3 Booster immunization of pre-school-age children, school-age children, adolescents and adults

For the evaluation of diphtheria vaccines intended for booster immunization, the age of the participant and the interval since the last dose of diphtheria vaccine should be taken into account when designing and analysing the studies, since these factors may have a significant impact on the response to a booster dose. Criteria for the evaluation of booster doses of diphtheria vaccines should reflect the fact that prior to booster vaccination, a substantial proportion of the study population may have diphtheria antibody levels equal to or above those that may have been specified to evaluate responses to primary immunization. Assessing antibody levels both prior to and following immunization is recommended to optimize the interpretation of the data. In some cases, analysing the proportion of subjects who achieve a specified booster response (based on a comparison of pre-vaccination and post-vaccination antibody levels) may be more meaningful than using criteria based on the proportion of subjects reaching a prespecified antibody level. Decisions about whether to use an antibody threshold or booster response as the primary end-point should take into consideration the expected proportion of subjects who may have antibody levels that exceed the threshold prior to vaccination. If this proportion is high, the booster response may be a more meaningful primary end-point.

Where an antibody threshold is specified as a primary end-point for evaluating booster vaccination, it is recommended that the threshold value of 0.1 IU/ml should be used, even when analyses use the Vero cell assay. In comparative studies, noninferiority should be evaluated; noninferiority is demonstrated if the upper limit of the 95% confidence interval of the difference in seroprotection rates (i.e. for the comparator vaccine minus the investigational vaccine) is less than the prespecified margin (usually 10%, although a margin of 5% may be recommended if the expected rates of seroprotection are very high). In noncomparative studies, an acceptability criterion for the proportion of subjects who achieve the specified threshold post-vaccination (e.g. 90% or 95%) should be used, based on the 95% confidence interval for the observed proportion.

If the booster response is used as the primary end-point, the definition of booster response, based on an increase in diphtheria antibody concentration from pre-vaccination to post-vaccination, should be prespecified. For subjects with low pre-vaccination levels of antibody, the definition of booster response should include a requirement that the post-vaccination level exceeds an appropriate threshold by a specified amount. For instance, in subjects with a pre-vaccination antibody level less than 0.1 IU/ml, a booster response might be defined as a post-vaccination concentration of 0.4 IU/ml or greater (i.e. at least 4 times higher than the clinically relevant threshold of 0.1 IU/ml). In subjects with a pre-vaccination concentration of 0.1 IU/ml or greater, a booster response might be defined as a post-vaccination concentration that is at least 4 times higher than the pre-vaccination concentration. Using a lower-fold rise in antibody concentration to define the booster response in persons with specified high levels of pre-existing antibody may be appropriate, but this lower level should be prespecified and justified.

In comparative studies, booster responses should be compared between groups, and should be evaluated using a predefined noninferiority limit (e.g. the upper limit of the 2-sided 95% confidence interval of the observed difference – that is, the comparator vaccine minus the investigational vaccine – should be less than a prespecified margin, which is usually 10%). In noncomparative studies, an acceptability criterion should be used for the proportion of subjects who achieve a booster response (e.g. 80%), and this should be based on the 95% confidence interval of the observed proportion (see section C.2.2.1).

As an indicator of long-term protection, the proportion of subjects with a post-vaccination antibody level of 1.0 IU/ml or greater may be evaluated as a secondary end-point. The post-vaccination GMT of antidiphtheria antibody may be evaluated as a secondary end-point. In comparative studies, the GMT of the ratio of the investigational vaccine to the comparator vaccine may be evaluated using a predefined margin of noninferiority (e.g. the lower limit of the 95% confidence interval of the observed ratio of the investigational vaccine to the comparator vaccine should be greater than a prespecified limit, which is usually 0.67).

The choice of end-points and criteria for evaluation (including the need for a comparator vaccine) should be justified by the manufacturer, and approved by the NRA.

### C.2.3 Antibody persistence

Where possible, subsets of subjects should be identified for longer-term follow-up of the persistence of immunity in order to determine the need for booster doses. Alternatively, population surveillance studies should be carried out to determine the prevalence of diphtheria antibody in different age groups, and to guide recommendations on the need for booster doses.

## C.3 Safety evaluation

The clinical assessment of diphtheria vaccines should include a thorough assessment of the vaccine's safety using comparative prelicensure studies. In some cases, the evaluation of safety may be the primary (or coprimary) objective of a clinical study (e.g. when a change to the vaccine's formulation has been made to lower the antigen dose with the intention of reducing the frequency of local reactions). The assessment of safety should follow the general principles outlined in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (*30*). Safety data should be collected throughout the duration of clinical development. For a new vaccine, it is generally recommended that the overall safety database should consist of a minimum of 3000 subjects who have received the investigational vaccine. This number allows for the detection of adverse events that occur at a rate of 1 in 1000 subjects. However, the minimum acceptable size of the safety database needed to support licensure will vary according to several factors, including the formulation of the vaccine and prior experience with vaccines that have the same or similar composition. The size of the database should be justified by the manufacturer, and approved by the NRA. For diphtheria vaccines, in cases in which efficacy has been inferred from serological correlates of protection, the number of subjects that should be evaluated for safety is likely to exceed the number required for the evaluation of immunogenicity.

The frequency of adverse reactions following diphtheria immunization may vary according to the vaccine's formulation (e.g. according to the dose of the diphtheria antigen) and subjects' characteristics (e.g. prior vaccination history, time since previous dose and pre-vaccination level of diphtheria antibody). Careful attention should be paid to documenting and evaluating safety associated with the administration of booster doses, since higher rates of local adverse reactions have been observed following booster immunization with diphtheria toxoid compared with primary immunization. Consideration should also be given to the potential for the increased reactogenicity that may occur when diphtheria vaccines are administered at the same time as, or shortly after, polysaccharide conjugate vaccines containing CRM197 or diphtheria toxoid as the carrier protein.

Commonly occurring adverse reactions expected after diphtheria immunization include pain, redness and swelling at the injection site. Post-vaccination fever may also occur. Serious adverse reactions associated with diphtheria vaccine occur too infrequently to be reliably evaluated in most clinical trials. Although serious adverse events should be monitored during prelicensure clinical trials, post-marketing surveillance must also be performed to monitor such events.

### C.4 Post-marketing studies and surveillance

Monitoring the effectiveness, safety and quality of licensed vaccines consists of post-marketing surveillance and post-marketing studies (phase IV studies). The purpose of post-licensure monitoring is to assess the performance of a vaccine in the target population under conditions of routine use, and to monitor rare adverse events. Post-marketing studies may also be useful for assessing antibody persistence and the need for booster doses. Marketing authorization holders should be committed to presenting a post-marketing surveillance programme at the time of licensure. The programme should be based on criteria for assessing the quality, safety and effectiveness of a particular vaccine to gain marketing approval.

In many cases, comprehensive post-marketing safety and effectiveness data cannot be collected by manufacturers alone, and close cooperation between manufacturers and public-health authorities is required. All data collected should be submitted to the NRA at regular intervals so that action can be taken if there are implications for the marketing authorization.

Post-marketing surveillance may be the only means of detecting rare adverse events that occur too infrequently to have been detected during clinical trials. For the collection of safety data, surveillance may be conducted by active or passive processes. Voluntary reporting of serious adverse events (passive surveillance) is most commonly used.

## Part D. Recommendations for NRAs

### D.1 General

The general recommendations for NRAs and national control laboratories (NCLs) given in Guidelines for national authorities on quality assurance for biological products (*35*) and Guidelines for independent lot release of vaccines by regulatory authorities (*12*) apply.

The details of production and quality control procedures, as well as any significant changes in them that may affect the quality, safety or efficacy of diphtheria vaccines, should be discussed with and approved by the NRA. For control purposes, the international standards currently in use (see the section on General considerations) should be obtained for the purpose of calibrating national, regional and working standards (*25*). The NRA may obtain the product-specific or working references from the manufacturer to be used for lot release until an international or national standard preparation has been established.

Consistency in production has been recognized as an essential component in the quality assurance of diphtheria vaccines. In particular, NRAs should carefully monitor production records and the results of quality control tests on clinical lots as well as results from tests on a series of consecutive lots of the vaccine.

### D.2 Release and certification by the NRA

A vaccine should be released only if it fulfils all national requirements or satisfies Part A of these Recommendations, or both (*12*).

A protocol based on the model given in Appendix 1, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for the release of a vaccine for use.

A statement signed by the appropriate official of the NRA should be provided to the manufacturing establishment, and should certify that the lot of vaccine in question meets all national requirements as well as Part A of the present Recommendations. The certificate should provide sufficient information about the vaccine lot. A model certificate is given in Appendix 2. The official national release certificate should be provided to importers of the vaccine. The purpose of the certificate is to facilitate the exchange of vaccines between countries.

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# Appendix 1

## Model protocol for the manufacturing and control of diphtheria vaccines (adsorbed)

The following protocol is intended for guidance. It indicates the information that should be provided as a minimum by a manufacturer to the NRA. Information and tests may be added or omitted as necessary, with the authorization of the NRA.

It is possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO Recommendations for a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet that will accompany the vaccine container. If the protocol is being submitted in support of a request to permit importation, it should also be accompanied by a lot-release certificate from the NRA or from the NCL in the country where the vaccine was produced or released stating that the product meets national requirements as well as the recommendations in Part A of this annex.

### 1. Summary information on finished product (final lot)

International name: _____
Trade name/commercial name: _____
Product licence (marketing authorization) number: _____
Country: _____
Name and address of manufacturer: _____
Name and address of licence holder, if different: _____
Final packaging lot number: _____
Type of container: _____
Number of containers in this packaging: _____
Final container lot number: _____
Number of filled containers in this final lot: _____
Date of manufacture: _____
Description of final product (adsorbed): _____
Preservative, and nominal concentration: _____
Volume of each single human dose: _____
Number of doses per final container: _____

Summary of the composition (include a summary of the qualitative and quantitative composition of the vaccine in each human dose, including any adjuvant used and other excipients):

_____

_____

Shelf-life approved (months): _____
Expiry date: _____
Storage conditions: _____

The following sections are intended for recording the results of the tests performed during the production of the vaccine so that the complete document will provide evidence of consistency in production. If any test has to be repeated, this must be indicated. Any abnormal result must be recorded on a separate sheet.

## 2. Detailed information on manufacture and quality control

### Starting materials

Identity of *C. diphtheriae* strain used for
vaccine production: _____
Reference number of seed lot: _____
Date(s) of reconstitution (or opening) of
seed lot ampoule(s): _____

### Single harvests used for preparing the bulk purified toxoid

Name of the culture medium: _____
Date of inoculation: _____
Temperature of incubation: _____
Control of bacterial purity
Methods: _____
Result: _____
Date: _____
Date of harvest: _____
Volume of harvest: _____
Yield (Lf/ml): _____
Volume after filtration: _____
Toxin purification method: _____
Toxin content yield (Lf/ml): _____

Method of detoxification: _____
Date started: _____
Date finished: _____

Volume used for inactivation: _____
Temperature: _____
Concentration of detoxification agent: _____

*Confirmation of detoxification (before or after purification)*
Method: _____
Dose of inoculation (Lf): _____
Route of inoculation (in vivo only): _____
Date of inoculation: _____
Date of observation: _____
Result: _____

Purification methods used for toxoid: _____
Yield of purified toxoid
Volume: _____
Toxoid content (Lf/ml): _____

## Bulk purified toxoid

Reference number: _____
Volume and Lf/ml: _____

*Sterility test*
*Tests for bacteria and fungi*
Method: _____
Media: _____
Number of bulks tested: _____
Volume of inoculum per bulk: _____
Volume of medium per bulk: _____
Temperature of incubation: _____
Date of start of test: _____
Date of end of test: _____
Result: _____

Antigenic purity (Lf/mg of protein (nondialysable) nitrogen): _____
Method: _____
Date of test: _____

*Specific toxicity test*
Method: _____
Dose of inoculation (Lf): _____
Route of inoculation (in vivo only): _____

Date of inoculation: _____
Date of observation: _____
Result: _____

*Test of reversion to toxicity*

Lf /ml of test toxoid solution: _____
Temperature of incubation of toxoid: _____
Dates of beginning and end of incubation: _____
Method: _____
Dose of inoculation (Lf): _____
Route of inoculation (in vivo only): _____
Date of inoculation: _____
Date of observation: _____
Result: _____

## Final bulk

Identification (lot number): _____
Date of manufacture or blending: _____
Volume: _____
Lf/ml: _____

| Blending: | Prescription (SHD) | Added |
|---|---|---|
| Toxoid (Lf): | | |
| Adjuvant: | | |
| Preservative (specify): | | |
| Others (salt): | | |
| Final volume (ml): | | |

*Preservative content*

Method: _____
Date of test: _____
Result: _____

*Adjuvant content*

Method: _____
Date of test: _____
Result: _____

*Degree of adsorption*

Method: _____
Date of test: _____
Result: _____

*Sterility test*

Tests for bacteria and fungi
Method: _____
Media: _____
Number of containers tested: _____
Volume of inoculum per container: _____
Volume of medium per container: _____
Temperature of incubation: _____
Date of start of test: _____
Date of end of test: _____
Result: _____

*Specific toxicity test (when required)*

Method: _____
Dose of inoculation (Lf): _____
Route of inoculation (in vivo only): _____
Date of inoculation: _____
Date of observation: _____
Result: _____

*Potency test*

Challenge method (multiple-dilution or single-dilution assay)
If single dilution, date of last satisfactory multiple-dilution assay: _____
Species, strain and weight range of animals: _____
Number of animals per dilution: _____
Reference vaccine used (IU): _____
Date of immunization: _____
Route of injection and volume of dilutions administered: _____
Date of challenge: _____
Challenge method used (lethal or intradermal challenge): _____
Challenge toxin used: _____
Challenge dose(s) used: _____
Date of end of observation: _____

Results (see Table 4.1 for an example of how to report the results from a lethal method, and see Table 4.2 for an example of reporting an intradermal method)

Table 4.1
**Reporting results from a lethal challenge assay**

| Vaccine | Dilution | Lethal method<br>No. survivors/No. tested | Median effective dose ($ED_{50}$) |
|---|---|---|---|
| Reference vaccine<br>…… IU/ml | 1 | / | …… ml |
| | 2 | / | |
| | 3 | / | |
| | | | |
| Test vaccine<br>lot number<br>…… | 1 | / | …… ml |
| | 2 | / | |
| | 3 | / | |
| Potency of test vaccine in IU/SHD with 95% confidence limits: ……<br>95% confidence limits expressed as % of potency estimate: …… | | | |
| Single-dilution assays only: *P* value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD: …… | | | |

Table 4.2
**Reporting results from an intradermal challenge assay**

| Vaccine | Dilution | Mean score |
|---|---|---|
| Reference vaccine<br>…… IU/ml | 1 | |
| | 2 | |
| | 3 | |
| | | |
| Test vaccine<br>lot number<br>…… | 1 | |
| | 2 | |
| | 3 | |
| Potency of test vaccine in IU/SHD with 95% confidence limits: ……<br>95% confidence limits expressed as % of potency estimate: …… | | |
| Single-dilution assays only: *P* value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD: …… | | |

When a single-dilution assay is performed, only the responses or scores at the single dilution used are shown. For the intradermal challenge assay, the $ED_{50}$ is not applicable.

Serological method

Species, strain and weight range of animals: _____
Number of animals per dilution: _____
Reference vaccine used (IU): _____
Date of immunization: _____
Route of injection and volume of dilutions administered: _____
Date of bleeding: _____

Method for titration of immune serum samples: _____
Reference serum or antibody: _____

Results (See Table 4.3 for an example of reporting results from a serological method)

Table 4.3
**Reporting results from a serology assay**

| Vaccine | Dilution | Mean scores or response |
|---|---|---|
| Reference vaccine ..... IU/ml | 1 | |
| | 2 | |
| | 3 | |
| | | |
| Test vaccine lot number ..... | 1 | |
| | 2 | |
| | 3 | |
| Positive control | | |
| Negative control | | |
| Potency of test vaccine in IU/SHD with 95% confidence limits: .....<br>95% confidence limits expressed as % of potency estimate: ..... | | |
| Single-dilution assays only: *P* value indicating the probability that the test vaccine contains more than the minimum potency requirement per SHD: ..... | | |

*Test for amount of residual free detoxifying agent*

Detoxifying agent: _____
Method: _____
Result (g/1): _____
Date of test: _____

*pH*

Result: _____

Date of test: _____

## Final product

Identification: _____

Volume: _____

*Identity test*

Method: _____

Result: _____

Date of test: _____

*Sterility test*

*Tests for bacteria and fungi*

Method: _____

Media: _____

Number of containers tested: _____

Volume of inoculum per container: _____

Volume of medium per container: _____

Temperature of incubation: _____

Date of start of test: _____

Date of end of test: _____

Result: _____

*Potency test*

If this test was not performed on the final bulk, indicate this and report the data obtained for the final product in the space provided for potency tests in the "final bulk" section.

*Innocuity test (when required)*

*Tests in mice*

Date of start of test: _____

Date of end of test: _____

Number of animals tested: _____

Route of injection: _____

Volume of injection: _____

Observation period: _____

Results (give details of deaths): _____

*Tests in guinea-pigs*

Date of start of test: _____
Date of end of test: _____
Number of animals tested: _____
Route of injection: _____
Volume of injection: _____
Observation period: _____
Results (give details of deaths): _____

*Test for adjuvant content*

Nature and concentration of adjuvant/SHD: _____
Method of testing: _____
Result: _____
Date of test: _____

*Test for degree of adsorption (when required)*

Method: _____
Desorption method and reagent: _____
Result: _____
Date of test: _____

*Test for preservative*

Nature and concentration of preservative: _____
Method of testing: _____
Result: _____
Date of test: _____

*pH*

Method of testing: _____
Result: _____
Date of test: _____

*Extractable content*

Result: _____
Date of test: _____

*Inspection of final containers*

Date of inspection: _____
Organoleptic characteristics: _____
Number of containers inspected: _____
% of containers rejected: _____

## 3. Certification by the manufacturer

Name of the manufacturer _____

Name of head of production (typed) _____

*Certification by the person from the control laboratory of the manufacturing company taking responsibility for the production and control of the vaccine*

I certify that lot no. _____ of diphtheria vaccine, whose number appears on the label of the final container, meets all national requirements and/or satisfies Part A[1] of the WHO Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (adsorbed) (2014).[2]

Signature _____
Name (typed) _____
Date _____

## 4. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA (as shown in Appendix 3), a label from a final container and an instruction leaflet for users.

[1] With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

[2] WHO Technical Report Series, No. 980, Annex 4.

# Appendix 2

## Model certificate for the release of diphtheria vaccines (adsorbed) by NRAs

### Lot release certificate

Certificate no. _____

The following lot(s) of diphtheria vaccine (adsorbed) produced by _____[1] in _____,[2] whose numbers appear on the labels of the final containers, complies with the relevant national specifications and provisions for the release of biological products[3] and Part A[4] of the WHO Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (adsorbed) (2014)[5], and complies with WHO good manufacturing practices: main principles for pharmaceutical products;[6] Good manufacturing practices for biological products;[7] and Guidelines for independent lot release of vaccines by regulatory authorities.[8]

The release decision is based on _____[9]

The certificate may include the following information:

- name and address of manufacturer
- site(s) of manufacturing
- trade name and common name of product
- marketing authorization number
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary)

[1] Name of manufacturer.

[2] Country of origin.

[3] If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA.

[4] With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

[5] WHO Technical Report Series, No. 980, Annex 4.

[6] WHO Technical Report Series, No. 961, Annex 3.

[7] WHO Technical Report Series, No. 822, Annex 1.

[8] WHO Technical Report Series, No. 978, Annex 2.

[9] Evaluation of the summary protocol, independent laboratory testing, or procedures specified in a defined document etc., as appropriate.

- type of container used
- number of doses per container
- number of containers or lot size
- date of start of period of validity (e.g. manufacturing date) and/or expiry date
- storage conditions
- signature and function of the person authorized to issue the certificate
- date of issue of certificate
- certificate number.

The Director of the NRA (or other appropriate authority)

Name (typed) _____
Signature _____
Date _____