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**Guidelines on the Quality, Safety and Efficacy of Dengue Tetravalent
Vaccines (Live, Attenuated)**

Proposed replacement of TRS No. 932, Annex 1

This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). **The text in its present form does not necessarily represent an agreed formulation of the Expert Committee. Comments proposing modifications to this text MUST be received by 23 September 2011** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Quality Safety and Standards (QSS). Comments may also be submitted electronically to the Responsible Officer: Dr Jinho Shin at email: shinj@who.int.

The outcome of the deliberations of the Expert Committee will be published in the WHO Technical Report Series. The final agreed formulation of the document will be edited to be in conformity with the "WHO style guide" (WHO/IMD/PUB/04.1).

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Recommendations and guidelines published by WHO are intended to be scientific and advisory in nature. It is recommended that modifications be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the recommendations set out below. To facilitate the international distribution of vaccine made in accordance with these Guidelines, a summary protocol for the recording of results of the tests is given in Appendix 1.

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Introduction

These Guidelines are intended to provide national regulatory authorities and vaccine manufacturers with guidance on the quality, safety and efficacy of live-tetravalent dengue vaccines currently under clinical development to facilitate their international licensure and use.

These Guidelines update the *Guidelines for the production and quality control of candidate tetravalent dengue virus vaccines (live)* [66]. They should be read in conjunction with other WHO guidelines that are referred in each part.

These Guidelines cover dengue tetravalent vaccines (live, attenuated). Other types of dengue virus vaccines under development, e.g. subunit or inactivated vaccines, are outside the scope of these Guidelines. However, some guiding principles provided in these Guidelines, e.g. Part C clinical evaluation, may be useful for the evaluation of other types of dengue vaccines. For quality control aspects, guiding principles applicable to other types of vaccines such as inactivated or subunit vaccines are available elsewhere if the product in development shares similar manufacturing process. For example, guidelines for human papillomavirus and hepatitis B vaccines may be useful for subunit vaccines for dengue as well.

These Guidelines are based on experience gained from candidate live-attenuated dengue tetravalent vaccines that have been developed, as described below, and will need to be updated as new data becomes available from additional studies.

Part A sets out guidelines for manufacture and quality control. Guidelines specific to the nonclinical, clinical evaluation and environmental risk assessment are provided in Parts B, C, and D, respectively. Part E provides guidelines for national regulatory authorities. In the following section, brief overviews of dengue disease and dengue vaccine development at the time of preparing this document are provided as scientific basis for each part.

General considerations

Dengue viruses

Dengue is a mosquito-borne disease and represents a major public health problem throughout the tropical world. The causative dengue viruses (DENVs) are members of the genus *Flavivirus*, within the family *Flaviviridae*. There are four serotypes (termed DENV-1 to -4) and at least 3 genetic groups (genotypes) within each serotype.

All flaviviruses are lipid-enveloped, positive-sense, single-stranded RNA viruses, approximately 55 nm in diameter. The genome is capped at the 5' terminus but does not have a poly A tract at the 3' terminus, and is approximately 11,000 nucleotides in length. The virion RNA encodes a single open reading frame (ORF) that is flanked by a 5' untranslated region (UTR) and 3'UTR. The ORF is translated into a polyprotein that is

co- and post-translationally cleaved to yield at least 10 proteins. Three structural proteins are derived by cleavages of the amino-terminal one-third of the polyprotein: the capsid or core protein forms a “nucleocapsid” complex with virion RNA that lies within the lipid envelope. The premembrane (prM) and envelope (E) proteins are embedded in the lipid envelope via carboxy-terminal transmembrane domains and are displayed on the surface of virions. Cleavage of the carboxy-terminal two-thirds of the polyprotein yields seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. NS3 encodes a serine protease in the N-terminal 180 amino acids and helicase, nucleotide triphosphatase, RNA 5'-triphosphatase activities in the C-terminal region while NS5 encodes two functions; the first one-third encodes a methyltransferase that sequentially methylates the N7 and 2'-O positions of the viral RNA cap using S-adenosyl-l-methionine as a methyl donor, and the remainder a RNA-dependent RNA polymerase. NS1 plays various roles in the virus replication cycle while NS2A, NS2B, NS4A and NS4B are all small hydrophobic proteins with the central region of NS2B required for the functioning of the NS3 protease.

Host range and transmission

DENVs are most commonly transmitted to humans by the bite of infected *Aedes (Ae.) aegypti* mosquitoes, which are highly domesticated and the primary mosquito vector; however, *Ae. albopictus* can also sustain human-to-human transmission. The drastic increase in the incidence of DENV infection in the Americas during the past 30 years is primarily due to the geographical spread of *Ae. aegypti* following decline in vector-control efforts. The DENV that infects and causes disease in humans is maintained in a human-to-mosquito-to-human cycle and does not require a sylvatic cycle in non-human primates. Certain strains of DENV are known to be transmitted to non-human primates in western Africa and Malaysia; however, transmission to humans via mosquitoes from non-human primates is believed to be very limited.

Clinical and pathologic manifestation in humans

Following infection by the bite of an infected mosquito, the virus is thought to replicate in local dendritic cells. Subsequent infection of macrophages and lymphocytes is followed by entry into the blood stream. Haematogenous spread is the likely mechanism for seeding of peripheral organs and the occasional reports of central nervous system (CNS) infections, which can lead to symptomatic illness.

Most DENV infections are either asymptomatic or only mildly symptomatic. The incubation period of dengue can range from 3 to 14 days, but is generally 4 to 7 days. Most symptomatic DENV infections present with a sudden onset of fever accompanied by headache, pain behind the eyes, generalized myalgia and arthralgia, flushing of the face, anorexia, abdominal pain and nausea. Rash is common in dengue and can be macular, maculopapular, morbilliform, scarlatiniform or petechial in character. Rash is most often seen on the trunk, insides of the arms and thighs, and plantar and palmar surfaces. Laboratory abnormalities that can be observed in dengue infection include leukopenia and thrombocytopenia.

Dengue illness is classified as (i) dengue with or without warning signs and (ii) severe dengue. A presumptive diagnosis of dengue can be made in a patient living in or traveling from a dengue-endemic area who has fever and at least two of the following clinical signs or symptoms: anorexia and nausea, rash, body aches and pains, warning signs, leukopenia, and a positive tourniquet test. "Warning signs" include abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy or restlessness, liver enlargement of > 2 cm, or an increase in haematocrit concurrent with a rapid decrease in platelet count. Dengue illness should be classified as severe in a patient with presumptive dengue and any of the following: severe plasma leakage leading to shock or respiratory compromise, clinically significant bleeding, or evidence of severe organ involvement. Detailed case classification of dengue is provided in a separate document (WHO/HTM/NTD/DEN/2009.1 or its update) [70].

Non-human primate dengue virus infection

Natural hosts for DENV infection are humans and mosquitoes. Serological evidence from nonhuman primate studies indicates a sylvatic DENV cycle involving several species of mosquitoes and several monkey species exists. Although monkeys develop a viremia and a neutralizing antibody response to DENV infection, they do not develop the hematologic abnormalities seen in humans. However, in non-human primate (NHP) (and AG129 mouse, see paragraph below) primary DENV infections cause a leukopenia.

Thrombocytopenia has been observed after a secondary infection [5, 8, 16, 19]. In the rhesus macaque, viremia typically begins 2-6 days after infection and lasts for 3-6 days [5, 7, 16]. Virus spreads to regional lymph nodes and can be isolated from the skin, distant lymph nodes, and rarely from spleen, thymus and other body organs. The NHP model for DENV is useful to measure protection from viremia conferred by vaccination or passively acquired antibody. Disadvantages of the NHP model include the lack of overt clinical signs of disease [8, 16, 19, 51].

Mouse dengue virus infection

Clinical isolates of DENV do not replicate well in genetically normal mice. However, mouse-brain-adapted DENVs can induce fatal encephalitis after intracranial inoculation of suckling mice. It has been demonstrated that adaptation of a DENV2 isolate to neurovirulence in suckling mice correlated positively with attenuation of virulence in humans [43]. Because of this ambiguity, the suckling mouse/encephalitis model is probably not useful for studying candidate DEN vaccine safety or efficacy. Nevertheless, it could be used to assess lot consistency (see A.3.2.5.5.2 and A.4.2.4.7). In recent years, both chimeric mice that are transplanted with human cells and severely immunocompromised strains of mice have been used to elucidate the immune response to DEN infection and to study pathogenesis [8, 55, 76]. Interferon receptor -deficient AG129 mice support replication of selected DENV strains which infect relevant cell and tissue types comparable to human infection [47, 55]. AG129 mice have been used to investigate antibody-mediated protection. A strain of DENV2 that has been adapted to AG129 mice by serial passage between mice and mosquito cells has a viscerotropic

phenotype, causing thrombocytopenia and vascular leakage in the infected animals. The phenomenon of antibody-dependent enhancement (ADE) of virus infection was observed in AG 129 mice following passive transfer of anti-DENV1 antibodies and challenge with the adapted strain of DENV2 [2, 47, 55]. The relevance of such immunocompromised mouse model may be however limited with regard to vaccine evaluation (see B.4.3).

Mosquito dengue virus infection

Vector competence refers to the efficiency with which the vector transfers infection between hosts. Typically, this is a product of vector susceptibility to infection, replication efficiency of the pathogen in the vector, and the sensitivity of the host to infection transmitted by vector contact. *Aedes aegypti* mosquitoes exhibit global variation in vector competence for flaviviruses. For example, in sub-Saharan Africa, a black “sylvan” subspecies, *Ae. formosus* predominates. This mosquito has a low vector competence for flaviviruses due primarily to a midgut infection barrier [3]. Once ingested in an infectious blood meal, DENVs should replicate in the midgut and disseminate to the salivary glands to facilitate transmission to a new host during feeding. In this process, the virus should overcome any midgut barrier that would limit replication and prevent spread of the virus to other tissues in the mosquito [19, 44, 45].

None of the live DEN vaccine preparations currently in the clinical trial phase of development is effectively transmitted by mosquito vectors [29, 45], because vaccine viruses replicate poorly in mosquito midgut epithelium, and/or do not disseminate efficiently to the salivary glands, thereby interdicting transmission to humans [19, 22, 50]. In addition, the low peak titre and very short duration of viremia induced by these candidates in humans has been shown to render vaccinees relatively non-infectious for feeding mosquitoes. The net effect of these two phenomena is a drastic reduction in vector competence.

At-risk populations and global health importance

Dengue is the most rapidly spreading mosquito-borne viral disease in the world. Since 1955, the incidence of dengue and severe dengue reported to WHO has increased approximately 30-fold with increasing geographic expansion to new countries and from urban to rural settings. Approximately 3.5 billion people live in dengue endemic countries which are located in the tropical and subtropical regions of the world. An estimated 50 million dengue infections occur annually and the number of cases reported annually to WHO ranged from 0.4 to 1.3 million in the decade 1996 - 2005 [70].

Justification for vaccine development

Prevention of dengue by vector control has proven to be very difficult and costly. While vector control efforts should be sustained, vaccination holds substantive potential in the control of the disease. Hence, there is an urgent need to develop dengue vaccines, especially to protect people from disease in endemic countries.

Development of candidate dengue vaccines

Efforts to develop a vaccine against dengue have primarily focused on live attenuated viruses, derived by serial passage of virulent viruses in tissue culture or via recombinant DNA technology that permits site-directed mutagenesis of the genome of a virulent parent strain. Success in the development, licensure, and clinical use of live attenuated flavivirus vaccines, such as yellow fever 17D vaccines and Japanese encephalitis SA14-14-2, suggests that a suitably attenuated live dengue vaccine could be highly efficacious. Other vaccine candidates based on inactivated whole virus, subunits that include E protein, virus-like particles comprised of prM and E proteins, and DNA vaccines that induce expression of DENV prM/E proteins and are in preclinical or early clinical stages of development.

There are no animal models that completely mimic the protean manifestations of dengue. The lack of a suitable animal model makes it difficult to assess efficacy of vaccine candidates and to identify/establish possible correlates of protection *in vivo*. Therefore, the protective capacity of any vaccine candidate will be finally defined by its ability to protect humans from dengue febrile illness (DFI). Results of non-clinical studies using monkeys and susceptible mouse strains suggest, however, that protection from dengue is best correlated with the presence of virus-neutralizing antibodies [1, 5, 17, 26, 27, 30, 42, 54]. Studies in which vaccinated volunteers were challenged with dengue viruses have been conducted in the past but are not a required part of currently recommended clinical development programs.

There is general agreement that DENV vaccines should ideally induce protective neutralizing antibodies to each of the four serotypes simultaneously. In theory, a tetravalent immune response would protect against all DFI and would also reduce or eliminate the risk of a phenomenon termed antibody-dependent enhancement of disease, which is thought to be one of the mechanisms that predispose to severe forms of dengue.

Several strategies have been employed to derive candidate live attenuated vaccines. There are four candidates that are in clinical development at the present time.

The Walter Reed Army Institute of Research (WRAIR) developed attenuated DENV strains by empirical serial passage in primary dog kidney (PDK) cells and produced vaccine candidates in fetal rhesus lung (FRhL) cells. Tetravalent formulations of these attenuated vaccine candidates have been evaluated in Phase I and Phase II clinical trials conducted by WRAIR and GlaxoSmithKline [21, 23, 51, 52].

The other three candidates were developed using recombinant DNA technology which involves first the generation of a full-length DNA copy of the DENV genome. Site-specific mutations expected to affect virulence are then introduced into the DNA, and mutant full-length DNAs can then be copied *in vitro* to produce infectious RNA transcripts that can be used to generate mutant DENVs in tissue culture. The Laboratory of Infectious Diseases of the U.S. National Institute of Allergy and Infectious Diseases (NIAID) has thus derived a total of five candidate DEN vaccine viruses that have been

tested in clinical trials. Two were generated by introduction of a 30-nucleotide deletion (termed $\Delta 30$) into the 3' - UTR of the DENV-4 and DENV-1 genomes [21, 23, 51, 52]. These DENV-1 and DENV-4 vaccine candidates were shown to be attenuated and immunogenic in nonhuman primates. A DENV-2 candidate vaccine was developed by replacing the gene segments encoding the prM and E proteins of the DEN4 $\Delta 30$ candidate vaccine with those of DENV-2. An additional DENV-3 candidate vaccine was developed by replacing the 3' UTR of a DENV-3 wild-type virus with that of the DEN4 $\Delta 30$ UTR. A third DENV-3 candidate vaccine was developed by introducing a 30 nucleotide deletion into the 3' UTR homologous to that of the DEN4 $\Delta 30$ vaccine virus and a second non-contiguous 31 nucleotide deletion, also in the 3' UTR [6]. Phase I trials have been conducted with " $\Delta 30$ " monovalent vaccines [7, 21, 51], and Phase I trials with the tetravalent formulation were initiated in 2010.

Thai scientists at Mahidol University developed a candidate DENV-2 vaccine empirically by 53 serial passages of the virus in PDK cells, designated DENV-2 strain PDK53, which was found to be highly attenuated and immunogenic in Phase I and Phase II clinical trials. The U.S. Centers for Disease Control and Prevention (CDC) determined that the attenuation mutations of DENV-2 PDK53 virus were not located in the prM or E proteins, and in collaboration with InViragen, Inc. used this genetic background to derive chimeric DENV-1, -3 and -4 vaccines expressing the respective prM/E genes in the context of the DENV-2 PDK53 genome "backbone" [21, 51]. A tetravalent formulation is in Phase I clinical trials.

Finally, a candidate live vaccine was developed by Acambis/Sanofi Pasteur using the live attenuated yellow fever virus (YFV) vaccine, 17D, as the backbone for chimeric DENV vaccine candidate. In these viral genomes, the prM and E genes from each of the four DENV serotypes, respectively, are substituted for those of YFV in the context of the genetic background of the 17D vaccine. A tetravalent chimeric YFV-DENV vaccine has been evaluated in Phase I and II clinical trials for safety and immunogenicity. Phase IIb trials to investigate protective efficacy in children began late in 2009 [21, 51] and Phase III trials have been in progress since late 2010.

Part A. Guidelines on manufacturing and control of dengue tetravalent vaccines (live, attenuated)

A.1 Definitions

A.1.1 International name and proper name

Although there is no licensed dengue vaccine, the provision of a suggested international name at this early stage of development will aid harmonization of nomenclature if licensure is obtained. The international name should be “dengue tetravalent vaccine, live attenuated”. The proper name should be the equivalent to 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 specifications formulated below.

A.1.2 Descriptive definition

A live attenuated tetravalent dengue virus vaccine defined in <A.1.1> should contain live attenuated dengue viruses representing each of the four serotypes, or replication-competent viral vectors that express the major structural antigen genes of each of the four dengue serotypes, that have been separately prepared in cell culture. It may be presented as a sterile, aqueous suspension or as freeze-dried material. The preparation should satisfy all of the specifications given below.

A.1.3 International reference materials

As the prospective vaccines are very different in type, no international reference material for a candidate live dengue vaccine is available. However, an international reference panel of human antisera against all four dengue serotypes is available from the National Institute of Biological Standards and Control (NIBSC). The panel is intended to help calibrate the response to vaccines.

A.1.4 Expression of dose related to vaccine potency

Potency of a live vaccine most often is expressed in terms of the number of infectious units of virus contained in a human dose, using a specified tissue culture substrate and based on results of phase I and II clinical trials. In the case of a tetravalent dengue vaccine, potency will have to be assessed in terms of the individual titers of each of the four serotypes of vaccine virus contained in a human dose. When international reference standards for the vaccine type under production become available, the dose related to vaccine potency should be calculated against the international standard and expressed in international unit (IU) to reduce variation between laboratories. Until then, the use of PFU, IFU or CCID₅₀ to express the potency and doses of vaccine can be an alternative. The dose should also serve as a basis for establishing parameters for stability and for the expiry date.

A.1.5 Terminology

The definitions given below apply to the terms as used in these Guidelines. They may have different meanings in other contexts.

Adventitious agents

Contaminating microorganisms of the cell culture or source materials including bacteria, fungi, mycoplasmas/spiroplasmas, mycobacteria, rickettsia, protozoa, parasites, transmissible spongiform encephalopathies (TSE) agents, and viruses that have been unintentionally introduced into the manufacturing process of a biological product.

Cell bank

A cell bank is a collection of appropriate containers whose contents are of uniform composition stored under defined conditions. Each container represents an aliquot of a single pool of cells.

Cell culture infectious dose 50% (CCID₅₀)

A measure of the number of infectious viruses per unit volume of virus suspension as determined in an end-point dilution assay in monolayer cell cultures. One CCID₅₀ / mL corresponds to the quantity of viruses capable of producing a cytopathic effect in 50% of replicate cell cultures per millilitre of virus suspension.

Cell seed

A quantity of vials containing well-characterized cells derived from a single tissue or cell of human or animal origin stored frozen in liquid nitrogen in aliquots of uniform composition, one or more of which would be used for the production of a master cell bank.

Cell substrates

Cells used for the production of a vaccine. Cells are derived from a master cell bank or a working cell bank.

Dengue febrile illness (DFI)

DFI is defined by the detection of dengue virus in patients with two days of fever irrespective of severity of illness.

Final lot

A collection of sealed final containers of finished vaccine that is homogeneous with respect to the risk of contamination during filling and freeze-drying. All the final containers should, therefore, have been filled from one vessel of final tetravalent bulk and freeze-dried under standardized conditions in a common chamber in one working session.

Final tetravalent bulk

The homogeneous finished tetravalent vaccine prepared from one or more virus harvest pools in the vessel from which the final containers are filled.

Genetically modified organism (GMO)

An organism in which the genetic material has been altered in a way that does not occur naturally by mating and /or natural recombination.

Immunofocus-forming unit (IFU)

A measure of the number of infectious viruses capable of forming plaques that can be detected using dengue-specific antisera and a counter-stain in monolayer cell cultures per unit volume of virus suspension.

Master cell bank (MCB)

A quantity of well-characterized cells of animal or other origin, derived from a cell seed at a specific population doubling level (PDL) or passage level, dispensed into multiple containers, cryopreserved, and stored frozen under defined conditions, such as the vapour or liquid phase of liquid nitrogen in aliquots of uniform composition. The master cell bank is prepared from a single homogeneously mixed pool of cells. It is considered best practice that the master cell bank is used to derive working cell banks.

Virus master seed (VMS)

A suspension of vaccine virus that has been aliquoted into identical vials and stored at a temperature and under conditions deemed to stabilize the virus in each container. The VMS is used as a source of infectious virus for generation of each virus working seed lot.

Monovalent virus pool

A monovalent virus pool is a suspension of single serotype of dengue virus and may be the result of one or more single harvests or multiple parallel harvests of the same virus serotype collected into a single vessel before clarification.

Multiple parallel harvest

A pool of harvests coming from multiple cultures that are initiated in parallel from the same ampoule of the same working cell bank infected together by the same virus suspension of the same virus working seed lot

Plaque-forming unit (PFU)

A measure of the number of infectious viruses capable of forming plaques, as detected by virtue of a cytopathic effect, in monolayer cell cultures per unit volume of virus suspension.

Production cell culture (PCC)

A collection of cell cultures used for biological production that have been prepared together from one or more containers from the working cell bank or, in case of primary cell cultures, from the tissues of one or more animals.

Single harvest

A quantity of virus suspension harvested from production cell cultures inoculated with the same working seed lot and processed together in a single production run.

Working cell bank (WCB)

A quantity of well-characterized cells of animal or other origin, derived from the master cell bank at a specific PDL or passage level, dispensed into multiple containers, cryopreserved, and stored frozen under defined conditions, such as in the vapour or liquid phase of liquid nitrogen in aliquots of uniform composition. The working cell bank is prepared from a single homogeneously mixed pool of cells. One or more of the WCB containers is used for each production culture.

Virus working seed (VWS)

A quantity of virus of uniform composition, well characterized, derived from a virus master seed lot (see above) by passage, in an approved production cell line. The working seed lot is used for the production of single harvest.

A.2 General manufacturing requirements

The general requirements for manufacturing establishments contained in the WHO *Good manufacturing practices for biological products* [58] should be applied by establishments manufacturing dengue tetravalent vaccine.

Separate manufacturing areas for each of the four dengue serotypes as well as tetravalent vaccine formulation may be used. Alternatively, manufacturing areas may be used on a campaign basis with adequate cleaning between campaigns to ensure that cross-contamination does not occur.

Production steps and quality-control operations involving manipulations of live virus should be conducted under the appropriate biosafety level as agreed with the national regulatory authority and country biosafety laws.

A.3 Control of source materials

A.3.1 Cell cultures for virus production

A.3.1.1 Conformity with WHO recommendations

Dengue viruses used in producing tetravalent dengue vaccine should be propagated in cell substrates which meet the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks [74] and approved by the national regulatory authority. All information on the source and method of preparation of the cell culture system used should be made available to the national regulatory authority.

A.3.1.2 Types of cell culture

Dengue vaccine candidates have been produced in fetal rhesus lung diploid cells and in continuous cell lines. For fetal rhesus lung diploid cells and continuous cells, sections A.3.1.3 and A.3.1.4 apply.

A.3.1.3 Cell banks

The use of a cell line such as fetal rhesus lung diploid cells or Vero cells for the manufacture of dengue vaccines should be based on the cell bank system. The cell seed should be approved by the national regulatory authority. The maximum number of passages or population doubling (PDL) allowable between the cell seed, the WCB and the production passage levels should be established by the manufacturer and approved by the national regulatory authority. Additional tests may include:

- Propagation of the MCB or WCB cells to or beyond the maximum in vitro age for production; and
- Examination for the presence of retroviruses and tumorigenicity in an animal test system [74].

WHO has established a bank of Vero cells, designated as WHO Vero reference cell bank (RCB) 10-87 that has been characterized in accordance with the WHO *Requirements for continuous cell lines used for biologicals production*, WHO Technical Report Series 745, 1987 [57]. The cell bank is available to manufacturers as a well-characterized starting material for preparation of their own master and working cell banks on application to the

Coordinator, Quality, Safety and Standards, WHO, Geneva, Switzerland [74].

In normal practice a master cell bank is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer and approved by the national regulatory authority, at which point the cells are combined to give a single pool distributed into ampoules and preserved cryogenically to form the WCB.

The manufacturer's WCB is used for the preparation of production cell culture, and thus for production of vaccine batches

A.3.1.4 Characterizations of cell banks

The cell seed (if applicable), master and working cell banks and end of production cells (EOPC) or extended cell bank (ECB) should be characterized according to the WHO *Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks* [74].

A.3.1.5 Cell culture medium

Serum used for propagating cells should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the requirements given in Part A, sections 5.2 and 5.3 of the *General requirements for the sterility of biological substances (Requirements for biological substances, no. 6)* [56, 61], and from infectious viruses.

Detailed guidelines for detecting bovine viruses in serum for establishing MCB and WCB are given in Appendix 1 of the WHO *Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks* [74]. The principles outlined in the cell substrate recommendations should be applied as appropriate, and the guidelines for detecting bovine viruses in serum for establishing the cell banks may be applicable to production cell cultures as well. In particular, validated molecular tests for bovine viruses might replace the cell-culture tests of bovine sera if agreed by the national regulatory authority. As an additional monitor of quality, sera may be examined for freedom from phage and endotoxin. Gamma-irradiation may be used to inactivate potential contaminant viruses, recognizing that some viruses are relatively resistant to gamma-irradiation.

The sources of animal components used in culture medium should be approved by the national regulatory authority. These components should comply with current guidelines in relation to animal transmissible spongiform encephalopathies [63, 67].

Human serum should not be used. If human albumin is used, it should meet the revised *Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (Requirements for biological substances no. 27)* [60], as well as current guidelines in relation to human transmissible encephalopathies [63, 67].

The use of human albumin as a component of a cell culture medium requires careful consideration due to potential difficulties with the validity period of albumin (which is

based on the length of time for which it is suitable for use in clinical practice) in relation to the potential long-term storage of monovalent bulks of each dengue serotype. In addition, if human albumin is used, it should be tested according to the WHO *Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks* [74].

Penicillin and other beta-lactams should not be used at any stage of the manufacture. Other antibiotics may be used at any stage in the manufacture provided that the quantity present in the final product is acceptable to the national regulatory authority. Nontoxic pH indicators may be added, e.g. phenol red at a concentration of 0.002%. Only substances that have been approved by the national regulatory authority may be added.

If porcine or bovine trypsin is used for preparing cell cultures, it should be tested and found free of cultivable bacteria, fungi, mycoplasmas and infectious viruses as appropriate [74]. The methods used to ensure this should be approved by the national regulatory authority.

The source(s) of trypsin of bovine origin, if used, should be approved by the national regulatory authority and should comply with current guidelines in relation to animal transmissible spongiform encephalopathies [63, 67].

A.3.2 Virus seeds

A.3.2.1 Vaccine virus strains

The strains of DENV 1-4 viruses attenuated either by serial passage in cell cultures or by recombinant DNA technology used in the production of candidate tetravalent dengue vaccine should be thoroughly characterized. This will include their historical records, such as information on the origin of the strain, cell culture passage history, method of attenuation, results of preclinical and clinical studies demonstrating attenuation, whether the strains have been biologically or molecularly cloned prior to generation of the master seed; their genome sequence; and the passage level at which clinical trials were performed, as well as the results of the clinical studies.

Only strains approved by the national regulatory authority should be used.

Strains of dengue recombinant viruses used for master and working seeds to produce vaccine candidates should comply with the additional specifications given below (section *A.3.2.2 Strains derived by molecular methods*).

A.3.2.2 Strains derived by molecular methods

If vaccine seeds derived by recombinant DNA technology are used, and because this is a live attenuated vaccine, the candidate vaccine is considered a GMO in several countries and should comply with the regulations of the producing and recipient countries regarding GMOs. An environmental risk assessment should be undertaken according to Part D of these Guidelines.

The nucleotide sequence of any cDNA clone used to generate vaccine virus stocks should

be determined prior to transfection of DNA-derived RNA into the cell substrate. The cell substrate used for transfection to generate the virus should be appropriate for human vaccine production and approved by the national regulatory authority.

Pre-seed lot virus stocks derived from passaging of the primary virus stock should also be sequenced as part of preclinical evaluation.

Viral vaccine seeds that are directly re-derived from RNA extracted from virus, to reduce the risk of previous contamination by TSE or other adventitious agents, are considered as new vaccine seeds and should be appropriately characterized to demonstrate comparability with the starting virus seed.

A.3.2.3 Virus seed lot system

The production of vaccine should be based on the master and working seed lot system to minimize the number of tissue culture passages needed for vaccine production. Seed lots should be prepared in the same type of cells using the same conditions for virus growth (other than scale) as those used for production of final vaccine.

The virus working seed lot should be prepared by defined number of passages from the virus master seed lot by a method and a passage level from the original virus seed that is established through clinical and vaccine development studies. Once the passage level of the working seed lot is established, it may not be changed without approval from the national regulatory authority.

Virus seed lots should be stored in a dedicated temperature-monitored freezer at a temperature that ensures stability upon storage. It is recommended that a large working virus seed lot be set aside as the basic material for use by the manufacturer for the preparation of each batch of vaccine.

Full in vitro and in vivo testing for detecting adventitious agents should be conducted on either master or working seed lots.

A.3.2.4 Control cell cultures for virus seeds

In agreement with national regulatory authorities, tests on control cell cultures may be required and performed as described in section A.4.1.

A.3.2.5 Tests on virus master and working seed lots

A.3.2.5.1 Identity

Each virus master and working seed lot should be identified as the appropriate dengue virus serotype by immunological assay or by molecular methods.

A.3.2.5.2 Genetic/phenotypic characterization

Different live dengue vaccine viruses may have significantly different properties. Such differences may influence the tests to be used to examine their genetic and phenotypic

stability relevant to consistency of production. The applicable tests will be identified in the course of the nonclinical evaluation of the strains. Each seed should be characterized by full-length nucleotide sequence determination and by other relevant laboratory and animal tests, which will provide information on the consistency of each virus seed.

Mutations introduced during derivation of each vaccine strain should be maintained in the consensus sequence, unless spontaneous mutations induced during tissue culture passage were shown to be innocuous in non-clinical and small-scale clinical trials. Some variations in the nucleotide sequence of virus population on passage are to be expected, but what is acceptable should be based on experience in production and clinical use.

For any new virus master and working seeds, it is recommended that the first three consecutive consistency bulk vaccine lots be analysed for consensus sequence changes from the virus master seed. The nucleotide sequence results should be used to demonstrate the consistency of the production process.

Routine nucleotide sequence analysis of bulk vaccine is not recommended.

A.3.2.5.3 Tests for bacteria, fungi mycoplasmas and mycobacteria

Each virus master and working seed lot should be shown to be free from bacterial, fungal and mycoplasmal contamination by appropriate tests as specified in Part A, sections 5.2 and 5.3, of the *General requirements for the sterility of biological substances (Requirements for biological substances, no. 6)* [56, 61]. Nucleic acid amplification techniques (NAAT) alone or in combination with cell culture, with an appropriate detection method, might be used as an alternative to one or both of the compendial mycoplasma detection methods after suitable validation and agreement from the national regulatory authority [74].

Seed lots should be shown to be free from mycobacteria by a method approved by the national regulatory authority. NAAT might be used as an alternative to mycobacteria microbiological culture method and/or to the in vivo guinea-pigs test for the detection of mycobacteria after suitable validation and agreement from the national regulatory authority [74].

A.3.2.5.4 Tests for adventitious agents

Each virus working seed lot and/or master seed lot should be tested in cell cultures for adventitious viruses relevant to the passage history of the seed virus. Where antisera are used to neutralize dengue virus or the recombinant dengue virus, the antigen used to generate the antisera should be produced in cell culture from a species different from that used for the production of the vaccine and free from extraneous agents. Simian and human cell cultures inoculated with the virus-antibody mixture should be observed microscopically for cytopathic changes. For virus grown in simian or human cells, the neutralized virus is tested on a separate culture of these cells. If other cell systems are used, cells of that species, but from a separate batch, are also inoculated. At the end of the observation period, the cells should be tested for haemadsorbing viruses.

Each virus master seed lot or working seed lot should also be tested in animals that include guinea pigs, adult mice, and suckling mice. For test details, refer to the section

B.11 of the WHO *Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks* [74]. Additional testing for adventitious viruses may be performed using validated NAAT.

New molecular methods with broad detection capabilities are being developed for adventitious agent detection. These methods include degenerate NAAT for whole virus families with analysis of the amplicons by hybridization, sequencing or mass spectrometry; NAAT with random primers followed by analysis of the amplicons on large oligonucleotide micro-arrays of conserved viral sequencing or digital subtraction of expressed sequences; and high throughput sequencing. These methods might be used in the future to supplement existing methods or as alternative methods to both in vivo and in vitro test after appropriate validation and agreement from the national regulatory authority.

A.3.2.5.5 Tests in experimental animals

A.3.2.5.5.1 Tests in non-human primates

All vaccine candidates should be evaluated at least once during pre-clinical development for neurotropism in non-human primates, as detailed in Part B (nonclinical evaluation). Candidate vaccines that are homogeneously “dengue” in terms of genetics are not expected to be neurotropic in such a test, but where attenuation has been achieved by recombination of dengue genes with those of a different virus species that itself displays neurotropism (e.g. dengue/yellow fever recombinants); the master seed should be tested in non-human primates. If these tests were not performed at the master seed level, they should be performed at working seed level.

National regulatory authorities may decide that such testing does not need to be repeated each time a novel Working seed lot is derived, if results of a well conducted monkey neurovirulence assay on the Master seed lot are negative. Recent data suggest that certain small animal models for neurovirulence may serve as a surrogate for non-human primates, at least where viruses expressing YF strain 17D genes are concerned [38]. National regulatory authorities may eventually wish to consider accepting results of such studies as a surrogate for studies using non-human primates to evaluate neurovirulence of novel dengue vaccines.

Test for neurotropism/neurovirulence

To provide assurance that a candidate vaccine virus is not unexpectedly neurovirulent, each vaccine strain of each serotype should be tested for neurovirulence in monkeys by inoculation of *Macaca mulatta* (rhesus), *Macaca fascicularis* (cynomolgus) or other susceptible species of monkey in the course of preclinical evaluation.

Prior to testing for neurotropism, the neutralizing antibody test should be used to assess the immune status of non-human primates to both dengue and yellow fever viruses. Tests should follow the WHO *Recommendations to assure the quality, safety and efficacy of*

live attenuated yellow fever vaccines [75].

A.3.2.5.5.2 Tests in suckling mice

The virulence of different vaccine candidates in mice will depend on the strains of virus and mouse. Novel vaccines that reach the clinical phase of development in many cases were tested for neurovirulence in suckling and adult mice during the pre-clinical phase of development.

While mice are not considered a good model for dengue, suckling and adult mice have been used to assess the neurovirulence of dengue/yellow fever recombinant vaccines [17, 18]. A mouse test might be considered in order to demonstrate consistency of characteristics of dengue/yellow fever recombinant viruses during production (*see A.4.2.4.7*).

A.3.2.5.6 Virus titration for infectivity

Each virus master and working seed lot should be assayed for infectivity in a sensitive assay in cell culture. Depending on the results obtained in preclinical studies, plaque assays, CCID₅₀ assays, IFU assays or CCID₅₀ with a molecular readout such as quantitative polymerase chain reaction (QPCR) may be used. All assays should be validated.

A.4 Control of vaccine production

A.4.1 Control of production cell cultures

Where the national regulatory authority requires the use of control cells, the following procedures should be followed. From the cells used to prepare cultures for production of vaccine, a fraction equivalent to at least 5 % of the total or 500 mL of cell suspension, or 100 million cells, should be used to prepare uninfected control cell cultures. These control cultures should be observed microscopically for cytopathic and morphologic changes attributable to the presence of adventitious agents for at least 14 days at a temperature of 35-37 °C after the day of inoculation of the production cultures, or until the time of final virus harvest, whichever comes last. At the end of the observation period, supernatant fluids collected from the control culture should be tested for the presence of adventitious agents as described below. Samples that are not tested immediately should be stored at -60 °C or lower, until such tests can be conducted.

If adventitious agent testing of control cultures yields a positive result, the harvest of virus from the parallel vaccine virus-infected cultures should not be used for production.

For the test to be valid, not more than 20 % of the control culture flasks should have been discarded, for any reason, by the end of the test period.

A.4.1.1 Test for haemadsorbing viruses

At the end of the observation period, a fraction of control cells comprising not less than 25% of the total should be tested for the presence of haemadsorbing viruses, using

guinea-pig red blood cells. If the guinea pig red blood cells have been stored prior to use in the haemadsorption assay, the duration of storage should not have exceeded 7 days, and the storage temperature should have been in the range of 2–8 °C.

In some countries, the national regulatory authority requires that additional tests for haemadsorbing viruses be performed using red blood cells from other species including those from humans (blood group O), monkeys, and chickens (or other avian species). For all tests, readings should be taken after incubation for 30 minutes at 0–4 °C, and again after a further incubation for 30 minutes at 20–25 °C. The test with monkey red cells should be read once more after an additional incubation for 30 minutes at 34–37 °C.

For the tests to be valid, not more than 20 % of the culture vessels should have been discarded for any reason by the end of the test period.

A.4.1.2 Tests for cytopathic, adventitious agents in control cell fluids

Supernatant culture fluids from each of control cell culture flasks or bottles collected at the time of harvest should be tested for adventitious agents. A 10-mL sample of the pool should be tested in the same cell substrate, but not the same cell batch as that used for vaccine production, and an additional 10-mL sample of each pool should be tested in both human and continuous simian cells.

Each sample should be inoculated into cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1:4. The surface area of the flask should be at least 3 cm² per mL of pooled fluid. At least one flask of the cells should remain uninoculated, as a control.

The inoculated cultures should be incubated at a temperature of 35–37 °C and should be examined at intervals for cytopathic effects over a period of at least 14 days.

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

For the tests to be valid, not more than 20 % of the culture vessels should have been discarded for any reason by the end of the test period.

A.4.1.3 Identity test

At the production level, the cells should be identified by means of tests approved by the national regulatory authority. Suitable methods are, but are not limited to, biochemical tests (e.g. isoenzyme analyses), immunological tests (e.g. major histocompatibility complex assays), cytogenetic tests (e.g. for chromosomal markers), and tests for genetic markers (e.g. DNA fingerprinting).

A.4.2 Production and harvest of monovalent virus

A.4.2.1 Cells used for vaccine production

On the day of inoculation with the working seed virus, each production cell culture flask (or bottle, etc) and/or cell culture control flask should be examined for cytopathic effect potentially caused by infectious agents. If such examination shows evidence of the presence in any flask of an adventitious agent, all cell cultures should be discarded.

If animal serum is used in growth medium, the medium should be removed from the cell culture either before or after inoculation of working virus seed. Prior to beginning virus harvests, the cell cultures should be rinsed and the growth medium replaced with serum-free maintenance medium.

Penicillin and other beta-lactam antibiotics should not be used at any stage of manufacture.

Minimal concentrations of other suitable antibiotics may be used if approved by the national regulatory authority.

A.4.2.2 Virus inoculation

Cell cultures are inoculated with dengue working seed virus at an optimal and defined multiplicity of infection. After viral adsorption, cell cultures are fed with maintenance medium and incubated at a temperature within a defined range and for a defined period.

The multiplicity of infection, temperature range and duration of incubation will depend on the vaccine strain and production method, and specifications should be defined by each manufacturer.

A.4.2.3 Monovalent virus harvest pools

Vaccine virus is harvested within a defined period post-inoculation. A monovalent harvest may be the result of one or more single harvests or multiple parallel harvests. Samples of monovalent virus harvest pools should be taken for testing and stored at a temperature of -60 °C or below. The sponsor should submit data to support the conditions chosen for these procedures.

The monovalent virus harvest pool may be clarified or filtered to remove cell debris and stored at a temperature that ensures stability before being used to prepare the tetravalent final bulk for filling. The sponsor should provide data to support the stability of the bulk over the duration of the chosen storage conditions as well as to support the choice of storage temperature .

Harvests derived from continuous cell lines should be subjected either to further purification to minimize the amount of cellular DNA and/or treatment with DNase to reduce the size of the DNA.

A.4.2.4 Tests on monovalent virus harvest pools

A.4.2.4.1 Identity

Each monovalent virus harvest pool should be identified as the appropriate dengue virus serotype by immunological assay on cell cultures using specific antibodies or by molecular methods (*see section A.6.1*) approved by the national regulatory authority.

A.4.2.4.2 Tests for bacteria, fungi, mycoplasmas, and mycobacteria

Each monovalent virus harvest pool should be shown to be free from bacterial, fungal, mycoplasmal, and mycobacterial contamination by appropriate tests. Sterility tests are specified in Part A, sections 5.2 (bacteria and fungi) and 5.3 (mycoplasmas), of the General requirements for the sterility of biological substances (Requirements for biological substances, no. 6) [56, 61].

NAAT alone or in combination with cell culture, with an appropriate detection method, might be used as an alternative to one or both of the pharmacopoeial mycoplasma detection methods after suitable validation and the agreement of the national regulatory authority [74].

For testing mycobacteria, the test method should be approved by the national regulatory authority. NAAT might be used as an alternative to mycobacteria microbiological culture method after validation and agreement of the national regulatory authority [74].

A.4.2.4.3 Tests for adventitious agents

Each monovalent virus harvest pool should be tested in cell culture for adventitious viruses by inoculation into continuous simian kidney cells, cell lines of human origin, and the cell line used for production, but from another batch. Where antisera are used to neutralize dengue virus or the recombinant virus, the antigen used to generate the antisera should be produced in cell culture from a species different from that used for the production of the vaccine and free from extraneous agents. The cells inoculated should be observed microscopically for cytopathic changes. At the end of the observation period, the cells should be tested for haemadsorbing viruses.

Additional testing for adventitious viruses may be performed using validated NAAT.

New molecular methods with broad detection capabilities are being developed. These methods include degenerate NAAT for whole virus families with analysis of the amplicons by hybridization, sequencing or mass spectrometry; DNA amplification with random primers followed by analysis of the amplicons on large oligonucleotide microarrays of conserved viral sequencing or digital subtraction of expressed sequences; and high throughput sequencing. These methods might be used in the future to supplement existing methods or as alternative methods to in vitro test after appropriate validation and agreement from the national regulatory authority.

A.4.2.4.4 Virus titration for infectivity

The titre for each monovalent virus harvest should be determined in a sensitive assay in cell culture. Depending on the results obtained in preclinical studies, plaque assays, CCID₅₀ assays, immunofocus formation assays or CCID₅₀ with a molecular readout such as QPCR may be used.

A.4.2.4.5 Tests for host cell proteins

The host cell protein profile should be examined as part of characterization studies [74].

A.4.2.4.6 Tests for residual cellular DNA

For viruses grown in continuous cell line cells, the monovalent harvest pool should be tested for the amount of residual cellular DNA, and the total amount of cell DNA per dose of vaccine should be not more than the upper limit agreed by the national regulatory authority. If this is technically feasible, the size distribution of the DNA should be examined as a characterization test, taking into account the amount of DNA detectable using state of the art methods [74] as approved by the national regulatory authority.

A.4.2.4.7 Test for consistency of virus characteristics

The dengue virus in the monovalent harvest pool should be tested to compare it with the working seed virus, or suitable comparator, to ensure that the vaccine virus has not undergone critical changes during its multiplication in the production culture system.

Relevant assays should be identified in preclinical studies and may include virus yield in tissue culture, plaque phenotype, or temperature sensitivity as examples. Other identifying characteristics might also be applicable.

Assays for the attenuation of dengue/yellow fever recombinants and others as appropriate include tests in suckling mice. Intracerebral inoculation of suckling mice with serial dilutions of vaccine and yellow fever 17D is followed by the determination of the mortality ratio and survival time. The results obtained with the vaccine are compared to the yellow fever 17D control results.

The test may be omitted as a routine test once the consistency of the production process has been demonstrated on a significant number of batches in agreement with the national regulatory authority. Where there is a significant change in the manufacturing process, the test should be reintroduced.

A.4.2.5 Storage

Monovalent virus harvest pools should be stored at a temperature that ensures stability until tetravalent vaccine formulation.

A.4.3 Final tetravalent bulk lot

A.4.3.1 Preparation of final tetravalent bulk lot

The final tetravalent bulk lot should be prepared from monovalent virus pools of the four dengue virus subtypes using a defined virus concentration of each component.

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

In preparing the final bulk, any excipients (such as diluents or stabilizer) that is added to the product should have been shown to the satisfaction of the national regulatory authority not to impair the safety and efficacy of the vaccine in the concentration used.

A.4.3.2 Tests on the final tetravalent bulk lot

A.4.3.2.1 Residual animal serum protein

If appropriate, a sample of the final bulk should be tested to verify that the level of serum is less than 50 ng per human dose.

A.4.3.2.2 Sterility

Except where it is subject to in-line sterile filtration as part of the filling process, each final bulk suspension should be tested for bacterial and fungal sterility according to Part A, section 5.2 of the *General requirements for the sterility of biological substances (Requirements for biological substances, no. 6)* [56], or by a method approved by the national regulatory authority.

A.4.3.3 Storage

Prior to filling, the final bulk suspension should be stored under conditions shown by the manufacturer to retain the desired viral potency.

A.5 Filling and containers

The requirements concerning good manufacturing practices for biological products [58] appropriate to a vaccine should apply.

Care should be taken to ensure that the materials from which the container and, if applicable, the closure are made do not adversely affect the infectivity (potency) of the vaccine under the recommended conditions of storage.

A final filtration could be included during the filling operations.

The manufacturer should provide the national regulatory authority with adequate data to prove the product is stable under appropriate conditions of storage and shipping.

A.6 Control tests on final lot

The following tests should be carried out on the final lot.

A.6.1 Vaccine

A.6.1.1 Inspection of final containers

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

A.6.1.1.1 Appearance

The appearance of the freeze-dried or liquid vaccine should be described with respect to its form and colour. In the case of freeze-dried vaccines, a visual inspection should be performed of the freeze-dried vaccine, the diluent, and the reconstituted vaccine.

A.6.1.2 pH

The pH of the final lot should be tested in a pool of final containers and an appropriate limit set to guarantee virus stability. In the case of freeze-dried vaccines, pH should be measured after reconstitution of the vaccine with the diluent.

A.6.1.3 Identity

Each monovalent component of a tetravalent dengue vaccine lot should be identified as dengue or recombinant virus type-1, -2, -3 or -4 by immunological assay using specific antibodies or by molecular methods. The methods used for the potency assay (A.6.1.5) may serve as the identity test.

A.6.1.4 Sterility

Vaccine should be tested for bacterial and fungal sterility according to the requirements in Part A, section 5.2 of the *General requirements for the sterility of biological substances (Requirements for biological substances, no. 6)* [56] by acceptable methods approved by the national regulatory authority.

A.6.1.5 Potency

At least three containers of each tetravalent vaccine lot should be assayed for infectivity in a validated assay in appropriate cell culture. The assay should include a working reference preparation to control the accuracy and reproducibility of the testing system. The titre of each individual serotype should be determined.

A.6.1.6 Thermal stability

The thermal stability test is to demonstrate consistency of production. Additional guidance on evaluation of vaccine stability is provided in the WHO *Guideline on stability evaluation of vaccines* [71]. At least three containers of tetravalent vaccine should be incubated at the appropriate elevated temperature for appropriate time (e.g. 37 °C for 7 days) depending on products. The geometric mean infectious virus titre (GMT) of the containers for each individual virus components that have been exposed should not have decreased during the period of exposure by more than an amount (e.g. 1 log) justified by production data and approved by the national regulatory authority. Titration of non-exposed and exposed containers should be done in parallel. A validity control reagent of each of the four virus components should be included in each assay to validate the assay.

A.6.1.7 General safety

Each filling lot should be tested for unexpected toxicity (sometimes called abnormal toxicity) using a general safety test approved by the national regulatory authority. This test may be omitted for routine lot release once consistency of production has been established to the satisfaction of the national regulatory authority and when good manufacturing practices are in place. Each lot, if tested, should pass a general safety test.

A.6.1.8 Residual moisture (if appropriate)

The residual moisture in each freeze-dried lot should be conducive to the stability of the product and the upper limit of the moisture content should be approved by the national regulatory authority based on results of stability testing.

A.6.1.9 Residual antibiotics (if applicable)

If any antibiotics are added in the vaccine production, the content of the residual antibiotics should be determined and be within limits approved by the national regulatory authority.

A.6.2 Diluent

The recommendations given in the *Good manufacturing practices for pharmaceutical products* [62] should apply for the manufacturing and control of diluents used to reconstitute live-attenuated dengue vaccines. An expiry date should be established for the diluent based upon stability data. For lot release of the diluent, tests for identity, appearance, pH, volume, sterility, and the content of key components should be done.

A.7 Records

The recommendations of the *Good manufacturing practices for biological products* [58] pp. 27–28, should apply, as appropriate for the level of development of the candidate vaccine.

A.8 Samples

A sufficient number of samples should be retained for future studies and needs. Vaccine lots that are to be used for clinical trials may serve as reference materials in the future, and a sufficient number of vials should be reserved and appropriately stored for that purpose.

A.9 Labelling

The recommendations of the *Good manufacturing practices for biological products* [58] pp. 26–27, appropriate for a candidate vaccine should apply, with the addition of the following:

The label on the carton enclosing one or more final containers, or the leaflet accompanying the container, should include:

- a statement that the candidate vaccine fulfils Part A of these Recommendations;
- a statement of the nature of the preparation, specifying the designation of the strains of dengue or recombinant viruses contained in the live attenuated tetravalent vaccine, the minimum number of infective units per human dose, the nature of any cellular systems used for the production of the vaccine, and whether the vaccine strains were derived by molecular methods;
- a statement of the nature and quantity, or upper limit, of any antibiotic present in the vaccine;
- an indication that contact with disinfectants is to be avoided;
- a statement concerning the photosensitivity of the vaccine, cautioning that both lyophilized and reconstituted vaccine should be protected from light;
- a statement indicating the volume and nature of diluent to be added to reconstitute the vaccine, and specifying that the diluent to be used is that supplied by the manufacturer; and
- a statement that after the vaccine has been reconstituted, it should be used without delay, or if not used immediately, stored between 2 °C and 8 °C and protected from light for a maximum period defined by stability studies.

A.10 Distribution and shipping

The recommendations given in the *Good manufacturing practices for biological products* [58] appropriate for a candidate vaccine should apply.

Shipments should be maintained at temperatures of 8 °C or below, and packages should contain cold-chain monitors [73].

A.11 Stability, storage and expiry date

The recommendations given in the *Good manufacturing practices for biological products*

[58] and the *Guidelines on stability evaluation of vaccines* [71] appropriate for a candidate vaccine should apply. The statements concerning storage temperature and expiry date that appear on the primary or secondary packaging should be based on experimental evidence and should be submitted for approval to the national regulatory authority.

A.11.1 Stability testing

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

The stability of the vaccine in its final container and at the recommended storage temperatures should be demonstrated to the satisfaction of the national regulatory authorities on at least three lots of final product. Accelerated thermal stability tests may be undertaken on each final lot to give additional information on the overall stability of a vaccine (*see section A.6.1.6*).

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

A.11.2 Storage conditions

Before being distributed by the manufacturing establishment or before being issued from a storage site, the vaccine should be stored at a temperature shown by the manufacturer to be compatible with a minimal loss of titre. The maximum duration of storage should be fixed with the approval of the national regulatory authority and should be such as to ensure that all quality specifications for final product including the minimum titre specified on the label of the container (or package) will still be maintained until the end of the shelf-life.

A.11.3 Expiry date

The expiry date should be defined on the basis of shelf-life and supported by the stability studies with the approval of the national regulatory authority and should relate to the date of the last satisfactory determination, of potency (infectious titer), performed in accordance with section A.6.1.5, i.e. the date on which the cell cultures were inoculated. If the vaccine is stored at a temperature lower than that used for stability studies and intended for release without re-assay, the expiry date is calculated from the date of removal from cold storage. The expiry dates for the vaccine and the diluent may be different.

Part B. Nonclinical evaluation of dengue tetravalent vaccines (live, attenuated)

B.1 General remarks

Nonclinical evaluation of a live dengue vaccine includes in vitro and in vivo testing required prior to initiation of the clinical phase of the vaccine development program. This testing should yield information suggesting the safety and potential for efficacy of a dengue vaccine candidate. Testing may continue in parallel with the clinical phase of product development. Tests should include product characterization at each stage of manufacture (including quantification of contaminants such as cellular proteins and DNA), proof of concept/immunogenicity studies (including dose ranging in animals, etc.), toxicology if required by the national regulatory authority, establishment of a test for potency to be used throughout, and safety testing in animals (see Table B1). These Guidelines should be read in conjunction with *the WHO Guidelines on nonclinical evaluation of vaccines* [65] and are specifically aimed at nonclinical evaluation of a live, attenuated dengue vaccine.

Although there is no animal model that precisely mimics dengue disease in humans, animal models have been and are being used in studies on immunogenicity, protective activity, toxicology, and safety. Animal models are briefly reviewed at the time of preparing these Guidelines to highlight the latest developments and to provide better understanding of their use in vaccine development.

Table B1. Nonclinical evaluation of dengue vaccines

Area of nonclinical evaluation	Primary concern	Scope of nonclinical evaluation
In vitro nonclinical evaluation		
Product characterization	Product risks are appropriate for the anticipated use	Mutations in the genome may impact infection efficiency and growth capacity in different cell types including cells of a monocyte lineage. Virus structural protein profiles, serotype identity. Consistency of manufacturing process; genetic stability of vaccine candidates.
Process development, quality control and quality assurance	Process meets all good manufacturing practices standards	Sources of all media, cells, and seed viruses; purification and virus concentration procedures; sources of all animal sera used to cultivate viruses and cells; demonstrate efficiency of purification processes; titration of virus dose; safety of excipients; standardize lab assays to measure immunogenicity, etc.
In vivo nonclinical evaluation		
Immunogenicity and protective activity in an animal model.	Demonstrate that the vaccine can protect from some aspect of DEN infection; estimate dose range for humans	DENV attenuated vaccines are immunogenic in nonhuman primates; candidate should induce limited viremia and should protect against viremia following wt DENV challenge in NHP. Interference between DENV serotypes may be evaluated in mice or NHP, but data may not always correlate with human.
Toxicity and Safety	Product risks are appropriate for the anticipated use	Focus on unexpected consequences of the effect of the vaccine dose and direct effects due to vaccine virus replication and tissue tropisms. The evaluation includes scoring and statistical analysis for histopathological lesions and clinical signs between treatment and control groups.

B.2 Product development and characterization

It is critical that vaccine production processes are standardized and controlled to ensure consistency of manufacture in support of nonclinical data suggesting potential safety and efficacy in humans. This is a pre-requisite for entering the clinical trial phase.

Each of the attenuated virus candidates in the tetravalent dengue vaccine formulation should be characterized to define as far as is practical the critical genetic and phenotypic markers associated with attenuation. Each vaccine virus should also be evaluated to determine whether the genetic basis of attenuation is stable enough to reduce the risk of reversion to wild-type virus (and consequently virulence), either during manufacture or during replication in a vaccinee following inoculation, using available *in vivo* and *in vitro* approaches. To this end, laboratory and animal studies should define genetic changes in the virus genome that are associated with phenotypic attenuation markers.

It is helpful when possible also to identify *in vitro* phenotypic markers that correlate with attenuation. Both types of markers are useful to detect reversion events and to differentiate vaccine strains from wild-type virus strains in epidemiological surveillance following human immunization.

Qualification of each attenuated vaccine strain should include obtaining the consensus nucleotide sequence of the entire genome of the vaccine candidate, using the consensus nucleotide sequence of the genome of the parent virus as a comparator. This is essential to map the sites of attenuating mutations. It may also be possible thereby to determine the genetic basis of any *in vitro* phenotypic markers that correlate with attenuation. Such markers include but are not limited to plaque size, replication efficiency in mosquito vectors, induction of viremia in non-human primates, suckling mouse neurovirulence, virulence in any other animal model, and temperature sensitivity [8, 11, 12, 25, 26]. Developers should bear in mind that consensus genome sequencing is unsuitable for identifying minor or quasi-species genomes in a vaccine seed or batch [19].

B.3 Nonclinical immunogenicity and protective activity

Assessment of innate and adaptive immune responses in animals can provide evidence that the DEN vaccine has replicated in the host. Animals, particularly mice, have also been valuable in assessing the various elements of the immune response to DENV. Although there is no specific immune correlate of protection, antibodies directed against the virus E protein neutralize the virus and have been shown to protect animals when actively induced by experimental vaccines or when passively administered, prior to challenge. Based on the accumulated data, it is generally accepted that protection in humans should require a DENV-specific neutralizing antibody response. However, a correlation between the titre of neutralizing antibodies in serum, as determined in an *in vitro* neutralizing antibody assay (e.g. PRNT₅₀), and protection has not been established for any of the four serotypes of virus.

While protective activity in an animal model does not necessarily predict the protective effect in humans, it provides useful information regarding the immunological potency of the vaccine.

The immune response or protective activity to each of the four serotypes in a tetravalent DENV vaccine should be assessed, including the quality of response and any potential virological/immunological interference between types.

B.4 Nonclinical toxicity and safety

B.4.1 Considerations

General guidance on the nonclinical safety assessment and design of preclinical studies that apply to dengue vaccines is provided in the WHO *Guidelines on nonclinical evaluation of vaccines* [65]. The term “toxicity” is generally associated with the untoward consequences of the administration of a nonreplicating drug or biological that relate to its direct dose-dependent effect in the test animal. Thus toxicity studies entail the careful analysis of all major organs, as well as tissues near to and distal from the site of administration, to detect unanticipated direct toxic effects typically of a drug or nonreplicating biological agent, over a wide range of doses, including doses sufficiently exceeding the intended clinically relevant dose amount. It is generally expected that if a live attenuated vaccine does not replicate in the test animal, direct toxic effects are very unlikely to be detected. However, single dose and repeated dose toxicity studies, which are recommended for inactivated vaccines (sometimes in combination with adjuvants) should be conducted to confirm the absence of direct toxicity and could be combined with nonclinical safety evaluation (e.g. distribution, tropism, shedding). For live vaccines the emphasis is on the demonstration of nonclinical safety as a consequence of vaccine virus replication.

Nonclinical safety studies of live vaccines should be required for live-attenuated vaccines in certain stages of development. Such studies are designed with the primary purpose to demonstrate the vaccine(s) are less “virulent” in the animal host than comparable wild-type viruses and that the vaccine does not exhibit any unexpected harmful tissue tropism and damage or the capacity to elicit a harmful immune response. There is no animal model that replicates human dengue disease adequately (*see B.2.1 and B.2.2*). However non human primates and mice may provide useful information to characterize the viruses (*see B.5.2 and B.5.3*). The design of preclinical safety studies should reflect route and frequency of administration as proposed in the protocol to support clinical trials [65].

If the live attenuated DENV vaccine is intended to be used to immunize women of childbearing age, developmental/reproductive toxicity studies should be performed according to WHO guidelines [65].

B.4.2 Assessment in the non-human primate

B.4.2.1 Neurotropism/neurovirulence in non-human primates

The consensus of current opinion is that all live dengue vaccines should be tested once for neurovirulence and/or neurotropism (whether vaccine viruses cause diseases in the CNS or they have affinity to nerve tissues). At this time, the most well-established model for vaccine neurotropism is the NHP, which has been historically used to evaluate new seeds of YF vaccines (17D substrains 17D204- or 17DD-derived) and live polio vaccines. Novel rodent (hamster and mouse) models for YF vaccine virulence are currently under development. A rodent model could eventually be considered in lieu of NHP testing [38] (see section A.3.2.5.5.1).

Involvement of the CNS in cases of dengue fever and dengue hemorrhagic fever has usually been diagnosed as secondary to vasculitis with resultant fluid extravasation. The rarity of reports of patients with dengue encephalitis suggest that the virus does not typically cross the blood-brain barrier and infect neuronal cells [32]. However, since dengue vaccine viruses are genetically altered compared to their wildtype parent viruses, it is advisable to ensure that candidate vaccines have not acquired a neurotropic phenotype as an unintended consequence of the attenuation process. This is a particular issue as regards dengue vaccine viruses that contain YF 17D chimeric genomes, and it would be of similar special importance in the future, if novel dengue vaccines are derived from the genomes of any other known neuropathic viruses. This evaluation could be done once at an early stage of development, using a master seed or working seed lot of the vaccine. National regulatory authorities would need to decide whether each component of the tetravalent formulation needs to be tested separately for the property of neurovirulence or whether the tetravalent formulation could be tested initially, in which case no further testing of the individual vaccines would need to be done if results of the initial tests were within predefined specifications.

Testing for neurotropism/neurovirulence in the NHP model via the intracerebral route should follow the WHO recommendations for neurotropism testing of YF vaccines [31, 75]. A brief testing and evaluation procedure is provided below. In addition, neurotropism should be also evaluated as part of nonclinical safety study for distribution and tropism.

Groups of at least 10 monkeys, determined to be non-immune to DENV and YFV prior to inoculation with the DENV master seed, should be inoculated intracerebrally in the frontal lobe. A control group of 10 monkeys, also demonstrated to be non-immune to DENV and YFV, should receive YF-17D as the control group. All monkeys should be observed for 30 days for signs of encephalitis, prior to necropsy. If the number of monkeys, the observation period and/or time point(s) for necropsy for histological examination are different from these recommendations, they should be justified and in agreement with the national regulatory authority. Clinical scores, and the scores of histological lesions in the central nervous system should be recorded. Advanced histological scoring method such as automated image analysis [33] may be implemented to provide quantitative assessment of virus-induced histopathology in brain tissues if the

method has been properly validated and is acceptable by the national regulatory authority. The overall mean clinical and histological scores of test group should not exceed the scores of the control YF vaccine group. The significance level in statistical difference between test and control groups should be in agreement with the national regulatory authority.

B.4.2.2 Viremia in non-human primates

Non-human primates, humans and mosquitoes are the only natural hosts of DENV [8, 19, 51]. NHPs have been widely used to evaluate replication and immunogenicity of candidate dengue vaccines [5, 16, 55]. Primary infection of macaques with wildtype DENV results in moderate lymphadenopathy and a robust immune response [8, 19]. The NHP model has traditionally been used as an important guide for selecting vaccine strains for further development. In such studies, reduced peak titres and duration of viremia induced by a candidate vaccine, compared to those induced by the non-attenuated parent virus, is often, but not always, a correlate of attenuation. Consequently, if a dengue vaccine candidate causes viremia in NHPs comparable to that caused by its wild-type parent virus the vaccine, developers may wish to consider discontinuing further development.

B.4.3 Assessment in mouse models

DENV infection has been studied in many different mouse models [2, 8, 47, 55, 76]. When appropriate, a mouse model may be selected to evaluate the potential of a candidate vaccine to cause disease in comparison to its wild-type parent virus. In such an experiment, the titres of virus in blood, spleen, liver, lymph nodes, lungs, brain, and other tissues at various time points post-infection [8]. The AG129 interferon receptor-deficient mouse will support replication of DENVs of all serotypes [25, 26]. A DENV2 strain adapted to replicate in the AG129 induces a physiologically relevant disease in that strain [55]. At present, the AG129 mouse seems most suitable for safety studies, but National regulatory authorities should be aware of the pitfalls of interpreting results, since these animals do not possess an intact innate immune response. For this same reason, as mentioned earlier, it would not be advisable to use AG129 mice for classic toxicology studies. Other inbred mouse strains with genes knocked-out are under investigation as models of DENV infection and disease. One or more of these may have applicability to vaccine development in the future.

B.4.4 DENV replication in vector mosquitoes

Transmission of DENV to arthropod vectors from humans is essential to maintain the virus in nature. As noted previously, none of the DENV attenuated candidate vaccines studied to date induce a viremia in vaccinees that is sufficient in magnitude to infect feeding mosquitoes [19, 44, 50]. Further, if mosquitoes are infected with dengue vaccines, the viruses do not replicate sufficiently to permit transmission of the virus. For these two reasons, *Ae. Aegypti* mosquitoes are not expected to transmit dengue vaccine viruses [19,

22, 50]. As a measure of attenuation and safety, future novel candidate vaccines should be shown to have reduced ability to replicate and disseminate in *Ae aegypti* mosquitoes that have been infected in a controlled laboratory setting, using parent strains as controls [19, 22, 36, 45].

B.5 Environmental risk

For live dengue vaccines, the primary environmental risks relate to their capacity to be spread from human to human by vector mosquitoes and the risk that prolonged or repeated cycles of replication in mosquitoes could permit reversion to virulence. As previously noted, live vaccines currently under development have been shown to replicate poorly both in vaccinees and in mosquitoes, such that the risk for transmission by the mosquito vector is very low, if any risk exists at all [3, 22, 28, 44, 45, 50]. These factors should markedly reduce the chance that any of these vaccines could revert in mosquitoes to a virulent phenotype when used in a mass vaccination campaign in an endemic area. In addition, genetic stability during multiple sequential passages in mosquitoes has also been demonstrated for most existing live dengue vaccine candidates. For future candidate novel live vaccines, similar studies would need to be done.

Recently, some investigators have raised the issue, as regards live dengue vaccines, that vaccine viruses could revert to virulence in mosquitoes via intragenic recombination with endogenous wild-type flaviviruses. Such a phenomenon would seem to be highly unlikely due to factors noted above plus the controversial question of whether flaviviruses are able to undergo recombination even under ideal conditions in vitro. For further details see Part D guidelines for environmental risk assessment.

Part C. Clinical evaluation of dengue tetravalent vaccines (live, attenuated)

C.1 General considerations for clinical studies

The following should be read in conjunction with the:

- WHO *Guidelines on clinical evaluation of vaccines: regulatory expectations* (TRS 924, Annex 1) [64] and
- *Guidelines for the clinical evaluation of dengue vaccines in endemic areas* (WHO/IVB/08.12) [69].

C.1.1 Objectives of the clinical development programme

The clinical evaluation of a candidate live dengue tetravalent vaccine should aim to demonstrate that it:

- Elicits immune responses against all four dengue serotypes

- Prevents DFI of any severity caused by any of the serotype 1, 2, 3 and 4 viruses over an appropriate minimum period of observation
- Has an acceptable safety profile

In addition, the program should:

- Gather preliminary evidence that the immediate and longer-term immune response to a candidate dengue vaccine does not predispose vaccinated individuals to develop severe DFI (e.g. including haemorrhagic manifestations and systemic shock) during natural infections
- Attempt to examine the association between neutralizing antibody and protection against clinical disease (referred to as a surrogate marker for efficacy in this document)
- Attempt to identify a neutralizing antibody titre that predicts (in the short or longer-term) protection against clinical disease (referred to as an immunological correlate of protection in this document).

C.1.2 Outline of the clinical development program

In the initial clinical studies (i.e. Phase 1 studies) it is expected that relatively small numbers of healthy adults are vaccinated with investigational vaccine formulations and that the primary focus is on assessing safety. These studies may include exploration of immune responses to ascending doses of the four DENV serotypes when administered alone and in combination.

The subsequent clinical studies (i.e. Phase 2 studies) should be designed to select a dose of each DENV serotype for use in the tetravalent candidate vaccine formulation and to identify an appropriate primary immunization schedule for further study.

It is not currently possible to license candidate dengue vaccines based only on safety and immunogenicity data. because:

- No dengue vaccine has yet been licensed
- There is no established surrogate marker for protection and no immunological correlate of protection.

Therefore, candidate tetravalent dengue vaccines should be evaluated for protective efficacy against DFI.

Sponsors may decide to conduct at least one preliminary study of protective efficacy (i.e. sometimes referred to as a Phase 2b study) in order to identify a final candidate vaccine and immunization schedule for further study. Alternatively, depending on the data already accumulated (e.g. based on demonstration of a robust neutralizing antibody response), sponsors may consider it appropriate to omit such a study.

The selected candidate vaccine should be evaluated in at least one adequately sized study of protective efficacy (i.e. Phase 3 study) that compares numbers of cases of laboratory-confirmed DFI between groups of vaccinated and unvaccinated subjects. The total DFIs counted should include those due to any of the four DENV serotypes and of any degree of clinical severity that occur within a defined observation period.

Section C.3 gives more details of study designs and populations to be enrolled into studies conducted at each phase of development.

C.2 Immunogenicity

C.2.1 Measurement of immune responses to vaccination

Current evidence suggests that neutralizing antibody against each DENV serotype is likely to be the best surrogate marker for efficacy.

It is recommended that the methodology for determination of DENV serotype-specific neutralizing antibody titres should follow the WHO *Guidelines for the plaque reduction neutralization test* (PRNT) (WHO/IVB/07.07) [68]. If alternative methods for determining neutralizing antibody (e.g. high throughput microneutralization assays) are developed these should be validated against the PRNT.

Reference virus strains and cell substrates are available from the WHO and titres should be expressed in IU calibrated against the WHO reference sera. In-house strains may be used provided that the reference serum is employed and results expressed in IU. In addition, a comparison with results obtained with the WHO reference strains is advised.

An assessment of neutralizing antibody titres against a range of DENV serotypes and strains of those serotypes, including recent wild-type isolates, is encouraged. This would be valuable information to obtain due to worldwide strain diversity and because neutralizing antibody titres against specific isolates will be variable. Such additional assays could be applied to subsets of sera collected from vaccinees that have been selected randomly or based on a scientific justification (e.g. to select sera known to cover a range of neutralizing antibody titres against the reference or in-house strains).

The assay of DENV-specific antibody other than neutralizing antibody (e.g. IgM and IgG ELISA) may be of interest but is not considered to be essential for the assessment of potential vaccine efficacy.

It is considered unlikely that data on cell-mediated immunity (CMI) will provide an immunological correlate of protection. However, the exploration of CMI is encouraged since specific CMI assays may be useful for the assessment of immunological memory and durability of protection. Assessments of cytokine responses may assist in the evaluation of vaccine safety and provide some indication of the potential risk that

vaccination could predispose subjects to develop severe DFI during subsequent natural infection [49].

C.2.2 Investigation and interpretation of immune responses to vaccination

There is no established immunological correlate of protection against any DENV serotype. In the initial clinical studies of safety and immunogenicity, including the dose- and regimen-finding studies, it is essential to fully describe the pre- and post-vaccination neutralizing antibody titres that are observed against each of the four DENV serotypes (*see also section C.3.1*). Adequate data should be generated to describe the kinetics of the neutralizing antibody response in the short-term. Longer-term antibody persistence data may be collected in these and/or in later studies, as described below.

In a non-endemic population with no detectable pre-vaccination antibody in the majority of subjects a comparison of percentages with a detectable titre post-vaccination (which may be defined as seroconversion in such a population) against each DENV serotype should be made. The analyses should also look at proportions that seroconvert (in accordance with an appropriate definition of seroconversion stated in the protocol) to multiple serotypes (i.e. two, three or all four serotypes).

In an endemic population in which very high proportions of subjects are already seropositive with respect to at least one dengue type a comparison of pre- and post-vaccination geometric mean titres (GMTs) with respect to those types will be informative in addition to seroconversion rates.

In endemic and non-endemic populations detailed consideration of reverse cumulative distribution curves is important. For example it may be informative to compare percentages achieving a pre-defined high titre of neutralizing antibody.

In protective efficacy studies neutralizing antibody against DENV serotypes should be determined and followed over time in pre-defined subsets of the study population, including an assessment of antibody persistence after the protocol-defined period for the primary evaluation of protective efficacy. It is preferable that subsets of subjects to be included in these detailed immunogenicity evaluations should be identified at the time of randomization with stratification for age and any other factors that might have an important impact on immune responses to vaccination. In any case the data should be analysed according to pre-defined subsets. Immune responses should be determined for vaccinated and unvaccinated subjects so that the effects of background exposure to DENVs during the study period can be assessed.

Depending on the specific vaccine construct and taking into account any pertinent results of non-clinical studies, sponsors may wish to undertake some exploratory investigations of antibody against other antigens (e.g. those associated with the attenuated yellow fever virus backbone in a chimeric vaccine).

Long-term storage of sera is encouraged since future developments in the field and/or emerging data on longer-term safety or efficacy might point to a need for additional investigations that cannot be predicted at the time of conducting the study.

Subsets of subjects should also be identified for collection of peripheral blood mononuclear cells (PBMCs), taking into account feasibility issues, such as the blood volumes required from different age groups to produce adequate cell numbers for study and accessibility to adequate sample processing and storage facilities.

For the analysis of the relationship between neutralizing antibody titres and protection against laboratory-confirmed DFI, sera should be collected at timed intervals from a substantial cohort of subjects (and preferably the entire study population if feasible). Once the protocol-defined double-blind observation period has been completed, the analysis of the relationship between immune response and protection against DFI should follow according to:

a) A cohort study in which one or more measures of immune response are related to disease in all or a large subset of immunized subjects. This design is potentially the more informative if there are enough cases among immunized subjects to allow for a quantitative estimation of disease risk as a function of immune response(s);

OR

b) A case-control study in which immune responses are compared between immunized subjects who developed disease and a subset of immunized subjects who did not develop disease. This design enables establishment of an association between disease and measure(s) of immune response but it does not allow for a quantitative estimation of disease risk as a function of immune response.

The use of serology to help identify infections with dengue viruses (whether or not clinically apparent) is a quite separate issue that is discussed in section C.3.3.

C.3 Clinical studies

C.3.1 Phase 1 studies

The Phase 1 studies should be designed to provide an early indication of whether severe local and/or systemic adverse events (AEs) may occur commonly after vaccination. These studies may also provide preliminary data on immune responses to assist in the selection of DENVs (or constructs) and doses to be included in candidate tetravalent vaccine formulations for further study.

Subjects enrolled into these initial studies should be healthy adults who are naïve to flaviviruses (based on medical and vaccine history and serological studies). It is preferred that subjects are resident in non-endemic areas so that they are not at risk of natural infection with dengue or other flaviviruses. Eligible subjects should not be in need of vaccination against other flaviviruses at least throughout the duration of the study.

Sponsors may choose to commence studies with monovalent (i.e. containing a single live attenuated DENV serotype) before progressing to evaluate multivalent versions (which may include bivalent, trivalent and then tetravalent formulations) of a candidate dengue vaccine.

If a candidate tetravalent vaccine formulation elicits a much lower antibody titer to one (or more than one) DENV serotype than to others it is important that consideration is given to modification of the vaccine (e.g. by modifying the infectious titers of serotypes) and/or the immunization schedule due to the potential implications for safety and efficacy.

If a likely candidate tetravalent vaccine is identified it may be appropriate that a preliminary exploration of safety and immunogenicity should be conducted in healthy adult residents of an endemic area (i.e. including subjects with evidence of some pre-existing immunity to dengue or other flaviviruses). Such a study could provide further reassurance regarding the ability of the candidate vaccine to elicit immune responses to all four DENV serotypes before progressing to studies in larger numbers of subjects.

C.3.2 Phase 2 studies

The Phase 2 studies should extend the information on safety and immunogenicity of candidate tetravalent vaccine formulations. They should include studies in residents of endemic areas who are therefore at risk of natural infection with dengue and may have some degree of pre-existing immunity to one or more DENV serotypes and to other flaviviruses.

While the first data may be obtained in adults there should be a plan to move down to younger age groups in a stepwise fashion. The age range should reflect that proposed for the evaluation of protective efficacy of the tetravalent vaccine candidate.

Depending on the findings of the Phase 1 studies, the first Phase 2 studies may further explore dose-response relationships. The data generated on safety and immunogenicity should be sufficient to support the selection of one or more candidate tetravalent vaccines and immunization schedules (i.e. number of doses and dose intervals) for further evaluation.

If the sponsor chooses to undertake a preliminary (i.e. Phase 2b) study of safety and efficacy this should be of an appropriate design and of adequate size to support a robust decision regarding the vaccine formulation and schedule to be further evaluated (*see* C.3.3). Even in a Phase 2b study it is recommended that subjects should be followed up for approximately 3-5 years from the time of completion of vaccination to collect data on safety and to document antibody to DENV serotypes in subsets of each treatment group.

The total number of subjects enrolled into Phase 2 studies should be sufficient to describe at least common adverse reactions to vaccination with some degree of confidence.

Therefore it is expected that several hundred subjects should have been exposed to candidate tetravalent vaccines containing the final or near final doses of DENVs of each serotype. If any unusual, severe or serious adverse reactions are documented it may be appropriate that further studies include the assessment of safety as one of the primary objectives provided that these reactions would not preclude further vaccine development.

C.3.3 Phase 3 studies

It is not currently possible to license dengue vaccines based only on safety and immunogenicity data because there is no licensed dengue vaccine and no immunological correlate of protection has been established for any DENV serotype. Each tetravalent candidate vaccine should be evaluated in at least one study that is of an appropriate design and adequate size to estimate vaccine efficacy (VE). This situation may change in the future (see section C.3.3.7).

C.3.3.1 General issues for study design

VE is estimated by comparing the total numbers of laboratory-confirmed cases of DFI of any degree of severity and due to any of the four DENV serotypes between the vaccinated and unvaccinated (control) groups. The primary analysis of VE should be conducted at the conclusion of a protocol-defined double-blind observation period. Each study should be of sufficient size and duration to provide a robust estimation of VE and to provide preliminary evidence that the vaccine does not predispose recipients to develop one of the severe forms of DFI following natural infection.

Studies of protective efficacy should be performed in endemic areas where a proportion of the population is likely to have some naturally-acquired immunity to one or more of the four DENV serotypes and/or other flaviviruses. It is assumed that in most, if not all, cases each study will evaluate a single tetravalent candidate dengue vaccine and immunization schedule. However, the study design may be adapted as necessary if more than one possible active vaccination group is to be included.

Studies that involve vaccination of a large proportion of subjects at any one study locality carry the potential to significantly interrupt DENV transmission during the observation period. The result could be a reduced likelihood of demonstrating a difference in the numbers of laboratory-confirmed cases of DFI between the vaccine and control groups. Consideration should be given to this possibility when designing the study.

Randomization should be performed using a centralized system. When using a 1:1 randomization ratio the block size should be selected with the aim of enrolling approximately equal numbers in test and control groups at each of the study sites so that subjects in each group are at the same risk of developing mild and severe DFI throughout the observation period. It is also possible to consider the use of unbalanced randomization (e.g. 3 : 2 or 2 : 1 vaccine : control) provided that care is taken to ensure the desired ratio

is applied at each study site (or geographically localized sites) and the sample size is calculated to provide adequate power.

The decision to use unbalanced randomization should take into consideration the possible advantages and disadvantages. Advantages include a larger safety database and possibly easier enrolment due to the greater chance that any one subject would receive the candidate dengue vaccine. Disadvantages include the possibility that a larger proportion vaccinated against dengue could increase the risk of achieving a reduction in DENV transmission sufficient to impact on the chance of obtaining a conclusive study result.

Whenever possible, subjects randomized to the control group should receive an alternative active vaccine (selected to provide an anticipated benefit to study participants) rather than injections of placebo. If the active control vaccine cannot be given at the same schedule as the candidate dengue vaccine, then placebo injections can be used within the schedule as necessary to maintain a double-blind design. If the active control vaccine has a different presentation or appearance to the candidate dengue vaccine then study personnel who administer the vaccinations should not have any other involvement in the conduct of the study. Vaccine recipients should not be allowed to observe preparation of the vaccines for injection (e.g. any reconstitution steps that may or may not be necessary) to avoid the risk of them sharing this information and so identifying themselves with one of the study groups.

If there is no suitable active control vaccine that can be given by the same route of administration as the candidate dengue vaccine then the use of a placebo control is necessary to achieve a double blind design. In such cases, the protocol could plan to administer a suitable licensed vaccine to all subjects in the study (i.e. those who do and do not receive the candidate dengue vaccine) at some time after completion of the assigned study treatments and during the double-blind follow-up period. In this way all study subjects can derive some potential benefit from participation in the study without compromising the study integrity.

It is expected that several different production lots of vaccine will be used during protective efficacy studies. Whether or not a formal lot-to-lot consistency study should be built into the protocol, with the specific aim of comparing safety and immunogenicity between subjects who receive different lots (usually 3 of the total used) according to pre-defined criteria, must be decided on a case by case basis. If such a formal comparison is to be made then additional measures will be needed to ensure that adequately sized subsets of subjects are randomized to receive each of the vaccine lots identified for this comparison.

C.3.3.2 Study location and duration

The geographic areas selected for study should have background rates of DFI that are sufficient to provide enough cases in the control group during the observation period to facilitate the estimation of VE. To assess background rates efficacy studies should be preceded by the collection of epidemiological information to document the expected

incidences of DENV serotype-specific and all DFI preferably over several years. The data should include information on seasonality of disease to identify periods of transmission and case demographics (e.g. age and gender) so that populations at highest risk of DFI can be targeted for enrolment.

There should also be an assessment of the likely extent of exposure of the population to other species of flaviviruses at potential study sites because such exposure may confound the interpretation of dengue-specific serological data and possibly affect the clinical course of DFI. This assessment should take into account any available epidemiological data, serological studies and information on rates of vaccination against other flaviviruses.

Study sites should be endemic for one or more DENV serotypes with transmission expected each season during the observation period, which would likely range from 1-3 years from the time of the first vaccination. Nevertheless, even if the study is conducted over several seasons and at geographically dispersed study sites there may not be sufficient numbers of cases of DFI to support an estimation of serotype-specific VE for some or all of the four serotypes. Additional evidence for protective efficacy against individual DENV serotypes should be sought from post-licensure (i.e. effectiveness) studies as discussed in sections C.3.3.7 and C.4.

There should be a plan to follow-up subjects for safety and efficacy for at least 3-5 years from the time of completion of primary vaccination. During this period it is possible that an efficacious dengue vaccine may be offered to subjects originally assigned to the control group with potential implications for interpretation of the data that can be collected (see C.3.3.7).

C.3.3.3 Study population

Since protective efficacy studies should be performed in endemic areas there is a need to consider that the ultimate target group for vaccination may range from a subgroup (e.g. infants) to the entire population. However, even in areas where a substantial proportion of hospitalized cases of severe DFI are aged less than one year there are likely to be concerns regarding inclusion of infants in protective efficacy studies because of the possible risk of DFI that has been reported in association with waning maternal antibody against one or more DENV serotypes and the unknown effects of vaccination in the presence of maternal antibody.

Therefore it is expected that protective efficacy studies would likely exclude subjects aged less than one year but should enrol children across a wide age range subject to satisfactory results from the safety and immunogenicity studies. Section C.3.3.7 considers bridging the observed VE to populations that were not included in efficacy studies.

C.3.3.4 Objectives, endpoints and analyses

The primary objective of a protective efficacy is to estimate VE against DFI. The primary analysis should seek to demonstrate superiority for the vaccinated group versus the control group in terms of the total numbers of cases of laboratory-confirmed DFI in subjects who have been fully vaccinated in accordance with the protocol and have been followed up for the required time with no major protocol deviations. In this analysis counting of cases should commence from a designated time point after the last dose of protocol-assigned doses has been administered.

VE is estimated by comparing the total numbers of laboratory-confirmed cases of DFI (i.e. summation of cases due to any DENV serotype and of any degree of severity) that occur in vaccinated and unvaccinated (control) groups during a protocol-defined double-blind observation period. VE should be calculated using the standard formula $VE (\%) = 100 \times (1 - r1/r0)$ [where $r1$ = incidence rate in the vaccine group and $r0$ = incidence rate in the control group].

The assessment of DENV serotype-specific VE should be a major secondary objective and the subject of a planned secondary analysis. It is not expected that the study would be powered to support a formal statistical analysis of DENV serotype-specific efficacy.

The statistical analysis plan should explain how multiple episodes of DFI in any one study participant will be handled in the analyses.

Other important secondary analyses should include the estimation of:

- Efficacy based on counting all DFI that occur after administration of the first dose of protocol-assigned treatment;
- Efficacy in all vaccinated subjects regardless of protocol deviations (including those with incomplete vaccination courses and missing data);
- Efficacy according to pre-vaccination flavivirus serological status, which might be determined in a randomized subset of enrolled subjects who are followed serologically;
- Efficacy according to severity of laboratory-confirmed DFI (with adequate protocol definitions);
- Efficacy that includes prevention of “possible” or “probable” dengue infection (e.g. applied to patients in whom serology is used as the basis for dengue diagnosis without a virologically-confirmed diagnosis). The justification for this secondary analysis is based on expectation that a dengue vaccine may reduce the viremia so making it more difficult to detect in patients who may also have abbreviated clinical signs and symptoms. Thus, serological secondary end-points may help assess overall efficacy assuming that serological assays are equally sensitive and specific to DENV (but not to individual serotypes) in detecting dengue infection in vaccine and control groups; or

- The effect of vaccination on the duration of hospitalization and/or need for specific interventions to manage the clinical illness.

If more than one study of protective efficacy is performed with a single candidate vaccine (e.g. perhaps covering different geographical regions) using the same or a very similar study protocol, it may be appropriate to pre-define a pooled analysis of the data. This pooled analysis could provide additional insight into serotype-specific VE and the risk of severe DFI in vaccine and control groups.

Each study should have in place a data and safety monitoring board (DSMB) consisting of persons with no involvement in study conduct and analysis and including a statistician. The DSMB charter should enable it to unblind treatment assignments as necessary and to recommend that enrolment is halted or the study is terminated based on pre-defined criteria designed to protect subjects from harm. In addition, studies may include one or more planned interim analyses with pre-defined stopping rules.

C.3.3.5 Case definitions

The case definitions for the primary and for the various secondary analyses, with details of the criteria to be met, should be defined in the protocol and should be in accordance with the latest WHO recommendations (WHO/HTM/NTD/DEN/2009.1) [70].

Clinical diagnosis

The most commonly diagnosed form of clinically apparent dengue virus infection is characterized by the sudden onset of fever lasting at least two and up to seven days. Fever is commonly accompanied by severe headache, pain behind the eyes, gastrointestinal symptoms, muscle, joint and bone pain and a rash. These cases are usually self-limiting and result in complete recovery.

For the purposes of classification of cases it is important to characterize the severity of each DFI. The criteria used to assess severity should be those described by WHO that are current when the protocol is finalized (WHO/HTM/NTD/DEN/2009.1 and updates of this guidance) [70]. These criteria should be used to determine the features of DFI that are captured in the case report form.

Virological diagnosis

All methods used for the virological component of the case definition should be fully validated. Virological confirmation of the clinical diagnosis can be based on direct detection of dengue viremia by isolation. However, the use of alternative virological methods to confirm the diagnosis (e.g. detection of NS1 to demonstrate the presence of DENV and the use of reverse transcription-polymerase chain reaction (RT-PCR) to

determine the serotype) is acceptable. The standardization of viral diagnostic methods is encouraged. Every effort should be made to conduct testing in one or a small number of designated central laboratories with appropriate expertise.

Obtaining specimens to attempt virological confirmation of the diagnosis should be triggered by a set of clinical features laid down in the study protocol that aim to identify all potential cases of DFI of any severity and as early as possible, taking into account the observation that virological diagnostic methods (including virus isolation and PCR-based assays) are more sensitive during the first five days of infection.

Serological diagnosis

There is at least a theoretical possibility of a reduced likelihood of obtaining virological confirmation of the clinical diagnosis in vaccinated subjects due to levels of viremia that are below detection limits. If this were to occur in a significant proportion of clinical cases there could be a bias in numbers of virologically-confirmed DFI in favour of the vaccinated group.

Commercial and/or in-house serological assays (e.g. enzyme immunoassay [EIA], immunofluorescence and virus neutralization tests) may be performed on paired acute and convalescent sera. The results may be used to identify possible cases of DFI in which a virological diagnosis was not confirmed and the numbers may be compared between vaccinated and control groups in an additional secondary analysis of vaccine efficacy.

For example, using the IgM capture EIA an acute primary infection may be implied from a rise in IgM levels during the first two weeks post-infection. The results need to be interpreted with some caution because the IgM response to acute infection may be blunted in vaccinated subjects and in those infected previously by wildtype DENV or by another flavivirus. Depending on the timing of the illness, the results may also be confounded by the fact that IgM and IgG responses may reflect recent dengue vaccination rather than acute infection with wild type dengue. In this regard, the ratio of IgM and IgG may assist in the differentiation of primary and secondary infections.

The interpretation of serological data is also complicated by cross-reacting antibody among flaviviruses. In those instances where cross-reaction with other flaviviruses does not occur, a four-fold or greater rise in dengue neutralizing antibodies makes it possible to attribute recent infection to a dengue virus presumptively, but not definitively.

C.3.3.6 Case detection and description

It is essential that there is adequate surveillance to detect any possible case of DFI as early as possible to optimize the chances of virological confirmation of the diagnosis. The surveillance mechanisms (e.g. including arrangements for periodic home visits or telephone calls and involvement of hospitals serving the study catchment areas) should be tested before the study is initiated at each study site. It is essential that study subjects are

educated regarding the need to contact or directly present to the designated study healthcare facilities whenever they develop signs or symptoms that may be indicative of DFI. A check list of these signs and symptoms should be provided to all study participants at the time of enrolment.

In addition, measures should be in place to follow each possible case of DFI for any change in disease course (e.g. progression from mild to severe DFI, onset of complications) and to document the outcome, including the time to recovery or death. In case of death before collection of specimens or in the absence of virological confirmation of the diagnosis permission should be sought to perform needle puncture of the liver for virological study and a post-mortem examination.

In some study sites that are otherwise considered suitable it may not be possible to identify a local healthcare facility willing to participate in the study. These sites should not be initiated unless there is at least agreement from local healthcare providers to notify study staff of possible cases of DFI within a sufficient timeframe to allow for specimens to be collected and transported for virological diagnosis. Subjects should carry a study participant card with contact names and numbers to ensure that study personnel are alerted and can arrange for the collection of all the necessary clinical data and the transport of specimens for virological diagnosis.

Despite taking the steps described, there will still be some cases of possible DFI that are not confirmed virologically and for which serological testing is inconclusive. In addition, some subjects may not comply with the study requirement to present to a designated healthcare facility when they have signs and symptoms indicative of a possible DFI or they may be so ill that they are immediately admitted to a hospital not directly participating in the study and/or may die without notification of study personnel in time to collect data and specimens. It is important that as much information as possible is collected on these cases whenever and however they come to light and that they are taken into account in a “worse-case scenario” analysis of vaccine efficacy that counts all cases (proven and unproven and regardless of protocol deviations).

C.3.3.7 Need for additional studies of efficacy

Once the efficacy of at least one candidate dengue vaccine has been satisfactorily demonstrated (and perhaps already licensed and introduced into the routine vaccination program in at least one country) there will be a need to reassess the content of clinical development programs for other candidate dengue vaccines. In particular, there will be a need to consider the appropriateness and/or feasibility of conducting studies in which subjects are randomized to a control (i.e. unvaccinated) group.

It is not currently possible to make a definitive recommendation regarding what could or should be required in this scenario. Much will depend on the findings reported from the first completed efficacy study of a candidate vaccine or from ongoing studies with other candidate vaccines.

Some issues to be taken into account include the following:

- i) Once a vaccine has been licensed and introduced into the routine vaccination program in one or more countries the inclusion of an unvaccinated group in subsequent studies in these (and possibly many other) countries is likely to be considered unethical. In addition, studies that include an unvaccinated group are not likely to be feasible in some regions after approval of the first vaccine (with or without introduction into the routine vaccination program) because subjects are unlikely to risk assignment to an unvaccinated group. However, if there remains considerable uncertainty about vaccine efficacy against specific DENV serotype(s) then another study with an unvaccinated control group might be possible in a region where such serotype(s) are predominant;
- ii) The use of licensed dengue vaccine(s) in the routine vaccination program in a country or region may reduce the incidence of DFI down to levels that are too low to permit the estimation of VE from further studies of reasonable and manageable size and duration;
- iii) There should be a careful scientific evaluation of the arguments for and against extrapolation of the efficacy observed for a particular vaccine to other populations. For example, to other age groups, to subjects at highest risk of dengue and severe dengue, to geographical areas with predominant circulating DENV serotypes for which serotype-specific efficacy may not have been established and to populations with high rates of immunity to other flaviviruses;
- iv) It is very possible that a definitive immunological correlate of protection cannot be established from pre-licensure protective efficacy studies. Moreover, accumulating evidence suggests that a neutralizing antibody titer that seems to correlate with protection against DFI due to one DENV serotype may not predict protection against DFI caused by other dengue serotypes. Therefore, there needs to be a discussion of the validity of using immunogenicity data to bridge the efficacy of a dengue vaccine that was demonstrated in one test population to other populations, with or without identification of correlates of protection;
- v) There also needs to be a discussion regarding the use of bridging studies to support the extrapolation of efficacy observed with one vaccine to subsequent candidate vaccines that may or may not be live dengue vaccines. For reasons already discussed above, there may be no alternative to the use of bridging studies because the following approaches are unlikely to be feasible;

- vi) To continue to request evaluation of subsequent candidate dengue vaccines in pre-licensure protective efficacy studies that include an unvaccinated group;
- vii) To request a relative efficacy study i.e. that compares rates of febrile dengue illness in groups assigned to either a licensed or candidate vaccine;
or
- viii) After consideration of all these issues it is expected that at some future time point, which will depend on the safety and efficacy that is observed with the first or first few vaccines to be licensed, the inclusion of unvaccinated control groups in efficacy studies will no longer be possible. Once this time point is reached there will have to be recommendations made regarding:
 - Additional data to be obtained after initial approval of a vaccine for which VE has been estimated based on numbers of laboratory-confirmed DFI in vaccinated versus unvaccinated groups.
 - Data that should be generated before and after licensure of subsequent candidate vaccines.

In both instances it is important that vaccines should be evaluated in post-approval studies of safety and effectiveness as discussed in section C.4.

C.3.3.8 Documentation of safety during pre-licensure studies

The routine monitoring of safety during all pre-licensure clinical studies should follow the usual principles taking into account issues relevant to live attenuated vaccines. In addition to providing study-specific safety data there should be an analysis of safety data pooled across all study groups that received the final selected vaccine formulation.

In particular, there is a need to assess whether DFI (which could be of any degree of severity, including a very mild illness) may be caused by vaccine strains. In studies conducted in non-endemic areas there is a risk that vaccine-associated DFI, which would be expected to occur only within about 30 days post-dose, could be confused with an intercurrent infection while in studies in endemic areas there is a need to distinguish vaccine-associated from naturally-occurring DFI. Therefore in all cases it is essential that laboratory studies should be undertaken to determine whether or not the clinical picture is associated with vaccine virus viremia.

The safety considerations also include the possible risk that vaccination could predispose recipients to develop a severe form of DFI. The monitoring and investigation of all subjects who develop signs or symptoms potentially indicative of DFI during pre-licensure studies in endemic regions should provide a preliminary assessment of this risk. If no undue risk is identified and the vaccine is licensed it is essential that there is

adequate follow-up of study subjects together with further assessment of the risk in the post-licensure period (see section C.4).

The total safety database derived from all pre-licensure studies should be sufficient to describe uncommon adverse reactions. Based on considerations outlined in section C.3.3.7, it may be that vaccines that are developed subsequent to the approval of the first vaccine(s) will not be evaluated in pre-licensure studies of protective efficacy, in which case the pre-licensure safety database would usually be much smaller. In these instances the minimum acceptable safety database should be determined according to the nature of the vaccine and in the light of experience amassed with other similar vaccines.

C.4 Post-licensure investigations

As with all medicinal products, including all vaccines, there is a need to ensure that adequate surveillance is in place and is maintained to detect adverse reactions during the post-licensure period in accordance with requirements of the countries in which approval has been obtained.

The need for, design and extent of specific studies of safety and/or effectiveness following approval of a dengue vaccine should be given careful consideration by sponsors and national regulatory authorities. However, at least in the case of the first dengue vaccines that reach the market there will be a clear need for well-designed studies, which should include long-term extension periods of pre-licensure efficacy studies as well as newly-initiated studies, to collect information at least on the following:

- Long-term evaluation of breakthrough cases of DFI to detect any waning of protective immunity against one or more DENV serotypes and the possible need for booster doses;
- Antibody persistence;
- Responses to booster doses, which may be planned for pre-defined subsets enrolled into studies or may be instituted as/when disease surveillance indicates a possible need;
- The possible increased risk of severe DFI in vaccine recipients; and
- Vaccine effectiveness

Depending on the countries/regions in which any one licensed vaccine is introduced into routine vaccination programs it is desirable that attempts should be made to estimate DENV serotype-specific effectiveness and effectiveness in populations that were not included in pre-licensure studies. There are several possible study designs and methods

for estimating vaccine effectiveness and it is essential that expert advice is sought. In addition, it is likely that such studies would need to be performed in close liaison with Public Health Authorities.

Sponsors and national regulatory authorities should consider the need to assess immune interference and safety on co-administration of a dengue vaccine with other vaccines that may be given at the same health care visits for convenience. In addition, if licensure is sought in non-endemic areas with the intent to protect travellers to endemic areas, sponsors and national regulatory authorities may consider it necessary to obtain additional safety and immunogenicity data with the final vaccine and schedule in flavivirus-naïve subjects.

Some or all post-licensure studies may be conducted as post-approval commitments made to individual national regulatory authorities. In this regard, both sponsors and national regulatory authorities that have approved a vaccine should communicate and co-operate to ensure that studies are well-designed to answer the questions posed and to avoid demands for numerous studies in individual countries that are likely to be too small to provide reliable results. Provisional plans for appropriate post-licensure studies should be submitted with the application dossier and these should be refined during the assessment of the national regulatory authority and as necessary after initial approval.

Part D. Environmental risk assessment of dengue tetravalent vaccines (live, attenuated) derived by recombinant DNA technology

D.1 Introduction

D.1.1 Scope

Some countries have legislation covering the environmental and other issues related to the use of live vaccines derived by recombinant DNA technology since they may be considered genetically modified organisms (GMO) including vaccines. Similar issues may be raised by live vaccines derived by other methods.

This section of the guideline considers the environmental risk assessment (ERA) that may be performed during DENV vaccine development. The ERA assesses the risk to public health and the global environment. It does not assess the risk to the intended recipient of the vaccine; this is assessed through clinical studies of the vaccine. Nor does it assess the risk to laboratory workers.

The environmental impact is not usually the responsibility of the national regulatory authority but of other agencies. Nonetheless the national regulatory authority should

receive a copy of the ERA and any associated decisions taken for information and to ensure that the appropriate procedures have been followed.

D.1.2 Principles and objectives

Live DENV vaccines in which the genetic material has been genetically modified by recombinant DNA technology, should be considered to be GMO. The manufacture, use and transboundary shipping of such live recombinant vaccines, for research or commercial use, should comply, when applicable, with any relevant legislation or regulations of the producing and recipient countries regarding GMOs. In some regulatory regimes, in order to comply with environmental regulations, an ERA should be undertaken if the live vaccine is being tested in a clinical trial or if it is placed on the market. It should be noted that this guidance on the ERA of live recombinant DENV vaccines does not intend to replace existing GMO legislation when already in place in certain countries.

Generally, the objective of an ERA is to identify and evaluate, on a case-by-case basis, potential adverse effects of a GMO on public health and the environment, direct or indirect, immediate or delayed. This means that for each different live recombinant dengue vaccine a case-by-case ERA should be performed. Direct effects refer to primary effects on human health or on the environment which is a result of the GMO itself and which occur through a short causal chain of events. Indirect effects refers to effects occurring through a more extended causal chain of events, through mechanisms such as interactions with other organisms, transfer of genetic material, or changes in use or management. Immediate effects are observed during the period of the release of the GMO, whereas delayed effects refer to effects which may not be observed during the period of release of the GMO, but become apparent as a direct or indirect effect either at a later stage or after termination of the release.

The ERA should be performed in a scientifically sound and transparent manner based on available scientific and technical data. Important aspects to be addressed in an ERA include the characteristics of: (a) the parental organism, (b) the recipient organism, (c) viral vector characteristics, (d) the donor sequence, (e) genetic modification, (f) the intended use and (g) the receiving environment. The data needed to evaluate the ERA do not have to derive solely from experiments performed by the applicant; data available in the scientific literature can also be used in the assessment. Regardless of the source of the data, it should be both relevant and of an acceptable scientific quality. The ERA could be based on data of experiments previously performed for other purposes, such as product characterization tests, toxicity and nonclinical safety studies.

Ideally, the ERA is based on quantitative data and expressed in quantitative terms. However, much of the information that is available for an ERA may be qualitative for the reason that quantification is often hard to accomplish, and may not be necessary to make a decision. The level of detail and information required in the ERA is also likely to vary according to the nature and the scale of the proposed release. Information requirements

may differ between licensure and clinical development and whether studies will be carried out in a single country or multiple countries.

Uncertainty is inherent in the concept of risk. Therefore, it is important to identify and analyse areas of uncertainty in the risk assessment. Since there is no universally accepted approach for addressing uncertainty, risk management strategies may be considered. Precise data on the environmental fate of the live vaccine in early clinical trials will in most cases be insufficient or lacking. However, at the market registration stage, the level of uncertainty is expected to be lower as identified gaps in available data should already have been addressed.

The need for risk management measures should be based upon the estimated level of risk. If new information on the GMO becomes available the ERA may need to be re-performed to determine whether the estimated level of risk has changed. This also holds true if the risks for the participating subjects have changed as these aspects can be translated to other individuals. It should be noted that the ERA will not deal with medical benefit for the subject or scientific issues such as proof-of-principle.

D.2 Procedure for environmental risk assessment

Risk assessment involves identification of novel characteristics of the GMO that may have adverse effects (hazard), evaluating the consequences of each potential adverse effect, estimation of the likelihood of adverse effects occurring, risk estimation, risk management and, in some methodologies, estimation of the overall risk for the environment. These processes should identify the potential adverse effects by comparing the properties of the GMO with non-modified organisms under the same conditions, in the same receiving environment. The principles and methodology of an ERA should be applicable irrespective of the geographic location of the intended environmental release of the GMO. Although the ERA should take into account the specificities associated with the mosquito vector being endemic or non-endemic in the region in which vaccine trials will be carried out, and/or where licensure is being requested. Depending upon local regulatory requirements, the ERA may be undertaken by the applicant or by the local competent authority on the basis of data supplied. In all cases, the local competent authority should use the ERA as a basis for deciding whether any identified environmental risks are acceptable. However, the decision on whether any identified risks are acceptable may vary from country to country.

There are several national and multinational documents addressing ERA issues, such as:

- EU Directive 2001/18/EC [14] (Annex II) and Commission Decision 2002/623/EC [15];
- Risk Analysis Framework of the Australian Office of the Gene Technology Regulator [40];
- New Substances Notification Regulations of Canadian Environmental Protection Act, 1999 [13];

- Recombinant DNA Safety Guidelines of Genetic Engineering Approval Committee in India; and
- Normative Resolution No.5 (12 March 2008) comprising provisions on rules for commercial release of GMOs and their derivatives in Brazil.

As an example, a detailed description of a six-step ERA module of EU Directive 2001/18/EC is described in the Appendix 3 of these Guidelines.

D.3 Special considerations for live recombinant dengue vaccines

The ERA of live recombinant DENV vaccines should be conducted according to the general principles described above taking into consideration in particular the vector responsible for disease transmission. The following aspects which could be developed include: the genetic stability of the live recombinant virus including reversion and recombination, potential transmission of vaccine virus among hosts by the vector and the immune status of the population, and these are further outlined below.

D.3.1 Genetic stability

DENV vaccines currently under clinical evaluation are attenuated DENV strains, intertypic chimeric vaccines or DENV/YF-17D vaccine chimeras. In the intertypic approach, the structural genes of an attenuated strain of DENV of a given serotype are replaced by the corresponding genes of a different DENV serotype. In the dengue/yellow fever chimeras, the prM/E structural genes of the dengue virus are cloned into the backbone of the YF-17D vaccine, in replacement of the corresponding structural YF-17D genes.

D.3.1.1 Reversion

After vaccination, there is the potential of reversion of attenuated live dengue virus vaccines to a virulent form of the dengue virus, although this has not been seen in clinical trials so far. The potential reversion is based on the stability of the attenuating mutation(s), the number of attenuating mutations, and the nature of attenuating mutation. Attenuating mutations that are dependent upon a single base change may be more susceptible to reversion than a mutation that is stabilized by multiple base substitutions. In addition, attenuating mutations that are derived by deletions of segments of RNA are generally more stable against reversion. Changes in virus genotype have the potential to influence disease transmission, tropism of vector vaccine, virulence, and/or patterns of disease, resulting in a virus with a previously unknown combination of properties. However, the

likelihood of such a reversion depends on the number of attenuation mutations present and the viral genes involved in the vaccine virus [9]

D.3.1.2 Recombination

Whether or not recombination takes place among flaviviruses is controversial. Theoretically, recombination between live DENV vaccines and wild type flaviviruses could produce a virus with an altered phenotype, but currently there is no evidence to support this [24, 34, 35, 37, 41, 46, 48].

The potential for recombination issues within and between flaviviruses has been widely discussed and challenged in the past years, based on existing literature [24, 34, 35, 37, 41, 48], and also from data obtained in specific experiments. In particular, a "recombination trap", has been recently designed to allow the products of rare recombination events to be selected and amplified, in the case of West Nile (WN), tick-borne encephalitis (TBE) and Japanese encephalitis (JE) viruses [48]. Intergenomic but aberrant recombination was observed only in the case of JE virus, and not for WN or TBE viruses. Moreover, its frequency appeared to be very low and generated viruses with impaired growth properties.

While their likelihood of appearance is very low, as stated above, the potential adverse effects of recombined DENVs should be evaluated in the ERA. In this respect, "worst-case" scenario chimeras have been constructed to address that risk [34, 35, 41].

These different studies showed that such recombinants constructed artificially from a wild type flavivirus and a chimeric vaccine [41], or from two wild type viruses, such as highly virulent YF Asibi virus and wild type DEN4 virus [35], were highly attenuated compared to their parental viruses. Attenuation was shown in culture *in vitro*, in mosquito vectors and in susceptible animal models, including monkeys. These data provide experimental evidence that the potential of recombinants, should they ever emerge, to cause disease or spread would probably be very low. Dual infection laboratory studies between vaccine and wildtype strains are not recommended because the predictive clinical value of such studies would be low.

D.3.2 Vector transmission

The presence of the DENV vectors such as *Ae. aegypti* and *Ae. albopictus* play a key role in the transmission of flaviviruses and potentially live DENV vaccines from the vaccinated subject to other individuals. Dengue does not usually spread directly from person to person, except via blood transfusion. Transmission of the dengue vaccine in regions in which the vector is absent is therefore highly unlikely. Dengue is currently restricted to the tropical regions. Due to climate change there is an increasing incidence of the detection of mosquito species not normally found in regions with traditionally milder climates. This could lead to the introduction of dengue in these areas and potentially change disease transmission and population immune status.

Recombination between live DENV vaccines and wild type flaviviruses could theoretically occur in the vaccinee (see above), but possibly also within an infected mosquito although neither has been reported. A recombined DENV could potentially, for instance in combination with climate change, use new vectors for transmission leading to previously unknown transmission characteristics. Therefore, the presence of a relevant mosquito vector and a 'dengue favorable climate' in the vaccination region should be taken into account in the ERA of live DENV vaccines.

To assess the likelihood of effective transmission of the vaccine from a vaccinated individual, one has to take into consideration two parameters: the level of viremia in the vaccinated hosts, and the ability of the mosquito vectors to transmit the live DENV vaccine to new hosts. The blood titer required for effective transmission is difficult to define. If the candidate vaccine strains replicate at low levels (1-2 logs) then possible initiation of the transmission cycle (mosquito infection) is reduced. For instance, in the case of the YFV17D chimeric vaccine, viremia in the hosts after tetravalent vaccination is very low [39]. In case the viremia titer is high enough and virus is intrinsically incubated in the mosquito, then the ability to transmit the virus to other hosts will be critical. For the recombinant live attenuated DENV vaccines currently in clinical trial, the likelihood of occurrence appears to be low to negligible. These viruses replicate to titers thousands-fold lower than that which results in transmission to mosquitoes. In addition, the ability of these viruses to infect, replicate, and escape the midgut is impaired [4, 6, 22, 28, 50, 53, 54].

The outcome of the ERA for clinical trials in regions in which the vector is absent, is that the environmental risk is negligibly small. The mosquito vector is not present and therefore the vaccine or possible created recombinants cannot be transmitted to other people. However, in endemic areas, it is important to perform an ERA as the vector is present, thus there is a possibility of transmission.

D.3.3 Immune status

Live DENV vaccines are able to replicate and form new viral particles in vaccinated persons. The immune status of the vaccinee and the rest of the population could influence the environmental risk of a live DENV vaccine. In general, the presence of pre-existing immunity due to earlier exposure to DENVs will reduce the extent and duration of vaccine virus replication and biodistribution. The potential for transmission of the vaccine is therefore considered to be greater in naïve or immunocompromised individuals.

An unvaccinated population with no pre-existing immunity will respond differently upon exposure to the vaccine compared to a population in which dengue is endemic. The immune status should therefore be taken into account in the ERA as it can influence the environmental impact of the vaccines, and the potential occurrence of adverse effects in contacts of the vaccinees. There is a potential for pre-existing heterotypic antibody to cause higher levels of vaccine virus. Enhanced illness in vaccine recipients who have pre-existing DENV antibody (antibody-dependent enhancement) has not been observed in clinical trials of live attenuated dengue vaccines to date and was not observed in a

clinical trial of live attenuated dengue vaccines designed to address this possibility [10, 20].

Part E. Guidelines for national regulatory authorities

E.1 General

The general recommendations for national regulatory authorities and national control laboratories given in the *Guidelines for national authorities on quality assurance for biological products* [59] should apply. In addition, the general recommendations for national regulatory authorities and national control laboratories provided in the *Guidelines for independent lot release of vaccines by regulatory authorities* [72] should be followed. These Guidelines specify that no new biological substance should be released until consistency of manufacturing and quality as demonstrated by a consistent release of batches has been established. The detailed production and control procedures and any significant changes in them should be discussed with and approved by the national regulatory authority. The national regulatory authority should obtain the working reference from manufacturers to establish a national working reference preparation until an international reference reagent is available.

E.2 Release and certification

A vaccine lot should be released only if it fulfils the national requirements and/or Part A of the present Guidelines. 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 national regulatory authority in support of a request for release of vaccine for use.

A statement signed by the appropriate official of the national regulatory authority should be provided if requested by a manufacturing establishment and should certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these Guidelines. The certificate should also state the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, the date of the last satisfactory determination of antigen concentration as well as assigned expiry date on the basis of shelf life should be stated. A copy of the official national release document should be attached. The certificate should be based on the model given in Appendix 2. The purpose of the certificate is to facilitate the exchange of dengue virus vaccines between countries.

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Appendix 1: Model summary protocol for manufacturing and control of dengue tetravalent vaccines (live, attenuated)

The following protocol is intended for guidance, and indicates the information that should be provided as a minimum by the manufacturer to the national regulatory authority. Information and tests may be added or deleted if necessary to be in line with the marketing authorization approved by the national regulatory authority. It is thus 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 license and with the relevant WHO guidelines of a particular product should be given in the protocol submitted. The section concerning the final product should be accompanied by a sample of the label and a copy of the leaflet that accompanies 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 national regulatory authority of the country in which the vaccine was produced and /or released stating that the product meets national requirements as well as Part A guidelines of this document published by WHO.

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

International name:	_____
Commercial name:	_____
Product license (marketing authorization) number :	_____
Country:	_____
Name and address of manufacturer:	_____
Name and address of product license holder if different:	_____
Virus strains:	_____
Origin and short history:	_____
Batch number(s):	_____
Finished product (final lot):	_____
Final bulk:	_____
Type of container:	_____
Number of filled containers in this final lot:	_____
Number of doses per container:	_____
Composition (antigen concentration) / volume of single human dose:	_____
Target group:	_____
Expiry date:	_____
Storage conditions:	_____

2. Summary Information on manufacture

Batch number of each monovalent bulk:	_____
Site of manufacture of each monovalent bulk:	_____
Date of manufacture of each monovalent bulk:	_____
Batch number of final bulk:	_____
Site of manufacture of final bulk:	_____
Date of manufacture of final bulk:	_____
Date of manufacture (filling or lyophilizing) of finished product (final vaccine lot):	_____
Date on which last determination of virus concentration was started:	_____
Shelf-life approved (months):	_____
Storage conditions :	_____
Volume of single dose :	_____
Prescribed virus concentration per human dose:	_____
Serotype 1:	_____
Serotype 2:	_____
Serotype 3:	_____
Serotype 4:	_____
Antibiotiques addenda :	_____
Release date :	_____

A genealogy of the lot numbers of all vaccine components used in the formulation of the final product will be informative.

The following sections are intended to report the results of the tests performed during the production of the vaccine

3. Control of source materials

3.1 Cell cultures

3.1.1 General information on cell banking system

Information and results of characterization tests on cell banking system from cell seed (if applicable), master cell bank (MCB), working cell bank (WCB), end of production cells (EOPC) or extended cell bank (ECB) should be provided according to the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks

Name and identification of cell substrate: _____

Origin and short history (attach a flowchart if necessary): _____

Lot number & date of preparation for each bank _____

Date established for each bank: _____

Date of approval by the national regulatory authority: _____

Total number of ampoules stored for each bank: _____

Passage/Population doubling level (PDL) of each bank _____

Maximum passage/ population doubling level approved for each bank: _____

Storage conditions: _____

Date of approval of protocols indicating compliance with the requirements of the relevant monographs and with the marketing authorization: _____

3.1.2. Characterization tests on cell seed (if applicable), MCB, WCB, EOPC or ECB

A summary table for characterization tests on each bank should be provided

Characterization tests performed on each bank

Methods: _____

Specifications: _____

Date tested: _____

Results: _____

3.1.3 Cell culture medium

Serum used in cell culture medium

Animal origin of serum: _____

Batch number: _____

Vendor: _____

Country of origin: _____

Certificate for TSE free: _____

Tests performed on serum:

Methods: _____

Specifications: _____

Date of test: _____

Results:

Trypsin used for preparation of cell cultures

Animal origin of trypsin:

Batch number:

Vendor:

Country of origin:

Certificate for TSE-free:

Tests performed on trypsin:

Methods:

Specifications:

Date of test:

Results:

Antibiotics

Nature and concentration of antibiotics or selecting agent (s) used in production cell culture maintenance medium:

Other source material

Identification and source of starting materials used in preparing production cells including excipients and preservatives (particularly any materials of human or animal origin e.g. albumin; serum):

3.2 Virus seeds

Vaccine virus strain(s) and serotype(s):

Substrate used for preparing seed lots:

Origin and short history:

Authority that approved virus strain(s):

Date approved:

3.2.1 Information on seed lot preparation

Virus master seed (VMS)

Source of virus master seed lot:

Virus master seed lot number:

Name and address of manufacturer: _____
Passage level: _____
Date of inoculation: _____
Date of harvest: _____
Number of containers: _____
Conditions of storage: _____
Date of establishment: _____
Maximum passage level approved for VMS: _____

Date approved by the national regulatory authority: _____

Virus working seed (VWS)

Virus working seed lot number: _____
Name and address of manufacturer: _____
Passage level from virus master seed lot: _____
Date of inoculation: _____
Date of harvest: _____
Number of containers: _____
Conditions of storage: _____
Date of establishment: _____
Date approved by the national regulatory authority: _____

3.2.3 Tests on VMS and VWS

Identity test

Method: _____
Specification: _____
Lot number of reference reagents : _____
Date of test (on, off): _____
Result: _____

Genetic/phenotypic characterizations

Method: _____
Reference reagents _____
Specification: _____
Date of test (on, off): _____
Result: _____

Tests for bacteria and fungi

Method: _____
Specification: _____

Media: _____
Number of containers tested : _____
Volume of inoculum per container: _____
Volume of medium per container: _____
Temperatures of incubation: _____
Date of test (on, off): _____
Result: _____

Test for mycoplasmas

Method: _____
Specification: _____
Media: _____
Volume tested: _____
Temperature of incubation _____
Positive controls: _____
Date of test (on, off): _____
Result: _____

Test for mycobacteria

Method: _____
Specification: _____
Media: _____
Volume tested: _____
Temperature of incubation _____
Date of test (on, off): _____
Result: _____

Adventitious agents:

Volume of virus seed samples for
neutralization and testing _____
Batch number(s) of antisera/antiserum used
for neutralization of virus seeds _____

Test in tissue cultures for adventitious agents

Test in simian cells:
Type of simian cells _____
Quantity of neutralized sample inoculated: _____
Incubation conditions: _____
Method: _____
Specification: _____
Date of test (on, off): _____
Ratio of cultures viable at end of test _____

Result: _____

Test in human cells

Type of simian cells _____

Quantity of neutralized sample inoculated: _____

Incubation conditions: _____

Method: _____

Specification: _____

Date of test (on, off): _____

Ratio of cultures viable at end of test _____

Result: _____

Other cell types:

Type of cells _____

Quantity of neutralized sample inoculated: _____

Incubation conditions: _____

Method: _____

Specification: _____

Date of test (on, off): _____

Ratio of cultures viable at end of test _____

Result: _____

Test in animals for adventitious agents

Method: _____

Specification: _____

Date of test (on, off): _____

Result: _____

Test by molecular methods for adventitious agents

Method: _____

Specification: _____

Date of test (on, off): _____

Result: _____

Tests in non-human primates (either master or working seed lot) for neurovirulence

(For details, please see Recommendations for yellow fever vaccine)

Method: _____

Specification: _____

Date of test (on, off): _____

Result: _____

Tests in suckling mice (either master or working seed lot; where necessary) for neurovirulence

(Detailed protocol should be developed)

Method: _____
Specification: _____
Date of test (on, off): _____
Result: _____

Virus titration for infectivity

Method: _____
Specification: _____
Date of test (on, off): _____
Result: _____

4. Control of vaccine production

4.1 Control of production cell cultures

4.1.1 Information on preparation

Lot number of MCB _____
Lot number of WCB _____
Date of thawing ampoule of WCB: _____
Passage number of production cells _____
Date of preparation of control cell cultures _____
Result of microscopic examination _____

4.1.2 Tests on control cell cultures

Amount or ratio of control cultures to production cell cultures: _____
Incubation conditions: _____
Period of observation of cultures: _____
Date started/ended: _____
Ratio of cultures discarded and reason: _____
Results of observation: _____
Date supernatant fluid collected: _____

Test for haemadsorbing viruses

Quantity of cells tested: _____
Method: _____
Specification: _____
Date of test (on, off): _____

Result: _____

Test for adventitious agents on supernatant culture fluids

Test in simian cells:

Type of simian cells _____
Quantity of pooled sample inoculated: _____
Incubation conditions: _____
Method: _____
Specification: _____
Date of test (on, off): _____
Ratio of cultures viable at end of test _____
Result: _____

Test in human cells

Type of simian cells _____
Quantity of pooled sample inoculated: _____
Incubation conditions: _____
Method: _____
Specification: _____
Date of test (on, off): _____
Ratio of cultures viable at end of test _____
Result: _____

Other cell types:

Type of cells _____
Quantity of pooled sample inoculated: _____
Incubation conditions: _____
Method: _____
Specification: _____
Date of test (on, off): _____
Ratio of cultures viable at end of test _____
Result: _____

Identity test:

Method: _____
Specification: _____
Date test on: _____
Date test off: _____
Result: _____

4.1.3 Cells used for vaccine production:

Observation of cells used for production

Specification: _____

Date: _____

Result: _____

4.2 Monovalent virus harvest pools

4.2.1 Information on manufacture

Information on each monovalent virus harvest pool should separately be provided

Batch number(s): _____

Date of inoculation: _____

Date of harvesting: _____

Lot number of Virus master seed lot _____

Lot number of virus working seed lot _____

Passage level from virus working seed lot _____

Methods, date of purification if relevant _____

Volume(s), storage temperature, storage
time and approved storage period: _____

4.2.2 Tests on monovalent virus harvest pools

Identity

Method: _____

Specification: _____

Lot number of reference reagents: _____

Specification: _____

Date of test _____

Result: _____

Test for bacteria and fungi

Method: _____

Specification: _____

Media: _____

Number of containers tested: _____

Volume of inoculum per container: _____

Volume of medium per container: _____

Temperatures of incubation: _____

Date of test (on, off): _____

Result: _____

Test for mycoplasma

Method: _____
 Specification: _____
 Media: _____
 Volume tested: _____
 Temperature of incubation _____
 Positive controls: _____
 Date of test (on, off) _____
 Result: _____

Test for mycobacteria

Method: _____
 Specification: _____
 Media: _____
 Volume tested: _____
 Temperature of incubation _____
 Date of test (on, off) _____
 Result: _____

Test for adventitious agentsTest in simian cells:

Type of simian cells _____
 Quantity of neutralized sample inoculated: _____
 Incubation conditions: _____
 Method: _____
 Specification: _____
 Date of test (on, off): _____
 Ratio of cultures viable at end of test _____
 Result: _____

Test in human cells

Type of simian cells _____
 Quantity of neutralized sample inoculated: _____
 Incubation conditions: _____
 Method: _____
 Specification: _____
 Date of test (on, off): _____
 Ratio of cultures viable at end of test _____
 Result: _____

Other cell types:

Type of cells _____
Quantity of neutralized sample inoculated: _____
Incubation conditions: _____
Method: _____
Specification: _____
Date of test (on, off): _____
Ratio of cultures viable at end of test _____
Result: _____

Virus titration for infectivity

Method: _____
Specification: _____
Date of test (on, off): _____
Result: _____

Test for host cell proteins

Method: _____
Specification: _____
Date of test _____
Result: _____

Test for residual cellular DNA

Method: _____
Specification: _____
Date of test _____
Result: _____

Consistency of virus characteristics

Method: _____
Specification: _____
Date of test (on, off): _____
Result: _____

4.3. Final tetravalent vaccine bulk

4.3.1 Information on manufacture

Batch number(s): _____
Date of formulation: _____
Total volume of final bulk formulated: _____
Monovalent virus pools used for
formulation _____

Serotype / lot number / volume added /
virus concentration _____
Name & concentration of added substances
(e.g. diluent, stabilizer if relevant): _____
Volume(s), storage temperature, storage
time and approved storage period: _____

4.3.2 Tests on final tetravalent bulk lot

Residual animal serum protein

Method: _____
Specification: _____
Date of test (on, off): _____
Result: _____

Test for bacteria & fungi

Method: _____
Specification: _____
Media: _____
Number of containers tested: _____
Volume of inoculum per container: _____
Volume of medium per container: _____
Temperatures of incubation: _____
Date of test (on, off): _____
Result: _____

5. Filling and containers

Lot number _____
Date of filling _____
Type of container: _____
Volume of final bulk filled _____
Filling volume per container _____
Number of containers filled (gross) _____
Date of lyophilization _____
Number of containers rejected during
inspection _____
Number of containers sampled _____
Total number of containers (net) _____
Maximum period of storage approved _____
Storage temperature and period _____

6. Control tests on final vaccine lot

6.1 Tests on vaccine lot

Inspection of final containers

Appearance	_____
Specification:	_____
Date of test	_____
Results	_____
Before reconstitution	_____
After reconstitution	_____
Diluent used	_____
Lot number of diluent used	_____

Test for pH

Method:	_____
Specification:	_____
Date of test	_____
Result:	_____

Identity test (each serotype)

Method:	_____
Specification:	_____
Date of test	_____
Result:	_____

Test for bacteria and fungi

Method:	_____
Specification:	_____
Media:	_____
Volume tested:	_____
Temperatures of incubation:	_____
Date of test (on, off):	_____
Result:	_____

Test for potency (each serotype):

Method:	_____
Batch number of reference vaccine and assigned potency:	_____
Specification:	_____
Date of test (on, off):	_____

Result for each serotype: _____

Thermal stability (each serotype)

Method: _____

Specification: _____

Date of test (on, off): _____

Result for each serotype: _____

General safety (unless deletion authorized)

Tests in mice

Date of inoculation _____

No. of animals tested _____

Volume and route of injection _____

Observation period _____

Specification: _____

Results (give details of deaths) _____

Tests in guinea-pigs

Date of inoculation _____

No. of animals tested _____

Volume and route of injection _____

Observation period _____

Specification: _____

Results (give details of deaths) _____

Residual moisture

Method _____

Specification _____

Date _____

Result _____

Residual antibiotics if applicable

Method _____

Specification _____

Date _____

Result _____

6.2 Diluent

Name and composition of diluent	_____
Lot number	_____
Date of filling	_____
Type of diluent container	_____
Filling volume per container	_____
Maximum period of storage approved	_____
Storage temperature and period	_____

Name (Typed): _____

Signature: _____

Date: _____

Appendix 2: Model certificate for the release of dengue vaccine (live, tetravalent) by national regulatory authorities

LOT RELEASE CERTIFICATE

The following lot(s) of dengue vaccine produced by _____⁽¹⁾ in _____⁽²⁾, whose numbers appear on the labels of the final containers, meet all national requirements⁽³⁾ and Part A⁽⁴⁾ of the WHO Guidelines to assure the quality, safety and efficacy of live attenuated (recombinant) dengue virus vaccines (_____)⁽⁵⁾, and comply with Good Manufacturing Practices for Pharmaceutical Products: Main Principles⁽⁶⁾ and Good Manufacturing Practices for Biological Products⁽⁷⁾. As a minimum, this certificate is based on examination of the summary protocol of manufacturing and control.

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, packaging lot numbers if necessary);
- Type of container;
- Number of doses per container;
- Number of containers/lot size;
- Date of start of period of validity (e.g. manufacturing date) and/or expiry date;
- Storage condition;
- Signature and function of the authorized person and authorized agent to issue the certificate;
- Date of issue of certificate; and
- Certificate number.

The Director of the National Regulatory Authority (or Authority as appropriate):

Name (Typed)

Signature

Date

1 Name of manufacturer

2 Country of origin

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

4 With the exception of provisions on distribution and shipping, which the national regulatory authority may not be in a position to assess.

5 WHO Technical Report Series, No. ____, YYYY, Annex __.

6 WHO Technical Report Series, No. 908, 2003, Annex 4.

7 WHO Technical Report Series, No. 823, 1992, Annex 1.

Appendix 3: Procedure for environmental risk assessment

This appendix provides an example of the steps to be followed in conducting an ERA, as exemplified by the EU Directive 2001/18/EC (Annex II) and Commission Decision 2002/623/EC.

Step 1:

Identification of characteristics that may cause potential adverse effects and evaluation of the potential consequences of each adverse effect (hazard identification)

A hazard is defined as a potential harmful effect on human health or the environment, caused by features of the GMO and its application. Effects on human health or the environment are differentiated into direct and indirect effects. Direct effects occur due to an interaction between the GMO itself and its direct environment. Indirect effects are the result of a more extensive causal chain of events. Moreover, both direct and indirect effects may be either immediate or delayed. Immediate effects occur during the time period of the release, and can usually be attributed to the release in a straightforward manner, whereas delayed effects may become apparent at a later stage, after termination of the release, and consequently they are usually less easily attributed to an effect caused by the GMO. Potential adverse effects may include disease to humans (other than the patient), animals or any other organism, or altered susceptibility to pathogens facilitating the dissemination of infectious diseases.

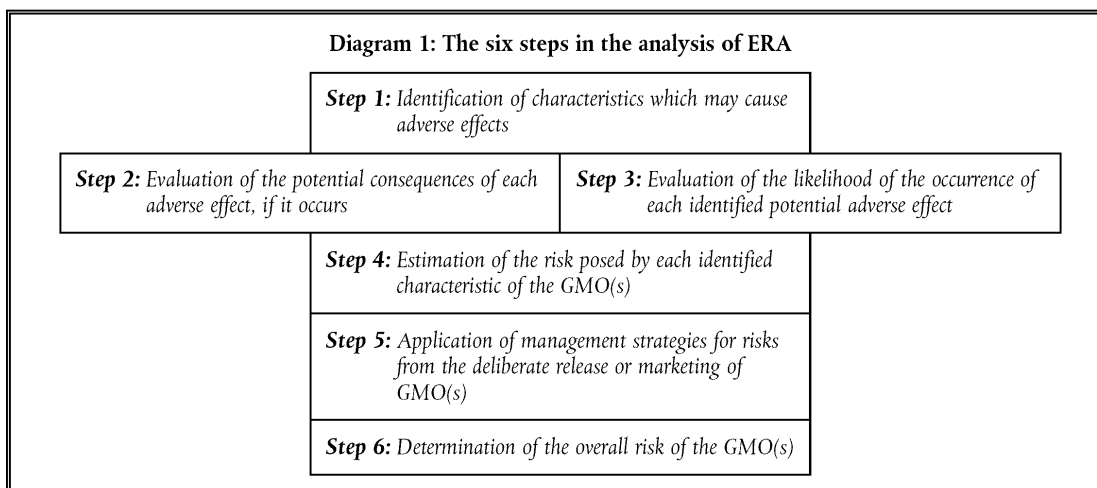


Figure 1. Steps in the analysis of ERA according to the guidelines in the Commission Decision 2002/623/EC and 2001/18/EC

Step 2:**Evaluation of the potential consequences of each adverse effect, if it occurs**

The magnitude of the consequences is likely to be influenced by the environment in which the GMO is released and the manner of release. For each adverse effect that is identified the consequences for the environment need to be qualified in qualitative terms ranging from high, moderate, low to negligible.

Step 3:**Evaluation of the likelihood**

The next step in the ERA deals with the evaluation of the likelihood of the occurrence of the described adverse effects and their consequences, i.e. whether the potential adverse effect associated with the application of the GMO will be effectuated. All characteristics that could contribute to the actual occurrence of the hazard should be identified. For the determination of the likelihood, it is important to consider the manner of release and the surrounding receiving environment. If no quantitative data are available to determine the likelihood, the likelihood should be described in qualitative terms ranging from high, moderate, or low to negligible, based on an expert judgment that considers the available data. In case the likelihood of a potential effect cannot be determined, a worst-case scenario will be applied that assumes the potential adverse effect will occur to the fullest extent.

Step 4:**Risk estimation**

An estimation of the risk to human health or the environment posed by each identified characteristic of the GMO which has the potential to cause adverse effects should be made by multiplying the likelihood of the adverse effect occurring and the consequences, were it to occur. The risk is usually characterized on scale, e.g. 'negligible', 'low', 'moderate', or 'high'. Usually it is concluded that risk management should be applied to reduce the risk, if the risk is characterized as higher than negligible.

Step 5:**Application of risk management strategies**

Where the estimated overall risk is higher than negligible, risk management strategies should be applied that are adequate to reduce the level of risk. In practice, risk management measures usually reduce the likelihood instead of the potential adverse effects. In most cases risk management measures aim at reducing spreading of the GMO into the environment, e.g. by hospitalization of the patient or bandaging of the application site. Note that risk management strategies to prevent transmission of dengue virus is likely to be ineffective or unfeasible in certain parts of the world. Therefore, it should be well considered to demand the use of mosquito nets and repellents for an appropriate period after vaccination as an effective strategy to reduce this risk.

Step 6:

Determination of the final risk

An evaluation of the final overall risk of the GMO should be made taking into account any proposed risk management strategies. The determination of the final risk should conclude an ERA based on the previous steps described under step 1 to 5. Steps 4 and 5 may be repeated in situations where the overall environmental impact is not acceptable. In that case, the ultimate risk management tool is not to perform the activity with the GMO until more scientific data are available and the ERA may be repeated on the basis of these data. It is up to the competent authority to decide whether risks are acceptable or not, and which risk management measurements are should be installed

In the assessment of clinical trials and marketing authorization approvals, environmental risk assessment and risk management strategies should be taken into account, but there should always be a well balanced risk and benefit consideration.

Abbreviations

ADE	antibody-dependent enhancement
<i>Ae</i>	<i>Aedes</i>
AEs	adverse events
CCID50	cell culture infectious dose 50%
CDC	Centers for Disease Control and Prevention
CMI	cell-mediated immunity
CNS	central nervous system
DENVs	dengue viruses
DFI	dengue febrile illness
DSMB	data and safety monitoring board
E	envelope
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EOPC	end of production cell
ECB	extended cell bank
ERA	environmental risk assessment
GMO	genetically modified organism
GMTs	geometric mean titres
IFU	immunofocus-forming unit
IU	international unit
JE	Japanese encephalitis
MCB	master cell bank
NAAT	nucleic acid amplification techniques
NHP	non-human primate
NIAID	National Institute of Allergy and Infectious Diseases
NIBSC	National Institute of Biological Standards and Control
NS	non-structural
ORF	open reading frame
PBMCs	peripheral blood mononuclear cells
PCC	production cell culture
PDK	primary dog kidney
PDL	population doubling
PFU	plaque-forming unit
prM	premembrane
PRNT	plaque reduction neutralization test
QPCR	quantitative polymerase chain reaction
RCB	reference cell bank
RT-PCR	reverse transcription-polymerase chain reaction
TBE	tick-borne encephalitis
TSE	transmissible spongiform encephalopathies
UTR	untranslated region
VE	vaccine efficacy
VMS	virus master seed

VWS	virus working seed
WCB	working cell bank
WN	West Nile
WRAIR	Walter Reed Army Institute of Research
YFV	yellow fever virus

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