

Annex 2

Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated)

Replacement of Annex 1 of WHO Technical Report Series, No. 932

Abbreviations	55
Introduction	55
General considerations	56
Part A. Guidelines on manufacturing and control of dengue tetravalent vaccines (live, attenuated)	62
A.1 Definitions	62
A.2 General manufacturing requirements	65
A.3 Control of source materials	65
A.4 Control of vaccine production	71
A.5 Filling and containers	76
A.6 Control tests on final lot	77
A.7 Records	79
A.8 Samples	79
A.9 Labelling	79
A.10 Distribution and shipping	80
A.11 Stability, storage and expiry date	80
Part B. Nonclinical evaluation of dengue tetravalent vaccines (live, attenuated)	81
B.1 General remarks	81
B.2 Product development and characterization	83
B.3 Nonclinical immunogenicity and protective activity	83
B.4 Nonclinical toxicity and safety	84
B.5 Environmental risk	87
Part C. Clinical evaluation of dengue tetravalent vaccines (live, attenuated)	87
C.1 General considerations for clinical studies	87
C.2 Immunogenicity	89
C.3 Clinical studies	91
C.4 Post-licensure investigations	102
Part D. Environmental risk assessment of dengue tetravalent vaccines (live, attenuated) derived by recombinant DNA technology	103
D.1 Introduction	103

D.2	Procedure for environmental risk assessment	105
D.3	Special considerations for live recombinant dengue vaccines	106
Part E. Guidelines for NRAs		109
E.1	General	109
E.2	Release and certification	109
Authors		110
Acknowledgements		113
References		114
Appendix 1		
	Summary protocol for manufacturing and control of dengue tetravalent vaccine (live, attenuated)	119
Appendix 2		
	Model certificate for the release of dengue tetravalent vaccine (live, attenuated) by NRAs	134

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 Guidelines 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.

Abbreviations

<i>Ae</i>	<i>Aedes</i>
CCID ₅₀	cell culture infectious dose 50%
CDC	Centers for Disease Control and Prevention
CMI	cell-mediated immunity
DENVs	dengue viruses
DFI	dengue febrile illness
E	envelope
ELISA	enzyme-linked immunosorbent assay
ERA	environmental risk assessment
GMO	genetically modified organism
IU	International Unit
NIAID	National Institute of Allergy and Infectious Diseases
NS	non-structural
PDK	primary dog kidney
prM	premembrane
PRNT	plaque-reduction neutralization test
RT-PCR	reverse transcription-polymerase chain reaction
TRS	Technical Report Series
TSE	transmissible spongiform encephalopathy
UTR	untranslated region
VE	vaccine efficacy
YFV	yellow fever virus

Introduction

These Guidelines are intended to provide national regulatory authorities (NRAs) 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) (1). They should be read in conjunction with other WHO guidelines that are referred to in each part. The 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 on clinical evaluation) may be useful for the evaluation of other types of dengue vaccine. For quality control, 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 processes. For example, guidelines for human papillomavirus and hepatitis B vaccines may also be useful for subunit vaccines for dengue.

These Guidelines are based on experience gained from candidate dengue tetravalent vaccines (live, attenuated) that have been developed as described below, and will need to be updated as new data become available from additional studies. Part A sets out guidelines for manufacture and quality control. Guidelines specific to the nonclinical and clinical evaluation and environmental risk assessment are provided in parts B, C and D, respectively. Part E provides guidelines for NRAs. In the following section, brief overviews of dengue disease and dengue vaccine development at the time of preparing this document are provided as a 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 DENV-4) and at least three 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 that is flanked by a 5' untranslated region (UTR) and a 3' UTR. The open reading frame 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, and RNA 5'-triphosphatase activities in the C-terminal region. 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 aegypti* mosquitoes, which are highly domesticated and the primary mosquito vector. However, *Aedes 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 the 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 nonhuman primates. Certain strains of DENV are known to be transmitted to nonhuman primates in western Africa and Malaysia. However, transmission to humans via mosquitoes from nonhuman primates is believed to be very limited.

Clinical and pathological manifestation in humans

Following infection resulting from 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 bloodstream. Haematogenous spread is the likely mechanism for seeding of peripheral organs and the occasional reports of central nervous system 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 4–7 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, on the insides of the arms and thighs, and on 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 travelling 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 WHO document (or its subsequent update) (2).

Nonhuman primate dengue virus infection

Natural hosts for DENV infection are humans and mosquitoes. Serological evidence from nonhuman primate studies indicates the existence of a sylvatic DENV cycle involving several species of mosquitoes and several monkey species. Although monkeys develop a viraemia and a neutralizing antibody response to DENV infection, they do not develop the haematological abnormalities seen in humans. However, in nonhuman primates (and AG129 mouse, see item below) primary DENV infections cause a leukopenia. Thrombocytopenia has been observed after a secondary infection (3–6).

In the rhesus macaque, viraemia typically begins 2–6 days after infection and lasts for 3–6 days (3, 5, 7). 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 nonhuman primate model for DENV is useful for measuring the protection from viraemia conferred by vaccination or passively acquired antibody. Disadvantages of the nonhuman primate model include the lack of overt clinical signs of disease (4–6, 8).

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 DENV-2 isolate to neurovirulence in suckling mice correlated positively with attenuation of virulence in humans (9). Because of this ambiguity, the suckling mouse/encephalitis model is probably not useful for studying the safety or efficacy of candidate dengue vaccines. Nevertheless, it could be used to assess lot consistency (see sections 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 dengue infection and to study pathogenesis (4, 10, 11). Interferon receptor-deficient AG129 mice support replication of selected DENV strains which infect relevant cell and tissue types comparable to human infection (10, 12). AG129 mice have been used to investigate antibody-mediated protection. A strain of DENV-2 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 of virus infection was observed in AG129 mice following passive transfer of anti-DENV-1 antibodies and challenge with the adapted strain of DENV-2 (10, 12, 13). The relevance of such an immunocompromised mouse model may, however, be limited with regard to vaccine evaluation (see section 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. *Ae. 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 (14). 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 (6, 15, 16).

None of the live dengue vaccine preparations currently in the clinical trial phase of development is effectively transmitted by mosquito vectors (16, 17), because vaccine viruses replicate poorly in mosquito midgut epithelium and/or do not disseminate efficiently to the salivary glands, thereby effectively precluding transmission to humans (6, 18, 19). In addition, the low peak titre and very short duration of viraemia 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.

Populations at risk 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 geographical 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 million to 1.3 million in the decade 1996–2005 (2).

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 substantial 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 focused primarily 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 or chimerization between flaviviruses. 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 composed of prM and E proteins, and DNA vaccines that induce expression of DENV prM/E proteins, are in the nonclinical 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 the efficacy of vaccine candidates and to identify or 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 nonclinical studies using monkeys and susceptible mouse strains suggest, however, that protection from dengue is best correlated with the presence of virus-neutralizing antibodies (3, 20–26). 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 programmes.

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 in clinical development at the present time. The Walter Reed Army Institute of Research developed attenuated DENV strains

by empirical serial passage in primary dog kidney (PDK) cells and produced vaccine candidates in fetal rhesus lung cells. Tetravalent formulations of these attenuated vaccine candidates have been evaluated in Phase 1 and Phase 2 clinical trials conducted by the Walter Reed Army Institute of Research and GlaxoSmithKline (8, 27–29).

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 United States National Institute of Allergy and Infectious Diseases (NIAID) has thus derived a total of five candidate dengue 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 (8, 27–29). 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 (30). Phase 1 trials have been conducted with “ $\Delta 30$ ” monovalent vaccines (31), and Phase 1 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 1 and 2 clinical trials. The United States 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 used this genetic background to derive chimeric DENV-1, DENV-3 and DENV-4 vaccines expressing the respective prM/E genes in the context of the DENV-2 PDK53 genome “backbone” (8, 27). A tetravalent formulation is in Phase 1 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 1 and 2 clinical trials for safety and immunogenicity. Phase 2b trials to investigate protective efficacy in children began late in 2009 (8, 27) and Phase 3 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 tetravalent dengue virus vaccine (live, attenuated), as defined in section 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 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, Potters Bar, England. 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 is usually 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 1 and Phase 2 clinical trials. In the case of a tetravalent dengue vaccine, potency will have to be assessed in terms of the individual titres 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 Units (IU) to reduce variation between laboratories. Until then, the use of plaque-forming unit, immunofocus-forming unit or cell culture infectious dose 50%

(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 encephalopathy (TSE) agents and viruses that have been unintentionally introduced into the manufacturing process of a biological product.

Cell bank: 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%: the amount of a virus sufficient to cause a cytopathic effect in 50% of inoculated replicate cell cultures, as determined in an end-point dilution assay in monolayer cell cultures.

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.

Dengue febrile illness (DFI): the virological confirmation of dengue virus infection in patients with two days of fever irrespective of the 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 finished tetravalent vaccine prepared from virus harvest pools in the vessel from which the final containers are filled.

Genetically modified organism: 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: the amount of a virus required to generate one focus of infected cells that can be detected by dengue-specific antisera and a counter-stain in monolayer cell cultures.

Master cell bank: a quantity of well-characterized cells of animal or other origin, derived from a cell seed at a specific population doubling level 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 for the master cell bank to be used to derive working cell banks.

Virus master seed: 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 virus master seed is used as a source of infectious virus for the generation of each virus working seed lot.

Neurovirulence: the capacity of a microorganism to cause disease of the nervous system, leading to paralysis or dysfunction of the nervous system. In animal experimental settings, clinical and pathological evaluations are often carried out after intracranial inoculation of a microorganism.

Neurotropism: the affinity of a microorganism for, or for localizing selectively in, nerve tissue.

Monovalent virus pool: a suspension of single serotype of dengue virus that 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: the amount of a virus sufficient to cause a single visible focus of infection due to cytopathic effect in a cell culture monolayer after proper staining of cells.

Production cell culture: 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 the 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 virus working seed and processed together in a single production run.

Working cell bank: a quantity of well-characterized cells of animal or other origin, derived from the master cell bank at a specific population doubling level 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 working cell bank containers is used for each production culture.

Virus working seed: a quantity of virus of uniform composition, well characterized and derived from a virus master seed lot (see above) in a production cell line. The working seed lot is used for the production of a single harvest.

A.2 General manufacturing requirements

The general requirements for manufacturing establishments contained in WHO's Good manufacturing practices for biological products (32) should be applied by establishments manufacturing dengue tetravalent vaccine. Separate manufacturing areas for each of the four dengue serotypes and for 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 NRA and in accordance with 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 Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (33) and should be approved by the NRA. All information on the source and method of preparation of the cell culture system used should be made available to the NRA.

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 NRA. The maximum number of passages or population doubling allowable between the cell seed, the working cell bank and the production passage levels should be established by the manufacturer and approved by the NRA. Additional tests may include, but are not limited to: propagation of the master cell bank or working cell bank 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 (33).

WHO has established a bank of Vero cells, designated as WHO Vero reference cell bank 10-87 that has been characterized in accordance with the

WHO Requirements for continuous cell lines used for biologicals production (34). The cell bank is available to manufacturers, as is well-characterized starting material for manufacturers to prepare their own master and working cell banks on application to the Coordinator, Quality, Safety and Standards, WHO, Geneva, Switzerland (33).

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 NRA, at which point the cells are combined to give a single pool distributed into ampoules and preserved cryogenically to form the working cell bank. The manufacturer's working cell bank is used for the preparation of production cell culture, and thus for the production of vaccine batches.

A.3.1.4 Characterization of cell banks

The cell seed (if applicable), master and working cell banks and end-of-production cells or extended cell bank 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 (33).

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) (35, 36), as well as from infectious viruses.

Detailed guidelines for detecting bovine viruses in serum for establishing a master cell bank and working cell bank 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 (33). 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 NRA. 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.

The sources of animal components used in culture medium should be approved by the NRA. These components should comply with current guidelines in relation to animal TSE (37, 38).

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) (39), as well as current guidelines in relation to human TSE (37, 38).

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 (33).

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 NRA. Nontoxic pH indicators may be added (e.g. phenol red at a concentration of 0.002%). Only substances that have been approved by the NRA may be added.

If porcine or bovine trypsin is used for preparing cell cultures, it should be prepared, tested and found free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, as described in 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 (33). The methods used to ensure this should be approved by the NRA. The source(s) of trypsin of bovine origin, if used, should be approved by the NRA and should comply with current guidelines in relation to animal TSE (37, 38).

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 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, and whether the strains have been biologically or molecularly cloned prior to generation of the master seed), their genome sequence, the passage level at which clinical trials were performed, and the results of clinical studies. Only strains approved by the NRA 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 in section A.3.2.2.

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 genetically modified organism (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 some time prior to any further nonclinical or clinical trial. The cell substrate used for transfection to generate the virus should be appropriate for human vaccine production and should be approved by the NRA.

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

Viral vaccine seeds that are directly re-derived from RNA extracted from virus in order to reduce the risk of previous contamination by TSEs or other adventitious agents are considered as new vaccine seeds, and they 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 should have a well-defined relationship to the virus master seed with respect to passage level and method of preparation, such that the virus working seed retains all of the *in vitro* and *in vivo* phenotypes and the genetic character of the virus master seed. Nonclinical and clinical data are needed to support this relationship. Once the passage level of the virus working seed with respect to the virus master seed is established, it may not be changed without approval from the NRA.

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 virus working seed lot should 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 NRAs, 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

The serotype of all dengue virus master seeds and working seeds should be confirmed 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 nonclinical and small-scale clinical trials. Some variations in the nucleotide sequence of the 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 seeds and working seeds, it is recommended that the first three consecutive consistency bulk vaccine lots be analysed for consensus sequence changes from 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 General requirements for the sterility of biological substances (Requirements for biological substances, no. 6) (35, 36). Nucleic acid amplification techniques alone or in combination with cell culture, with an appropriate detection method, may be used as an alternative to one or both of the compendial mycoplasma detection methods after suitable validation and agreement from the NRA (33).

Seed lots should be shown to be free from mycobacteria by a method approved by the NRA. Nucleic acid amplification techniques may be used as an alternative to the microbiological culture method for mycobacteria and/or to the

in vivo guinea-pig test for the detection of mycobacteria after suitable validation and agreement from the NRA (33).

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. Monkey and human cell cultures inoculated with the virus antibody mixture should be observed microscopically for cytopathic changes. For virus grown in monkey 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 or working seed lot should also be tested in animals that include guinea-pigs, adult mice, and suckling mice. For test details, refer to section B.11 of WHO's Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (33). Additional testing for adventitious viruses may be performed using validated nucleic acid amplification techniques.

New molecular methods with broad detection capabilities are being developed for adventitious agent detection. These methods include: (i) degenerate nucleic acid amplification techniques for whole virus families with analysis of the amplicons by hybridization, sequencing or mass spectrometry; (ii) nucleic acid amplification techniques 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 (iii) high throughput sequencing. These methods may be used in the future to supplement existing methods or as alternative methods to both in vivo and in vitro tests after appropriate validation and agreement from the NRA.

A.3.2.5.5 *Tests in experimental animals*

Tests in nonhuman primates: all vaccine candidates should be evaluated, at least once during nonclinical development, for neurovirulence in nonhuman 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 neurovirulence (e.g. dengue/yellow fever recombinants), the master seed should be tested in nonhuman primates. If these tests were not performed at the master seed level, they should be performed at working seed level.

NRAs 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 nonhuman primates, at least where viruses expressing yellow fever strain 17D genes are concerned (40). NRAs may eventually wish to consider accepting results of such studies as a surrogate for studies using nonhuman primates to evaluate neurovirulence of novel dengue vaccines.

Test for neurovirulence: to provide assurance that a candidate vaccine virus is not unexpectedly neurovirulent, each vaccine strain of each serotype, or a tetravalent formulation if agreed by the NRA, 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 neurovirulence, the neutralizing antibody test should be used to assess the immune status of nonhuman primates to both dengue and yellow fever viruses. For further details on the test for neurovirulence see Part B.

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 preclinical 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 (21, 41). A mouse test might be considered in order to demonstrate consistency of characteristics of dengue/yellow fever recombinant viruses during production (see section 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, immunofocus-forming unit assays or CCID₅₀ with a molecular readout such as quantitative polymerase chain reaction may be used. All assays should be validated.

A.4 Control of vaccine production

A.4.1 Control of production cell cultures

Where the NRA 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 morphological 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 seven days, and the storage temperature should have been in the range of 2–8 °C.

In some countries, the NRA requires that additional tests for haemadsorbing viruses should 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 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 the 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 (monkey) 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 NRAs 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 seven days. Furthermore, some NRAs 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 NRA. 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 the 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 the virus working seed. Prior to beginning virus harvests, the cell cultures should be rinsed and the growth medium should be 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 NRA.

A.4.2.2 Virus inoculation

Cell cultures are inoculated with dengue virus working seed at an optimal and defined multiplicity of infection. After viral adsorption, cell cultures are fed with maintenance medium and are 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 should be 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 throughout 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 to further purification to minimize the amount of cellular DNA, and/or to 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 NRA.

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

Each monovalent virus harvest pool should be shown by appropriate tests to be free from bacterial, fungal, mycoplasmal and mycobacterial contamination. 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) (35, 36).

Nucleic acid amplification techniques 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 NRA (33).

The method for testing mycobacteria should be approved by the NRA. Nucleic acid amplification techniques might be used as an alternative to the microbiological culture method for mycobacteria after validation and agreement by the NRA (33).

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 that is different from that used for the production of the vaccine and that is 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 nucleic acid amplification techniques.

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 quantitative polymerase chain reaction 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 (33).

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 NRA. 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 (33), as approved by the NRA.

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 virus working seed, or another 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 nonclinical studies and may include, for example, virus yield in tissue culture, plaque phenotype, or temperature sensitivity. Other identifying characteristics may also be applicable.

Assays for the attenuation of dengue/yellow fever recombinants and other vaccine viruses, if 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 for consistency 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 NRA. 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.

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 a manner that avoids contamination of the product.

In preparing the final bulk, any excipients (such as diluents or stabilizer) that are added to the product should have been shown to the satisfaction of the NRA 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) (35), or by a method approved by the NRA.

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 (32) 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 to assure sterility.

The manufacturer should provide the NRA with adequate data to prove that 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 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 should be 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 DENV-1, -2, -3 or -4 by immunological assay using specific antibodies or by molecular methods. The methods used for the potency assay (section 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 of Part A, section 5.2 of the General requirements for the sterility of biological substances (Requirements for biological substances, no. 6) (35), or by methods approved by the NRA.

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 serotype of dengue virus in the final tetravalent mixture should be determined.

A.6.1.6 Thermal stability

The purpose of the thermal stability test is to demonstrate consistency of production. Additional guidance on evaluation of vaccine stability is provided in WHO's Guidelines for stability evaluation of vaccines (42). At least three containers of tetravalent vaccine should be incubated at the appropriate elevated temperature for the appropriate time (e.g. 37 °C for seven days) depending on the products. The geometric mean titre of infectious virus in the containers for each individual virus serotype that has been exposed should not have decreased during the period of exposure by more than a specified amount (e.g. 1 log) that is justified by production data and approved by the NRA. Titration of non-exposed and exposed containers should be carried out 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 NRA. This test may be omitted for routine lot release once consistency of production has been established to the satisfaction of the NRA 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 NRA on the basis of the results of stability testing.

A.6.1.9 Residual antibiotics (if applicable)

If any antibiotics are added during the vaccine production, the content of the residual antibiotics should be determined and should be within limits approved by the NRA.

A.6.2 Diluent

The recommendations given in WHO's Good manufacturing practices for pharmaceutical products: main principles (43) should apply to the manufacturing and control of diluents used to reconstitute live, attenuated dengue vaccines. An expiry date should be established for the diluent on the basis of stability data.

For lot release of the diluent, tests should be done for identity, appearance, pH, volume, sterility, and the content of key components.

A.7 **Records**

The recommendations of Good manufacturing practices for biological products (32) should apply, as appropriate to 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 stored appropriately for that purpose.

A.9 **Labelling**

The recommendations of Good manufacturing practices for biological products (32) should apply, as appropriate for a candidate vaccine, 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 Guidelines;
- 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;

- a statement that after it has been reconstituted, the vaccine should be used without delay or, if not used immediately, stored at 2–8 °C and protected from light for a maximum period defined by stability studies.

A.10 **Distribution and shipping**

The recommendations given in Good manufacturing practices for biological products (32) appropriate for a candidate vaccine should apply.

Shipments should be maintained within specified temperature ranges and packages should contain cold-chain monitors (44).

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

The recommendations given in Good manufacturing practices for biological products (32) and in Guidelines for stability evaluation of vaccines (42) 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 NRA.

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 as appropriate 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 NRAs 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 NRAs.

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 NRA 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 should be supported by the stability studies with the approval of the NRA. 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 differ.

A.11.4 Expiry of reconstituted vaccine

For single-dose containers, the reconstituted vaccine should be used immediately. Multi-dose containers should be kept in the dark at 2–8 °C and the expiry time for use of an opened container should be defined by stability studies approved by the NRA, but should be not more than six hours.

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 that is required prior to initiation of the clinical phase of the vaccine development programme. 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 NRA, establishment of a test for potency to be used throughout, and safety testing in animals (see Table A2.1). These Guidelines, which are specifically aimed at nonclinical evaluation of a live, attenuated dengue vaccine, should be read in conjunction with the WHO Guidelines on nonclinical evaluation of vaccines (45).

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 were briefly reviewed at the time of preparing these Guidelines to highlight the latest developments and to provide a better understanding of their use in vaccine development.

Table A2.1
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 the manufacturing process; genetic stability of vaccine candidates.
Process development, quality control and quality assurance	Process meets all good manufacturing practice 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; demonstrated efficiency of purification processes; titration of virus dose; safety of excipients; standardized laboratory 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 dengue infection; estimate dose range for humans.	DENV live, attenuated vaccines are immunogenic in nonhuman primates; candidate should induce limited viraemia and should protect against viraemia following wild-type DENV challenge in nonhuman primates. Interference between DENV serotypes may be evaluated in mice or nonhuman primates, but data may not always correlate with data from humans.
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 prerequisite 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 markers of attenuation and phenotypic markers that suggest that the genome of a vaccine virus has remained stable following tissue culture passage. 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 virulence, either during manufacture or during replication in a vaccinee, using available *in vivo* and *in vitro* approaches. To this end, laboratory and animal studies should define genetic changes in the virus genome.

Phenotypic markers may be useful for detecting 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 for documenting the mutations in the vaccine virus genome that may correlate with its attenuated phenotype. It is also good practice to document any *in vitro* phenotypes of vaccine viruses that might serve as indicators of the stability of the mutations that differentiate the vaccine virus from its virulent parent. Such markers include, but are not limited to, plaque size, replication efficiency in mosquito vectors, induction of viraemia in nonhuman primates, suckling mouse neurovirulence, virulence in any other animal model, and temperature sensitivity (4, 22, 46–48). Developers should bear in mind that consensus genome sequencing is unsuitable for identifying minor or quasi-species genomes in a vaccine seed or batch (6).

B.3 Nonclinical immunogenicity and protective activity

Assessment of innate and adaptive immune responses in animals provides evidence that the dengue vaccine has replicated in the host, at the very least. Animals, particularly mice, have also been valuable for 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. On the basis of 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 potency of the vaccine.

The immune response to or protective activity of 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 (45). The term “toxicity” is generally associated with the untoward consequences of the administration of a nonreplicating medicine 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 amount of dose. 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. 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 of demonstrating that the vaccine(s) is 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 sections B.2.1 and B.2.2). However, nonhuman primates and mice may provide useful information for characterizing the viruses (see sections B.4.2 and B.4.3). The design of preclinical safety studies should reflect route and frequency of administration, as proposed in the protocol to support clinical trials (45).

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 (45).

B.4.2 Assessment in the nonhuman primate

B.4.2.1 Neurovirulence and neurotropism in nonhuman primates

The consensus of current opinion is that all live dengue vaccines should be tested once for neurovirulence. If any vaccine virus strain is determined to be neurovirulent to nonhuman primates on the basis of neurovirulence testing, neurotropism in nonhuman primates via the clinical or peripheral inoculation route should also be evaluated as part of the nonclinical safety study.

At this time, the most well-established model for vaccine neurovirulence is the nonhuman primate, which has historically been used to evaluate new seeds of yellow fever vaccines (17D substrains 17D204- or 17DD-derived) and live polio vaccines. Novel rodent (hamster and mouse) models for yellow fever vaccine virulence are currently under development. A rodent model could eventually be considered in place of nonhuman primate testing (40) (see section A.3.2.5.5.1).

Involvement of the central nervous system in cases of dengue fever and dengue haemorrhagic fever has usually been diagnosed as secondary to vasculitis with resultant fluid extravasation. The rarity of reports of patients with dengue encephalitis suggests that the virus does not typically cross the blood–brain barrier and infect neuronal cells (49). However, since dengue vaccine viruses are genetically altered compared to their wild-type 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 concern with regard to dengue vaccine viruses that contain yellow fever 17D chimeric genomes, and it would be of similar 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. NRAs 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 neurovirulence in the nonhuman primate model via the intracerebral inoculation route should follow the WHO recommendations for neurovirulence testing of yellow fever vaccines (50, 51) as appropriate (see a brief procedure below).

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 yellow fever 17D. 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 agreed with the NRA. Clinical scores, and the scores of histological lesions in the central nervous system, should be recorded. An advanced histological scoring method such as automated image analysis (52) may be implemented to provide quantitative assessment of virus-induced histopathology in brain tissues if the method has been properly validated and is acceptable to the NRA. The overall mean clinical and histological scores of the test group should not exceed the scores of the yellow fever vaccine control group. The significance level in statistical difference between test and control groups should be agreed by the NRA.

B.4.2.2 Viraemia in nonhuman primates

Nonhuman primates, humans and mosquitoes are the only natural hosts of DENV (4, 6, 8). Nonhuman primates have been widely used to evaluate replication and immunogenicity of candidate dengue vaccines (3, 5, 10). Primary infection of macaques with wild-type DENV results in moderate lymphadenopathy and a robust immune response (4, 6). The nonhuman primate 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 viraemia 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 viraemia in nonhuman primates 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 (4, 10–13). 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 post-infection time-points can be evaluated (4). The AG129 interferon receptor-deficient mouse will support replication of selected DENVs of all serotypes (22, 48). A DENV-2 strain adapted to replicate in the AG129 mouse induces a physiologically relevant disease in that strain (10). At present, the AG129 mouse seems suitable for safety studies, but NRAs 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 in maintaining the virus in nature. As noted previously, none of the DENV live, attenuated candidate vaccines studied to date induces a viraemia in vaccinees that is sufficient in magnitude to infect feeding mosquitoes (6, 15, 19). 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 (6, 18, 19). 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 (6, 16, 18, 53).

B.5 Environmental risk

The primary environmental risks of live dengue vaccines 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 (14–16, 18, 19, 54). 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.

Some investigators have recently raised a concern regarding live dengue vaccines, suggesting that vaccine viruses might revert to virulence in mosquitoes via intragenic recombination with endogenous wild-type flaviviruses. Such a phenomenon would seem to be highly unlikely due to the factors noted above plus the controversial question of whether flaviviruses are able to undergo recombination at all, even under ideal conditions *in vitro*.

Guidelines for live dengue vaccines derived by recombinant DNA technology are described in Part D below.

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: WHO's Guidelines on clinical evaluation of vaccines: regulatory expectations (55); and *Guidelines for the clinical evaluation of dengue vaccines in endemic areas* (56).

C.1.1 Objectives of the clinical development programme

The clinical evaluation of a candidate live dengue tetravalent vaccine should document:

- the immune responses elicited by the vaccine against all four dengue serotypes;
- vaccine efficacy in the prevention of DFI of any severity caused by any of the serotype 1, 2, 3 and 4 viruses over an appropriate minimum period of observation;
- the safety profile.

In addition, the programme 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 titres 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 programme

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 only on the basis of safety and immunogenicity data because there is no established surrogate marker for protection and, hence, no immunological correlate of protection has been identified.

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 (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 virologically confirmed DFI (see section C.3.3.5) 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 in 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 WHO guidelines for the plaque-reduction neutralization test (PRNT) (57). If alternative methods for determining neutralizing antibody (e.g. high throughput microneutralization assays) are developed, these should be validated against the PRNT.

Vero cells for dengue PRNT are available from the National Institute of Biological Standards and Control, England. Serum neutralization titres should be expressed in IUs calibrated against the reference panel of human antisera for dengue (see section A.1.3). In-house virus strains may be used.

An assessment of neutralizing antibody titres against each of the four serotypes of DENV is required. Additional testing against a range of 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 who have been selected randomly, or on the basis of 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 will provide an immunological correlate of protection. However, the exploration of cell-mediated immunity is encouraged since specific cell-mediated immunity 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 may provide some indication of the potential risk that vaccination could predispose subjects to develop severe DFI during subsequent natural infection (58).

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-finding and regimen-finding studies, it is essential to describe fully the pre-vaccination 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 neutralizing antibody in the majority of subjects, a comparison of percentages with a detectable neutralization titre post-vaccination (which may be defined as seroconversion in such a population) should be made against each DENV serotype. 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 for neutralizing antibody with respect to at least one dengue type, a comparison of pre-vaccination and post-vaccination geometric mean titres with respect to those types will be informative, in addition to analyses based on seroconversion rates and increments in antibody from pre- to post-vaccination.

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 predefined high titre of neutralizing antibody.

In protective efficacy studies, neutralizing antibody against DENV serotypes should be determined and followed over time in predefined 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 the 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 may have an important impact on immune responses to vaccination. In any case, the data should be analysed

according to predefined 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 nonclinical 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, may point to the 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, 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 virologically confirmed DFI, sera should be collected at timed intervals from a substantial cohort of subjects (and preferably from the entire study population, if feasible). Once the protocol-defined double-blind observation period has been completed, the initial analysis of the relationship between immune response and protection against DFI should follow. The most likely approach would be a cohort study in which one or more measures of the immune response to vaccination are related to disease in all, or in a large subset of, immunized subjects. Further analyses using longer-term follow-up data should be planned.

The use of serology to help identify infections with dengue viruses (whether or not clinically apparent) is a 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 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 in these initial studies should be healthy adults who are naive to flaviviruses (based on medical and vaccine history and serological studies). It is preferred that the 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 a monovalent vaccine (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 titre 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 titres 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 for a preliminary exploration of safety and immunogenicity to 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 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 section 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 in 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 for further studies to 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

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. This requirement may change in the future (see section C.3.3.7).

C.3.3.1 General issues for study design

Vaccine efficacy is estimated by comparing the total numbers of virologically 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 vaccine efficacy 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 estimate of vaccine efficacy 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 interrupt DENV transmission significantly during the observation period. The result could be a reduced likelihood of demonstrating a difference in the numbers of virologically 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. vaccine:control = 3:2 or 2:1) provided that care is taken to ensure that 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 the possible advantages and disadvantages into consideration. 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 influence the chance of obtaining a conclusive study result.

Whenever possible, subjects randomized to the control group should receive an alternative active vaccine (i.e. not a dengue vaccine) that can be given by the same route of administration as the candidate tetravalent dengue vaccine, rather than injections of placebo. The active vaccine should be selected to provide an anticipated benefit to study participants. However, such an appropriate vaccine may not always be available and there may be no option to using placebo injections to maintain the double-blind design. In addition, if the active control vaccine cannot be given at the same schedule as the candidate dengue vaccine, then placebo injections may need to be used within the schedule, as necessary, to maintain a double-blind design.

If the active control vaccine has a different presentation or appearance from those of the candidate dengue vaccine, 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 their sharing this information and so identifying themselves with one of the study groups.

If the use of a placebo control is necessary to achieve a double-blind design, the protocol could plan to administer a suitable licensed vaccine to all subjects in the study (i.e. those who do and who 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's integrity.

It is expected that several different production lots of vaccine will be used during protective efficacy studies. The decision whether 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 three of the total used) according to predefined criteria, must be made on a case-by-case basis. If such a formal comparison is to be made, 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 geographical 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 vaccine efficacy. In order to assess background rates, efficacy studies should be preceded by the collection of epidemiological information to document the expected incidences of DENV serotype-specific DFI, 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 sex), so that the 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 may 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 dengue disease. Site selection should be based on the information collected prior to study initiation regarding the expected number of cases of dengue within the study population each season during the observation period, which would probably range from one to three 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 vaccine efficacy 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 for follow-up of 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 section 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. a specific age range) to the entire population. There are likely to be concerns regarding the 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 probably exclude subjects aged under 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 vaccine efficacy to populations that were not included in efficacy studies.

C.3.3.4 Objectives, end-points and analyses

The primary objective of an efficacy study is to estimate vaccine efficacy 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 virologically 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.

Vaccine efficacy is estimated by comparing the total numbers of virologically 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. Vaccine efficacy should be calculated using the standard formula $VE (\%) = 100 \times (1 - r_1/r_0)$ (where VE = vaccine efficacy, r_1 = incidence rate in the vaccine group, and r_0 = incidence rate in the control group).

The assessment of DENV serotype-specific vaccine efficacy should be a major secondary objective and should be 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.

The following secondary analyses are suggested for inclusion in the study protocol (although some of the data needed to complete these analyses may not become available until some time after completion of the double-blind observation period that precedes the primary analysis):

- 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 virologically 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 viraemia, 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 predefine a pooled analysis of the data. This pooled analysis could provide additional insight into serotype-specific vaccine efficacy and the risk of severe DFI in vaccine and control groups.

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

C.3.3.5 Case definitions

The case definitions for the primary and various secondary analyses, with details of the criteria to be met, should be stated in the protocol and should be in accordance with the latest WHO recommendations (2).

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 (2) at the time of preparation of these Guidelines). 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 viraemia 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 detect dengue viraemia and/or 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 that are laid down in the study protocol and that aim to identify all potential cases of DFI of any severity 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: commercial and/or in-house serological assays (e.g. enzyme immunoassay, 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.

Nevertheless, although an acute primary infection may be implied from a rise in IgM levels during the first two weeks post-infection, such data need to be interpreted with considerable caution. For example, the IgM response to acute infection may be blunted in vaccinated subjects and in those infected previously by wild-type 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 fourfold 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 in order 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 health-care facilities whenever they develop signs or symptoms that may be indicative of DFI. A checklist 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 a postmortem examination or, if this is refused, to at least obtain a specimen for virological examination using needle puncture of the liver.

In some study sites that are otherwise considered suitable, it may not be possible to identify a local health-care facility willing to participate in the study. These sites should not be initiated unless there is at least agreement from local health-care providers to notify study staff of possible cases of DFI within a time frame that is sufficient 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 health-care 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 “worst-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 it is perhaps already licensed and introduced into the routine

vaccination programme in at least one country) there will be a need to reassess the content of clinical development programmes for other candidate dengue vaccines. For example, depending on the licensed dengue vaccine(s) available and the data that have been generated during their development, it may or may not be feasible or considered necessary to conduct studies that include an unvaccinated control group with subsequent candidate tetravalent vaccines.

It is not currently possible to make a definitive recommendation regarding what could or should be required in this scenario, since 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 pertinent issues are discussed below.

Once one or more dengue vaccine(s) has been licensed and introduced into the routine vaccination programme in one or more countries, the inclusion of an unvaccinated group in subsequent studies in these (and possibly other) countries may be considered unethical. However, studies that include an unvaccinated group may be feasible in some regions after approval of the first vaccine where there is appropriate justification and if approved by the NRA. In such a case, standard care and protection of study subjects should be provided as appropriate.

Efficacy studies using an unvaccinated control group and relative efficacy studies (i.e. studies in which the test vaccine is compared to a licensed vaccine) may not be feasible once a dengue vaccine has been introduced into the routine vaccination programme in a country or region, because this may reduce the incidence of DFI to levels that are too low to permit the estimation of vaccine efficacy from further studies of feasible size and duration.

However, if there remains considerable uncertainty about vaccine efficacy against one or more DENV serotype(s), efficacy studies that include an unvaccinated control group might be possible in regions where such serotype(s) are predicted to predominate.

It is very possible that immunological correlates of efficacy for each of the four serotypes of dengue virus cannot be established by analysis of early results of the first efficacy trials. Until surrogates and correlates of protection have been established, it may not be possible to determine the efficacy of a novel live vaccine by conducting a head-to-head comparison of its immunogenicity to that of a licensed live vaccine (i.e. in a bridging study). It may also be problematic to use bridging studies to support the extrapolation of efficacy observed with live virus vaccines to other types of dengue vaccine (e.g. killed virus vaccines, DNA vaccines, subunit vaccines). However, there may be no alternative to the use of bridging studies because of the factors described above.

Before resorting to bridging studies, there should be a careful scientific evaluation of the arguments for and against extrapolation of the efficacy observed

for a particular vaccine to populations that differ in character from the population in which the efficacy study was actually performed. Examples include populations that differ in age, risk for severe dengue, ethnicity, and/or prior or concurrent exposure to other flaviviruses.

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.

There is a particular need to assess whether DFI (which could be of any degree of severity, including very mild illness) may be caused by vaccine strains. In all cases of fever or other dengue-like signs or symptoms that occur following vaccination during clinical studies, it is essential that laboratory investigations are undertaken to determine whether or not the vaccine is responsible. Since the results will not always allow for a clear judgement of relatedness to vaccination, the protocol should provide criteria for ranking causality.

Low-grade and very transient fevers are to be expected and have been routinely observed in a small fraction of vaccinees after exposure to live dengue vaccines. NRAs will have to judge on a case-by-case basis whether the incidence, duration and/or severity of febrile episodes that cannot be ascribed to intercurrent illness that is observed during early studies in non-endemic areas are unacceptable. In these studies the level of vaccine viraemia (determined by RT-PCR and/or by direct culture methods) and level of vaccine virus NS1 antigenaemia should be determined in each vaccinee at one or more time-points post-vaccination in order to establish an average profile for the novel live vaccine under study. Levels higher than the predetermined average levels of vaccine virus or NS1 protein in blood detected during a febrile episode could be taken as evidence in favour of a direct causative role for the vaccine. During later studies in endemic areas there is a need to distinguish vaccine-associated from naturally occurring DFI, in addition to the other issues noted above.

There is a risk that vaccination could predispose recipients to developing a severe form of DFI. The risk may increase with time elapsed since vaccination in relation to waning titres of vaccine-induced antibodies in subjects who have not been naturally boosted in the interim period. 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. It is desirable to rule out events that occur at a frequency greater than 1:1000 vaccinees.

On the basis of the 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, with implications for the size of the safety database. NRAs will need to assess numbers that would constitute an adequate safety database before initial licensure. In addition, NRAs may make specific recommendations regarding the method of data collection, classification and scoring of severity of adverse events that are captured, as well as the post-vaccination duration of the safety data collection (i.e. after each dose and following the last dose of a course).

C.4 Post-licensure investigations

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, and the 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 NRAs. There will be a clear need to try to collect information 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 (there may be considerable practical difficulties in collecting reliable data in some settings; therefore it is recommended that sponsors and NRAs discuss ways in which such data could be obtained at least in some areas/regions);
- persistence of the immune response to vaccination (e.g. based on serial measurements of neutralizing antibody and detection of sensitized B-cells);
- responses to booster doses, which may be planned for predefined subsets enrolled in studies or may be instituted when disease surveillance indicates a possible need;
- the possible increased risk of severe DFI in vaccine recipients (e.g. in some areas/regions it may be possible to collect data on hospitalization of vaccinees to capture severe dengue cases);
- surveillance of dengue in areas/regions where routine vaccination is introduced and, if possible, collection of sufficient data for a formal estimate of vaccine effectiveness.

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.

There may be no information on vaccine co-administration at the time of initial licensure. Sponsors and NRAs should consider the need to assess the interaction of any novel dengue vaccine with other vaccines that are likely to be co-administered. For instance, in countries where dengue vaccination will become part of the routine childhood immunization programme, the interaction with the other vaccines used in the programme needs to be studied. In addition, if licensure is sought in non-endemic areas with the intention of protecting travellers, it is advisable to study the possible interactions of a novel dengue vaccine with other vaccines for travellers. Interaction studies should assess safety and the immune response to all co-administered antigens.

Some or all post-licensure studies may be conducted as post-approval commitments made to an individual NRA. In this regard, both sponsors and NRAs that have approved a vaccine should communicate and cooperate 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 by the NRA 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 environmental and other concerns related to the use of live vaccines derived by recombinant DNA technology since they may be considered as GMOs. However, similar concerns may be raised by live vaccines derived by conventional methods.

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

The environmental impact is not usually the responsibility of the NRA but of other agencies. Nonetheless the NRA should receive a copy of the ERA

and of any associated decisions taken, both for information and to ensure that the appropriate procedures have been followed.

D.1.2 Principles and objectives

Live DENV vaccine in which the genome has been genetically modified by recombinant DNA technology is considered a GMO. The manufacture, use and transboundary shipping of such live recombinant vaccines for research or commercial use should, when applicable, comply with relevant legislation or regulations on GMOs in the producing and recipient countries. 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 the following guidance on the ERA of live recombinant DENV vaccines is not intended to replace existing GMO legislation that is already in place in certain countries.

Generally, the objective of an ERA is to identify and evaluate, on a case-by-case basis, the potential adverse effects (direct or indirect, immediate or delayed) of a GMO on public health and the environment. This means that a separate ERA should be performed for each different live recombinant dengue vaccine. “Direct effects” are primary effects on human health or on the environment which result from the GMO itself and which occur through a short causal chain of events. “Indirect effects” are effects that occur 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” are those effects which may not be observed during the period of release of the GMO but which 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 and should be based on available scientific and technical data. Important aspects to be addressed in an ERA include the characteristics of: (i) the parental organism, (ii) the recipient organism, (iii) viral vector characteristics, (iv) the donor sequence, (v) genetic modification, (vi) the intended use and (vii) 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, data should be both relevant and of an acceptable scientific quality. The ERA may be based on data from experiments previously performed for other purposes, such as product characterization tests and nonclinical safety and toxicity 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 since quantification is often difficult 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 according to 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 stage of market registration, the level of uncertainty is expected to be lower as gaps identified in available data should already have been addressed.

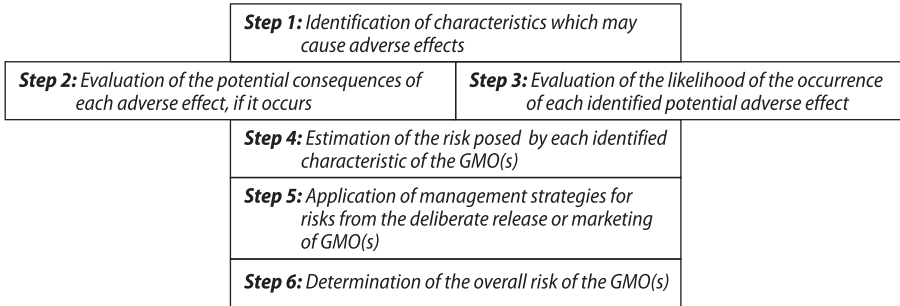
The need for risk management measures should be based on the estimated level of risk. If new information on the GMO becomes available, the ERA may need to be re-performed to determine whether the estimated level of risk has changed. This also holds true if the risks for the participating subjects have 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), evaluation of 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 to the environment. These processes should identify the potential adverse effects by comparing the properties of the GMO with those of non-modified organisms under the same conditions and in the same receiving environment. The principles and methodology of an ERA should be applicable irrespective of the geographical location of the intended environmental release of the GMO. However, 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 on local regulatory requirements, the ERA may be undertaken by the applicant or by the competent local authority on the basis of data supplied. In all cases, the competent local authority should use the ERA as a basis for deciding whether any identified environmental risks are acceptable. Nevertheless, the decision on whether any identified risks are acceptable may vary from country to country. Several national and international documents address ERA issues (59–62).

The general process for undertaking an ERA is shown in Figure A2.1 as an example (60, 61).

Figure A2.1
Typical steps in an environmental risk assessment



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. Aspects which could be developed include: the genetic stability of the live recombinant virus (including reversion and recombination), potential transmission of the vaccine virus among hosts by the vector, and the immune status of the population. These aspects are further outlined below.

D.3.1 Genetic stability

DENV vaccines currently under clinical evaluation are attenuated DENV strains, intertypic chimeric vaccines or DENV/yellow fever 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 yellow fever 17D vaccine, replacing the corresponding structural yellow fever 17D genes.

D.3.1.1 Reversion

After vaccination, there is potential for 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 on 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 (63).

D.3.1.2 Recombination

Whether or not recombination takes place among flaviviruses is controversial. In theory, recombination between live DENV vaccines and wild-type flaviviruses could produce a virus with an altered phenotype, but there is currently no evidence to support this (64–70).

The potential for recombination within and between flaviviruses has been widely discussed and challenged in the past, both on the basis of existing literature (64–67, 69, 70) and also of data obtained in specific experiments. In particular, a “recombination trap” has recently been designed to allow the products of rare recombination events to be selected and amplified, in the case of West Nile encephalitis, tick-borne encephalitis and Japanese encephalitis viruses (69). Intergenomic but aberrant recombination was observed only in the case of Japanese encephalitis virus, and not for West Nile or tick-borne encephalitis 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” scenarios for chimeras have been constructed to address that risk (65, 66, 70).

These different studies showed that such recombinants constructed artificially from a wild-type flavivirus and a chimeric vaccine (70), or from two wild-type viruses, such as highly virulent yellow fever Asibi virus and wild-type DEN-4 virus (66), 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 wild-type 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 of live DENV vaccines from the vaccinated subject to other individuals. Dengue does not spread directly from person to person, except via blood transfusion in very rare instances where a donor was dengue viraemic. Transmission of the dengue

vaccine in regions where the vector is absent is therefore highly unlikely. Dengue is currently restricted to the tropical and a few subtropical regions. Due to climate change there is the possibility of a geographical shift in mosquito populations which could conceivably lead to the spread of dengue to areas that are currently non-endemic.

Recombination between live DENV vaccines and wild-type flaviviruses could theoretically occur in a vaccinee (see above) and possibly also within an infected mosquito, although neither has been reported. A recombinant 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 favourable 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, two parameters should be taken into consideration: the level of viraemia in the vaccinated hosts, and the ability of the mosquito vectors to transmit the live DENV vaccine to new hosts. The blood titre required for effective transmission of dengue virus from human to mosquito via the bite has been studied in a laboratory setting. The typical level and duration of vaccine viraemia in inoculated volunteer subjects is also known for all live vaccines currently in the clinical phase of development. These data show that peak titres of vaccine viraemia are several orders of magnitude below those needed to infect a mosquito (71). In addition, the ability of the live vaccine viruses to replicate in mosquitoes and then to escape the midgut in order to render the mosquito infectious for humans by entering the salivary glands is also very impaired compared to wild-type dengue (18, 19, 26, 30, 54, 72, 73). Thus it is highly unlikely that vaccinated subjects could ever spread vaccine virus via mosquito transmission.

The outcome of the ERA for clinical trials in regions where the vector is absent is obviously that the environmental risk is negligibly small. The mosquito vector is not present and therefore the vaccine, or theoretical de novo recombinant viruses, cannot be transmitted to other people. However, in endemic areas, NRAs should decide whether or not to perform an ERA.

D.3.3 Immune status

Live DENV vaccines are able to replicate in vaccinated persons. The immune status against the vaccine antigens, the viral vectors and/or cross-reacting flaviviruses in the vaccinee may be a confounding factor in the assessment of 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 dissemination within a vaccinee. The potential for transmission of the vaccine is therefore considered to be greater in naive 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 both the environmental impact of the vaccines and the potential occurrence of adverse effects in contacts of the vaccinees. There is a theoretical 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 (74, 75).

Part E. Guidelines for NRAs

E.1 General

The general recommendations for NRAs and NCLs provided in the Guidelines for national authorities on quality assurance for biological products (76) should apply. In addition, the general recommendations for NRAs and NCLs provided in the Guidelines for independent lot release of vaccines by regulatory authorities (77) 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 NRA. The NRA 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 NRA in support of a request for release of vaccine for use.

A statement signed by the appropriate official of the NRA 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 the expiry date assigned 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.

Authors

The scientific basis for the revision of the Guidelines for the production and quality control of candidate tetravalent dengue virus vaccines (live) published in WHO Technical Report Series, No. 932, was discussed at the meeting of the WHO working group on technical specifications for manufacture and evaluation of dengue vaccines, which met in Geneva, Switzerland, 11–12 May 2009 and was attended by the following: Dr A. Barrett, University of Texas Medical Branch, Galveston, TX, USA; Dr D. Bleijs, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; Dr F. Denamur, GlaxoSmithKline Biologicals, Rixensart, Belgium; Dr A. Durbin, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA; Dr K. Eckels, Walter Reed Army Institute of Research, Silver Spring, MD, USA; Dr R. Edelman, University of Maryland School of Medicine, Baltimore, MD, USA; Dr D. Francis, Global Solutions for Infectious Diseases, South San Francisco, CA, USA; Dr M. Freire, Instituto Oswaldo Cruz, Manguinhos, Rio de Janeiro, Brazil; Dr J. Hombach, World Health Organization, Geneva, Switzerland; Dr H. Langar, World Health Organization Regional Office for the Eastern Mediterranean, Cairo, Egypt; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr C. Lecomte, GlaxoSmithKline Biologicals, Wavre, Belgium; Dr L. Mallet, Sanofi Pasteur, Marcy l'Étoile, France; Dr H. Margolis, International Vaccine Institute, Seoul, Republic of Korea; Dr L. Markoff, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA; Dr P. Minor, National Institute of Biological Standards and Control, Potters Bar, England (Chair); Dr S. Nishioka, World Health Organization, Geneva, Switzerland; Dr K. Peden, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England; Dr J. Robertson, National Institute of Biological Standards and Control, Potters Bar, England; Dr J. Roehrig, Centers for Disease Control and Prevention, Fort Collins, CO, USA; Dr A. Sabouraud, Sanofi Pasteur, Marcy l'Étoile, France; Dr J. Shin, World Health Organization, Geneva, Switzerland; Mrs P. Thanaphollert, Food and Drug Administration, Ministry of Public Health, Nonthaburi, Thailand; Dr D. Trent, University of Texas Medical Branch, Galveston, TX, USA (Rapporteur); Dr D. Wood, World Health Organization, Geneva, Switzerland.

The first draft of these Guidelines was developed by the following lead authors for the part indicated: (1) Part A – Dr L. Mallet, Sanofi Pasteur, Canada and Dr P. Minor, National Institute of Biological Standards and Control, Potters

Bar, England; (2) Part B – Dr D. Trent, University of Texas Medical Branch, Galveston, TX, USA; (3) Part C – Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England; (4) Part D – Dr D. Bleijs, National Institute for Public Health and the Environment, Bilthoven, the Netherlands and Dr J. Robertson, National Institute of Biological Standards and Control, Potters Bar, England.

The first draft was discussed in the meeting of the working group held on 29–30 April 2010 in Geneva, Switzerland attended by: Dr A. Barrett, University of Texas Medical Branch, Galveston, TX, USA; Dr D. Bleijs, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; Dr F. Denamur, GlaxoSmithKline Biologicals, Rixensart, Belgium; Dr A. Durbin, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA; Dr K. Eckels, Walter Reed Army Institute of Research, Silver Spring, MD, USA; Dr R. Edelman, University of Maryland School of Medicine, Baltimore, MD, USA; Dr D. Francis, Global Solutions for Infectious Diseases, South San Francisco, CA, USA; Dr M. Freire, Instituto Oswaldo Cruz, Manguinhos, Rio de Janeiro, Brazil; Dr N. Gallina, Buntan, Sao Paulo, Brazil; Mr M. Galves, National Agency of Health Surveillance, Brasília-DF, Brazil; Mrs F. Garnier, Agence Française de Sécurité sanitaire de Produits de Santé (French Agency for Safety of Health Products), Lyons, France; Dr J. Hombach, World Health Organization, Geneva, Switzerland; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr L. Mallet, Sanofi Pasteur, Toronto, Canada; Dr P. Minor, National Institute of Biological Standards and Control, Potters Bar, England (Chair); Dr L. Morgan, Sanofi Pasteur, Marcy l'Étoile, France; Dr Le Van Phung, National Institute for Control of Vaccine and Biologicals, Hanoi, Viet Nam; Dr L. Markoff, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA (Rapporteur for clinical working group); Dr J. Korimbocus, Agence Française de Sécurité sanitaire de Produits de Santé (French Agency for Safety of Health Products), Lyons, France; Dr S. Nishioka, World Health Organization, Geneva, Switzerland; Dr K. Peden, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA (Rapporteur for manufacture, nonclinical and environmental risk assessment group); Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England; Dr V. Quivy, GlaxoSmithKline Biologicals, Wavre, Belgium; Dr J. Roehrig, Centers for Disease Control and Prevention, Fort Collins, CO, USA; Ms M. Saville, Sanofi Pasteur, Marcy l'Étoile, France; Mrs P. Thanaphollert, Food and Drug Administration, Ministry of Public Health, Nonthaburi, Thailand; Dr C. Thomson, Inviragen, Capricorn, Singapore; Dr D. Trent, University of Texas Medical Branch, Galveston, TX, USA; Dr J-W. van der Laan, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; and Dr D. Wood, World Health Organization, Geneva, Switzerland.

On the basis of comments from the April 2010 meeting, a second draft was prepared by Dr D. Bleijs of the National Institute for Public Health and

the Environment, Bilthoven, the Netherlands; Dr L. Mallet of Sanofi Pasteur, Canada; Dr M. Powell of the Medicines and Healthcare Products Regulatory Agency, London, England; and Dr D. Trent of the University of Texas Medical Branch, Galveston, TX, USA. A modified draft of Part D of the Guidelines was further developed by Dr D. Bleijs of the National Institute for Public Health and the Environment, Bilthoven, the Netherlands, on the basis of comments from teleconferences held in January and February 2011 with an informal workgroup on environmental risk assessment for dengue vaccines in which additional members were: Dr M. Dornbusch, Office of the Gene Technology Regulator, Department of Health and Aging, Canberra, Australia; Dr A. Durbin, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA; Dr K. Eckels, Walter Reed Army Institute of Research, Silver Spring, MD, USA; Dr R. Edelman, University of Maryland School of Medicine, Baltimore, MD, USA; Dr L. Morgan, Sanofi Pasteur, Marcy l'Étoile, France; Dr J. Robertson, National Institute of Biological Standards and Control, Potters Bar, England; Dr J. Shin, World Health Organization, Geneva, Switzerland; and Dr V. Quivy, GlaxoSmithKline Biologicals, Wavre, Belgium.

The third draft was prepared by Dr J. Shin, World Health Organization, Geneva, Switzerland.

The fourth draft was prepared by Dr A. Barrett, University of Texas Medical Branch, Galveston, TX, USA; Dr D. Bleijs, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; Dr P. Minor, National Institute of Biological Standards and Control, Potters Bar, England; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England; Dr J. Shin, World Health Organization, Geneva, Switzerland; and Dr D. Trent, University of Texas Medical Branch, Galveston, TX, USA, taking into account suggestions for modification and comments by the participants in the informal consultation held on 11–12 April 2011 in Geneva, Switzerland, attended by: Dr B. Barrere, Sanofi Pasteur, Marcy l'Étoile, France; Dr A. Barrett, University of Texas Medical Branch, Galveston, TX, USA; Dr D. Bleijs, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; Dr A. Chawla, Greater Noida, Uttar Pradesh, India; Dr K. Dobbelaere, GlaxoSmithKline Biologicals, Rixensart, Belgium; Dr M. Dornbusch, Office of the Gene Technology Regulator, Department of Health and Aging, Canberra, Australia; Dr A. Durbin, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA; Dr K. Eckels, Walter Reed Army Institute of Research, Silver Spring, MD, USA; Dr R. Edelman, University of Maryland School of Medicine, Baltimore, MD, USA; Mr M. Galves, National Agency of Health Surveillance, Brasília-DF, Brazil; Dr E. Griffiths, Biologics and Genetic Therapies Directorate, Health Canada, Ottawa, Canada; Dr J. Hombach, World Health Organization, Geneva, Switzerland; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr L. Mallet, Sanofi Pasteur, Toronto, Canada; Dr L. Markoff, Center for Biologics Evaluation and Research,

Food and Drug Administration, Bethesda, MD, USA; Dr P. Minor, National Institute of Biological Standards and Control, Potters Bar, England (Chair); Dr L. Morgan, Sanofi Pasteur, Marcy l'Étoile, France; Dr J. Korimbocus, Agence Française de Sécurité sanitaire de Produits de Santé (French Agency for Safety of Health Products), Lyons, France; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England; Dr A. Precioso, Butantan, Sao Paulo, Brazil; Dr V. Quivy, GlaxoSmithKline Biologicals, Wavre, Belgium; Dr J. Roehrig, Centers for Disease Control and Prevention, Fort Collins, CO, USA; Dr J. Schmitz, World Health Organization, Geneva, Switzerland; Mrs P. Thanaphollert, Food and Drug Administration, Ministry of Public Health, Nonthaburi, Thailand; Dr D. Trent, University of Texas Medical Branch, Galveston, TX, USA; Dr J-W. van der Laan, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; and Dr S. Viviani, Sanofi Pasteur, Marcy l'Étoile, France. This fourth draft was posted on the WHO web site with a call for public comments for one month from 22 May to 23 June 2011.

The fifth draft was prepared by the same members of the drafting group that prepared the fourth, and was submitted to the Expert Committee on Biological Standardization for consideration. This draft was posted on the WHO web site with a call for public comments for two months from 21 July to 23 September 2011.

The document was further modified and then adopted by the WHO Expert Committee on Biological Standardization in October 2011.

Acknowledgements

Acknowledgements are also due to the following experts for their written comments on scientific and technical issues during the public consultations following web publication of amended drafts from 22 May to 23 June 2011 and from 21 July to 23 September 2011: Dr M. Alali, Therapeutic Goods Administration, Australian Capital Territory, Australia; Dr L. Bigger, International Federation of Pharmaceutical Manufacturers and Associations, Geneva, Switzerland; Dr R. Edelman, University of Maryland School of Medicine, Baltimore, MD, USA; Dr J. Hombach, World Health Organization, Geneva, Switzerland; Dr J. Korimbocus, Agence Française de Sécurité sanitaire de Produits de Santé (French Agency for Safety of Health Products), Lyons, France; Dr R. Krause, International Federation of Pharmaceutical Manufacturers and Associations, Geneva, Switzerland; Dr L. Markoff, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA; Dr S. Morgeaux, Agence Française de Sécurité sanitaire de Produits de Santé (French Agency for Safety of Health Products), Lyons, France; Dr F. Mortiaux, GlaxoSmithKline Biologicals, Rixensart, Belgium; Dr S. Phumiamorn, Ministry of Public Health, Nonthaburi,

Thailand; Dr J. Schmitz, World Health Organization, Geneva, Switzerland; Dr L. Slamet, National Agency of Drug and Food Control, Jakarta Pusat, Indonesia; Dr T.F. Tsai, Novartis Vaccines, Cambridge, MA, USA; Dr S. Whitehead, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA; and Dr K. Zoon, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA.

References

1. Guidelines for the production and quality control of candidate tetravalent dengue virus vaccines (live). In: *WHO Expert Committee on Biological Standardization. Fifty-fifth report*. Geneva, World Health Organization, 2006 (WHO Technical Report Series, No. 932), Annex 1.
2. *Dengue: guidelines for diagnosis, treatment, prevention and control*. Geneva, World Health Organization, 2009 (WHO/HTM/NTD/DEN/2009.1; http://whqlibdoc.who.int/publications/2009/9789241547871_eng.pdf, accessed 3 April 2012).
3. Blaney JE Jr et al. Recombinant, live-attenuated tetravalent dengue virus vaccine formulations induce a balanced, broad, and protective neutralizing antibody response against each of the four serotypes in rhesus monkeys. *Journal of Virology*, 2005, 79:5516–5528.
4. Cassetti MC et al. Report of an NIAID workshop on dengue animal models. *Vaccine*, 2010, 28:4229–4234.
5. Guirakhoo F et al. Construction, safety, and immunogenicity in nonhuman primates of a chimeric yellow fever-dengue virus tetravalent vaccine. *Journal of Virology*, 2001, 75:7290–7304.
6. Guy B et al. Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses. *Vaccine*, 2010, 28:632–649.
7. Blaney JE Jr et al. Vaccine candidates for dengue virus type 1 (DEN1) generated by replacement of the structural genes of rDEN4 and rDEN4Delta30 with those of DEN1. *Virology Journal*, 2007, 4:23.
8. Vaughn DW et al. Dengue. In: Barrett AD, Stanberry L, eds. *Vaccines for biodefense and emerging and neglected diseases*. New York, NY, Elsevier, 2009:287–324.
9. Sabin AB, Schlesinger RW. Production of immunity to dengue with virus modified by propagation in mice. *Science*, 1945, 101:640–642.
10. Williams KL et al. A mouse model for studying dengue virus pathogenesis and immune response. *Annals of the New York Academy of Sciences*, 2009, 1171 (Suppl. 1):E12–E23.
11. Yauch LE, Shresta S. Mouse models of dengue virus infection and disease. *Antiviral Research*, 2008, 80:87–93.
12. Shresta S et al. Murine model for dengue virus-induced lethal disease with increased vascular permeability. *Journal of Virology*, 2006, 80:10208–10217.
13. Balsitis SJ et al. Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. *PLoS Pathogens*, 2010, 6:e1000790.
14. Bennett KE et al. Quantitative trait loci that control dengue-2 virus dissemination in the mosquito *Aedes aegypti*. *Genetics*, 2005, 170:185–194.
15. Salazar MI et al. Dengue virus type 2: replication and tropisms in orally infected *Aedes aegypti* mosquitoes. *BMC Microbiology*, 2007, 7:9.
16. Schoepp RJ et al. Infection of *Aedes albopictus* and *Aedes aegypti* mosquitoes with dengue parent and progeny candidate vaccine viruses: a possible marker of human attenuation. *American Journal of Tropical Medicine and Hygiene*, 1991, 45:202–210.

17. Khin MM et al. Infection, dissemination, transmission, and biological attributes of dengue-2 PDK53 candidate vaccine virus after oral infection in *Aedes aegypti*. *American Journal of Tropical Medicine and Hygiene*, 1994, 51:864–869.
18. Higgs S et al. Growth characteristics of ChimeriVax-Den vaccine viruses in *Aedes aegypti* and *Aedes albopictus* from Thailand. *American Journal of Tropical Medicine and Hygiene*, 2006, 75:986–993.
19. Troyer JM et al. A live-attenuated recombinant dengue-4 virus vaccine candidate with restricted capacity for dissemination in mosquitoes and lack of transmission from vaccinees to mosquitoes. *American Journal of Tropical Medicine and Hygiene*, 2001, 65:414–419.
20. An J et al. Development of a novel mouse model for dengue virus infection. *Virology*, 1999, 263:70–77.
21. Guirakhoo F et al. Safety and efficacy of chimeric yellow fever-dengue virus tetravalent vaccine formulations in nonhuman primates. *Journal of Virology*, 2004, 78:4761–4775.
22. Huang CY et al. Dengue 2 PDK-53 virus as a chimeric carrier for tetravalent dengue vaccine development. *Journal of Virology*, 2003, 77:11436–11447.
23. Johnson AJ, Roehrig JT. New mouse model for dengue virus vaccine testing. *Journal of Virology*, 1999, 73:783–786.
24. Kinney RM et al. Construction of infectious cDNA clones for dengue 2 virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53. *Virology*, 1997, 230:300–308.
25. Putnak RJ et al. An evaluation of dengue type-2 inactivated, recombinant subunit, and live-attenuated vaccine candidates in the rhesus macaque model. *Vaccine*, 2005, 23:4442–4452.
26. Whitehead SS et al. Substitution of the structural genes of dengue virus type 4 with those of type 2 results in chimeric vaccine candidates which are attenuated for mosquitoes, mice, and rhesus monkeys. *Vaccine*, 2003, 21:4307–4316.
27. Halstead SB, Vaughn DW. Dengue vaccines. In: Plotkin SA et al., eds. *Vaccines*. New York, NY, Elsevier Inc., 2008:1155–1162.
28. Hombach J. Vaccines against dengue: a review of current candidate vaccines at advanced development stages. *Revista panamericana de salud pública (Pan American Journal of Public Health)*, 2007, 21:254–260.
29. Whitehead SS et al. Prospects for a dengue virus vaccine. *Nature Reviews Microbiology*, 2007, 5:518–528.
30. Blaney JE Jr et al. Dengue virus type 3 vaccine candidates generated by introduction of deletions in the 3' untranslated region (3'-UTR) or by exchange of the DENV-3 3'-UTR with that of DENV-4. *Vaccine*, 2008, 26:817–828.
31. Durbin AP et al. Development and clinical evaluation of multiple investigational monovalent DENV vaccines to identify components for inclusion in a live-attenuated tetravalent DENV vaccine. *Vaccine*, 2011, 29:7242–7250.
32. Good manufacturing practices for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 1.
33. Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. In: *WHO Expert Committee on Biological Standardization. Sixty-first report*. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 3.
34. Requirements for continuous cell lines used for biologicals production. In: *WHO Expert Committee on Biological Standardization. Thirty-sixth report*. Geneva, World Health Organization, 1987 (WHO Technical Report Series, No. 745), Annex 3.

35. General requirements for the sterility of biological substances (Requirements for biological substances No. 6, revised 1973). In: *WHO Expert Committee on Biological Standardization. Twenty-fifth report*. Geneva, World Health Organization, 1973 (WHO Technical Report Series, No. 530), Annex 4.
36. General requirements for the sterility of biological substances (Requirements for biological substances No. 6, revised 1973, amendment 1995). In: *WHO Expert Committee on Biological Standardization. Forty-sixth report*. Geneva, World Health Organization, 1998 (WHO Technical Report Series, No. 872), Annex 3.
37. *Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products*. Geneva, World Health Organization, 2003 (<http://www.who.int/biologicals/publications/en/whotse2003.pdf>, accessed 3 April 2012).
38. *WHO guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies*. Geneva, World Health Organization, 2006 (<http://www.who.int/bloodproducts/TSEPUBLISHEDREPORT.pdf>, accessed 3 April 2012).
39. Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (Requirements for biological substances No. 27). In: *WHO Expert Committee on Biological Standardization. Forty-third report*. Geneva, World Health Organization, 1994 (WHO Technical Report Series, No. 840), Annex 2.
40. Monath TP et al. Safety testing for neurovirulence of novel live, attenuated flavivirus vaccines: infant mice provide an accurate surrogate for the test in monkeys. *Biologicals*, 2005, 33:131–144.
41. Guirakhoo F et al. Recombinant chimeric yellow fever-dengue type 2 virus is immunogenic and protective in nonhuman primates. *Journal of Virology*, 2000, 74:5477–5485.
42. Guidelines for stability evaluation of vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-seventh report*. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 962), Annex 3.
43. Good manufacturing practices: main principles for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report*. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 3.
44. Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products (jointly with the Expert Committee on Biological Standardization). In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report*. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 9.
45. WHO guidelines on nonclinical evaluation of vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-fourth report*. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 1.
46. Durbin AP et al. rDEN4delta30, a live attenuated dengue virus type 4 vaccine candidate, is safe, immunogenic, and highly infectious in healthy adult volunteers. *Journal of Infectious Diseases*, 2005, 191:710–718.
47. Edelman R et al. Phase I trial of 16 formulations of a tetravalent live-attenuated dengue vaccine. *American Journal of Tropical Medicine and Hygiene*, 2003, 69:48–60.
48. Huang CY et al. Chimeric dengue type 2 (vaccine strain PDK-53)/dengue type 1 virus as a potential candidate dengue type 1 virus vaccine. *Journal of Virology*, 2000, 74:3020–3028.
49. Lum LC et al. Dengue encephalitis: a true entity? *American Journal of Tropical Medicine and Hygiene*, 1996, 54:256–259.

50. Levenbook IS et al. The monkey safety test for neurovirulence of yellow fever vaccines: the utility of quantitative clinical evaluation and histological examination. *Journal of Biological Standardization*, 1987, 15:305–313.
51. Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines. In: *WHO Expert Committee on Biological Standardization. Sixty-first report*. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 5.
52. Maximova OA et al. High-throughput automated image analysis of neuroinflammation and neurodegeneration enables quantitative assessment of virus neurovirulence. *Vaccine*, 2010, 28:8315–8326.
53. Miller BR et al. Dengue-2 vaccine: oral infection, transmission, and lack of evidence for reversion in the mosquito, *Aedes aegypti*. *American Journal of Tropical Medicine and Hygiene*, 1982, 31:1232–1237.
54. Johnson BW et al. Analysis of the replication kinetics of the ChimeriVax-DEN 1, 2, 3, 4 tetravalent virus mixture in *Aedes aegypti* by real-time reverse transcriptase-polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene*, 2004, 70:89–97.
55. Guidelines on clinical evaluation of vaccines: regulatory expectations. In: *WHO Expert Committee on Biological Standardization. Fifty-second report*. Geneva, World Health Organization, 2004 (WHO Technical Report Series, No. 924), Annex 1.
56. *Guidelines for the clinical evaluation of dengue vaccines in endemic areas*. Geneva, World Health Organization, 2008 (WHO/IVB/08.12; http://whqlibdoc.who.int/hq/2008/WHO_IVB_08.12_eng.pdf, accessed 3 April 2012).
57. *Guidelines for plaque-reduction neutralization testing of human antibodies to dengue viruses*. Geneva, World Health Organization, 2007 (http://whqlibdoc.who.int/hq/2007/WHO_IVB_07.07_eng.pdf, accessed 3 April 2012).
58. Thomas SJ et al. Scientific consultation on cell mediated immunity (CMI) in dengue and dengue vaccine development. *Vaccine*, 2009, 27:355–368.
59. *The New Substances Notification Regulations of the Canadian Environmental Protection Act, 1999*. Gatineau, QC, Environment Canada, 1999.
60. Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. *Official Journal of the European Communities*, 2002, L106:1–39.
61. Commission Decision 2002/623/EC of 24 July 2002 establishing guidance notes supplementing Annex II to Directive 2001/18/EC. *Official Journal of the European Communities*, 2002, L106:22–33.
62. *Risk analysis framework. (Version 3, April 2009)*. Canberra, ACT, Commonwealth of Australia, 2009 (<http://www.health.gov.au/internet/ogtr/publishing.nsf/Content/riskassessments-1>, accessed 3 April 2012).
63. dos Santos CN et al. Complete nucleotide sequence of yellow fever virus vaccine strains 17DD and 17D-213. *Virus Research*, 1995, 35:35–41.
64. Hombach J et al. Arguments for live flavivirus vaccines. *Lancet*, 2004, 364:498–499.
65. McGee CE et al. Recombinant chimeric virus with wild-type dengue 4 virus premembrane and envelope and virulent yellow fever virus Asibi backbone sequences is dramatically attenuated in nonhuman primates. *Journal of Infectious Diseases*, 2008, 197:693–697.
66. McGee CE et al. Substitution of wild-type yellow fever Asibi sequences for 17D vaccine sequences in ChimeriVax-dengue 4 does not enhance infection of *Aedes aegypti* mosquitoes. *Journal of Infectious Diseases*, 2008, 197:686–692.

67. Monath TP et al. Recombination and flavivirus vaccines: a commentary. *Vaccine*, 2005, 23:2956–2958.
68. Seligman SJ, Gould EA. Safety concerns with regard to live attenuated flavivirus vaccines. *Journal of Infectious Diseases*, 2008, 198:794–795.
69. Taucher C et al. A trans-complementing recombination trap demonstrates a low propensity of flaviviruses for intermolecular recombination. *Journal of Virology*, 2010, 84:599v611.
70. Pugachev KV et al. Construction and biological characterization of artificial recombinants between a wild type flavivirus (Kunjin) and a live chimeric flavivirus vaccine (ChimeriVax-JE). *Vaccine*, 2007, 25:6661–6671.
71. Morrison D et al. A novel tetravalent dengue vaccine is well tolerated and immunogenic against all 4 serotypes in flavivirus-naïve adults. *Journal of Infectious Diseases*, 2010, 201:370–377.
72. Blaney JE Jr et al. Genetically modified, live attenuated dengue virus type 3 vaccine candidates. *American Journal of Tropical Medicine and Hygiene*, 2004, 71:811–821.
73. Whitehead SS et al. A live, attenuated dengue virus type 1 vaccine candidate with a 30-nucleotide deletion in the 3' untranslated region is highly attenuated and immunogenic in monkeys. *Journal of Virology*, 2003, 77:1653–1657.
74. Durbin AP et al. Heterotypic dengue infection with live attenuated monotypic dengue virus vaccines: implications for vaccination of populations in areas where dengue is endemic. *Journal of Infectious Diseases*, 2011, 203:327–334.
75. Guy B et al. Development of Sanofi Pasteur tetravalent dengue vaccine. *Human Vaccines*, 2010, 6.
76. Guidelines for national authorities on quality assurance for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 2.
77. Guidelines for independent lot release of vaccines by regulatory authorities. In: *WHO Expert Committee on Biological Standardization. Sixty-first report*. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 2.

Appendix 1

Summary protocol for manufacturing and control of dengue tetravalent vaccine (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 NRA. Information and tests may be added or omitted as necessary to be in line with the marketing authorization approved by the NRA. It is therefore possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO guidelines on 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 NRA of the country in which the vaccine was produced and/or released stating that the product meets national requirements as well as Part A of the Guidelines of this document published by WHO.

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

International name: _____
 Commercial name: _____
 Product licence (marketing authorization) number: _____
 Country: _____
 Name and address of manufacturer: _____
 Name and address of product licence 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: _____

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 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 the cell banking system from cell seed (if applicable), master cell bank, working cell bank, end-of-production cells or extended cell bank should be provided according to WHO's 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 and date of preparation for each bank: _____
 Date each bank was established: _____
 Date of approval by the NRA: _____
 Total number of ampoules stored for each bank: _____
 Passage/population doubling level 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), master cell bank, working cell bank, end-of-production cells, or extended cell banks

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 of 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 of 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

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 virus master seed: _____

Date approved by the NRA: _____

Virus working seed

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 NRA: _____

3.2.2 Tests on virus seeds

Identity test

Method: _____
 Specification: _____
 Lot number of reference reagents: _____
 Dates of test (start, end): _____
 Result: _____

Genetic/phenotypic characterizations

Method: _____
 Reference reagents: _____
 Specification: _____
 Dates of test (start, end): _____
 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: _____
 Dates of test (start, end): _____
 Result: _____

Test for mycoplasmas

Method: _____
 Specification: _____
 Media: _____
 Volume tested: _____
 Temperature of incubation: _____
 Positive controls: _____

Dates of test (start, end): _____

Result: _____

Test for mycobacteria

Method: _____

Specification: _____

Media: _____

Volume tested: _____

Temperature of incubation: _____

Dates of test (start, end): _____

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 monkey cells

Type of monkey cells: _____

Quantity of neutralized sample inoculated: _____

Incubation conditions: _____

Method: _____

Specification: _____

Dates of test (start, end): _____

Ratio of cultures viable at end of test: _____

Result: _____

Test in human cells

Type of human cells: _____

Quantity of neutralized sample inoculated: _____

Incubation conditions: _____

Method: _____

Specification: _____

Dates of test (start, end): _____

Ratio of cultures viable at end of test: _____

Result: _____

Other cell types

Type of cells: _____

Quantity of neutralized sample inoculated: _____
 Incubation conditions: _____
 Method: _____
 Specification: _____
 Dates of test (start, end): _____
 Ratio of cultures viable at end of test: _____
 Result: _____

Test in animals for adventitious agents

Method: _____
 Specification: _____
 Dates of test (start, end): _____
 Result: _____

Test by molecular methods for adventitious agents

Method: _____
 Specification: _____
 Dates of test (start, end): _____
 Result: _____

Tests in nonhuman primates (either master or working seed lot) for neurovirulence

For details, please see Recommendations for yellow fever vaccine (51)

Method: _____
 Specification: _____
 Dates of test (start, end): _____
 Result: _____

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

(Detailed protocol should be developed)

Method: _____
 Specification: _____
 Dates of test (start, end): _____
 Result: _____

Virus titration for infectivity

Method: _____
 Specification: _____
 Dates of test (start, end): _____
 Result: _____

4. Control of vaccine production

4.1 Control of production cell cultures

4.1.1 Information on preparation

Lot number of master cell bank: _____

Lot number of working cell bank: _____

Date of thawing ampoule of working cell bank: _____

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: _____

Dates 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: _____

Dates of test (start, end): _____

Result: _____

Test for adventitious agents on supernatant culture fluids

Test in monkey cells

Type of monkey cells: _____

Quantity of pooled sample inoculated: _____

Incubation conditions: _____

Method: _____

Specification: _____

Dates of test (start, end): _____

Ratio of cultures viable at end of test: _____

Result: _____

Test in human cells

Type of human cells: _____

Quantity of pooled sample inoculated: _____

Incubation conditions: _____
 Method: _____
 Specification: _____
 Dates of test (start, end): _____
 Ratio of cultures viable at end of test: _____
 Result: _____

Other cell types

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

Identity test

Method: _____
 Specification: _____
 Dates of test (start, end): _____
 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 be provided separately.

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: _____
Dates of test (start, end): _____
Result: _____

Test for mycoplasma

Method: _____
Specification: _____
Media: _____
Volume tested: _____
Temperature of incubation: _____
Positive controls: _____
Dates of test (start, end): _____
Result: _____

Test for mycobacteria

Method: _____
Specification: _____
Media: _____
Volume tested: _____
Temperature of incubation: _____
Dates of test (start, end): _____
Result: _____

Test for adventitious agents

Test in monkey cells

Type of monkey cells: _____

Quantity of neutralized sample inoculated: _____
 Incubation conditions: _____
 Method: _____
 Specification: _____
 Dates of test (start, end): _____
 Ratio of cultures viable at end of test: _____
 Result: _____

Test in human cells

Type of human cells: _____
 Quantity of neutralized sample inoculated: _____
 Incubation conditions: _____
 Method: _____
 Specification: _____
 Dates of test (start, end): _____
 Ratio of cultures viable at end of test: _____
 Result: _____

Other cell types

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

Virus titration for infectivity

Method: _____
 Specification: _____
 Dates of test (start, end): _____
 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: _____

Dates of test (start, end): _____

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 and 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: _____

Dates of test (start, end): _____

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: _____

Dates of test (start, end): _____

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: _____
Dates of test (start, end): _____
Result: _____

Test for potency (each serotype)

Method: _____
Batch number of reference vaccine and
assigned potency: _____
Specification: _____
Dates of test (start, end): _____
Result for each serotype: _____

Thermal stability (each serotype)

Method: _____
Specification: _____
Dates of test (start, end): _____
Result for each serotype: _____

General safety (unless deletion authorized)

Tests in mice

Date of inoculation: _____
Number of animals tested: _____
Volume and route of injection: _____
Observation period: _____
Specification: _____
Results (give details of deaths): _____

Tests in guinea-pigs

Date of inoculation: _____
Number 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: _____

7. Certification by the manufacturer

Name of head of production (typed) _____

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

I certify that lot no. _____ of dengue vaccine, whose number appears on the label of the final containers, meets all national requirements and satisfies Part A¹ of the WHO Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated) (2013)² (if applicable)

Name (typed) _____
 Signature _____
 Date _____

8. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA (as shown in Appendix 2), a label from a final container, and an instruction leaflet for users.

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

² WHO Technical Report Series, No. 979, Annex 2.

Appendix 2

Model certificate for the release of dengue tetravalent vaccine (live, attenuated) by NRAs

This certificate is to be provided by the NRA of the country where the vaccine has been manufactured, on request by the manufacturer.

Lot release certificate

Certificate no. _____

The following lot(s) of dengue vaccine produced by _____¹ in _____,² whose numbers appear on the labels of the final containers, complies with the relevant specification in the marketing authorization and provisions for the release of biological products³ and Part A⁴ of the WHO Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated) (2013)⁵ and comply with WHO good manufacturing practices: main principles for pharmaceutical products;⁶ Good manufacturing practices for biological products;⁷ and Guidelines for independent lot release of vaccines by regulatory authorities.⁸

The release decision is based on _____⁹

The certificate may include the following information:

- name and address of manufacturer;
- site(s) of manufacturing;
- trade name and common name of product;

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 NRA.

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

⁵ WHO Technical Report Series, No. 979, Annex 2.

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

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

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

⁹ Evaluation of summary protocol, independent laboratory testing, and/or specific procedures laid down in defined document etc., as appropriate.

- 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;
- certificate number.

The Director of the NRA (or other authority as appropriate):

Name (typed) _____

Signature _____

Date _____

