ANNEX III A IN ACCORDANCE WITH DIRECTIVE 2001/18/EC

INFORMATION REQUIRED IN NOTIFICATIONS CONCERNING RELEASES OF GENETICALLY MODIFIED ORGANISMS OTHER THAN HIGHER PLANTS

INDEX

I. GENERAL INFORMATION

A. Name and address of the notifier (company or institute)

Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc.
(hereafter referred to as the Sponsor or MSD)
Legal Registered Address:
One Merck Drive
P.O. Box 100
Whitehouse Station, New Jersey, 08889-0100, U.S.A.

B. Name, qualifications and experience of the responsible scientist(s)

Site 1: UZ Gent – CEVAC – Center For Vaccinology
Site 2: CHU Saint-Pierre
Site 3: Insituut voor Tropische Geneeskunde – Department Clinial Services
Site 4: ANIMA Research Center

C. Title of the project

A Phase 2, Randomized, Double-Blind, Multicenter Study to Evaluate the Safety and Immunogenicity of Three Different Potency Levels of V181 (Dengue Quadrivalent Vaccine rDENVΔ30 [live, attenuated]) in Healthy Adults

II. INFORMATION RELATING TO THE GMO

A. Characteristics of (a) the donor, (b) the recipient or (c) (where appropriate) parental organism(s):

1. scientific name,

Dengue virus (DENV) Type 1,2,3,4
Strain and isolate:…… DENV1: Western Pacific
DENV2 M-E: New Guinea C
DENV3: Slemen/78
DENV4: Dominica/81 strain 814669

The parental viral strains are not attenuated.

2. taxonomy

Taxonomy of Organism:

(i)	order and/or higher taxon (for animals)	…
(ii)	genus	Flavivirus
(iii)	species	dengue virus (DENV)
(iv)	subspecies	…
(v)	strain	4 Strains (DENV1, DENV2, DENV3, DENV4)
(vi)	pathovar (biotype, ecotype, race, etc.)	…
(vii)	common name	Dengue

3. other names (usual name, strain name, etc.),

dengue virus (DENV)

DENV1: Western Pacific
DENV2: prM-E DENV2 New Guinea C/DENV4 backbone 814669
DENV3: Slemen/78
DENV4: Dominica/81 strain 814669

4. phenotypic and genetic markers,

Dengue Virus (DENV) is an enveloped (+)ssRNA arbovirus of the Flaviviridae family and Flavivirus genus, with four serotypes (DENV1 – DENV4) sharing 70–80% a.a. sequence homology. The genus flavivirus also includes yellow fever virus and West Nile virus (WNV). Dengue is 40-60 nm in size, with an isometric nucleocapsid of 25-30 nm. The positive-sense RNA genome contains ~10.7 kb and is linear. Dengue virus is genetically related to other flaviviruses such as yellow fever and tick-borne encephalitis viruses {07WZTX}.

5. degree of relatedness between donor and recipient or between parental organisms,

The homology of the vaccine strains is identical to the parental strains with the exception of the following genetic modifications: for the rDENV1Δ30, rDENV3Δ30/31, and rDENV4Δ30 components of V181, the vaccine strains include a modification (deletion) of a portion of the dengue virus (Δ30) as the mechanism of attenuation. The deletions involve stem-loop structures in the 3'-non-coding region of the genome. The deletion of the stem-loop structures results in reduced replication of the viruses and corresponding attenuation. The entire coding region of the genome for the DENV1, DENV3, and DENV4 components are identical to the parental organisms {07WTYT}.

For the rDENV2/4Δ30(ME) component of V181, the rDENV4Δ30 backbone is utilized and the pre-M and E genes are deleted from the backbone and the homologous pre-M and E genes from DENV2 are inserted in its place. As with the other constructs the coding regions of the rDENV2/4Δ30(ME) is identical to the parental organisms.

6. description of identification and detection techniques,

The viral components of V181 are detected using validated RT-PCR assays sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques,

7. sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques,

The validated RT-PCR assays are specific for each of the 4 dengue serotypes. The assays can identify which serotype is present in a sample and whether it is a vaccine strain or wild type strain.

8. description of the geographic distribution and of the natural habitat of the organism including information on natural predators, preys, parasites and competitors, symbionts and hosts,

Dengue is widespread throughout the tropics, with local variations in risk influenced by rainfall, temperature, relative humidity and unplanned rapid urbanization. The virus is not endemic to the EU but travelers visiting the tropics, including EU territories, have been known to return to the EU with infection.

The host range is restricted to humans, simians and mosquitoes {04VB3R}. Dengue virus is transmitted to humans primarily through *Aedes aegypti* mosquito bites. Virus can be communicated from human-to-human via transfusion of tainted blood. Humans are infective for mosquitoes a few days before and after the febrile period and mosquitoes become infective for humans 8-12 days after infection. Other mosquito species in the genus *Aedes* — including *Aedes albopictus*, *Aedes polynesiensis,* and *Aedes scutellaris* — have a limited ability to serve as dengue vectors {07WZTX}.

9. organisms with which transfer of genetic material is known to occur under natural conditions,

None. DENV replicates in the cytoplasm. As it is an RNA virus, there is no DNA intermediate, and it does not integrate into the host cell DNA.

10. verification of the genetic stability of the organisms and factors affecting it,

Attenuation of the four viral components of V181 (comprising the four serotypes of dengue) is driven by the deletion of 30 nucleotides in the 3' non-coding region (Δ30) of each dengue virus genome. WHO in their guidance on live, attenuated, tetravalent dengue vaccine states that a potential reversion is based on the stability of the attenuating mutation(s), the number of attenuating mutations, and the nature of attenuating mutation {05C0FR}. Attenuating mutations that are derived by deletions of segments of RNA are generally more stable against reversion than attenuations based on single nucleotide mutations. As the Δ30 deletions encoded in the viral genomes and resulting in the attenuation of each of the 4 viral components are a large segment and not a single point mutation, the risk of reversion is low.

In addition, because of the presence of the Δ30 deletion in all components, reversion back to a wild type (wt) phenotype via a recombination event is also unlikely and would require co-infection with wild type(wt) dengue. Since wt-dengue is not typically present in non-tropical regions such as the USA and Europe, there is very low probability of co-infection of wt-dengue at the moment of vaccination. Furthermore, even for regions where wt-dengue virus is present, co-infection with wt-Dengue would have to happen within the short time period (up to 3 days) of viremia in vaccinated individuals which is very unlikely.

Positive strand RNA viruses other than picornaviruses, coronaviruses, togaviruses and noroviruses recombine only inefficiently. In different species of arthropod borne flaviviruses differences in observable recombination frequencies in nature are attributable to differences in mechanism of vectoring by ticks and mosquitoes and by differences in both host and vector ecology {07WZQN}. Studies on intra-typic recombination among flaviviruses that occurred on an evolutionary scale in the wild demonstrated that there was little or no recombination in cell culture, and that substitution of heterologous envelope proteins into a virulent flavivirus backbone resulted in viruses with properties of attenuation matching those of an attenuated vaccine vector {07WZQN}. Intertypic recombination occurs at a frequency 100-fold lower than in the case of intratypic recombination {07WZLF}.

Virtually all vaccine vectors or vaccine strains are either naturally or artificially attenuated for pathogenicity in their target populations and thus demonstrate reduced replication. Recombination between a vaccine vector or vaccine strain and a wild type virus, should it happen, should not lead to a construct more virulent than the wild type virus itself {04RHB3}.

The probability of non-homologous recombination, such as between V181 and another non-related RNA virus, is substantially lower than homologous recombination between related viruses. The non-homologous recombination mechanism involves a cleavage-joining or joining of RNA fragments, generally occurring without replication or a requirement for the viral RNA polymerase. Non-homologous recombinations are rarely detected principally because they are deleterious. They have been demonstrated as relatively rare event even under forced experimental conditions {04RH9W}.

WHO in their 2013 report on the quality, safety and efficacy of dengue tetravalent vaccines states that 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 {05C0FR}.

For verification of genetic stability, the Sponsor performed Illumina-based high-throughput sequencing of master virus seeds, working virus seeds, and clinical bulks (or production harvests) manufactured to date. Sequence analysis includes confirmation of full-length consensus sequence and evaluation of minor single-nucleotide variants.

11. pathological, ecological and physiological traits:

Infection with dengue viruses leads to a diverse clinical picture ranging from an inapparent or mild febrile illness, to classic dengue fever (DF) characterized by high fever, headache, joint and muscle pain, rash, lymphadenopathy, and leukopenia, to life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF and DSS). The clinical picture of DHF and DSS is characterized by hemorrhagic manifestations ranging from the presence of petechiae and ecchymosis to spontaneous severe hemorrhage and profound shock which may, if untreated, result in death. These more severe forms of the disease occur most often after secondary dengue infection, when infection with one serotype of dengue virus is followed sometime later by a second infection with another serotype. The severity of secondary dengue infections has been observed to increase with a longer interval between the first and second infection {03RK08}.
Although DF is less severe than DHF/DSS, a substantial burden of illness is associated with DF.
There are no specific therapies licensed for dengue virus and treatment is supportive.
The major approach for mitigating the possible risk of vaccine-induced sensitization for DHF is development of a tetravalent vaccine that will simultaneously and durably protect against disease caused by any of the 4 dengue serotypes {05KZ00}.

a) classification of hazard according to existing Community rules concerning the protection of human health and/or the environment;

EC Directive 2000/54/EU classifies wild type dengue virus as human pathogen Risk group 3.
Each monovalent virus strain includes a genetic modification of the dengue genome [i.e. deletion of 30 nucleotides in the 3' non-coding region (Δ30)]. rDENV1Δ30, rDENV3Δ30/31, and rDENV4Δ30 are full-length homotypic genomes. The DENV3 component has an additional 31-nucleotide deletion in the 3' non-coding region (Δ30/31). rDENV2/4Δ30(ME) is a chimeric virus with the pre-M and E proteins from DENV2 inserted into an attenuated rDENV4Δ30 backbone where the DENV4 pre-M and E proteins have

been deleted. All 4 strains have been fully characterized and their attenuation confirmed through testing in vitro and in vivo studies {07WTYT}.

The absence of a clinically apparent dengue-like illness with V181 is likely a result of the high degree of attenuation of the National Institute of Health (NIH) parental vaccine viruses. Peak viremia titers are more than ≥100-fold lower than those observed with symptomatic wild type dengue infection {04PXH0} {04F8TT}.

V181, NIH live attenuated tetravalent vaccine (LATV) and Butantan-dengue vaccine (Butantan-DV) were well tolerated by study participants in Phase 1 and Phase 2 studies with these vaccines. The main adverse effects that occurred more frequently in vaccinees compared with placebo recipients was a mild transient rash, mild to moderate myalgia, headache and fatigue and an occasional mild transient leukopenia, which were either self-resolved or easily managed.
The low-level viral replication of the attenuated strains and lack of infectivity for mosquitoes mean that the risk of dissemination is negligible since that is the key mechanism for dengue spread.

Information on the previous use of the GMO in children is provided in Section II.C.2.h. *History of previous releases or uses of the GMO*. The GMO has not been tested in pregnant women or immunosuppressed individuals; therefore such participants are excluded from the proposed study.

b) generation time in natural ecosystems, sexual and asexual reproductive cycle;

The host range is restricted to humans, simians and mosquitoes {04VB3R}. The 50% human infectious dose (HID_{50}) for the four serotypes in V181 is ≤10 PFU {04F8TT}.

Dengue virus is primarily transmitted to humans through *Aedes aegypti* mosquito bites. Virus can be communicated from human-to-human via transfusion of tainted blood. Humans are infective for mosquitoes a few days before and after the febrile period and mosquitoes become infective for humans 8-12 days after infection {03RHH4}. Other mosquito species in the genus *Aedes* — including *Aedes albopictus*, *Aedes polynesiensis*, and *Aedes scutellaris* — have a limited ability to serve as dengue vectors Dengue is widespread throughout the tropics, with local variations in risk influenced by rainfall, temperature, relative humidity and unplanned rapid urbanization {07WZTX}.

c) information on survival, including seasonability and the ability to form survival structures;

Dengue is an enveloped virus. These viruses are known to be easily inactivated by routine surface cleaning and disinfection. Their lipid envelope can be easily destroyed by alcohols and a wide range of commercially available disinfectants tested for inactivation of these viruses are available. The virus is stable in dried blood for up to 9 weeks at room temperature.

The biological profile of the V181 viral vaccine candidate including the host range, host specificity, and tissue or cell tropism is expected to be identical to the parental virus except that the viruses comprising V181 replicate less efficiently in human subjects and mosquitoes compared to the parental virus strains leading to an attenuated phenotype. The route of transmission is also expected to be the same but with lower viral replication in human subjects and mosquitoes, and the available data suggests that the attenuated viruses would not be efficiently transmitted via mosquitoes.

d) pathogenicity: infectivity, toxigenicity, virulence, allergenicity, carrier (vector) of pathogen, possible vectors, host range including non-target organism. Possible activation of latent viruses (proviruses). Ability to colonise other organisms;

While no specific data are available on shedding of V181 in urine or feces, these are not implicated as an environmental source for dengue spread.

Dengue virus is only believed to infect humans via direct exposure to blood/blood products or via mosquito bite {03RK0R}. Dengue viruses are transmitted by Aedes Sp. mosquitos which are day-biting mosquitos found commonly in urban environments. The host range of the Aedes Sp. is limited mostly to humans and simians. Among the species that can be infected with dengue viruses, overt symptomatic disease and severe outcomes of infection are limited to humans. Human to human transmission of dengue is mediated primarily by the mosquito vector. Humans are infectious to mosquitoes starting a few days prior to the febrile phase of illness through a few days following defervescence. A mosquito acquiring dengue via a blood meal from an infected human typically become infectious to humans within 10- 14 days. The overall burden of dengue can be related to the abundance and density of the mosquito vector as well as the population size and density of the community in which the definitive hosts (i.e., humans) reside.

Importantly studies conducted by the NIH suggest that the attenuated viruses comprising V181 are not transmitted from human to human via mosquitos. Experimental infection and transmission studies using vaccine virus and mosquito vectors have shown that the peak virus titer of all the V181 live attenuated dengue vaccine viral strains tested thus far in humans were at least ≥100-fold below the viremia level required for transmission to mosquitoes. Furthermore, for the vaccine virus to be transmitted from one human to another the following series of events would have to take place: (1) The subject would have to be viremic with a peak virus titer greater than 10^5 PFU/mL; (2) The viremic subject would then have to be bitten by a viable vector mosquito at the peak of viremia; (3) This mosquito would have to live for a 10 – 14 day period following the blood meal to make the mosquito infectious for the vaccine virus; (4) The same mosquito would then have to bite another individual. Therefore, the risk that the DENV vaccine will be transmitted from vaccinated to nonvaccinated populations is very low.

The NIH DENV4 component was evaluated for its transmissibility from vaccinees to mosquito, and its capacity to grow within mosquitos. Transmissibility to mosquitos was evaluated in 10 vaccinees who received a 10^5 dose of rDENV4Δ30. A. albopictus mosquitoes were fed on vaccinated subjects on days when the subjects were expected to be viremic. Five of the 10 vaccinees had detectable viremia on at least 1 of those days, with titers ranging from 1.0 log10 PFU/mL to 2.3 log10 PFU/mL. Vaccine virus was not detected in any of the 352 mosquitoes that fed on the subjects. Studies in A aegypti mosquitos showed that, compared with wild-type virus, rDENV4Δ30 was found to be restricted in its capacity to infect the midgut and to disseminate further. This lack of transmissibility was attributed to both the low level of viremia in subjects and to the restricted capacity of rDENV4Δ30 to disseminate from the midgut of the mosquito to its head{04QYLH}.

In summary, the monovalent components of the tetravalent vaccine have multiple barriers to transmission by mosquitos, resulting in an extremely low risk of transmission of vaccine virus between close contacts/other humans via mosquitos. The participants will be instructed to not donate blood or fluid products for 6 weeks after vaccination, further minimizing any possibility that V181 would be transmitted to other humans.

e) antibiotic resistance, and potential use of these antibiotics in humans and domestic organisms for prophylaxis and therapy;

The GMO is a virus, and therefore resistance to antibiotics is not relevant.

f) involvement in environmental processes: primary production, nutrient turnover, decomposition of organic matter, respiration, etc.

Dengue viruses are not implicated in environmental processes such as nutrient turnover, decomposition of organic matter, respiration, etc. Therefore, this is not applicable.

12. Nature of indigenous vectors:

The V181 attenuated viral strains were rescued from plasmids (DENV1, DENV2, and DENV4 strains) or derived from a clinical grade viral stock manufactured by the NIH (DENV3 strain).

The attenuated viral strains that comprise V181 were developed at the US National Institutes of Health (NIH). The NIH spent more than 20 years designing the individual vaccine components and demonstrating safety and immunogenicity of the monovalent vaccine candidates in completed nonclinical and Phase 1 clinical studies in the US and Phase 2 clinical studies in Thailand and Bangladesh.

Dengue virus (DENV) Type 1,2,3,4
Strain and isolate:…… DENV1: Western Pacific
DENV2 M-E: New Guinea C
DENV3: Slemen/78
DENV4: Dominica/81 strain 814669
The parental viral strains are not attenuated.

a) sequence;

V181 is a live attenuated dengue tetravalent vaccine (rDENV1Δ30, rDENV2/4Δ30(ME), rDENV3Δ30/31, and rDENV4Δ30). Each monovalent serotype of the vaccine includes a genetic modification of the dengue genome [i.e. deletion of 30 nucleotides in the 3′ non-coding region (Δ30)]. Two of the four vaccine constructs have additional modifications. DENV2 is the only serotype that is not a full-length homotypic genome but is instead a chimeric virus with the Pre-M and E protein from DENV2 inserted into an attenuated rDENV4Δ30 DENV4 backbone and is designated as rDENV2/4Δ30(ME). DENV3 has an additional 31-nucleotide deletion in the 3′ non-coding region and is designated as rDENV3Δ30/31. The viruses have been shown to be attenuated in nonclinical and clinical trials. A diagram illustrating the constructs comprising V181 is provided in Figure 1 below.

The attenuation is driven by the deletion of 30 nucleotides in the 3' non-coding region (Δ30) of the dengue genome. WHO in their guidance on live, attenuated, tetravalent dengue vaccine {Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated) WHO Technical Report Series, No. 932, WHO Technical Report Series No. 979, 2013) {05C0FR} states that a 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 derived by deletions of segments of RNA are generally more stable against reversion than attenuations based on single nucleotide mutations.
The use of the same Δ30 deletion in all components makes these mutations unlikely to revert to wild type.

b) frequency of mobilisation;

The attenuated virus strains are replication competent. The viruses replicate in the cytoplasm of cells, do not include a DNA intermediate, and do not integrate into the DNA of the cells.

c) specificity;

The host range is restricted to humans, simians and mosquitoes and of those hosts dengue is only known to be pathogenic for humans. Mosquitoes are the vector for transmission from human to human.

d) presence of genes which confer resistance.

There are no resistance genes in the vaccine viral strains.

13. History of previous genetic modifications.

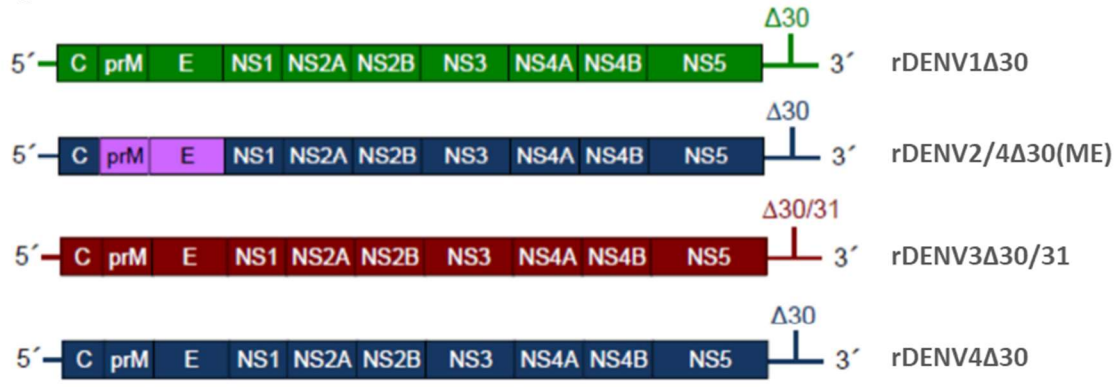
The viral sequences are derived from wild-type dengue viruses that have been passaged in cell culture with no specific genetic modifications introduced. In some cases, single point mutations that appear to be related to adaptation to Vero cells have been reported.

B. Characteristics of the vector

1. nature and source of the vector,

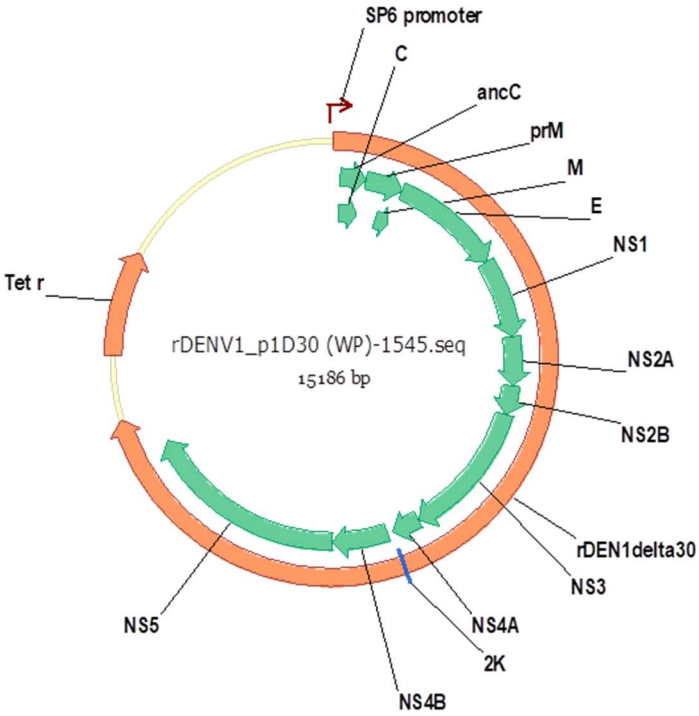
The viral strains comprising V181 were rescued from cDNA plasmids. A diagram illustrating the constructs comprising V181 is provided below (Figure 1). The map of the plasmids is also provided below (Figure 2). Once the virus is rescued there are no residual elements originating from the plasmid (e.g. tetracycline resistance gene) remaining.

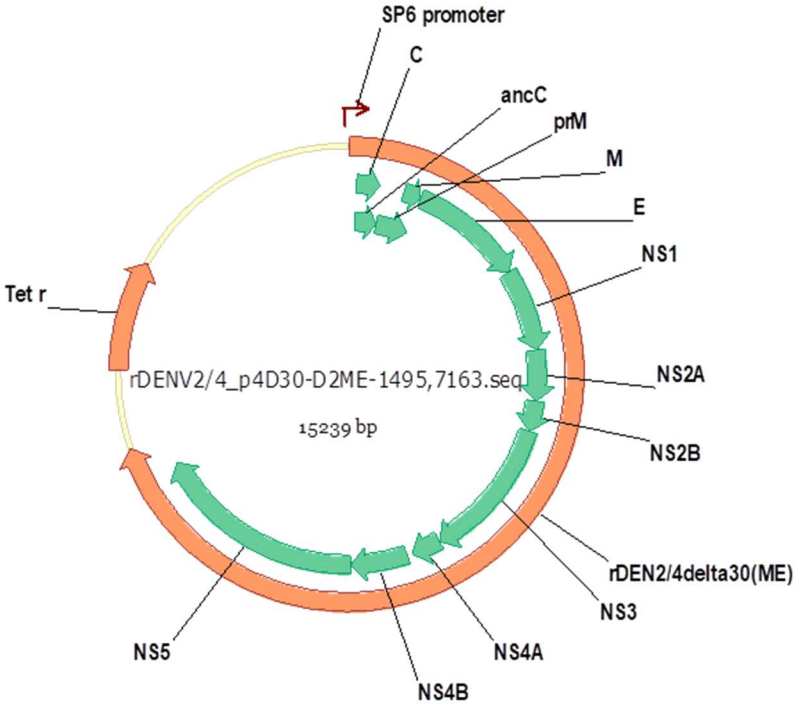
Figure 1:



Circular renditions of the plasmids are displayed below.

Figure 2:


SP6 promoter
C
ancC
prM
M
E
NS1
NS2A
NS2B
rDEN1delta30
NS3
NS4A
2K
NS4B
NS5
Tet r
rDENV1_p1D30 (WP)-1545.seq
15186 bp


SP6 promoter
C
ancC
prM
M
E
NS1
NS2A
NS2B
rDEN2/4delta30(ME)
NS3
NS4A
NS4B
NS5
Tet r
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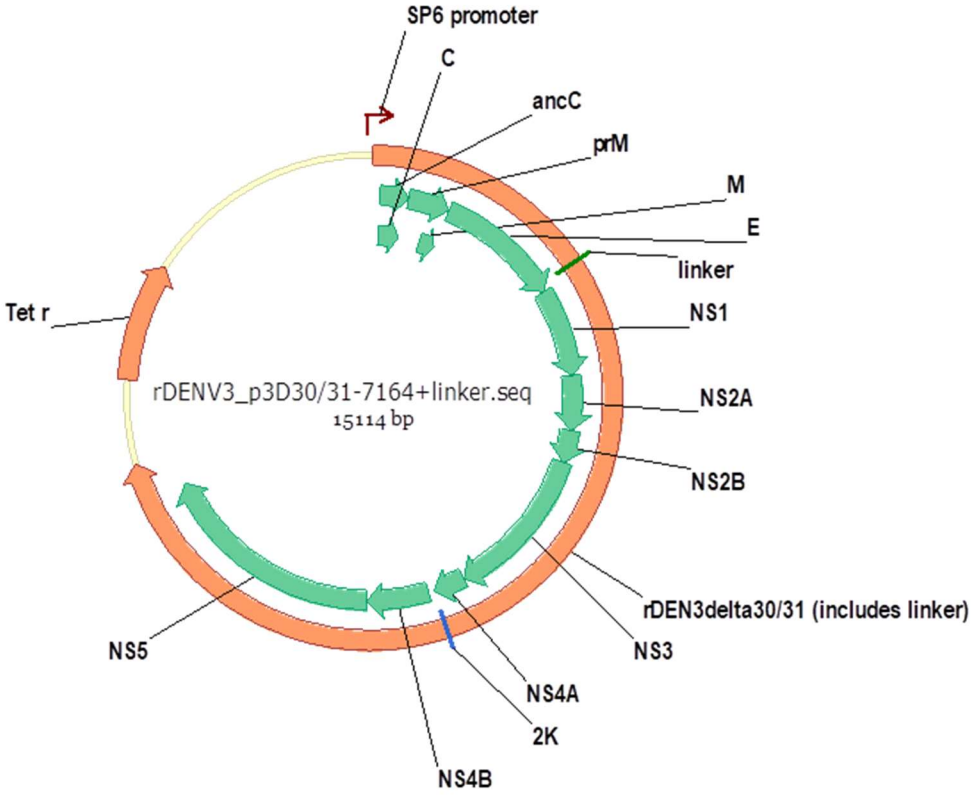
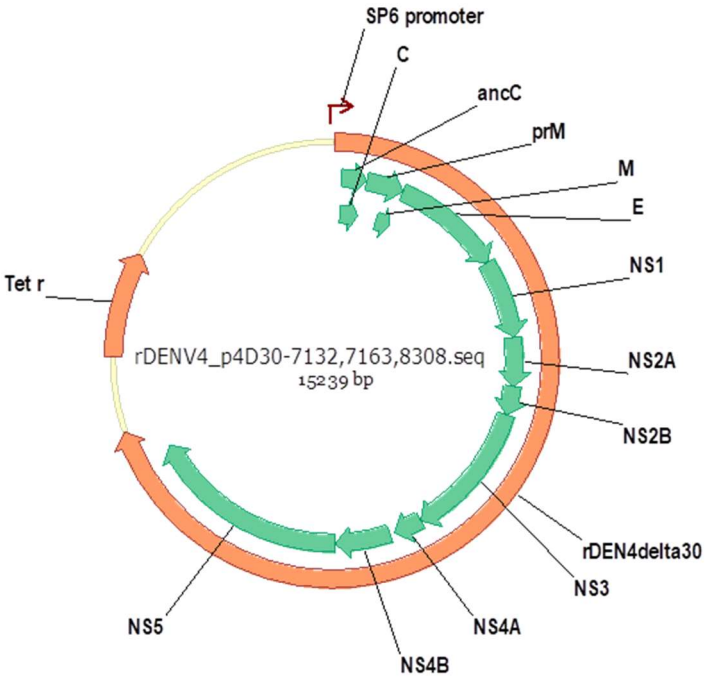
15239 bp





The attenuated viral strains comprising V181 are produced in Vero cells (ATCC CCL-81.2) which are monkey kidney cells derived from Cercopithecus aethiops monkeys. Vero cells do not contain sequences that could complement or recombine with the attenuating mutations in the viral constructs comprising V181.

2. sequence of transposons, vectors and other non-coding genetic segments used to construct the GMO and to make the introduced vector and insert function in the GMO,

Maps of the plasmid vectors used to generate the infectious recombinant viruses comprising V181 including all the key regulatory elements of the plasmid is provided in response to previous question.

3. frequency of mobilisation of inserted vector and/or genetic transfer capabilities and methods of determination,

The GMO replicates in the cytoplasm. As it is an RNA virus, it does not have a DNA intermediate and does not integrate into the host cell DNA. Therefore, the capacity for gene transfer is highly unlikely.

4. information on the degree to which the vector is limited to the DNA required to perform the intended function.

The GMO encodes an RNA genome, and there is no DNA intermediate.

C. Characteristics of the modified organism

1. Information relating to the genetic modification:

a) methods used for the modification;

The vaccine was developed at the NIH with recombinant DNA technology. An attenuating deletion of 30 contiguous nucleotides (Δ30) from the 3′ UTR of wild-type (wt) cDNA clones of DENV-1 and DENV-4 was used to generate vaccine candidates for each serotype. The Δ30 mutation attenuated DENV-1 and DENV-4, but not DENV-2 and DENV-3, was sufficiently based on data obtained from non-clinical studies.

Therefore, additional approaches were adopted for the other serotypes. Using a second method, antigenic chimeric viruses were generated by replacing wt M and E structural genes of rDEN4Δ30 with those from DENV-2 and DENV-3). The resulting vaccine candidates, DEN2/4Δ30 and DEN3/4Δ30, were found to be attenuated in SCID mice transplanted with human liver tumor cells (SCID-HuH-7) and Rhesus monkeys, and clinical development of these 2 chimeric vaccines was initiated. DEN2/4Δ30(ME) was found to be safe, immunogenic and highly infectious in healthy adult volunteers. DEN3/4Δ30 was safe in adult volunteers but proved to be insufficiently infectious so that an alternative DENV-3 construct was developed.

Using a third approach, an additional DENV-3 vaccine candidate (rDEN3Δ30/31-7164) was developed by introducing a second deletion into the 3′ untranslated region of the Sleman/78 strain of DENV-3. The first 30 nucleotide deletion removed a stem-loop structure homologous to the attenuating deletion in rDEN1Δ30 and rDEN4Δ30. The second 31 nucleotide deletion removed another stem-loop structure in the 3′ UTR {07WTYT}. When administered to healthy adult volunteers at a dose of 10^3 PFU, rDEN3Δ30/31was found to be safe and elicited an 80% seroconversion rate to wild type DENV-3.

b) methods used to construct and introduce the insert(s) into the recipient or to delete a sequence;

The deletions and insertions comprising the GMO dengue viral strains in V181 were generated using standard molecular biology techniques for manipulating sequenced encoded in a cDNA plasmid.

c) description of the insert and/or vector construction;

The plasmid construction is described above in Section II.B.1.*Characteristics of the vector, nature and source of the vector.*

d) purity of the insert from any unknown sequence and information on the degree to which the inserted sequence is limited to the DNA required to perform the intended function;

There are no unknown sequences in the GMO.

e) methods and criteria used for selection;

The V181 attenuated viral strains were rescued from plasmids as described previously. The viruses were originally selected by the NIH scientists for their ability to replicate, in vitro growth characteristics, performance in non-clinical models, and ultimately their performance in clinical trials {05C0FR}.

Upon transfer to MSD, the V181 viral strains were subjected to multiple rounds of terminal dilution and the purified viral clones characterized for their growth and sequence attributes.

f) sequence, functional identity and location of the altered/inserted/deleted nucleic acid segment(s) in question with particular reference to any known harmful sequence.

The deleted nucleic acid segments that result in attenuation of the viral strains comprise a stem-loop structure in the 3' non-coding region of the dengue genome. The stem-loop structure is implicated in viral replication and the deletion results in a lower level of viral replication compared to the wild type parent dengue viruses.

2. Information on the final GMO:

a) description of genetic trait(s) or phenotypic characteristics and in particular any new traits and characteristics which may be expressed or no longer expressed;

V181 is a live-attenuated dengue quadrivalent vaccine. Each monovalent serotype of the vaccine is a genetic modification of the dengue genome (ie, deletion of 30 nucleotides in the 3′ noncoding region (Δ30)). Two of the 4 vaccine constructs have additional modifications. DENV2 is the only serotype that is not a full-length homotypic genome but is instead a chimeric virus with the Pre-M and E protein from DENV2 inserted into an attenuated DENV4 backbone and is designated as rDENV2/4Δ30(ME). DENV3 has an additional 31- nucleotide deletion in the 3′ noncoding region and is designated as rDENV3Δ30/31 (Figure 1).

The viruses derived from the plasmids do not contain sequence elements from the vector beyond the viral sequences themselves.

b) structure and amount of any vector and/or donor nucleic acid remaining in the final construction of the modified organism;

The viruses derived from the plasmids do not contain sequence elements from the vector beyond the viral sequences themselves.

c) stability of the organism in terms of genetic traits;

Because of the presence of the Δ30 deletion in all components reversion back to a wild type (wt) phenotype via a recombination event would require the presence of wt-dengue. Since wt-dengue is not typically present in non-tropical regions such as the USA and Europe, there is very low probability of co-infection of wt-dengue at the moment of vaccination. Furthermore, even for regions where wt-dengue virus is present, co-infection with wt-Dengue would have to happen within the short time period (up to 3 days) of viremia in vaccinated individuals which is very unlikely.

For details, please refer to Section II.A.10.. *Verification of the genetic stability of the organisms and factors affecting it*, above.

d) rate and level of expression of the new genetic material. Method and sensitivity of measurement;

The only expression of the new genetic material are the live, attenuated viruses comprising V181 and the levels are measured via RT-PCR as described previously.

e) activity of the expressed protein(s);

Not applicable

f) description of identification and detection techniques including techniques for the identification and detection of the inserted sequence and vector;

The GMO is evaluated by RT-PCR. The details for RT-PCR are provided in Section V.A.1.

g) sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques;

As above. The details for RT-PCR are provided in Section V.A.1.

h) history of previous releases or uses of the GMO;

Recombinant DNA technology was used to generate the components of V181. Nonclinical studies have been performed on the components of the NIH dengue LATV admixture, which supports the V181 clinical program given that the vaccines are similar in design. The two vaccines have identical consensus sequences except for minor differences and are produced by a similar manufacturing process. Thus, results from NIH studies with NIH LATV are considered applicable for Merck-manufactured V181. A short summary of clinical studies is given below.

Human clinical trials: The NIH has conducted numerous clinical trials of the individual components as well as quadrivalent formulations of the viruses comprising V181. Peak viremia titers are ≥100 fold lower than those observed with symptomatic wild type dengue infection {04PXGX} {04PXGZ}{06DWPN}{04PXH0}

The NIH LATV has been well tolerated by flavivirus-naïve vaccinees. The main adverse effect that occurred significantly more frequently in vaccinees compared with placebo recipients was a mild transient rash and a rare transient leukopenia. A single dose of NIH LATV induced a tri- or tetravalent response in

>90% of flavivirus-naïve vaccinees {04DX4G}. A second dose given at 6 months did not significantly boost the antibody titers. In addition, the NIH LATV induced protection against viremia and rash associated with subsequent rDEN2Δ30-7169 challenge in 100% of subjects {04DX4J}. The low-level viral replication of the attenuated strains and lack of infectivity for mosquitoes mean the risk of dissemination is negligible since that is the key mechanism for dengue spread.

A brief summary of completed clinical studies is provided below.

MSD V181 clinical studies
Results from the V181-001 Phase 1 study indicated V181 is highly immunogenic through 6 months post dose 1 and generally safe and well tolerated in flavivirus-naïve and flavivirus experienced participants. AEs were generally assessed as mild to moderate in intensity by the participants.

NIH Dengue LATV clinical studies
To date, the NIH has administered the dengue LATV monovalent components or tetravalent formulations comprising the same parental strains as V181 to more than 1000 participants with studies completed or ongoing in the US, Thailand and Bangladesh, going down to 1 year of age. The NIH monovalent and tetravalent vaccines were generally well-tolerated in both nonclinical and human studies conducted by the NIH.

Butantan-DV clinical studies
V181 and Butantan-DV are derived from the same parental strains originally developed by the NIH, are produced using similar methods, have the same antigenic composition and are therefore fundamentally the same. Therefore, data from Butantan-DV clinical trials are relevant for V181.

Butantan-DV was evaluated in a Phase 2 study of 300 participants (of which 210 received Butantan-DV) in Brazil. The vaccine was found to be immunogenic and generally safe and well-tolerated in both dengue-naïve and dengue-exposed participants {05G8ZY}. The vaccine is currently being investigated in a large Phase 3 safety and efficacy study with approximately 17,000 participants enrolled in Brazil, about 9,000 of whom are children 2 to < 18 years of age, of which >6,000 will have received the Butantan-DV vaccine (NCT02406729).

i) considerations for human health and animal health, as well as plant health:

(i) toxic or allergenic effects of the GMOs and/or their metabolic products;

There are no known toxic or allergenic effects from exposure of humans or animals to the GMO.

(ii) comparison of the modified organism to the donor, recipient or (where appropriate) parental organism regarding pathogenicity;

The viruses comprising V181 replicate less efficiently and are less pathogenic compared to the parental strains. While wild type dengue virus infection is associated with fever, headache, body aches, rash, and other symptoms, the V181 vaccine strain viruses have demonstrated very few clinical manifestations in the clinical trials conducted to date. The main adverse effects that occurred more frequently in vaccinees compared with placebo recipients was a mild transient rash, mild to moderate myalgia, headache and fatigue and an occasional mild transient leukopenia, which were either self-resolved or easily managed.
The low-level viral replication of the attenuated strains and lack of infectivity for mosquitoes mean that the risk of dissemination is negligible since that is the key mechanism for dengue spread.

(iii) capacity for colonisation;

Wild-type dengue infection has rarely been reported to result in shedding of the virus in saliva and urine {07X3TC}; while no specific data are available on shedding of V181 in saliva, urine or feces, these are expected to be negligible given the lower level of viremia induced by V181 compared to wild-type dengue infection. Additionally, these routes are not implicated as an environmental source for dengue spread. Even in the event of incidental shedding of genetically modified organism (GMO) in wastewater, the establishment of GMO in such system is not to be expected due to the low volumes released and the destruction of the GMO by wastewater treatment techniques (e.g., temperature, chlorination, etc.).

(iv) if the organism is pathogenic to humans who are immunocompetent:

- diseases caused and mechanism of pathogenicity including invasiveness and virulence,

DENV is a positive-sense RNA virus belonging to the Flavivirus genus of the family Flaviviridae. The approximately 10600 base genome of DENV contains a single ORF encoding a polyprotein which is processed by proteases of both viral and cellular origin into 3 structural proteins, namely the C, prM, and E proteins, and 7 NS proteins. Each end of the DENV genome consists of an UTR, which is predicted to be highly structured, and the overall genome organization is 5´-UTR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4BNS5-UTR-3´. DENV infects predominantly monocytes, macrophages, and lymphocytes, however, subclinical infection of the liver also appears common {04R24K} {04QYM8}. Four distinct DENV serotypes exist (DENV1, DENV2, DENV3, and DENV4); each serotype is widely distributed and each is capable of causing the full spectrum of DENV-induced disease ranging from an inapparent or mild febrile illness, to classic DF characterized by high fever, headache, joint and muscle pain, rash, lymphadenopathy, and leukopenia, to life threatening DHF and DSS.

Primary DENV infection results in the induction of virus-neutralizing antibodies that are broadly cross-reactive early after infection, and become more type-specific over time (at least 6 to 24 months and longer). Primary dengue infection confers long-lasting immunity to the infecting serotype, but only short-term protection against the other dengue serotypes. The more severe forms of the disease occur most often after secondary dengue infection, when infection with 1 serotype of DENV is followed sometime later by a second infection with another serotype. The severity of secondary dengue infections has been observed to increase with a longer interval between the first and second infection. Infection with a second DENV type typically results in very robust and broadly cross-reactive immune responses, including high-titer virus-neutralizing antibodies reactive against all 4 virus types {03RJZY} {03RK0C}. Disease associated with a third or fourth DENV infection is only rarely reported, suggesting that most individuals are protected against all virus serotypes following secondary infection {0403J6}.

Though the majority of primary and secondary infections with dengue are asymptomatic, in some cases following an incubation period of approximately 1 week, the self-limiting acute illness, dengue fever, occurs and is characterized by a febrile period of about 5 days. This is accompanied by systemic symptoms such as headache, malaise, anorexia, arthralgia and myalgia. Rash (including petechial hemorrhages), lymphadenopathy, leukopenia, and thrombocytopenia may accompany the fever, and elevated liver enzymes. Persistent viral

infection is not established, and the virus is usually eliminated by the end of the second week. Severe manifestations of dengue illness can include hemorrhagic manifestations ranging from the presence of petechiae and ecchymosis to spontaneous severe hemorrhage and profound shock, which may, if untreated, result in death {05BSBH} {03RK0R}.

Severe dengue is much less common than dengue fever and occurs predominantly in children living in hyperendemic regions who experience a second infection with a DENV of a different serotype from that which caused the primary infection. Severe dengue is also seen during primary infection of 6 to 12 month old infants coinciding with declining maternally acquired dengue virus specific antibodies. Severe dengue typically develops at the time of defervescence and is characterized by an increased tendency to bleed into the skin or from mucous membranes and by a marked increase in vascular permeability resulting in hemoconcentration and shock that can be fatal in about 1% of even properly managed patients.

The low-level viral replication of the attenuated strains and lack of infectivity for mosquitoes mean the risk of disease in recipients or dissemination to others is negligible since high level viral replication is the key mechanism for dengue spread.

- **communicability,**

As above.

- **infective dose,**

The 50% human infectious dose (HID_{50}) for the four serotypes in V181 is ≤10 PFU {04F8TT}.

- **host range, possibility of alteration,**

The host range is restricted to humans, simians and mosquitoes. There is no evidence for an altered host range through natural dengue virus spread over hundreds of years and the GMO contains native coding sequences for dengue so there is no altered tropism expected.

- **possibility of survival outside of human host,**

Dengue is an enveloped virus. These viruses are known to be easily inactivated by routine surface cleaning and disinfection. Their lipid envelope can be easily destroyed by alcohols and a wide range of commercially available disinfectants tested for inactivation of these viruses are available. The virus is stable in dried blood for up to 9 weeks at room temperature.

- **presence of vectors or means of dissemination,**

The study will be performed in Belgium, where the primary vector for transmission of dengue, Aedes mosquito, is not endemic. In addition, the low-level viral replication of the attenuated strains and lack of infectivity for mosquitoes mean the risk of dissemination is negligible since that is the key mechanism for dengue spread.

- **biological stability,**

Dengue is an enveloped virus. These viruses are known to be easily inactivated by routine surface cleaning and disinfection. Their lipid envelope can be easily destroyed by alcohols and a wide range of commercially available disinfectants tested for inactivation of these viruses are available. The virus is stable in dried blood for up to 9 weeks at room temperature.

- **antibiotic resistance patterns,**

Not applicable

- **allergenicity,**

There is no evidence of allergenicity in non-clinical or clinical studies conducted to date

- **availability of appropriate therapies.**

V181, Butantan-DV, and NIH-LATV vaccines which are all derived from the same constructs have been shown to be well tolerated. The main adverse effects that occurred more frequently in vaccinees compared with placebo recipients was a mild transient rash, mild to moderate myalgia, headache and fatigue and an occasional mild transient leukopenia, which were either self-resolved or easily managed.

(v) other product hazards.

None identified

III. INFORMATION RELATING TO THE CONDITIONS OF RELEASE AND THE RECIEVING ENVIRONMENT

A. Information on the release

1. description of the proposed deliberate release, including the purpose(s) and foreseen products,

The GMO is developed as a vaccine to protect against dengue virus, and it has no other intended use. The proposed deliberate release is administration by injection in a clinical trial setting. The foreseen product is the GMO itself, and no by-products are expected.

The GMO will be shipped frozen and stored frozen at -15° to -25°C in a limited-access area with a temperature monitoring device. Containment is suitable for work involving agents of low to no potential hazard to personnel and the environment.

Additionally, after the injection, the vaccination site will be covered with an adequate bandage (e.g. any adhesive bandage or gauze and tape) that provides a physical barrier to protect against direct contact. The bandage may be removed after the 30 minute safety observation period if there is no visible fluid leakage, and they will be disposed in biohazard waste containers.

foreseen dates of the release and time planning of the experiment including frequency and duration of releases,

The study is expected to commence on the 09 August 2022 in Belgium. The study will have an enrollment target of 185 participants on the 4 Belgian sites (60 subjects at two sites, 40 subjects at one site & 25 subjects at one site). Chances for the patients to receive any dose of the GMO against Placebo are 10:1 (2:4:4:1 for High Potency Level: Mid Potency Level: Low Potency Level: Placebo, respectively).

The proposed period of release is from 09 August 2022 until 05 May 2023. The sponsor estimates approximately 20 months to complete the trial starting with the first informed consent signed subject and finalizing with the last call phone or visit related to the study of the last subject.

The V181 study vaccine potency levels to be evaluated in this trial are $10^{4.0}$ to $10^{4.95}$ pfu/serotype for the V181 High Potency Level Group, $10^{2.8}$ to $10^{3.8}$ pfu/serotype for the V181 Mid Potency Level Group, and $10^{2.0}$ to $10^{2.5}$ pfu/serotype for the V181 Low Potency Level Group. Final potency levels, within those specified ranges, are to be determined. Each participant will receive a 1 dose (0.5mL) of the GMO/Placebo (Day1).

2. preparation of the site previous to the release,

All necessary study approvals must be place before the study can commence. The clinical trial site(s) at which the GMO will be administered to participants will be initiated according to Good Clinical Practice (GCP) and according to documented legal and local procedures and guidelines prior to study initiation. Investigator site staff will be given trial-specific training.

3. size of the site,

The proposed release will be conducted at the 4 Belgian sites as indicated under Section I.B.

4. method(s) to be used for the release,

The proposed deliberate release is administration by injection in a clinical trial setting. The non-deliberate release would only be expected to occur by incidental shedding from subjects. Even in the event of incidental shedding of GMO in wastewater, the establishment of GMO in such system is not to be expected due to the low volumes released and the destruction of the GMO by wastewater treatment techniques (e.g., temperature, chlorination, etc.).

Wild-type dengue infection has rarely been reported to result in shedding of the virus in saliva and urine {07X3TC}; while no specific data are available on shedding of V181 in saliva, urine or feces, these are expected to be negligible given the lower level of viremia induced by V181 compared to wild-type dengue infection. Additionally, these routes are not implicated as an environmental source for dengue spread. Even in the event of incidental shedding of genetically modified organism (GMO) in wastewater, the establishment of GMO in such system is not to be expected due to the low volumes released and the destruction of the GMO by wastewater treatment techniques (e.g., temperature, chlorination, etc.).

V181 has a very limited host range (non-human primates and humans) and is not transmitted to humans via arthropod bites, the almost exclusive mode of transmission.

5. quantities of GMOs to be released,

The study will have an enrollment target of 185 participants on the 4 Belgian sites (60 subjects at two sites, 40 subjects at one site & 25 subjects at one site). The GMO will be supplied in a 2mL glass vial with a vaccine potency level ranging from $10^{4.0}$ to $10^{4.95}$ pfu/serotype for the V181 High Potency Level Group, $10^{2.8}$ to $10^{3.8}$ pfu/serotype for the V181 Mid Potency Level Group, to $10^{2.0}$ to $10^{2.5}$ pfu/serotype for the V181 Low Potency Level. The actual dose for each participant will depend on the dose level that is assigned (2:4:4:1 for High Potency Level: Mid Potency Level: Low Potency Level: Placebo, respectively). Each participant will receive 1 dose (0.5mL) of the GMO/Placebo (Day1). The non-deliberate release into the environment would only be expected to occur by incidental shedding from patients, the probability of which is very low.

6. disturbance on the site (type and method of cultivation, mining, irrigation, or other activities),

Not applicable.

7. worker protection measures taken during the release,

All personal should be wearing gloves during preparation and administration of the investigational medical product (IMP).

8. post-release treatment of the site,

Decontamination will be performed according to standard operating procedures of the site. Contaminated surfaces and areas will be decontaminated with the appropriate disinfectants as well as used instruments. The personnel follow all local protection measurements as per local standard.

9. techniques foreseen for elimination or inactivation of the GMOs at the end of the experiment,

Where local discard and destruction of the IMP is appropriate, the site is responsible for ensuring that a local discard/destruction procedure is documented. Refer to local requirements with regards to GMO disposal.
If local discard/destruction is appropriate, it should occur after the clinical research associate has performed full accountability of un-used vials.
Delivery system components (injection needle and syringe) will be disposed of in a manner consistent with the standard practice of the institution for biohazardous sharps. In addition, any disposable surgical instruments or other materials used during the administration procedure or collection of body fluids will be disposed according to standard biosafety practice of the institution.

10. information on, and results of, previous releases of the GMOs, especially at different scales and in different ecosystems.

The GMO has not been studied or released in Europe.

The proposed trial will be submitted for countries Australia, Taiwan, South Korea, Israel, US, Canada.

Previous release of the GMO:

V181:

V181 study in the US (continental US and Puerto Rico)

NIH dengue LATV:

V181 is similar by design to the NIH dengue LATV TV003 based on using the same starting viral materials and very similar manufacturing processes. The NIH dengue monovalent vaccines and different formulations of the LATV were tested in Phase 1 trials in the US and two formulations of the LATV, TV003 and TV005, were studied by the NIH in Thailand and Bangladesh.

TV003 and TV005 are the same GMO and only differ in the potency of the DENV2 serotype included in the formulation: TV003 targets 10^3 plaque forming units of each component whereas TV005 targets 10^3 plaque forming units for DENV1, DENV3, DENV4 components and 10^4 plaque forming units of DENV2 component.

TV003 has been evaluated in 5 different Phase 1 studies in the US:

ClinicalTrials.gov NCT01072786 {04DX4G}

ClinicalTrials.gov NCT01436422 {04DX4G}

ClinicalTrials.gov NCT01506570 {05BZ23}

ClinicalTrials.gov NCT01782300 {05BZ22}

ClinicalTrials.gov NCT02021968 {04DX4J}

TV005 has been evaluated in 5 different Phase 1 studies in the US:

ClinicalTrials.gov NCT01072786 {04DX4G}

ClinicalTrials.gov NCT01436422 {04DX4G}

ClinicalTrials.gov NCT02873260

ClinicalTrials.gov NCT02879266

TV003 and TV005 have been evaluated in 2 Phase 2 studies:

ClinicalTrials.gov NCT02332733: Thailand

ClinicalTrials.gov NCT02678455: Bangladesh

Butantan dengue LATV (Butantan-DV) Instituto:

Butantan in Brazil used the same dengue viral strains from the NIH to manufacture a dengue LATV analogous to the NIH dengue LATV TV003. Butantan-DV is comprised of the same NIH attenuated viruses, contains the same antigenic composition, is produced using similar methods, and is fundamentally the same as V181.

Butantan-DV was evaluated in 2 trials in Brazil

ClinicalTrials.gov NCT02406729

ClinicalTrials.gov NCT01696422 {05G8ZY}

B. Information on the environment (both on the site and in the wider environment):

1. geographical location and grid reference of the site(s) (in case of notifications under part C the site(s) of release will be the foreseen areas of use of the product),

Site 1: UZ Gent – CEVAC – Center For Vaccinology
Site 2: CHU Saint-Pierre
Site 3: Instituut voor Tropische Geneeskunde – Department Clinical Services
Site 4: ANIMA Research Center

2. physical or biological proximity to humans and other significant biota,

Not applicable.

3. proximity to significant biotopes, protected areas, or drinking water supplies,

Not applicable.

4. climatic characteristics of the region(s) likely to be affected,

Not applicable.

5. geographical, geological and pedological characteristics,

Not applicable.

6. flora and fauna, including crops, livestock and migratory species,

The GMO is administration by injection in a clinical trial setting. The release into the environment would only be expected to occur by incidental shedding from patients.

Wild-type dengue infection has rarely been reported to result in shedding of the virus in saliva and urine {07X3TC}; while no specific data are available on shedding of V181 in saliva, urine or feces, these are expected to be negligible given the lower level of viremia induced by V181 compared to wild-type dengue infection. Additionally, these routes are not implicated as an environmental source for dengue spread. Even in the event of incidental shedding of genetically modified organism (GMO) in wastewater, the establishment of GMO in such system is not to be expected due to the low volumes released and the destruction of the GMO by wastewater treatment techniques (e.g., temperature, chlorination, etc.).
Additionally, as part of an inpatient portion of the study, a mosquito-transmissibility study was conducted to determine whether or not the vaccine virus could be transmitted to mosquitoes from infected volunteers 04QYLH}. It was found that the rDEN4VΔ30 vaccine virus was not transmitted from 10 infected vaccinees to more than 300 mosquitoes that fed on the vaccinees during the period of viremia. This indicated that the rDENV4Δ30 vaccine virus was poorly transmissible.
Therefore, the potential interaction of flora and fauna with the GMO will be very limited.

7. description of target and non-target ecosystems likely to be affected,

While no specific data are available on shedding of V181 in urine or feces, these are not implicated as an environmental source for Dengue spread. V181 has a very limited host range (non-human primates and humans) and is transmitted to these hosts under environmental conditions through arthropod bites, only.
Additionally, as part of an inpatient portion of the study, a mosquito-transmissibility study was conducted to determine whether or not the vaccine virus could be transmitted to mosquitoes from infected volunteers {04QYLH}. It was found that the rDEN4VΔ30 vaccine virus was not transmitted from 10 infected vaccinees to more than 300 mosquitoes that fed on the vaccinees during the period of viremia. This indicated that the rDENV4Δ30 vaccine virus is poorly transmissible from vaccinated to unvaccinated individuals.

8. a comparison of the natural habitat of the recipient organism with the proposed site(s) of release,

The GMO has a very limited host range (non-human primates and humans) and it was shown to not be transmissible via mosquitos, the almost exclusive mode of transmission.
The GMO is planned to be administered subcutaneously to study subject in a clinical trial setting.

9. any known planned developments or changes in land use in the region which could influence the environmental impact of the release.

None

IV. INFORMATION RELATING TO THE INTERACTIONS BETWEEN THE GMOS AND THE ENVIRONMENT

A. Characteristics affecting survival, multiplication and dissemination

1. biological features which affect survival, multiplication and dispersal,

Because of the presence of the Δ30 deletion in all components reversion back to a wild type (wt) phenotype via a recombination event would require the presence of wt-dengue. Since wt-dengue is not typically present in non-tropical regions such as the USA and Europe, there is very low probability of co-infection of wt-dengue at the moment of vaccination. Furthermore, even for regions where wt-dengue virus is present, co-infection with wt-Dengue would have to happen within the short time period (up to 3 days) of viremia in vaccinated individuals which is very unlikely. Therefore, it is extremely unlikely that V181 will facilitate the dissemination of infectious disease and/or create a new reservoir or vector.

2. known or predicted environmental conditions which may affect survival, multiplication and dissemination (wind, water, soil, temperature, pH, etc.),

Dengue is an enveloped virus. These viruses are known to be easily inactivated by routine surface cleaning and disinfection. Their lipid envelope can be easily destroyed by alcohols and a wide range of commercially available disinfectants tested for inactivation of these viruses are available. The virus is stable in dried blood for up to 9 weeks at room temperature.

3. sensitivity to specific agents.

Enveloped viruses are easily inactivated. Bleach, quaternary ammonium- and phenolic-based disinfectants will inactivate the virus. A freshly made 10% household bleach solution will inactivate the virus.

B. Interactions with the environment

1. predicted habitat of the GMOs,

Modifications in the GMO do not change the host range. Therefore, the predicted habitat of the GMO corresponds to that of wtDENV.

2. studies of the behaviour and characteristics of the GMOs and their ecological impact carried out in simulated natural environments, such as microcosms, growth rooms, greenhouses,

Not applicable

3. genetic transfer capability

Negligible due to low infectivity of mosquitoes.

a) post-release transfer of genetic material from GMOs into organisms in affected ecosystems;

Negligible due to low infectivity of mosquitoes.

b) post-release transfer of genetic material from indigenous organisms to the GMOs;

Very low – would require coinfection and recombination which is very unlikely given short period and very low level of vaccine viremia observed. Recombination between a vaccine virus and a wild type virus, should it happen, should not lead to a more virulent virus than the wild type virus itself {04RHB3}.

4. likelihood of post-release selection leading to the expression of unexpected and/or undesirable traits in the modified organism,

Given the nature of the mutation that attenuates the virus (Δ30 deletion) there is very little risk of selection leading to reversion post-release. Furthermore, recombination of flaviviruses is very rare and would require co-infection with a wild-type virus and adequate viral loads to support recombination. The low infectivity of mosquitoes further reduces the risk of spread. Therefore, any kind of post-release selection leading to unexpected or undesirable traits is very unlikely.

5. measures employed to ensure and to verify genetic stability. Description of genetic traits which may prevent or minimise dispersal of genetic material. Methods to verify genetic stability,

Again, the nature of the mutation that attenuates the virus contributes to the genetic stability of the virus and leads to very low rates of viral replication in humans or mosquitoes which will minimize dispersal of genetic materials. For verification of genetic stability, the Sponsor performed Illumina-based high-throughput sequencing of master virus seeds, working virus seeds, and clinical bulks (or production harvests) manufactured to date. Sequence analysis includes confirmation of full-length consensus sequence and evaluation of minor single-nucleotide variants.

6. routes of biological dispersal, known or potential modes of interaction with the disseminating agent, including inhalation, ingestion, surface contact, burrowing, etc.

Refer to Section II.A.11.d

7. description of ecosystems to which the GMOs could be disseminated,

Even in the event of incidental shedding of GMO in wastewater, the establishment of GMO in such system is not to be expected due to the low volumes released and the destruction of the GMO by wastewater treatment techniques (e.g., temperature, chlorination, etc.).

Possible inoculation of mosquitos with vaccine virus(es) comprising V181 if the mosquito was to bite a person who was viremic postvaccination. The probability of this is very low as viremia levels are very low (≤ 10 pfu) and studies conducted to date suggest very low infectivity for mosquitoes or in mosquito cells.

8. potential for excessive population increase in the environment,

Increased competitiveness or invasiveness is unlikely due to the attenuated nature of the vaccine and low-level viral replication. The low-level viral replication of the attenuated strains and lack of infectivity for mosquitoes mean the risk of dissemination is negligible since that is the key mechanism for dengue spread. Thus, the potential for excessive population increase of the GMO in the environment is very low.

9. competitive advantage of the GMOs in relation to the unmodified recipient or parental organism(s),

Not applicable. There is no competitive advantage compared to the unmodified parent.

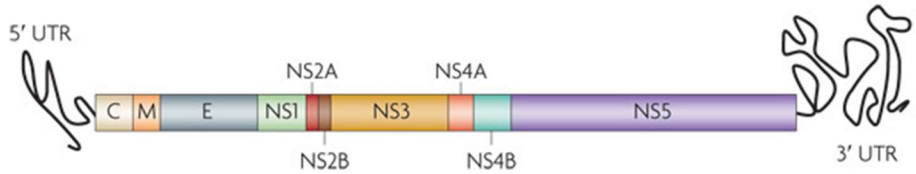
10. identification and description of the target organisms if applicable,

The GMO is planned to be administered subcutaneously to study subject in a clinical trial setting.

11. anticipated mechanism and result of interaction between the released GMOs and the target organism(s) if applicable,

Key cellular mechanisms of the GMO include:
Dengue virus may undergo 2 different cycles of transmission and amplification, sylvan and urban. In the sylvan cycle the virus undergoes rounds of infection, amplification, and re-infection between nonhuman primates and arthropod vectors. It is believed that infected arthropod vectors then migrate from jungle environments and initiate the urban cycle in which the cycles of infection, amplification, and re-infection occur between humans and vector species {03RHGW}.
The envelope protein of the virus (E-protein) plays important role in attachment of virus to target cells and their interaction with a wider range of cellular receptors whose affinity for DENV binding can be serotype specific. Following the bite of a DENV infected mosquito, the resident skin dendritic cells (DC), are amongst the first cells to be infected with DENV though the dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN). DENV has been shown to diffuse along the cell surface and to associate with pre-existing clathrin-coated pits prior to cell entry. Subsequently, the internalized virions are delivered to early endosomes. Membrane fusion is facilitated by the E glycoprotein and is triggered by the low pH and lipid environment of endosomes. The viral genome is translated by ribosomes on the endoplasmic reticulum (ER) and subsequently remodels this organelle to facilitate viral replication and assembly. The dengue virus genome is shown in Figure 2. The positive sense RNA genome encodes for a single polyprotein, which is subsequently processed by virally encoded and cellular proteases to yield seven non-structural proteins (NS1, 2A, 2B, 3, 4A, 4B, and 5), as well as three structural proteins: C (capsid), prM (precursor membrane protein) and E (envelope). The non-structural proteins assemble in a sequential manner to initiate RNA replication. The prM and E proteins form heterodimers that are oriented into the lumen of the endoplasmic reticulum (ER). Newly assembled immature particles have 60 hetero-oligomeric spikes, a single spike consisting of a trimer of prM/E heterodimers. Immature virus particles mature by furin-mediated cleavage of prM while passing through the Golgi network. Subsequently, dengue virus-particles are secreted into the extra-cellular space {03RK08}

Figure 2. Dengue virus genome

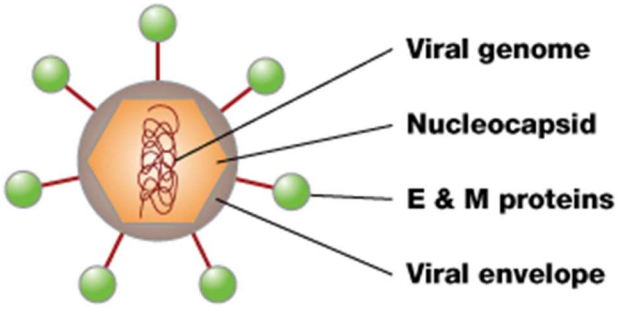


The dengue virus genome encodes three structural (capsid [C], membrane [M], and envelope [E]) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins.

Both ends of the dengue virus genome contain an untranslated region (UTR). The 3'UTR is nearly 400 bases in length and is predicted to contain several stem-loop structures conserved among dengue virus serotypes.
The four dengue virus serotypes are similar — they share approximately 65% of their genomes — but even within a single serotype, there is some genetic variation. Within each of the four dengue virus serotypes, phylogenetic studies have identified genetic subtypes that differ in nucleotide sequence by up to 12% in the envelope (E) gene, which determines most antigenic characteristics of the virus. DENV1 virus comprises five known subtypes (I–V), and DENV2 virus comprises six, although DENV2 virus subtype III has been further divided into sublineages IIIa and IIIb. DENV3 and DENV4 viruses currently are classed into four and two different subtypes, respectively {07WZTX}.

The structure of the dengue virus is roughly spherical, with a diameter of approximately 50 nm (Figure 3) (The core of the virus is the nucleocapsid, a structure that is made of the viral genome along with C proteins. The nucleocapsid is surrounded by the viral envelope, a lipid bilayer that is taken from the host. Embedded in the viral envelope are 180 copies of the E and M proteins that span through the lipid bilayer (https://www.nature.com/scitable/content/dengue-virus-structure-22401481/).

Figure 3: Dengue virus structure



12. identification and description of non-target organisms which may be adversely affected by the release of the GMO, and the anticipated mechanisms of any identified adverse interaction,

None. The vaccine viruses have been shown to not efficiently infect mosquitoes or mosquito cells.

13. likelihood of post-release shifts in biological interactions or in host range,

The V181 vaccine viruses are attenuated for pathogenicity in their target populations and demonstrate reduced replication. Recombination between a vaccine virus and a wild type virus, should it happen, should not lead to a more virulent virus than the wild type virus itself.
The probability of non-homologous recombination, such as between V181 and another non-related RNA virus, is substantially lower than homologous recombination between related viruses. The non-homologous recombination mechanism involves a cleavage-joining or joining of RNA fragments, generally occurring without replication or a requirement for the viral RNA polymerase. Non-homologous recombinations are rarely detected principally because they are deleterious. They have been demonstrated as relatively rare event even under forced experimental conditions {04RH9W}.

14. known or predicted interactions with non-target organisms in the environment, including competitors, preys, hosts, symbionts, predators, parasites and pathogens,

Increased competitiveness or invasiveness is unlikely due to the attenuated nature of the vaccine and low-level viral replication. The low-level viral replication of the attenuated strains and lack of infectivity for mosquitoes mean the risk of dissemination is negligible since that is the key mechanism for dengue spread. The likelihood to exchange genetic material is negligible due to low infectivity of mosquitoes.

15. known or predicted involvement in biogeochemical processes,

Not applicable

16. other potential interactions with the environment.

Not applicable

V. INFORMATION ON MONITORING, CONTROL, WASTE TREATMENT AND EMERGENCY RESPONSE PLANS

A. Monitoring techniques

1. methods for tracing the GMOs, and for monitoring their effects,

Viral nucleic acids are isolated from serum samples using a magnetic-bead based extraction procedure followed by target amplification and detection using TaqMan chemistry.
The DENV-WT (dengue Virus wildtype) RT-PCR and the DENV-Vacc (Dengue Virus vaccine) RT-PCR reactions are performed first. The DENV-WT RT-PCR reaction, targeting the Δ30 sequence deleted in the 3'-UTR of vaccines, selectively detects all wild-type strains, regardless of serotype, and the DENV-Vacc RT-PCR reaction bridges the Δ30 deletion present only in vaccines to selectively detect the four DENV vaccine serotypes. These two reactions qualitatively determine if a test sample is positive for wild-type and/or vaccine dengue virus. If a sample is determined to contain wild-type and/or vaccine virus by the DENV-WT and DENV-Vacc RT-PCR reactions, the extracted nucleic acid is then subjected to the DENV1/3 (Dengue Virus serotypes 1 and 3) and DENV2/4 (Dengue Virus serotypes 2 and 4) RT-PCR reactions. The DENV1/3 and DENV2/4 RT-PCR reactions determine serotype identity and quantity and are used to quantify the total viral load of a sample by extrapolation from the appropriate external standard curve(s) generated from each of the four wild-type and vaccine calibrators run in these assays. Quantitative results are reported as copies/mL.

2. specificity (to identify the GMOs, and to distinguish them from the donor, recipient or, where appropriate, the parental organisms), sensitivity and reliability of the monitoring techniques,

The viral components of V181 are detected using validated RT-PCR. The assays are designed to distinguish the viral vaccine strains from wild type strains.

3. techniques for detecting transfer of the donated genetic material to other organisms,

The likelihood of transfer of donated genetic material from the GMO to other organisms is low. Therefore, techniques for detecting transfer of donated genetic material to other organisms is deemed unnecessary and have not been developed.

4. duration and frequency of the monitoring.

Blood samples for viremia testing for each of the 4 dengue serotypes are taken at the post-dose timepoints at Day 7 and Day 12. These samples are evaluated by RT-PCR.

B. Control of the release

1. methods and procedures to avoid and/or minimize the spread of the GMOs beyond the site of release or the designated area for use,

To mitigate against the risk of unintentional release, V181 will be appropriately contained and labelled during transport. The risk from exposure to a healthy worker appears to be low to no risk. Staff handling V181 should wear gloves and follow other universal precautions as apply to the country.

In the event of an accidental spill, staff will follow their site standard operating procedure (SOP) for spill response and cleanup. In the event of an accidental spill, staff will follow their site SOP for spill response and cleanup. Bleach, quaternary ammonium- and phenolic-based disinfectants will inactivate the virus.

2. methods and procedures to protect the site from intrusion by unauthorized individuals,

All areas where IMP is stored is access protected via restricted access. Only authorized personnel are able to enter treatment or storage areas. The laboratory follows the rules for S2-labs and uses regular disinfection and decontamination procedures. Windows and doors have kept closed during the work process.

3. methods and procedures to prevent other organisms from entering the site.

All GMO stocks are assessed for purity and microbial growth as part of the GMP manufacturing process. All personal should be wearing gloves during preparation and administration of the IMP. In addition, aseptic technique when handling the GMO will minimize the risk of other organisms entering the facility.

C. Waste treatment

1. type of waste generated,

Types of waste include empty vials, used vials, delivery system components (injection needle and syringe), gauzes, gloves and bandages after administration.

2. expected amount of waste,

The amount of waste will be typical for the clinical site and laboratory operations amounting to a few clinical waste bags and bins per day waste. The amount of expected waste will be managed by standard operating procedures currently in place at the site.

3. description of treatment envisaged.

Delivery system components (injection needle and syringe) will be disposed of in a manner consistent with the standard practice of the institution for biohazardous sharps. In addition, any disposable materials used during the administration procedure will be disposed according to standard biosafety practice of the institution.

D. Emergency response plans

1. methods and procedures for controlling the GMOs in case of unexpected spread,

In the case of accidental spills during administration, appropriate disinfection is applied to prevent release into the environment.

In case of transport accidents, packaging should prevent release of GMO into the environment. Detailed decontamination procedures will accompany each shipment. In order to reduce the likelihood of packages that are damaged or leaking, and thereby prevent accidental exposure to personnel who handle the material during its shipment, the IMP is shipped according to the applicable national & international regulations; UN3245.

In the event of incidental shedding of GMO in wastewater, the establishment of GMO in such system is not to be expected due to the low volumes released and the destruction of the GMO by wastewater treatment techniques (e.g., temperature, chlorination, etc.).

V181 has a very limited host range (non-human primates and humans) and is not transmitted between humans under environmental conditions through arthropod bites

2. methods for decontamination of the areas affected, for example eradication of the GMOs,

In the event of an accidental spill, staff will follow their site SOP for spill response and cleanup. Bleach, quaternary ammonium- and phenolic-based disinfectants are all capable of destroying the virus. A freshly made 10% bleach solution will inactivate the GMO but can damage stainless steel.

3. methods for disposal or sanitation of plants, animals, soils, etc., that were exposed during or after the spread,

As no exposure and thus no spread is expected, disposal or sanitation plans for plants, animals and soils are not required.

4. methods for the isolation of the area affected by the spread,

Large spills are not expected in the clinic.

5. plans for protecting human health and the environment in case of the occurrence of an undesirable effect.

Staff handling the GMO and samples that could potentially contain the GMO should be wearing gloves. If breakage/spillage were to occur, bleach, quaternary ammonium- and phenolic-based disinfectants are proven to reduce viral infection potential after only a few minutes. In addition, as a precaution, upon vaccination with V181, it will be required to cover the vaccination injection site with an adequate bandage (e.g. any adhesive bandage or gauze and tape) that provides a physical barrier to protect against direct contact for the 30 minutes after vaccination that the participant is required to remain on site. The bandage may be removed when there is no visible fluid leakage, and it will be disposed of in a biohazardous waste container.