

**LIVE-ATTENUATED YELLOW FEVER VIRUS STRAIN ADAPTED
TO GROW ON VERO CELLS AND VACCINE COMPOSITION COMPRISING
THE SAME**

5 **FIELD OF THE INVENTION**

The present invention relates to a live-attenuated yellow fever virus (YFV) strain and uses thereof for the preparation of a vaccine composition against an infection by a YFV.

In particular, the live-attenuated YFV strain is adapted to grow on Vero cells,
10 and has been obtained from a parent live-attenuated YFV strain that is not adapted to grow on Vero cells, but rather that is adapted to grow on embryonated eggs. The live-attenuated YFV strain is further characterized by a reduced neurovirulence, as compared to the parent live-attenuated YFV strain.

15 **BACKGROUND OF THE INVENTION**

The yellow fever is a virus-mediated and lethal disease that is spread over 50 countries in the tropical areas of Africa, Central and South America.

The yellow fever is an acute viral haemorrhagic disease, some patients being affected by a jaundice, which explains the use of the term "yellow". The featured symptoms of yellow fever may include fever, headache, jaundice, muscle pain, nausea,
20 vomiting and fatigue. Moreover, a small proportion of patients who contract the virus develop severe symptoms and approximately half of those die within 7 to 10 days.

The yellow fever virus (YFV) belongs to the family of *Flaviviruses*, among which Dengue virus (DV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV) and Zika virus (ZV) are other members. The YFV
25 consists of a lipoprotein envelope surrounding a nucleocapsid composed of the capsid protein and a single-stranded, positive-sense RNA, which has a length of 10862 nucleotides. In between a 5' untranslated (5' UTR) and a 3' untranslated regions (3' UTR), the RNA encodes, from the 5' end to the 3' end, three structural proteins, namely a capsid protein (C protein), a premembrane/membrane protein (prM/M protein), an envelope protein (E protein) and eight non-structural (NS) proteins, namely NS1, NS2A, NS2B,
30 NS3, NS4A, P2k peptide, NS4B and NS5 proteins.

Wild-type YFV is principally vectored by *Aedes* spp. mosquitoes in Africa and *Haemogogus* and *Sabethes* spp. in South America, and there are non-human primate hosts which differ by geographic region. The YFV transmission is mainly achieved according to two epidemiological patterns, (1) the urban pattern and (2) the forest pattern (also known
5 as the jungle or sylvan cycle). Despite the two patterns of transmission, only one clinically relevant disease has been identified, which accounts for the same virus being involved. In the American continent, the YFV circulates today by means of an endemic, forest pattern that results in up to several hundred reports of infection in non-immune forest workers per year. In parallel, the virus circulates in Africa by means of both urban and forest patterns
10 and periodically breaks out of its endemic pattern to infect large numbers of non-immune persons in the course of major epidemics.

Currently, there are no antivirals for yellow fever disease, and vaccination is critical in preventing the disease. In this regard, as early as in the 1930's, two kinds of live-attenuated YFV vaccines were developed.

15 The first one corresponds to the French neurotropic vaccine (FNV), that was prepared from the wild-type French viscerotropic virus (FVV, isolated from Françoise Mayali in Senegal in 1928) and was passaged in mouse brain. However, the FNV proved rapidly to be too neurovirulent, having an exacerbated incidence of post-vaccinal encephalitis in children, and was abandoned in the early 1980's (Barrett, 2017).

20 The second approach corresponds to the "17D" strain, that was prepared from the wild-type strain Asibi (isolated from a mild human case – "Mr. Asibi" - in Ghana in 1927) and was passaged in mouse and chicken tissues. The vaccine strain 17D has lost both viscerotropism and neurovirulence (Monath, 2005).

Currently, six countries are producing live-attenuated YFV vaccine
25 compositions from substrains derived from the 17D strain, namely Brazil (17DD substrain), China (17D-204 substrain), France (17D-204 substrain Stamaril®), Russia (17D-213 substrain), Senegal (17D-204 substrain) and USA (17D-204 substrain YF-VAX®) (Barrett, 2017).

To date, all currently commercialized vaccines are produced in embryonated
30 hen eggs, a production process that has been complicated by robustness issues in the past (Barrett, 2017). Particularly, there is often a shortage of YFV vaccines due to manufacturing issues. Indeed, during the 2016 epidemics in Angola and the Democratic

Republic of Congo, a shortage of available vaccine lots resulted, for the first time, in the necessity of fractioning doses in order to adapt to the emergency context (Barrett; 2017). Further, YFV vaccine produced on embryonated hen eggs is contraindicated in people allergic to eggs.

5 An alternative to vaccine production on the basis of embryonated eggs is the use of suitable cell lines for passaging the virus, such as mammalian cell lines. Among the mammalian cell lines, the Vero cell line is one of the most studied, while providing stability and well-documented performance in quality and quantity of viral yield. The Vero cells have received FDA approval and are used throughout the world. For example, Vero
10 cells have been used for the preparation of a vaccine against the Japanese encephalitis (commercialized under the brand IXIARO®), against *Influenza* virus, against poliovirus and against rabies.

 Past and current strategies to take advantage of Vero cells to prepare YFV vaccines have emerged, and it is noteworthy to mention that these strategies are merely
15 oriented towards the feasibility of preparing YFV vaccines on the basis of inactivated virus (Hayes, 2010; Beasley et al., 2013; Pereira et al., 2015). Nevertheless, while a yellow fever inactivated vaccine may theoretically seem safer, it is unlikely to fully match the long-term protection provided by a single dose of the current live-attenuated yellow fever vaccines (Hayes, 2010). Further, in the context of the recent yellow fever epidemics, live-attenuated
20 vaccines seem better suited to provide high population coverage of long lasting protective immunity against yellow fever in endemic areas.

 The specific constraints of a live-attenuated virus for use in a vaccine is to maintain its attenuation, *i.e.* for a yellow fever virus to be at least as attenuated in terms of neurovirulence and viscerotropism as the current marketed live-attenuated yellow fever
25 vaccines; while being immunogenic enough to protect patients from the corresponding disease. In this regard, achieving both characteristics, *i.e.* attenuation and immunogenicity, for a given yellow fever strain has not been easy as can be seen, for instance, in Monath, 2005.

 Accordingly, due to the various drawbacks associated with producing live-
30 attenuated YFV vaccines based on embryonated hen eggs, there remains a need for alternative production methods for providing live-attenuated YFV vaccines.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a live-attenuated yellow fever virus strain adapted to grow on Vero cells from a parent yellow fever virus 17D substrain that is not adapted to grow on Vero cells, wherein said live-attenuated yellow fever virus strain is less neurovirulent than said parent yellow fever virus 17D substrain.

In another aspect, the invention further relates to a live-attenuated yellow fever virus strain comprising a nucleic acid comprising:

- i) a mutation in the codon for the amino acid at position 480 of the envelope protein (E) which results in an amino acid change from valine to leucine, or
- 10 ii) a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a) which results in an amino acid change from methionine to valine.

Another aspect of the invention relates to a live-attenuated yellow fever virus strain which comprises an envelope protein comprising a mutation at position 480 which results in an amino acid change from valine to leucine.

Another aspect of the invention relates to a live-attenuated yellow fever virus strain which comprises an envelope protein comprising a leucine residue at the position within the protein that corresponds to position 480 of **SEQ ID NO. 15**.

In another aspect, the invention further relates to a live-attenuated yellow fever virus strain comprising a nucleic acid molecule encoding:

- (i) an envelope protein comprising a mutation at position 480 which results in an amino acid change from valine to leucine, and
- (ii) a NS2a protein comprising a mutation at position 65 which results in an amino acid change from methionine to valine.

Another aspect of the invention relates to a live-attenuated yellow fever virus strain comprising a nucleic acid molecule encoding:

- (i) an envelope protein which comprises a leucine residue at the position within the protein that corresponds to position 480 of **SEQ ID NO. 15**, and
- (ii) an NS2a protein which comprises a valine residue at the position within the protein that corresponds to position 65 of **SEQ ID NO. 16**.

Another aspect of the invention also relates to an immunogenic composition comprising a live-attenuated yellow fever virus strain according to the present invention and a pharmaceutically acceptable vehicle.

In a still other aspect, the invention further relates to a method for obtaining a
5 live-attenuated yellow fever virus strain adapted to grow on Vero cells, comprising the steps of:

- a) purifying the viral genomic RNA of a parent live-attenuated yellow fever virus strain that is not adapted to grow on Vero cells, and that is optionally adapted to grow on eggs;
- 10 - b) transfecting Vero cells with the viral genomic RNA purified in step a), whereby transfected Vero cells are obtained;
- c) growing the transfected Vero cells obtained in step b) in a culture medium, whereby a first yellow fever virus population is obtained and further recovered;
- d) amplifying the recovered first yellow fever virus population obtained at the
15 end of step c) 2 times or more on fresh Vero cells, whereby a second yellow fever virus population is obtained;
- e) cloning the second yellow fever virus population obtained in step d) by two or more successive plaque purifications on Vero cells whereby a plurality of yellow fever virus clones is obtained;
- 20 - f) amplifying separately each of the recovered yellow fever virus clones obtained at the end of step e) 2 times or more on fresh Vero cells, whereby a plurality of yellow fever virus strains is obtained; and
- g) selecting from the said plurality of yellow fever virus strains recovered in step f) one or more live-attenuated yellow fever virus strain that is less neurovirulent than
25 the parent live-attenuated yellow fever virus strain, in a mouse lethal dose 50 (MLD₅₀) test.

Another aspect of the invention also relates to a live-attenuated yellow fever virus strain obtainable by a method according to the present invention.

In another aspect, the invention also relates to a live-attenuated yellow fever virus strain according to the present invention for use in the preparation of a vaccine.

30 A further aspect of the invention relates to a vaccine comprising a live-attenuated yellow fever virus strain according to the present invention for use in preventing an infection by a yellow fever virus.

LEGENDS OF THE FIGURES

Figure 1: Diagram illustrating the preparation of a live-attenuated yellow fever virus strain adapted to grow on Vero cells (vYF), at the pre Master Seed Lot (pMSL) stage.

5 **Figure 2:** Diagram illustrating the viscerotropism assay on an A129 mouse model.

10 **Figure 3:** Plots illustrating the viremia measured by YF-NS5 qRT-PCR in sera collected at D4 and D6 from A129 mice immunized at D0 with PBS (white bar); Stamaril® reference (black bars) or with vYF pMSL candidates deriving from the Stamaril® lineage (TV2212, TV2232 and TV2241; dark grey bars) or from the YF-VAX® lineage (TV3111, TV3112 and TV4221; light grey bars).

15 **Figure 4:** Plots illustrating the viral load measured by YF-NS5 qRT-PCR in liver samples collected at D6 and D11 from A129 mice immunized at D0 with PBS (white bar); Stamaril® reference (black bars) or with vYF pMSL candidates deriving from the Stamaril® lineage (TV2212, TV2232 and TV2241; dark grey bars) or from the YF-VAX® lineage (TV3111, TV3112 and TV4221; light grey bars).

20 **Figure 5:** Plots illustrating the viral load measured by YF-NS5 qRT-PCR in brain samples collected at D6 and D11 from A129 mice immunized at D0 with PBS (white bar); Stamaril® reference (black bars) or with vYF pMSL candidates deriving from the Stamaril® lineage (TV2212, TV2232 and TV2241; dark grey bars) or from the YF-VAX® lineage (TV3111, TV3112 and TV4221; light grey bars).

25 **Figure 6:** Plots illustrating the viral load measured by YF-NS5 qRT-PCR in spleen samples collected at D6 and D11 from A129 mice immunized at D0 with PBS (white bar); Stamaril® reference (black bars) or with vYF pMSL candidates deriving from the Stamaril® lineage (TV2212, TV2232 and TV2241; dark grey bars) or from the YF-VAX® lineage (TV3111, TV3112 and TV4221; light grey bars).

30 **Figure 7:** Plots illustrating the Kaplan-Meier survival curves after a single immunization of A129 mice with Stamaril®, vYF strains TV221, TV2241, TV3111, TV3112, TV4221 (dotted lines) or TV2232 (plain line).

30 **Figure 8:** Diagram illustrating the immunogenicity assay on a hamster model.

Figure 9: Plots illustrating the neutralizing antibody titers specific to live-attenuated yellow fever virus strain measured by seroneutralization assay on Vero cells in

sera collected at D26 from hamsters immunized at D0 with 2.5 or 5.5 log₁₀ CCID₅₀/dose of vYF strains (TV2212, TV2232, TV2241, TV3111, TV3112 and TV4221) or Stamaril® reference vaccine. The horizontal line represents the responder threshold.

Figure 10: Plots illustrating the neutralizing antibody titers specific to live-attenuated yellow fever virus strain measured by seroneutralization assay on Vero cells in sera collected at D41 from hamsters immunized at D0 and D26 with 2.5 or 5.5 log₁₀ CCID₅₀/dose of vYF strains (TV2212, TV2232, TV2241, TV3111, TV3112 and TV4221) or Stamaril® reference vaccine. The horizontal line represents the responder threshold.

Figure 11: Plots illustrating the neutralizing antibody response in monkeys vaccinated with vYF strain TV3112 in comparison with current vaccines Stamaril® and YF-VAX®. The horizontal line represents the limit of detection.

Figure 12: Plots illustrating the YF-specific IgM responses from the B memory cells, in peripheral blood from monkeys vaccinated with the live-attenuated vYF strain TV3112 in comparison with the current reference vaccines Stamaril® and YF-VAX®. The results are expressed as the percentage of IgM antibody-secreting cells in total IgM population.

Figure 13: Plots illustrating the YF-specific IgG responses from the B memory cells, in peripheral blood from monkeys vaccinated with the live-attenuated vYF strain TV3112 in comparison with the current reference vaccines Stamaril® and YF-VAX®. The results are expressed as the percentage of IgG antibody-secreting cells in total IgG population.

Figure 14: Plots illustrating the IFN-γ (upper panels) and IL-2 (lower panels) specific T-cell responses in peripheral blood from monkeys vaccinated with vYF strain TV3112 upon stimulation with the envelope protein (ENV; left panels) or stimulation with the non-structural protein 3 (NS3; right panels), and comparison with current vaccines Stamaril® and YF-VAX®.

Figure 15: Plots illustrating the viral load in organs from monkeys vaccinated with the live-attenuated vYF strain TV3112 in comparison with the current reference vaccines Stamaril® and YF-VAX®. Light grey bars and circles represent results from monkeys vaccinated with Stamaril®; medium grey bars and squares represent results from monkeys vaccinated with YF-VAX®; dark grey bars and triangles represent results from

monkeys vaccinated with the live-attenuated vYF strain TV3112. The horizontal line represents the limit of detection.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention provides live-attenuated YFV strains adapted to grow on Vero cells, which have been obtained from a parent live-attenuated YFV strain that is adapted to grow on embryonated eggs. The live-attenuated YFV strains have been selected for their reduced neurovirulence in a mouse LD₅₀ test (MLD₅₀), as compared to the parent live-attenuated YFV strain.

10 As it will emerge from the present invention, the production of YFV by passaging on Vero cells allows providing stable, highly reproducible, high standard quality and quantity live-attenuated YFV strains that are subsequently suitable for preparing a vaccine against a YF infection.

• Miscellaneous definitions

15 Within the scope of the present invention, “YFV” relates to a yellow fever virus”, whereas the term “vYF” denotes a Vero cells-adapted yellow fever virus, *i.e.* a yellow fever virus adapted to grow on Vero cells.

 Therefore, within the scope of the present invention, “Vero cells-adapted yellow fever virus” (vYV) and “yellow fever virus adapted to grow on Vero cells” are
20 intended to be interchangeable expressions.

 Within the scope of the present invention, a virus adapted to grow on Vero cells is a virus which has undergone at least 3 successive passages on Vero cells. In some embodiments, the virus has undergone about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 successive passages on Vero cells.

25 By “passage”, one may understand any step in which the virus undergoes at least one replication cycle in Vero cells, in particular any step of transfection, amplification or cloning of the virus in Vero cells.

 The expression “live-attenuated yellow fever virus”, as used herein, has the common meaning known by a man skilled in the art. In some embodiments, this expression
30 refers to a live yellow fever virus having an attenuated neurovirulence and/or an attenuated viscerotropism.

Within the scope of the present invention, the term “neurovirulence” is intended to refer to the capacity of the virus to pass across the blood-brain barrier (neuro-invasiveness), to replicate in the brain tissue (neurotropism) and cause inflammation, neuronal damage and encephalitis (neurovirulence *stricto sensu*).

5 Within the scope of the present invention, the term “viscerotropism” refers to the capacity of the virus to replicate in extraneural tissues, cause viremia and damage vital organs, including the liver (Monath, 2005).

 In some embodiments, said live-attenuated yellow fever virus is at least as attenuated as one of the current commercialized live-attenuated yellow fever vaccine
10 strains, for instance Stamaril® or YF-VAX®.

 In some embodiments, said live-attenuated yellow fever virus has a neurovirulence at least as attenuated as one of the current commercialized live-attenuated yellow fever vaccine strains, for instance Stamaril® or YF-VAX®.

 In some embodiments, said live-attenuated yellow fever virus has a
15 viscerotropism at least as attenuated as one of the current commercialized live-attenuated yellow fever vaccine strains, for instance Stamaril® or YF-VAX®.

 In some embodiments, said live-attenuated yellow fever virus has a neurovirulence and a viscerotropism at least as attenuated as one of the current commercialized live-attenuated yellow fever vaccine strains, for instance Stamaril® or YF-
20 VAX®.

 The terms “comprising”/“comprises”/“comprise”/“comprised” encompass “including”/“includes”/“include”/“included” respectively as well as “consisting”/“consists”/“consist”/“consisted” respectively, *e.g.* a composition “comprising” component X may consist exclusively of component X or may include one
25 or more additional components, *e.g.* component X and component Y.

 As used herein, “CCID₅₀” refers to cell culture infectious dose 50%, *i.e.* 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.

 Following the standard definitions from the World Health Organization
30 (WHO), the present invention refers to the below definitions (WHO Technical report series, No. 872, 1998).

A “master seed lot” (“MSL”), or “primary seed lot”, as used herein, refers to a quantity of virus suspension that has been processed in a single production run and has a uniform composition.

5 A “working seed lot” (“WSL”), or “secondary seed lot”, as used herein, refers to a quantity of virus suspension that has been processed in a single production run, and that is uniform with respect to composition, is fully characterized and is only one passage from a MSL. Within the scope of the present invention, material drawn from WSL is used for inoculating embryonated eggs, or suitable cell lines, in the preparation of vaccine.

10 A “plaque-forming unit” (PFU) as used herein, refers to the smallest quantity of a virus suspension that will produce a plaque in monolayer cell cultures.

A “median mouse lethal dose” (mouse LD₅₀ or MLD₅₀) as used herein, refers to the quantity of a virus suspension that will kill 50% of the mice injected with it.

15 • Live-attenuated YFV adapted to grow on Vero cells (also referred as to “vYF virus” for Vero cells adapted YF virus)

In one aspect, the invention relates to a live-attenuated yellow fever virus strain adapted to grow on Vero cells from a parent yellow fever virus 17D substrain that is not adapted to grow on Vero cells. In various embodiments said live-attenuated yellow fever virus strain is less neurovirulent than said parent yellow fever virus 17D substrain.

20 In some embodiments, the parent yellow fever virus strain is a live-attenuated yellow fever virus strain adapted to grow on eggs.

In some embodiments, the eggs are embryonated hen eggs.

A “17D substrain” is a yellow fever strain having in its ancestors the 17D strain.

25 The “17D strain” has the common meaning known by a man skilled in the art. In some embodiments, “17D strain” refers to the yellow fever strain that was isolated from a mild human case, “Mr. Asibi”, in Ghana in 1927 and was passaged 18 times in minced murine embryo tissue and then passaged 58 times in minced chicken embryo tissue as described in Monath (2005).

30 In some embodiments, the 17D substrain encompasses the 17D-204 substrain, the 17DD substrain, and/or the 17D-213 substrain as described in Monath (2005). In an exemplary embodiment, the RNA sequence of the YFV 17D-204 strain (Genbank

accession number X03700), as previously disclosed by Rice et al., in 1985, may be represented by the RNA sequence **SEQ ID NO. 1**.

In some embodiments, the parent yellow fever virus strain is a yellow fever virus 17D-204 substrain.

5 In some embodiments, the parent YFV virus strain is the YFV 17D-204 derived YF-VAX® strain, the reference YFV strain used in the commercialized vaccine YF-VAX®.

In an exemplary embodiment, the RNA sequence of the YFV 17D-204 derived YF-VAX® strain may be represented by the RNA sequence **SEQ ID NO. 2**.

10 In some embodiments, the parent YFV virus strain is the YFV 17D-204 derived Stamaril® strain, the reference YFV strain used in the commercialized vaccine Stamaril®.

In an exemplary embodiment, the RNA sequence of the YFV 17D-204 derived Stamaril® strain may be represented by the RNA sequence **SEQ ID NO. 3**.

15 In an exemplary embodiment, the parent yellow fever virus 17D substrain comprises a RNA sequence of **SEQ ID NO. 2**.

In an exemplary embodiment, the parent yellow fever virus 17D substrain comprises a RNA sequence of **SEQ ID NO. 3**.

20 In an exemplary embodiment, the RNA sequence of the YFV 17D-213 strain (Genbank accession number U17067), as previously disclosed by Dos Santos et al., in 1995, may be represented by the RNA sequence **SEQ ID NO. 4** and the RNA sequence of the YFV 17DD strain (Genbank accession number U17066), as also previously disclosed by Dos Santos et al., in 1995, may be represented by the RNA sequence **SEQ ID NO. 5**.

25 In an exemplary embodiment, the RNA sequence of the Asibi strain (Genbank accession number KF769016) may be represented by the RNA sequence **SEQ ID NO. 6**.

In some embodiments, the live-attenuated yellow fever virus strain is less neurovirulent than the parent yellow fever virus 17D-substrain in a mouse lethal dose 50 (MLD₅₀) test.

30 In some embodiments, a suitable mouse lethal dose 50 (MLD₅₀) test is performed according to the protocol disclosed in page 68 of the WHO Technical report series, No. 872, 1998 (incorporated by reference).

Within the scope of the present invention, the MLD₅₀ is the quantity of virus suspension estimated to produce fatal, specific encephalitis in 50% of intracerebrally inoculated mice.

In some embodiments, appropriate serial dilutions of the reconstituted vaccine
5 are performed in phosphate-buffer, 0.75% serum albumin.

In an exemplary embodiment, 4-6 weeks old mice are injected intracerebrally under anaesthesia with an extemporaneous vaccine dilution. Groups of at least 6 mice are used for each dilution, and the series of dilutions should result in mortality rates after inoculation spanning the range 0-100%. Occurrences of death are recorded over a time
10 period of 21 days. Mice dying from unrelated causes are removed from both the numerator and denominator of mortality calculations. Mice paralysed on the twenty-first day are counted as alive.

In certain embodiments, the neurovirulence in a mouse lethal dose 50 (MLD₅₀) test may be measured by the parameter log₁₀MLD₅₀/mL.

15 In some embodiments, the live-attenuated YFV strain according to the present invention achieves a log₁₀MLD₅₀/mL of lower or equal to 4, lower or equal to 3.5, lower or equal to 3, or lower or equal to 2.5, in a mouse lethal dose 50 (MLD₅₀) test.

In an embodiment, a live-attenuated yellow fever virus strain according to the present invention is adapted to grow on VERO cells, is less neurovirulent than its parent
20 yellow fever virus 17D substrain and is at least as attenuated in viscerotropism as its parent yellow fever virus 17D substrain.

In an embodiment, a live-attenuated yellow fever virus strain according to the present invention is adapted to grow on VERO cells, is less neurovirulent than its parent yellow fever virus 17D substrain, is at least as attenuated in viscerotropism as its parent
25 yellow fever virus 17D substrain and is at least as immunogenic as its parent yellow fever virus 17D substrain.

In various embodiments, the present invention provides a live-attenuated YFV strain comprising a RNA sequence **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**, wherein one or more nucleotide is mutated.

30 Within the scope of the present invention, the expression “one or more nucleotide” is intended to encompass 2, 3, 4, 5 or more nucleotides.

In other words, the expression “one or more nucleotide” is intended to encompass 1 nucleotide, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15, or more nucleotides.

In some embodiments, a mutation is a nucleotide substitution.

5 In some other embodiments, a mutation does not encompass a nucleotide insertion and a nucleotide deletion.

In some embodiments, the nucleotide substitution is silent. Alternatively, the nucleotide substitution may promote an amino acid substitution.

In one embodiment, two nucleotides are mutated in the RNA sequence **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5.**

In another embodiment, three nucleotides are mutated in the RNA sequence **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5.**

In a further embodiment, four nucleotides are mutated in the RNA sequence **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5.**

15 In a further embodiment, five nucleotides are mutated in the RNA sequence **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5.**

Another aspect of the invention also relates to a live-attenuated yellow fever virus strain, which comprises a RNA sequence selected from **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5**, wherein at least the nucleotide at position 2411, position 3701 or position 6496 is mutated.

In some embodiments, the live-attenuated YFV strain comprises a RNA sequence selected from **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5**, wherein at least the nucleotide at position 2411 and the nucleotide at position 3701 are mutated.

25 In some embodiments, the live-attenuated YFV strain comprises a RNA sequence selected from **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5**, wherein at least the nucleotide at position 2411 and the nucleotide at position 6496 are mutated.

In some embodiments, the live-attenuated YFV strain comprises a RNA sequence selected from **SEQ ID NO. 1, SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5**, wherein at least the nucleotide at position 3701 and the nucleotide at position 6496 are mutated.

In some embodiments, the live-attenuated YFV strain comprises a RNA sequence selected from **SEQ ID NO. 1, SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4** and **SEQ ID NO. 5**, wherein at least the nucleotide at position 2411, the nucleotide at position 3701 and the nucleotide at position 6496 are mutated.

5 In certain embodiments, the nucleotide G (guanosine) at position 2411 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced by nucleotide U (uridine).

 In certain embodiments, the nucleotide A (adenosine) at position 3701 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced
10 by nucleotide G (guanosine).

 In certain embodiments, the nucleotide A (adenosine) at position 6496 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced by nucleotide G (guanosine).

 In some embodiments, the live-attenuated YFV strain is characterized as
15 follows:

 - (i) the nucleotide G (guanosine) at position 2411 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced by nucleotide U (uridine), and

 - (ii) the nucleotide A (adenosine) at position 3701 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced by nucleotide G (guanosine).
20

 In some other embodiments, the live-attenuated YFV strain is characterized as follows:

 - (i) the nucleotide G (guanosine) at position 2411 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced by nucleotide U (uridine), and
25

 (ii) the nucleotide A (adenosine) at position 6496 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced by nucleotide G (guanosine).
30

 In some other embodiments, the live-attenuated YFV strain is characterized as follows:

- (i) the nucleotide A (adenosine) at position 3701 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**, is replaced by nucleotide G (guanosine), and

5 - (ii) the nucleotide A (adenosine) at position 6496 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**, is replaced by nucleotide G (guanosine).

In certain embodiments, the live-attenuated YFV strain is characterized as follows:

10 - (i) the nucleotide G (guanosine) at position 2411 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**, is replaced by nucleotide U (uridine),

- (ii) the nucleotide A (adenosine) at position 3701 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**, is replaced by nucleotide G (guanosine); and

15 - (iii) the nucleotide A (adenosine) at position 6496 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**, is replaced by nucleotide G (guanosine).

In some embodiments, the live-attenuated YFV strain further comprises a mutation located at position 1408 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**.

In some embodiments, the live-attenuated YFV strain comprises a RNA sequence selected in a group comprising **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**, wherein at least the nucleotide at position 1408 and the nucleotide at position 2411 are mutated.

25 In some embodiments, the live-attenuated YFV strain comprises a RNA sequence selected in a group comprising **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5**, wherein at least the nucleotide at position 1408 and the nucleotide at position 3701 are mutated.

30 In some embodiments, the live-attenuated YFV strain comprises a RNA sequence selected in a group comprising **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5**, wherein at least the nucleotide at position 1408 and the nucleotide at position 6496 are mutated.

In some embodiments, the live-attenuated YFV strain comprises a RNA sequence selected in a group comprising **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4** and **SEQ ID NO. 5**, wherein at least the nucleotide at position 1408, the nucleotide at position 2411 and the nucleotide at position 3701 are mutated.

5 In some embodiments, the live-attenuated YFV strain comprises a RNA sequence selected in a group comprising **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4** and **SEQ ID NO. 5**, wherein at least the nucleotide at position 1408, the nucleotide at position 2411 and the nucleotide at position 6496 are mutated.

10 In some embodiments, the live-attenuated YFV strain comprises a RNA sequence selected in a group comprising **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4** and **SEQ ID NO. 5**, wherein at least the nucleotide at position 1408, the nucleotide at position 3701 and the nucleotide at position 6496 are mutated.

15 In some embodiments, the live-attenuated YFV strain comprises a RNA sequence selected in a group comprising **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4** and **SEQ ID NO. 5**, wherein at least the nucleotide at position 1408, the nucleotide at position 2411, the nucleotide at position 3701 and the nucleotide at position 6496 are mutated.

20 In some embodiments, the nucleotide A (adenine) at position 1408 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced by nucleotide U (uridine).

In certain embodiments, the live-attenuated YFV strain is characterized as follows:

25 - (i) the nucleotide G (guanosine) at position 2411 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced by nucleotide U (uridine),

- (ii) the nucleotide A (adenosine) at position 3701 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced by nucleotide G (guanosine);

30 - (iii) the nucleotide A (adenosine) at position 6496 of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced by nucleotide G (guanosine); and

- (iv) the nucleotide A (adenine) at position 1408 of **SEQ ID NO. 1**, **SEQ ID NO. 2**, **SEQ ID NO. 3**, **SEQ ID NO. 4**, or **SEQ ID NO. 5**, is replaced by nucleotide U (uridine).

In some embodiments, a live-attenuated YFV strain according to the present invention comprises a RNA sequence selected from **SEQ ID NO. 1**, **SEQ ID NO. 2**, **SEQ ID NO. 3**, **SEQ ID NO. 4** and **SEQ ID NO. 5**, wherein at least one or more nucleotide at position 2411, 3701, 6496 and optionally 1408 is/are mutated, with the proviso that no nucleotide is mutated in a way that results into a reversion to the Asibi genotype (which may be represented by the RNA sequence **SEQ ID NO. 6**). In other words, if a nucleotide in **SEQ ID NO. 1**, **SEQ ID NO. 2**, **SEQ ID NO. 3**, **SEQ ID NO. 4**, or **SEQ ID NO. 5** is different from the nucleotide at the same position in the Asibi genome (in **SEQ ID NO. 6**), this nucleotide in the RNA sequence of the live-attenuated YFV strain according to the present invention is not mutated in a way to become the nucleotide at the same position in the Asibi genome (in **SEQ ID NO. 6**). The nucleotides from **SEQ ID NO. 1**, **SEQ ID NO. 2**, **SEQ ID NO. 3**, **SEQ ID NO. 4**, or **SEQ ID NO. 5** which are different from the nucleotides at the same positions in the Asibi genome may easily be identified by a sequence alignment (Needleman and Wunsch, (1970)) between **SEQ ID NO. 1**, **SEQ ID NO. 2**, **SEQ ID NO. 3**, **SEQ ID NO. 4**, or **SEQ ID NO. 5** and Asibi sequence (**SEQ ID NO. 6**).

In some embodiments, a live-attenuated YFV strain according to the present invention comprises a RNA sequence comprising **SEQ ID NO. 2**, wherein at least one or more nucleotide at position 2411, 3701, 6496 and optionally 1408 is/are mutated, with the proviso that the nucleotides at the following positions in **SEQ ID NO. 2** are not mutated in a way that results into a reversion to the Asibi genotype (**SEQ ID NO. 6**): 304, 370, 854, 883, 1127, 1140, 1431, 1482, 1491, 1572, 1750, 1819, 1870, 1887, 1946, 1965, 2112, 2193, 2219, 2356, 2687, 3371, 3613, 3817, 3860, 3925, 4007, 4013, 4022, 4054, 4056, 4289, 4387, 4505, 4507, 4612, 4864, 4873, 5153, 5194, 5362, 5431, 5473, 5926, 6023, 6448, 6876, 7171, 7496, 7571, 7580, 7642, 7701, 7945, 8008, 8629, 10142, 10285, 10312, 10338, 10367, 10418, 10550 and 10800.

Table 1: Nucleotide differences between **SEQ ID NO. 2** and Asibi genomic RNA sequence (**SEQ ID NO. 6**).

Nucleotide position in SEQ	Nucleotide in Asibi	Nucleotide in SEQ ID	Protein-Amino acid	Amino acid in Asibi	Amino acid in SEQ ID NO.
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ID NO. 2	KF769016	NO. 2	position	KF769016	2
304	G	A	C-62	T	T
370	U	C	C-84	V	V
854	C	U	M-36	L	F
883	A	G	M-45	T	T
1127	G	A	E-52	G	R
1140	C	U	E-56	A	V
1431	A	C	E-153	N	T
1482	C	U	E-170	A	V
1491	C	U	E-173	T	I
1572	A	C	E-200	L	T
1750	C	U	E-258	T	T
1819	C	U	E-281	S	S
1870	G	A	E-299	M	I
1887	C	U	E-305	S	F
1946	C	U	E-325	P	S
1965	A	G	E-331	K	R
2112	C	G	E-380	T	R
2193	C	U	E-407	A	V
2219	G	A	E-416	A	T
2356	C	U	E-460	L	L
2687	C	U	NS1-79	L	F
3371	A	G	NS1-307	I	V
3613	G	A	NS2a-35	V	V
3817	A	G	NS2a-103	V	V
3860	A	G	NS2a-118	R	V
3925	A	U	NS2a-139	V	V
4007	A	G	NS2a-167	T	A
4013	C	U	NS2a-169	P	F
4022	A	G	NS2a-172	T	A
4054	C	U	NS2a-182	N	N
4056	C	U	NS2a-183	S	F
4289	A	C	NS2b-37	I	L
4387	A	G	NS2b-69	G	G
4505	A	C	NS2b-108	I	L
4507	U	C			
4612	U	C	NS3-14	I	I
4864	A	G	NS3-98	Q	Q
4873	U	G	NS3-101	A	A
5153	A	G	NS3-195	I	V
5194	U	C	NS3-208	F	F
5362	C	U	NS3-264	A	A
5431	C	U	NS3-287	I	I
5473	C	U	NS3-301	A	A
5926	C	U	NS3-452	R	R
6023	G	A	NS3-485	D	N

6448	G	U	NS4a-3	A	A
6876	U	C	P2k-20	V	A
7171	A	G	NS4b-95	I	M
7496	U	C	NS4b-204	L	L
7571	C	A	NS4b-229	R	R
7580	U	C	NS4b-232	Y	H
7642	U	C	NS5-2	S	S
7701	A	G	NS5-22	Q	R
7945	C	U	NS5-103	F	F
8008	U	C	NS5-124	I	I
8629	C	U	NS5-331	Y	Y
10142	G	A	NS5-836	E	K
10285	U	C	NS5-883	Y	Y
10312	A	G	NS5-892	R	R
10338	C	U	NS5-901	P	L
10367	U	C	3'UTR	-	-
10418	U	C	3'UTR	-	-
10550	U	C	3'UTR	-	-
10800	G	A	3'UTR	-	-

In some embodiments, a live-attenuated YFV strain according to the present invention comprises a RNA sequence **SEQ ID NO. 7**. Advantageously, the live-attenuated YFV strain according to the present invention comprises a RNA sequence which differs by a limited number of mutations, e.g. no more than 5, no more than 4, no more than 3, or no more than 2, from **SEQ ID NO. 7**. Advantageously, the live-attenuated YFV strain according to the present invention comprises a RNA sequence which differs by a limited number of mutations, e.g. no more than 5, no more than 4, no more than 3, or no more than 2, from **SEQ ID NO. 7**, with the proviso that no nucleotide is mutated in a way that results into a reversion to the Asibi genotype. In an exemplary embodiment, the genomic RNA sequence of a live-attenuated YFV strain according to the present invention may consist of the nucleotide sequence **SEQ ID NO. 7**.

In some embodiments, a live-attenuated YFV strain according to the present invention comprises a RNA sequence **SEQ ID NO. 8**. Advantageously, the live-attenuated YFV strain according to the present invention comprises a RNA sequence which differs by a limited number of mutations, e.g. no more than 5, no more than 4, no more than 3, or no more than 2, from **SEQ ID NO. 8**. Advantageously, the live-attenuated YFV strain according to the present invention comprises a RNA sequence which differs by a limited number of mutations, e.g. no more than 5, no more than 4, no more than 3, or no more than

2, from **SEQ ID NO. 8**, with the proviso that no nucleotide is mutated in a way that results into a reversion to the Asibi genotype. In an exemplary embodiment, the genomic RNA sequence of a live-attenuated YFV strain according to the present invention may consist of the nucleotide sequence **SEQ ID NO. 8**.

- 5 As mentioned above, the YFV nucleic acid encodes 11 proteins, as follows:
- the capsid protein (C protein), which precursor is 121 aa in length, and the mature protein is 101 aa in length,
 - a pre-membrane protein (prM protein) of 164 aa in length, which is the precursor of the membrane protein (M protein), of 75 aa in length,
 - 10 - an envelope protein (E protein), which is 493 aa in length,
 - non-structural protein 1 (NS1), which is 352 aa in length,
 - non-structural protein 2a (NS2a), which is 224 aa in length,
 - non-structural protein 2b (NS2b), which is 130 aa in length,
 - non-structural protein 3 (NS3), which is 623 aa in length,
 - 15 - non-structural protein 4a (NS4a), which is 126 aa in length,
 - non-structural peptide P2k, which is 23 aa in length,
 - non-structural protein 4b (NS4b) and which is 250 aa in length,
 - non-structural protein 5 (NS5), which is 905 aa in length.

 In some embodiments, the live-attenuated yellow fever virus strain comprises a
20 nucleic acid comprising a mutation in the codon for the amino acid at position 480 of the envelope protein (E), a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a), a mutation in the codon for the amino acid at position 19 of the non-structural protein 4A (NS4a) and/or a mutation in the codon for the amino acid at position 145 of the envelope protein (E).

25 In some embodiments, the live-attenuated yellow fever virus strain comprises a nucleic acid comprising a mutation in the codon for the amino acid at position 480 of the envelope protein (E) and a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a).

 In some embodiments, the live-attenuated yellow fever virus strain comprises a
30 nucleic acid comprising a mutation in the codon for the amino acid at position 480 of the envelope protein (E), a mutation in the codon for the amino acid at position 65 of the non-

structural protein 2A (NS2a) and a mutation in the codon for the amino acid at position 19 of the non-structural protein 4A (NS4a).

In some embodiments, the live-attenuated yellow fever virus strain comprises a nucleic acid comprising a mutation in the codon for the amino acid at position 480 of the envelope protein (E), a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a), a mutation in the codon for the amino acid at position 19 of the non-structural protein 4A (NS4a) and a mutation in the codon for the amino acid at position 145 of the envelope protein (E).

In some embodiments, the live-attenuated yellow fever virus strain comprises a nucleic acid comprising:

i) a mutation in the codon for the amino acid at position 480 of the envelope protein (E) which results in an amino acid change from valine to leucine, or

ii) a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a) which results in an amino acid change from methionine to valine.

In some embodiments, the live-attenuated yellow fever virus strain according to the present invention comprises a nucleic acid comprising:

i) a mutation in the codon for the amino acid at position 480 of the envelope protein (E) which results in an amino acid change from valine to leucine, and

ii) a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a) which results in an amino acid change from methionine to valine.

In some embodiments, the nucleic acid further comprises a mutation in the codon for the amino acid at position 19 of the non-structural protein 4A (NS4a) which results in a codon change from AAA to AAG.

In some embodiments, the mutation in the codon for the amino acid at position 480 of the envelope protein (E) results in a codon change from GUA to UUA, UUG, CUU, CUC, CUA or CUG. In an embodiment, the codon change is from GUA to UUA.

In some embodiments, the mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a) results in a codon change from AUG to GUG, GUU, GUC or GUA. In an embodiment, the codon change is from AUG to GUG.

In some embodiments, the nucleic acid further comprises a mutation in the codon for the amino acid at position 145 of the envelope protein (E) which results in a codon change from GUA to GUU.

In some embodiments, the live-attenuated yellow fever virus strain according to the present invention comprises a nucleic acid comprising:

i) a mutation in the codon for the amino acid at position 480 of the envelope protein (E) which results in an amino acid change from valine to leucine;

ii) a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a) which results in an amino acid change from methionine to valine; and

iii) a mutation in the codon for the amino acid at position 19 of the non-structural protein 4A (NS4a) which results in a codon change from AAA to AAG.

In some embodiments, the live-attenuated yellow fever virus strain according to the present invention comprises a nucleic acid comprising:

i) a mutation in the codon for the amino acid at position 480 of the envelope protein (E) which results in an amino acid change from valine to leucine;

ii) a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a) which results in an amino acid change from methionine to valine;

iii) a mutation in the codon for the amino acid at position 19 of the non-structural protein 4A (NS4a) which results in a codon change from AAA to AAG; and

iv) a mutation in the codon for the amino acid at position 145 of the envelope protein (E) which results in a codon change from GUA to GUU.

In some embodiments, the live-attenuated yellow fever virus strain comprises an envelope protein comprising a mutation at position 480. In particular, the live-attenuated yellow fever virus strain according to the present invention comprises an envelope protein comprising a mutation at position 480 which results in an amino acid change from valine to leucine.

In some embodiments, the live-attenuated yellow fever virus strain comprises an envelope protein comprising a leucine residue at the position within the protein that corresponds to position 480 of **SEQ ID NO. 15**. In particular, said envelope protein

comprises a sequence at least 90%, 95%, 98% or 100% identical to the sequence of **SEQ ID NO. 15**.

In particular in the nucleic acid of the live-attenuated yellow fever virus strain of the invention no nucleotide is mutated in a way that results into a reversion to the Asibi genotype (**SEQ ID NO. 6**). For instance the nucleic acid of the live-attenuated yellow fever virus strain comprises no mutation for the nucleotides at the following positions in **SEQ ID NO. 2** in a way that results into a reversion to the Asibi genotype (**SEQ ID NO. 6**): 304, 370, 854, 883, 1127, 1140, 1431, 1482, 1491, 1572, 1750, 1819, 1870, 1887, 1946, 1965, 2112, 2193, 2219, 2356, 2687, 3371, 3613, 3817, 3860, 3925, 4007, 4013, 4022, 4054, 4056, 4289, 4387, 4505, 4507, 4612, 4864, 4873, 5153, 5194, 5362, 5431, 5473, 5926, 6023, 6448, 6876, 7171, 7496, 7571, 7580, 7642, 7701, 7945, 8008, 8629, 10142, 10285, 10312, 10338, 10367, 10418, 10550 and 10800.

In some embodiments, the live-attenuated yellow fever virus strain comprises a nucleic acid molecule encoding:

- (i) an envelope protein comprising a mutation at position 480, and
- (ii) a NS2a protein comprising a mutation at position 65.

In particular, the nucleic acid molecule of the live-attenuated yellow fever virus strain further comprises a mutation in the codon for the amino acid at position 19 of the non-structural protein 4A (NS4a) and/or a mutation in the codon for the amino acid at position 145 of the envelope protein (E).

In some embodiments, the live-attenuated yellow fever virus strain according to the present invention comprises a nucleic acid molecule encoding:

- (i) an envelope protein comprising a mutation at position 480 which results in an amino acid change from valine to leucine, and
- (ii) a NS2a protein comprising a mutation at position 65 which results in an amino acid change from methionine to valine.

In particular, the nucleic acid further comprises a mutation in the codon for the amino acid at position 19 of the non-structural protein 4A (NS4a) which results in a codon change from AAA to AAG and/or a mutation in the codon for the amino acid at position 145 of the envelope protein (E) which results in a codon change from GUA to GUU.

In particular in the nucleic acid of the live-attenuated yellow fever virus strain of the invention no nucleotide is mutated in a way that results into a reversion to the Asibi

genotype (**SEQ ID NO. 6**). For instance the nucleic acid of the live-attenuated yellow fever virus strain comprises no mutation for the nucleotides at the following positions in **SEQ ID NO. 2** in a way that results into a reversion to the Asibi genotype (**SEQ ID NO. 6**): 304, 370, 854, 883, 1127, 1140, 1431, 1482, 1491, 1572, 1750, 1819, 1870, 1887, 1946, 5 1965, 2112, 2193, 2219, 2356, 2687, 3371, 3613, 3817, 3860, 3925, 4007, 4013, 4022, 4054, 4056, 4289, 4387, 4505, 4507, 4612, 4864, 4873, 5153, 5194, 5362, 5431, 5473, 5926, 6023, 6448, 6876, 7171, 7496, 7571, 7580, 7642, 7701, 7945, 8008, 8629, 10142, 10285, 10312, 10338, 10367, 10418, 10550 and 10800.

In some embodiments, the live-attenuated yellow fever virus strain according to the present invention comprises a nucleic acid molecule encoding:

(i) an envelope protein which comprises a leucine residue at the position within the protein that corresponds to position 480 of **SEQ ID NO. 15**, and

(ii) an NS2a protein which comprises a valine residue at the position within the protein that corresponds to position 65 of **SEQ ID NO. 16**.

15 In particular, said envelope protein comprises a sequence at least 90%, 95%, 98% or 100% identical to the sequence of **SEQ ID NO. 15** and said NS2a protein comprises a sequence at least 90%, 95%, 98% or 100% identical to the sequence of **SEQ ID NO. 16**.

The nucleic acid of the live-attenuated yellow fever virus strain of the invention may further comprise a G nucleotide at position within the nucleic acid coding for the non-structural protein 4A (NS4a) that corresponds to position 57 of **SEQ ID NO. 17** and/or a U nucleotide at position within the nucleic acid coding for the envelope protein (E) that corresponds to position 435 of **SEQ ID NO. 18**. In particular, the live-attenuated yellow fever virus strain of the invention may comprise a nucleic acid molecule 25 comprising a nucleic acid coding for the non-structural protein 4A (NS4a) which comprises a sequence at least 90%, 95%, 98% or 100% identical to the sequence of **SEQ ID NO. 17** and/or a nucleic acid coding for the envelope protein (E) which comprises a sequence at least 90%, 95%, 98% or 100% identical to the sequence of **SEQ ID NO. 18**.

In particular in the nucleic acid of the live-attenuated yellow fever virus strain 30 of the invention no nucleotide is mutated in a way that results into a reversion to the Asibi genotype (**SEQ ID NO. 6**). For instance the nucleic acid of the live-attenuated yellow fever virus strain comprises no mutation for the nucleotides at the following positions in

SEQ ID NO. 2 in a way that results into a reversion to the Asibi genotype (**SEQ ID NO. 6**): 304, 370, 854, 883, 1127, 1140, 1431, 1482, 1491, 1572, 1750, 1819, 1870, 1887, 1946, 1965, 2112, 2193, 2219, 2356, 2687, 3371, 3613, 3817, 3860, 3925, 4007, 4013, 4022, 4054, 4056, 4289, 4387, 4505, 4507, 4612, 4864, 4873, 5153, 5194, 5362, 5431, 5473, 5926, 6023, 6448, 6876, 7171, 7496, 7571, 7580, 7642, 7701, 7945, 8008, 8629, 10142, 10285, 10312, 10338, 10367, 10418, 10550 and 10800.

In some embodiments, the nucleic acid comprises the RNA sequence of a 17D substrain, comprising the mutations according to the present invention as described above.

In some embodiments, the nucleic acid comprises the RNA sequence of a 17D-204 substrain, comprising the mutations according to the present invention as described above.

In some embodiments, the nucleic acid comprises the RNA sequence **SEQ ID NO. 1**, **SEQ ID NO. 2**, **SEQ ID NO. 3**, **SEQ ID NO. 4**, or **SEQ ID NO.5**, comprising the mutations according to the present invention as described above.

In an exemplary embodiment, the nucleic acid comprises the RNA sequence **SEQ ID NO. 2**, comprising the mutations according to the present invention as described above.

As it will emerge from the example section hereunder, the mutations as defined above allow providing YFV strains adapted to grow on VERO cells and that have an attenuated virulence, such as an attenuated neurovirulence, as compared to the parent YFV strain, and which virulence is compatible with the use of these strains as vaccines or in vaccine compositions. In an embodiment, the mutations as defined above allow providing YFV strains adapted to grow on VERO cells and that are less neurovirulent, as compared to the parent YFV strain, and are at least as attenuated in viscerotropism, as compared to the parent YFV strain. In an embodiment, the mutations as defined above allow providing YFV strains adapted to grow on VERO cells and that are less neurovirulent, as compared to the parent YFV strain, are at least as attenuated in viscerotropism, as compared to the parent YFV strain, and are at least as immunogenic, as compared to the parent YFV strain.

30 • **Immunogenic, vaccine and pharmaceutical compositions**

In another aspect, the invention also relates to an immunogenic composition comprising a live-attenuated YFV strain according to the present invention.

Within the scope of the present invention, the term “immunogenic” refers to the capability of the composition to promote an antibody-mediated and/or a cell-mediated immunity and/or an immunological memory.

In some embodiment, the immunogenic composition may be employed in order
5 to generate neutralizing antibodies against a yellow fever virus.

In another aspect, the invention further relates to an immunogenic composition comprising a live-attenuated yellow fever virus strain according to the present invention and a pharmaceutically acceptable vehicle.

In some embodiments, the invention also relates to a vaccine composition
10 comprising a live-attenuated virus strain according to the present invention, and/or a vaccine composition comprising the immunogenic composition according to the present invention.

In some embodiments, a vaccine composition may not comprise any adjuvant.

Within the scope of the present invention, an “adjuvant” refers to any
15 substance intended to enhance relevant immune response and subsequent clinical efficacy of a vaccine.

Alternatively, a vaccine composition may further comprise one or more adjuvants.

In some embodiments, the adjuvant may include a mineral salt, an emulsion, a
20 microbial natural or synthetic derivative, a combination adjuvant, a cytokine-derived or accessory molecules-derived adjuvant, a particulate formulation, and the like. The preparation and use of adjuvants are well known in the art.

In some embodiments, the present invention provides an immunogenic composition comprising a live-attenuated YFV strain as described herein and a
25 pharmaceutically acceptable carrier.

In some embodiments, the present invention provides a pharmaceutical composition comprising a live attenuated YFV strain as described herein and a pharmaceutically acceptable carrier.

In the context of the invention, the expression “pharmaceutically acceptable
30 vehicle” refers to a vehicle that is physiologically acceptable for administration to a human being, while retaining the physiological activity of the immunogenic composition according to the invention, *i.e.* its ability to induce an immune response. One exemplary

pharmaceutically acceptable vehicle is a physiological saline buffer. Other physiologically acceptable vehicles are known to those skilled in the art and are described, for instance, in Remington's Pharmaceutical Sciences (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, Pa. An immunogenic composition as described herein may
5 optionally contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like. In addition, the vaccine composition may optionally comprise pharmaceutically acceptable additives including, for example, diluents, binders, stabilizers, and preservatives.

10 In various embodiments, the pH of the immunogenic composition is between 5.5 and 8, such as between 6.5 and 7.5 (e.g. about 7). Stable pH may be maintained by the use of a buffer. Thus, in some embodiments, the immunogenic composition includes a buffer. Immunogenic compositions may be isotonic with respect to humans. The immunogenic composition may also comprise one or several additional salts, such as NaCl.
15 The preparation and use of pharmaceutically acceptable carriers are well known in the art.

In practice, the immunogenic composition and/or the vaccine composition and/or the pharmaceutical composition comprising a live-attenuated YFV strain according to the present invention may be prepared using the conventional and good practices in the field.

20 In some embodiments, the immunogenic composition, the vaccine composition and/or the pharmaceutical composition according to the present invention may comprise one or more suitable diluent and/or excipient.

In various embodiments, the pharmaceutical compositions, the immunogenic compositions and the vaccine compositions may be sterilized by conventional sterilization
25 techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged and stored in liquid form or lyophilized, the lyophilized preparation being reconstituted with a sterile aqueous carrier prior to administration. In an exemplary embodiment, the pharmaceutical compositions, the immunogenic compositions and the vaccine compositions are packaged and stored as micropellets *via* a prilling process as described in
30 WO 2009/109550. In an embodiment the pharmaceutical compositions, the immunogenic compositions, and/or the vaccine compositions are lyophilized or spray-freeze dried.

• **Method for obtaining a live-attenuated YFV strain**

A further aspect of the invention relates to a method for obtaining a live-attenuated yellow fever virus strain adapted to grow on Vero cells, comprising the steps of:

- a) purifying the viral genomic RNA of a parent live-attenuated yellow fever virus strain that is not adapted to grow on Vero cells, and that is optionally adapted to grow on eggs;
- b) transfecting Vero cells with the viral genomic RNA purified in step a), whereby transfected Vero cells are obtained;
- c) growing the transfected Vero cells obtained in step b) in a culture medium, whereby a first yellow fever virus population is obtained and further recovered;
- d) amplifying the recovered first yellow fever virus population obtained at the end of step c) 2 times or more on fresh Vero cells, whereby a second yellow fever virus population is obtained;
- e) cloning the second yellow fever virus population obtained in step d) by two or more successive plaque purifications on Vero cells whereby a plurality of yellow fever virus clones is obtained;
- f) amplifying separately each of the recovered yellow fever virus clones obtained at the end of step e) 2 times or more on fresh Vero cells, whereby a plurality of yellow fever virus strains is obtained; and
- g) selecting from the said plurality of yellow fever virus strains recovered in step f) one or more live-attenuated yellow fever virus strain that is less neurovirulent than the parent live-attenuated yellow fever virus strain, in a mouse lethal dose 50 (MLD₅₀) test.

In some embodiments, step d) of the method of the present invention above is conducted 2, 3, 4, 5, 6, or more times. In some embodiments, the cloning at step e) of the method of the present invention above is conducted by 2, 3, 4, 5, 6, or more successive plaque purifications on Vero cells. In some embodiments, step f) of the method of the present invention above is conducted 2, 3, 4, 5, 6, or more times.

A further aspect of the invention relates to a method for obtaining a live-attenuated yellow fever virus strain adapted to grow on Vero cells, comprising the steps of:

- a) purifying the viral genomic RNA of a parent live-attenuated yellow fever virus strain that is not adapted to grow on Vero cells, and that is optionally adapted to grow on eggs;

- b) transfecting Vero cells with the viral genomic RNA purified in step a), whereby transfected Vero cells are obtained;

- c) growing the transfected Vero cells obtained in step b) in a culture medium, whereby a first yellow fever virus population is obtained and further recovered;

5 - d) amplifying the recovered first yellow fever virus population obtained at the end of step c) 2 times or more on fresh Vero cells, whereby a second yellow fever virus population is obtained;

10 - e) cloning the second yellow fever virus population obtained in step d) by two or more successive plaque purifications on Vero cells whereby a plurality of yellow fever virus clones is obtained;

- f) amplifying separately each of the recovered yellow fever virus clones obtained at the end of step e) 2 times or more on fresh Vero cells, whereby a plurality of yellow fever virus strains is obtained; and

15 - g) selecting from the said plurality of yellow fever virus strains recovered in step f) one or more live-attenuated yellow fever virus strain comprising a nucleic acid comprising:

i) a mutation in the codon for the amino acid at position 480 of the envelope protein (E) which results in an amino acid change from valine to leucine, or

20 ii) a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a) which results in an amino acid change from methionine to valine. Such selection is easily conducted with sequencing methods well known in the art.

In some embodiments, step g) may comprise selecting one or more live-attenuated yellow fever virus strain comprising a nucleic acid comprising:

25 i) a mutation in the codon for the amino acid at position 480 of the envelope protein (E) which results in an amino acid change from valine to leucine, and

ii) a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a) which results in an amino acid change from methionine to valine.

30 In some embodiments, step g) may comprise selecting one or more live-attenuated yellow fever virus strain as described above comprising a nucleic acid further comprising a mutation in the codon for the amino acid at position 19 of the non-structural protein 4A (NS4a) which results in a codon change from AAA to AAG.

In some embodiments, step g) may comprise selecting one or more live-attenuated yellow fever virus strain as described above comprising a nucleic acid further comprising a mutation in the codon for the amino acid at position 145 of the envelope protein (E) which results in a codon change from GUA to GUU.

5 In some embodiments, step g) may comprise selecting one or more live-attenuated yellow fever virus strain comprising a nucleic acid comprising:

i) a mutation in the codon for the amino acid at position 480 of the envelope protein (E) which results in an amino acid change from valine to leucine,

10 ii) a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a) which results in an amino acid change from methionine to valine,

iii) a mutation in the codon for the amino acid at position 19 of the non-structural protein 4A (NS4a) which results in a codon change from AAA to AAG, and/or

15 iv) a mutation in the codon for the amino acid at position 145 of the envelope protein (E) which results in a codon change from GUA to GUU.

In an embodiment, the parent live-attenuated yellow fever virus strain of step a) is a yellow fever 17D substrain, such as a yellow fever 17D-204 substrain.

20 In practice, Vero cells are available in cell collections, such as ATCC. The methods suitable to grow Vero cells in *in vitro* cell culture, including methods using serum-free medium, are well known to the man skilled in the art (Kolell K. *et al.* 2007). In an embodiment, the Vero cells are adapted to grow on serum-free medium before any viral culture.

In some embodiments, the culture medium used to grow the Vero cells is serum-free, and is optionally free of any human or animal-derived substance.

25 Within the scope of the present invention, the expression “human or animal-derived substance” refers to a substance, such as a protein, a lipid, a glycoprotein, a lipoprotein, glycolipid, a monosaccharide or a polysaccharide, originating from a human or non-human animal, e.g. a growth factor, a hormone, that is obtained from, e.g. extracted from, a human or non-human animal. Recombinant molecules are not considered as human or animal-derived substance. Such serum-free media and/or media free of any human or animal-derived substance are easily available on provider catalogues (for instance THERMOFISHER SCIENTIFIC® catalogue).

30

In some embodiments, the culture medium used to grow the Vero cells is also devoid of antibiotics.

In some embodiments, the culture medium used to grow the Vero cells may comprise one or more extract originating from a bacterium, yeast and/or plant.

5 In some embodiments, the genome of the parent live-attenuated yellow fever virus strain not adapted to grow on Vero cells may be in the form of a cDNA encoding the genomic RNA.

In certain embodiments, the cDNA is carried by a suitable vector, such as e.g. a plasmid.

10 In some other aspect, the invention relates to a vector comprising a nucleic acid comprising the RNA sequence **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**, in which the mutation(s) described herein is/are present.

In some other aspect, the invention relates to a vector comprising a nucleic acid comprising the cDNA sequence corresponding to sequence **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**, in which mutations corresponding to the mutation(s) described herein is/are present.

In a further aspect, the live-attenuated yellow fever virus strain according to the present invention is obtained by mutation of the genomic sequence of a yellow fever virus, in order to introduce in that said genomic sequence the mutations described in the present invention. In some embodiments, the genomic sequence of a yellow fever 17D-substrain may be mutated in order to introduce in that said genomic sequence the mutations described in the present invention. In some embodiments, the nucleic acid comprising the RNA sequence of **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**, or the corresponding cDNA sequence, may be mutated in order to introduce mutations described in the present invention. The mutations may be introduced in the genomic sequence by site-directed mutagenesis *via* methods well known by the skilled person, including use of any suitable gene-editing technology. The genomic sequence in which the mutations described in the present invention are introduced may be a cDNA encoding the genomic RNA of the yellow fever virus, such as, a cDNA encoding the genomic RNA of a yellow fever 17D-substrain, for instance a cDNA encoding **SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, or SEQ ID NO. 5**. In some embodiments, the cDNA is carried by a suitable plasmid. The mutations described in the

present invention that may be introduced in the genomic sequence of a yellow fever virus are selected from a mutation of the nucleotide at position 2411, position 3701 or position 6496 of the genomic sequence; or any combination thereof. In some embodiments, these mutations may comprise the nucleotide G (guanosine) at position 2411 of the genomic sequence being replaced by nucleotide U (uridine), the nucleotide A (adenosine) at position 3701 of the genomic sequence being replaced by nucleotide G (guanosine); or the nucleotide A (adenosine) at position 6496 of the genomic sequence being replaced by nucleotide G (guanosine), or any combination thereof. In some embodiments, a further mutation is introduced located at position 1408 of the genomic sequence. In some embodiments, this further mutation is the nucleotide A (adenine) at position 1408 of the genomic sequence being replaced by nucleotide U (uridine). In some embodiments, other mutations may be introduced in the genomic sequence of the yellow fever virus, with the proviso that no nucleotide is mutated in a way that results into a reversion to the Asibi genotype. In another aspect, the mutations described in the present invention that may be introduced in the genomic sequence of a yellow fever virus are selected from a mutation in the codon for the amino acid at position 480 of the envelope protein (E) which results in an amino acid change from valine to leucine, a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a) which results in an amino acid change from methionine to valine, or a mutation in the codon for the amino acid at position 19 of the non-structural protein 4A (NS4a) which results in a codon change from AAA to AAG, or any combination thereof. In some embodiments, a further mutation is introduced in the codon for the amino acid at position 145 of the envelope protein (E) which results in a codon change from GUA to GUU. In some embodiments, other mutations may be introduced in the genomic sequence of the yellow fever virus, with the proviso that no nucleotide is mutated in a way that results into a reversion to the Asibi genotype. In particular, the mutations described in the present invention that may be introduced in the genomic sequence of a yellow fever virus are a mutation in the codon for the amino acid at position 480 of the envelope protein (E) which results in an amino acid change from valine to leucine and a mutation in the codon for the amino acid at position 65 of the non-structural protein 2A (NS2a) which results in an amino acid change from methionine to valine.

In a further aspect, the invention also relates to a live-attenuated yellow fever virus strain obtainable by a method according to the present invention.

It is also disclosed herein a live-attenuated yellow fever virus strain obtained by a method according to the present invention.

5 • **Miscellaneous methods and uses**

The instant invention also relates to a method for immunizing an individual in need thereof against an infection by an YFV comprising the administration to the said individual of a vaccine composition according to the present invention.

10 Within the scope of the present invention, the expression “individual in need thereof” is intended to refer to an individual at risk of being infected by an YFV.

A further aspect of the invention also relates to the use of a live-attenuated YFV strain according to the present invention for the preparation of a vaccine. In some embodiments, the invention also relates to the use of a live-attenuated YFV strain according to the present invention as a pMSL, as a MSL or as a WSL. In particular, the invention also relates to the use of a live-attenuated YFV strain according to the present invention as a pMSL, as a MSL or as a WSL, in a vaccine preparation process.

In another aspect, the invention relates to a live-attenuated YFV strain according to the present invention for use in the preparation of a vaccine.

20 A still further aspect of the invention also relates to a vaccine composition according to the present invention for use in preventing an infection by an YFV.

In some embodiments, the present invention relates to a method for preventing an infection by a YFV in an individual comprising the administration to the said individual of an efficient amount of a live-attenuated YFV, an immunogenic composition, a pharmaceutical composition or a vaccine composition according to the present invention.

25 In some embodiments, the present invention relates to a method for generating neutralizing antibodies against a yellow fever virus in an individual comprising the administration to the said individual of an efficient amount of a live-attenuated YFV, an immunogenic composition, a pharmaceutical composition or a vaccine composition according to the present invention.

30 In some embodiments, the present invention relates to the use of a live-attenuated virus according to the present invention for preparing a medicament for preventing an infection by an YFV.

In some embodiments, the present invention relates to a live-attenuated virus according to the present invention for use in preventing an YFV infection.

In some embodiments, the present invention relates to the use of an immunogenic composition according to the present invention, for preparing a medicament
5 for preventing an infection by a YFV.

In some embodiments, the present invention relates to the use of an immunogenic composition according to the present invention, for preparing a vaccine composition for preventing an infection by a YFV.

In some embodiments, the present invention relates to an immunogenic
10 composition according to the present invention, for use in preventing an infection by an YFV.

The vaccine composition and the immunogenic composition according to the present invention may be administered to an individual in need thereof by any suitable route of administration.

15 The immunogenic composition or the vaccine, according to the invention can be administered *via* any suitable route, such as by mucosal administration (e.g. intranasal or sublingual), parenteral administration (e.g. intramuscular, subcutaneous, transcutaneous, or intradermal route), or oral administration. As appreciated by the man skilled in the art, a vaccine of the present invention is suitably formulated to be compatible with the intended
20 route of administration. In exemplary embodiments, the composition of the invention is administered intramuscularly or subcutaneously.

A vaccine according to the present invention may be administered in multiple doses. For example, a vaccine according to the present invention may be administered in one, two or three doses. In an embodiment the vaccine according to the present invention is
25 administered in a single dose.

The vaccine according to the present invention can be administered in amounts that can readily be determined by persons of ordinary skill in this art. In some embodiments, the vaccine dose is between 4 and 6 log₁₀CCID₅₀.

30 **EXAMPLES**

EXAMPLE 1: Preparation of live-attenuated YFV strains by adaptation on Vero cells (premaster seed lots (pMSLs))

1.1/ Choice of the method - Principles

The overall strategy for the pre Master Seed Lots (pMSLs) is displayed in **Figure 1**.

Both YF-VAX® and Stamaril® vaccines were developed from un-cloned
5 YF17D-204 strain preparations and contain heterologous populations of virus as visualized
by plaque size phenotype. In addition, both vaccines were produced on eggs.

In order to generate homogeneous, well-defined, virus strains adapted to grow
on Vero cells, and to ensure sterility and absence of adventitious agents in the final
pMSLs:

- 10 - (1) the viral genomic RNA of YF-VAX® and Stamaril® viruses were purified;
- (2) and then transfected into Vero cells to recover yellow fever viruses that were
then amplified twice on Vero cells in order to adapt the viruses for growing on this cell
substrate;
- (3) the viruses were then cloned by two plaque purification cycles. For that
15 purpose, the viral preparation was diluted for infection of Vero cells and grown under a
semi-solid overlay in order to get well separated virus plaques. For each transfection, 2
individual plaques, corresponding each to a single virus population were picked through
the overlay, diluted and used for a second cycle of plaque-purification leading to the
generation of viral clones;
- 20 - (4) these clones were then amplified to obtain a sufficient viral stock to constitute
a pMSL.

All media and solutions used for the pMSL production were animal- and human-
component free.

1.2/ Methods

25 1.2.1/ In vitro transcription from the YF-VAX® genomic cDNA

In vitro transcription of the genomic cDNA of YFV from the YF-VAX®
(plasmid pJSY2374.5, as disclosed in WO 2014/016360) was performed with mMessage
mMachine™ SP6 Kit (AMBION®, reference AM1340) according to the supplier's
recommendations. From the plasmid pJSY2374.5, 2 *in vitro* transcriptions were performed
30 in parallel.

Briefly, after thawing at room temperature, 10 µg of plasmid were linearized
by digestion for 2 hours at $37 \pm 2^\circ\text{C}$ with restriction enzyme NruI (30 U/10 µg). The

enzyme was then inactivated by incubating at 65°C for 20 minutes. The linearization of the plasmid was verified by electrophoresis on a 0.5% agarose gel. A 40 µL reaction mixture comprising the reaction buffer of the kit, the ribonucleotides (ATP, CTP, UTP and a mixture of GTP and 7-methyl-GTP), the enzyme and 1 µg of plasmid was prepared. The
5 resulting mixture was incubated for 2 hours at 37 ± 2°C.

1.2.2/ RNA purification

a) From the Stamaril® vaccine working seed lot

Two purifications of the viral RNA were performed in parallel.

Four vials of working seed lot of the Stamaril® vaccine (lot # FA238667,
10 infectious titer 6.38 log₁₀ PFU/vial) were each suspended in 200 µl of lysis buffer of the RNeasy® kit (QIAGEN®) and then pooled. The RNA was then purified by two series of extraction with a phenol/chloroform/isoamyl alcohol (125:24:1; pH 4.5).

2 mL Phase Lock Gel Heavy tubes (5PRIME®) were centrifuged for 30 seconds at 11,000 x g. 750 µL of RNA/lysis buffer mixture were introduced into each tube.
15 An equal volume (750 µl) of phenol/chloroform/IAA solution was then added to each tube. After vigorously mixing the organic and aqueous phases to form a homogeneous transient suspension, the tubes were centrifuged at 11,000 x g for 5 minutes to separate the phases. The upper phase (aqueous phase) was then recovered. The operation was renewed on new Phase Lock Gel 2mL tubes. Then the operation was again performed with a mixture of
20 chloroform and Isoamyl alcohol (24:1) to remove all traces of phenol. The RNA was then concentrated and cleaned of any trace of organic solvent by purification on silica column with RNeasy® kit (QIAGEN®) following the recommendations of the provider. The purified RNA was then eluted in nuclease-free water.

b) From the *in vitro* transcription of the YF-VAX® genomic cDNA

25 The plasmidic DNA contaminating the RNA obtained by *in vitro* transcription (see above) was eliminated by 4 U of DNase for 15 minutes at 37 ± 2°C. The SP6 polymerase was then inactivated by incubation for 10 minutes at 70°C.

The RNA obtained by *in vitro* transcription was mixed with 60 µl of RNase-free water and 350 µl of lysis buffer from the RNeasy® kit (QIAGEN®). The RNA was
30 then purified by two series of extraction with a phenol/chloroform/isoamyl alcohol (125:24:1; pH 4.5). For this, Phase Lock Gel Heavy 1.5 mL tubes were centrifuged for 30 seconds at 11,000 x g. 750 µL of RNA/lysis buffer mixture was introduced into each tube.

An equal volume (750 µl) of phenol/chloroform/isoamyl alcohol solution was then added to each tube. After vigorously mixing the organic and aqueous phases to form a homogeneous transient suspension, the tubes were centrifuged at 11,000 x g for 5 minutes in order to separate the phases. The upper phase (aqueous phase) was then recovered. The operation was renewed on new Phase Lock Gel 1.5 mL tubes. Then the operation was again performed with a mixture of chloroform and isoamyl alcohol (24: 1) to remove all traces of phenol. The RNA was then purified on silica column with RNeasy® kit (QIAGEN®) following the supplier's recommendations. The purified RNA was then eluted with nuclease-free water.

10 **1.2.3/ Transfection**

Two transfections were conducted in parallel for each RNA purification.

a) Preparation of the RNA/Lipofectamine™ mixture

10 or 15 µL of Lipofectamine™ 2000 CD (LIFE TECHNOLOGIES®)- were mixed with 1 mL of OptiPro SFM medium (LIFE TECHNOLOGIES®) and incubated for 5 minutes at room temperature. About 10 log₁₀Geq (genome equivalent titer determined by YF-NS5 qRT-PCR as described in Mantel *et al.* (2008)) of purified RNA were then added. These mixtures were incubated for 10 minutes at room temperature.

b) Vero cells preparation

Before transfection, serum-free Vero cells from Sanofi Pasteur's GMP bank previously seeded in 6-well plates (9.10⁵ cells in 3 mL of VP-SFM (THERMOFISHER SCIENTIFIC) per well) were rinsed with 2 mL per well of OptiPro SFM medium.

c) Transfection reaction

In the 6-well plate, after removal of the rinsing medium from the cells, the mixtures of transfection containing the RNA were deposited in two wells (1 mL/well) for each preparation. A well was brought into contact with an OptiPro SFM /Lipofectamine™ containing no RNA and the last well was kept as a cell control in OptiPro SFM medium alone. Two plates were prepared in parallel, one with the mixtures containing 10 µl of Lipofectamine™ and one with the mixtures containing 15 µl of Lipofectamine™. The mixture containing Lipofectamine™ and the RNA was left in contact with the Vero cells for 4 hours at 37 ± 2°C at 5 ± 2% CO₂ and then 2 mL of preheated VP-SFM medium were added to each well. The 6-well plates were incubated at 37 ± 2°C; 5 ± 2% CO₂ for 16 h. The medium was then renewed and the plates re-incubated at 37 ± 2°C, 5 ± 2% CO₂.

Transfection supernatants were collected when the cytopathic effect (cell lysis) was visible and when the genomic titer determined by YF-NS5 qRT-PCR from the culture supernatant (as described in Mantel *et al.* (2008)) was greater than $8.0 \log_{10}$ Geq/mL. Replacement of the culture medium by fresh medium was performed on D5 and D8 if culture times needed
5 to allow harvest were greater than these times. The harvested supernatants were divided into aliquots.

1.2.4/ Amplification of the virus

a) Amplification n°1 (viral passage n°2)

Two days before virus amplification n°1, 2.10^5 Vero cells were seeded in 25
10 cm^2 flasks containing 5 mL of VP-SFM medium. Then the viral suspensions resulting from the transfection were diluted in VP-SFM medium in order to obtain a multiplicity of genome (m.o.g) of 2 (*i.e.* 2 Geq per cell, estimated from the RNA concentration obtained by qRT-PCR). The culture medium of the Vero cells previously seeded was eliminated and the cells were brought into contact with 1 mL of the diluted viral suspension or 1 mL of
15 VP-SFM medium alone (cell control). The flasks were incubated for 2h at $37 \pm 2^\circ\text{C}$; $5 \pm 2\%$ CO_2 . The viral inoculum was then removed and replaced with 10 mL of VP-SFM medium and the cells were incubated at $37 \pm 2^\circ\text{C}$; $5 \pm 2\%$ CO_2 for 2 days. The culture medium was then renewed by new VP-SFM medium preheated to $37 \pm 2^\circ\text{C}$ and the flasks re-incubated at $37 \pm 2^\circ\text{C}$; $5 \pm 2\%$ CO_2 for 2 to 3 days. After a total of 4 to 5 days of
20 incubation, the culture supernatant containing the virus was recovered. The viral suspension was clarified by centrifugation for 10 minutes at 1200 rpm at 4°C and then distributed in aliquots. 140 μL of this viral suspension were used to extract total RNA with a QiaAmp viral mini kit (QIAGEN®; according to the supplier's protocol), and to quantify the viral RNA by YF-NS5 qRT-PCR (as described in Mantel *et al.* (2008)).

25 One or more aliquots, depending on the viral RNA titer, were stored to make the second amplification step if it was performed the same day, the others were frozen at $\leq -70^\circ\text{C}$ in the presence of 10% final sorbitol.

b) Amplification n°2 (viral passage n°3)

Two days before virus amplification n°2, 5.10^5 Vero cells were seeded in 75
30 cm^2 flasks containing 20 mL of VP-SFM medium. Then the viral suspension from the first amplification was diluted in VP-SFM medium so as to infect Vero cells at a rate of m.o.g of 2 (*i.e.* Geq per cell, estimated from the concentration of RNA obtained by qRT-PCR).

The other steps of the amplification n°2 were performed as detailed for the amplification n°1 (see section a) above).

c) Viral cloning - plate purification (viral passages 4 and 5)

Two 6-well plates were required per viral suspension obtained after
5 transfection and amplification.

An aliquot of viral suspension obtained after amplification n°2 was diluted so as to obtain a suspension at about $2.0 \log_{10}$ PFU/mL and a suspension at $1.7 \log_{10}$ PFU/mL. Vero cells previously seeded in 6-well plates (9.10^5 cells in 3 mL of VP-SFM per well) were observed for verifying the integrity of the cells and the absence of contamination,
10 then the culture medium was removed. For each dilution, 5 wells of a plate were infected by 500 μ L of the diluted virus in each well ($2.0 \log_{10}$ PFU/mL or $1.7 \log_{10}$ PFU/mL dilutions) and, a cell control well contained 500 μ L of VP-SFM only. The plates were incubated for 2 hours at $37 \pm 2^\circ\text{C}$; $5 \pm 2\%$ CO_2 . Then the inoculum was removed and replaced by 4 mL of an overlay mixture, *i.e.* a solution of VP-SFM 2X preheated to 42°C
15 and mixed extemporaneously volume to volume with a 2% agarose solution. After solidification of the overlay mixture, the plates were incubated in the reverse position (lid downward) for 3 to 6 days at $37 \pm 2^\circ\text{C}$; $5 \pm 2\%$ CO_2 . The plates were observed every day. As soon as a cytopathic effect appeared a second overlay mixture which was identical to the first one but further contains 0.008% neutral red was added to each well (2 mL), and
20 the plates were incubated in the reverse position for 1 to 2 days at $37 \pm 2^\circ\text{C}$; $5 \pm 2\%$ CO_2 .

Infection of a cell with a viral particle (clone) remained in these conditions limited to immediately surrounding cells and caused local lysis, creating a white spot (lysis plaque) rich in viruses over an otherwise red-colored cellular monolayer. For each amplified viral dilution, two clones were recovered through the cover medium using a
25 micropipette and a 1000 μ L cone. The viral clone thus obtained was suspended in 1 mL of VP-SFM medium and then mixed vigorously.

Each of these suspensions was diluted in cascade steps from 1:2 to 1:200,000 to perform a second series of plate purification. At the end of this second cloning run, two clones per plate were again harvested. Sixteen clones maximum per lineage were obtained,
30 *i.e.* 16 clones from Stamaril® parent strain and 16 clones from YF-VAX® parent strain.

d) Amplification n°3 (viral passage n°6)

For each clone (up to 32), the viral suspension obtained by re-suspension of the viral matter contained in agarose was diluted 1/4 or 1/2 (depending on the size of the collected plaque) in VP-SFM.

5 The amplification n°3 was performed according to the same protocol as amplification n°1 (see section a) above). The amplified virus was harvested when a cytopathic effect was visible and the genomic titer in qRT-PCR was above 8.0 log₁₀ Geq/mL.

10 After a total of 4 to 5 days of incubation, the culture supernatant containing the virus was recovered and divided into aliquots. 140 µL of this viral suspension were used to extract the total RNA with QiaAmp viral mini kit (QIAGEN®), according to the protocol of the supplier, and to quantify RNA by YF-NS5 qRT-PCR (as described in Mantel *et al.* (2008)). One or more aliquots, depending on the viral RNA titer, were retained to carry out the next step amplification, the others were frozen at ≤-70°C in the presence of 10% final sorbitol.

15 e) Amplification n°4 (viral passage n°7)

The viral suspension resulting from the amplification n°3 (see section d) above) was diluted in VP-SFM medium so as to infect Vero cells previously seeded at a rate of m.o.g of 2 and further processed according to the same protocol as the amplification n°2 (see section b) above).

20 The amplified virus was harvested when a cytopathic effect was visible and the genomic titer in qRT-PCR was above 8.0 log₁₀ Geq/mL. After a total of 4 to 5 days of incubation, the supernatant was recovered, the viral suspension was clarified by centrifugation for 10 minutes at 1200 rpm at 4°C, and then divided into frozen aliquots at ≤-70 °C in the presence of 10% final sorbitol.

25 The viral suspension thus obtained was used to carry out infectious titration and sequencing of the viral genome.

30 From these data three strains from each lineage (*i.e.* the three TV2212, TV2232 and TV2241 strains from Stamaril® parent strain and the three TV3111, TV3112 and TV4221 strains from YF-VAX® parent strain) were selected according to the following criteria: infectious titer ≥ 6 log₁₀ CCID₅₀/mL and a genomic sequence exhibiting no reversion to the Asibi original strain sequence.

Viral suspension infectious titration was performed using CCID₅₀ method on Vero cells. Briefly, the viral suspensions were serially 4-fold diluted in IMDM (THERMOFISHER SCIENTIFIC) + 4% FCS starting from -4.6 log₁₀ to -8 log₁₀ in a 96-deep well plate. A control virus (Stamaril® virus amplified once on Vero cells, batch MLE-JPO-000089) was included in each test as a positive reference. One hundred microliters of each virus dilution were added into 10 wells containing Vero cells seeded in flat bottom 96-well plates three days before the assay (8000 cells/well). After 4 days of incubation at +37°C, 5% CO₂, supernatants were discarded and cells were fixed for 15 minutes at -20±3°C with 150 µL acetone 85% then saturated with 2.5% milk PBS-Tween buffer solution before immunostaining with the pan-flavivirus E-specific 4G2 mouse monoclonal antibody (RD BIOTECH®, lot #130726-4G2) at 2 µg/mL (dilution 1/2,000). Infected foci stained with 4G2 antibody were then revealed after incubation with a Goat Anti-Mouse IgG alkaline-phosphatase conjugated antibody (CLINISCIENCES® SA, ref #1030-04, lot #A7013-Z145) diluted 1/1000 and then with an alkaline-phosphatase substrate (BCIP/NBT, SIGMA-ALDRICH®, ref# B5655, lot #SLBN0689V and levamisole, SIGMA-ALDRICH®, ref# L9756, lot #091M1227V).

Positive wells, *i.e.* wells containing at least one plaque stained in black were counted and the final titer was calculated using the Least Square regression method.

f) Amplification n°5 (viral passage n°8) – Premaster candidate (pMSL)

The viral suspension from each of the 6 strains selected from the amplification n° 4 (see section e) above) was diluted in VP-SFM medium so as to infect Vero cells at a m.o.i of 0.01.

Two days before virus amplification n°5, 12.10⁶ Vero cells were seeded in 175 cm² flasks containing 30 mL of VP-SFM medium. As previously performed, the culture medium was removed and replaced with 12 mL of diluted virus suspension or VP-SFM alone (control cells). The flasks were incubated during 2h at 37 ± 2°C; 5 ± 2% CO₂. The viral inoculum was then removed and replaced with 50 mL of VP-SFM medium. The flasks were incubated for 2 days at 37 ± 2°C; 5 ± 2% CO₂. The culture medium was then renewed with fresh VP-SFM medium preheated to 37 ± 2°C and the flasks were re-incubated at 37 ± 2°C; 5 ± 2% CO₂ for 1 to 3 days. The amplified virus was harvested when a cytopathic effect was visible and the genomic titer in qRT-PCR was above 8.0 log₁₀ Geq/mL.

After a total of 3 to 5 days of incubation, the supernatant was recovered, the viral suspension was clarified by centrifugation for 10 minutes at 1200 rpm at 4°C, then distributed in aliquots that were frozen at $\leq -70^{\circ}\text{C}$ in the presence of 10% final sorbitol. The amplified viruses obtained from the 6 selected strains constituted the 6 candidate pMSLs.

5

EXAMPLE 2: Neurovirulence of the candidates in a mouse model

2.1/ Neurovirulence of the pMSL candidates in a mouse model

The neurovirulence of the vYF (Vero cells adapted-Yellow fever virus) pre-master seed lot (pMSL) candidates was assessed through determination of Mouse Lethal Dose 50% (MLD₅₀) as described in WHO TRS 872, annex 2 (1998).

For the study of neurovirulence of the pMSL candidates, groups of 8 female OF1 mice (4-weeks old at inoculation), were injected by the intra-cerebral route with 30 μL of 5 to 7 virus dilutions in 0.4% NaCl 2.5% human serum albumin (HSA) buffer. Four vYF pMSL candidates TV2212, TV3111, TV3112 and TV4221 were evaluated for their neurovirulence and compared to Stamaril® and YF-VAX® reference vaccines. The mice were monitored for 21 days and the number of surviving mice was recorded at day 21. Three independent experiments were performed with a random distribution of the samples. Injected quantities were checked by CCID₅₀ back-titration on the day of inoculation for each experiment.

The clinical monitoring was performed daily to record each day the survival rate. The MLD₅₀ were calculated as the dose inducing 50% of surviving mice using the last square regression and expressed in log₁₀ MLD₅₀/mL. The MLD₅₀ of each strain was determined as a pondered mean of the 3 determinations and 95% confidence intervals, except for TV3111 and TV3112 for which no MLD₅₀ could be calculated, as 100% of surviving mice were recorded for groups administered with TV3111 and TV3112 strains even with the highest dose (30 μL of 0.7 log₁₀ dilution).

The results are presented in Table 2 below.

Table 2: Characterisation of the vYF strains at the pMSL stage, in a mouse neurovirulence test (MLD₅₀ test)

YFV strain	log ₁₀ CCID ₅₀ /mL	log ₁₀ MLD ₅₀ /mL
Stamaril®	6.3	6.0
TV2212	6.8	6.7

YF-VAX®	7.5	4.7
TV3111	7.2	<2.2
TV3112	7.2	<2.2
TV4221	7.9	4.8

With respect to vYF strain TV2212 issued from the Stamaril® lineage, it exhibited similar neurovirulence as compared to Stamaril® reference vaccine.

vYF strain TV4221 issued from the YF-VAX® lineage exhibited similar neurovirulence as compared to YF-VAX® reference vaccine. Finally, vYF strains
5 TV3111 and TV3112, both issued from the YF-VAX® lineage exhibited no neurovirulence effect as compared to YF-VAX® reference vaccine. No MLD₅₀ titer can be calculated for these 2 vYF strains (at least <2.2 log₁₀ MLD₅₀/mL).

As a result, 2 vYF strains TV2212 and TV4221 presented similar neurovirulence profile and MLD₅₀ titers than their respective parent references Stamaril®
10 and YF-VAX®. Two other vYF strains, TV3111 and TV3112 both issued from the YF-VAX® lineage, presented a noticeable attenuation of neurovirulence compared to their YF-VAX® parent strain and their MLD₅₀ titers could not be assessed.

2.2./ Neurovirulence of the TV3112 strain MSL and WSL in a mouse model

2.2.1/ TV3112 strain MSL and WSL

All media and solutions used for the MSL and WSL production were animal-
and human-component free.

After Vero cell amplification in static conditions, the cells were seeded in a bioreactor. After 3 days of cell growth in the bioreactor, the medium was changed from
20 cell growth medium to virus production medium. The virus was inoculated by adding the seed lot (TV3112 pMSL to produce TV3112 MSL or TV3112 MSL to produce TV3112 WSL) in the bioreactor. After 2 days of virus multiplication, the virus production medium was discarded and replaced by the same volume of fresh virus production medium. 4 days after virus inoculation, the content of the bioreactor was harvested, clarified, stabilized,
25 filled and stored frozen.

2.2.2/ Neurovirulence of the TV3112 strain MSL and WSL

The same protocol as described in Example 2, sub section 2.1 above was used.

Table 3: Characterisation of the TV3112 strain at the MSL and WSL stages, in a mouse virulence test (MLD₅₀ test)

YFV strain	log ₁₀ CCID ₅₀ /mL	log ₁₀ MLD ₅₀ /mL
TV3112 MSL	7.0	<2.2
TV3112 WSL	8.1	<2.2

As for TV3112 pMSL, TV3112 MSL and TV3112 WSL exhibited no neurovirulence effect. No MLD₅₀ titer can be calculated for TV3112 MSL and TV3112 WSL (at least <2.2 log₁₀ MLD₅₀/mL).

TV3112 MSL and TV3112 WSL presented a noticeable attenuation of neurovirulence compared to their YF-VAX® parent strain and their MLD₅₀ titers could not be assessed.

EXAMPLE 3: Viscerotropism and neurotropism of the vYF strain candidates in a mouse model

Viscerotropism and neurotropism of the 6 vYF (Vero cells adapted-Yellow fever virus) pre-master seed lot (pMSL) candidates were assessed in assays based on inoculation to type I IFN receptors deficient mice that were developed to allow the discrimination between pathogenic and attenuated vaccine strains (Meier et al., 2009; Erickson and Pfeiffer, 2015). The A129 immuno-deficient mice KO for type I IFN receptors are described to mimic the wild-type YF virus infection in primates and humans (Meier et al., 2009). Therefore, such a mouse model appears suitable to study viscerotropic disease caused by non-attenuated yellow fever viruses.

3.1/ Methods

3.1.1/ Group Definition

Fifteen groups (group A to O) of six 4-8 week old female A129 mice were administered with 4 log₁₀CCID₅₀/dose of each of the 6 pMSL candidates or of the Stamaril® reference vaccine as described in Table 4 below (no adjuvant; subcutaneous administration route; 200 µl at D0).

Table 4: Group definition

Group	Mice number per group	Product under test	
		Active substance	
		Name	Dose
A	6	PBS 1X	-
B	6	Stamaril®	4 log PFU

C	6	Stamaril®	4 log PFU
D	6	TV2212	4 log ₁₀ CCID ₅₀
E	6	TV2212	4 log ₁₀ CCID ₅₀
F	6	TV2232	4 log ₁₀ CCID ₅₀
G	6	TV2232	4 log ₁₀ CCID ₅₀
H	6	TV2241	4 log ₁₀ CCID ₅₀
I	6	TV2241	4 log ₁₀ CCID ₅₀
J	6	TV3111	4 log ₁₀ CCID ₅₀
K	6	TV3111	4 log ₁₀ CCID ₅₀
L	6	TV3112	4 log ₁₀ CCID ₅₀
M	6	TV3112	4 log ₁₀ CCID ₅₀
N	6	TV4221	4 log ₁₀ CCID ₅₀
O	6	TV4221	4 log ₁₀ CCID ₅₀

3.1.2/ Study Schedule

The study schedule is described in **Figure 2**.

The 6 mice of groups C,E,G,I,K,M,O were euthanized and their organs sampled at D6 and the 6 mice of groups B,D,F,H,J,L,N were euthanized and their organs sampled at D11. Intermediate blood sampling was collected in groups A, B, D, F, H, J, L and N at D4. For the PBS control, only 6 mice were included and sampled at D11 (group A).

3.1.3/ Mice Clinical Observations and Scoring

Animals were observed daily during 11 days post-inoculation according to the scoring grid described in Table 5 below. Body temperature was monitored and recorded every day from D3 to the end of the experiment at D11.

Table 5: Scoring table

Parameters	Description	Score
General aspect (GA)	Normal	0
	Fur/Spiked	1
	Arched back	2
	shiver	3
Neurological signs (NS)	Normal	0
	On tiptoe	1
	Motor complication	2
	Convulsion	3
Reaction to Stimuli (RS)	Normal	0
	Stationary	1
	Reduced	2
	Excessive/Prostration	3
	No reaction	4
Breathing	Normal	0

(B)	Speed or Irregular	1
	Respiratory distress syndrome	2

During the course of the experiment, animals were euthanized if any of the following events occur:

- Signs of suffering (cachexia, weakening, difficulty to move or to eat)
- Compound toxicity (hunching, convulsions)
- 5 - General Aspect score = 3 + Reaction to Stimuli= 3
- Body weight loss >20%

Any animal found dead was necropsied.

3.1.4/ Biological Sampling

10 a) On D4, intermediate blood samples were taken under anaesthesia from the submandibular vein. Around 200 µL of blood were collected in vials containing clot activator and serum separator (BD Microtainer SST).

b) On Day 6 and D11, blood samples were taken after exsanguination by carotid section from all the animals under anaesthesia. Around 1mL of blood was collected in vials containing clot activator and serum separator (BD Vacutainer SST).

15 c) Organ collections were performed under sterile conditions. Instruments used for the animal's dissection were previously rinsed with RNaseZap™ decontamination solution. All the organs listed below were sampled for all mice as soon as possible after the exsanguination followed by animal's euthanasia by cervical dislocation under anaesthesia: brain, liver and spleen.

20 For liver, two biopsy punches of 7 mm diameter dedicated to viral load detection were placed into vials containing 1mL of RNAlater™ solution.

For brain and spleen, 2 half sections dedicated to viral load detection were placed into vials containing 1 mL of RNAlater™ solution.

3.1.7/ Analytical Tests

25 a) Viremia

Total genomic RNA was extracted from 140 µL of each individual serum sample with the Macherey Nagel NucleoSpin® 96 virus kit on Tecan Evoware automated RNA extraction workstation according to the manufacturer's instructions and eluted in two steps into a final volume of 140 µL of nuclease-free water.

Immediately after extraction, RNA quantification was performed by YF-NS5 qRT-PCR (as described in Mantel *et al.* (2008)). The qRT-PCR targets a conserved region of the YF NS5 gene to detect the presence of YF viral genome.

b) Viral Load in Organs

5 Biopsy punches were frozen at -80°C in RNA later™ solution. At thawing, each sample of organ was weighted.

Total RNA was extracted from the punches of organs using a combined Trizol™ (Invitrogen®)/RNeasy™ (Qiagen®) method as prescribed by the supplier's recommendations.

10 The presence of viral RNA in the purified RNA samples was then quantified using the YF-NS5 qRT-PCR assay as described in Mantel *et al.* (2008). The qRT-PCR targets a conserved region of the YF NS5 gene to detect the presence of YF viral genome.

Each qRT-PCR run included two non-template controls (negative qRT-PCR controls) and two positive controls based on CYD-3 viral suspension.

15 To validate a run, all negative controls had to be below the limit of detection (LOD) and the positive controls had to be included in the control charts.

Due to the dilutions factors and for a sample of 100mg of organ, the limit of detection was calculated at 1 Geq/mg of organ.

20 3.2/ Results

3.2.1/ Clinical signs

All the animals were observed daily post-inoculation according to the scoring grid described in Table 5 above: all mice from groups A to O were scored daily from day 3 up to day 6 and all mice from groups A, B, D, F, H, J, L and N were further scored daily up
25 to day 11.

The mean scores were calculated for each criterion, *i.e.* General Aspect (GA), Reaction to Stimuli (RS), Neurological Signs (NS) and breath (B), from day 3 to day 11 for each group at each time-point As expected, for all the A129 mice injected with saline control (PBS, group A), no specific clinical score was recorded for any animal during all
30 the monitoring period.

For all the A129 groups administered either with Stamaril® reference vaccine or with one of the vYF pMSL candidates, the clinical signs were mild with means score for

each criterion inferior to 1.5 whatever the time-point and whatever the criterion (GA < 1.5; RS, NS and B <1).

No specific clinical score was recorded for days 3, 4 and 5; then some scores 1/0/0/0 or 2/0/0/0 (GA/RS/NS/B) were recorded for few mice by days 6 and 7. At day 10 and 11, all the A129 mice administered either with Stamaril® reference vaccine or with one of the vYF pMSL candidates exhibited low scores (some mice with GA score =1 or 2 and RS, NS as well as B scores= 1) except for one mouse administered with TV2232 (group F) presenting on day 10 some shivering phenotype, motor complication, prostration and respiratory distress (score 3/2/3/2) and that was euthanized for ethical reasons.

10 3.2.2/ Weight Monitoring

All mice (groups A to O) were weighed on days 0, 3, 4, 5 and 6; on days 7, 10 and 11 all mice from the remaining groups (A, B, D, F, H, J, L and N) were weighed. The percentages of weight loss compared to day 0 were calculated for each individual mouse at each time-point.

15 After immunization with Stamaril® reference vaccine, a slight weight loss was observed during the 11 days monitoring period (mean of less of 5% weight loss at D11).

After immunization with vYF pMSL candidates coming from the Stamaril® lineage, as for the Stamaril control, no drastic loss of weight was observed except for one mouse immunized with clone TV2232 at day 10 that loose more than 20% of its weight.

20 This mouse had to be euthanized for ethical reasons (see 3.2.1 above).

After immunization with vYF pMSL candidates coming from the YFVAX® lineage, TV3111, TV3112 and TV4221, stable weights were observed and recorded up to days 5 to 6 and a slight weight gain was observed up to the end of the monitoring period (mean of less of 5% weight gain at D11).

25 3.2.3/ Viral Load in Sera and Organs

a) In Sera – Figure 3

Individual viremia as well as geometric mean titers (GMT) and standard deviations calculated for each group and time points are depicted in **Figure 3**.

30 As expected, no viremia was detected at day 4 in A129 mice administered at day 0 with PBS (< LOD of 3 log₁₀Geq/mL) whereas geometric mean viremia titers between 4 and 5 log₁₀Geq/mL at day 4 and day 6 were detected in A129 mice administered with Stamaril® reference vaccine.

After immunization with the vYF pMSL candidates, no significant superiority of viremia was observed compared to the viremia induced after immunization with the Stamaril® control (all p-values > 0.2 for TV2212, TV2232, TV2241, TV3111 and TV3112 whatever the time point) except for TV4221 coming from the YF-VAX® lineage that
5 induced significantly higher viremia than the Stamaril® control at day 4 after injection (p-value = 0.001).

b) In Liver – Figure 4

Results are expressed in log₁₀Geq/mg of organ. Individual viral load as well as geometric means and standard deviations calculated for each group and time points are
10 depicted in **Figure 4**.

As expected, no liver viral load was detected at day 11 in A129 mice administered at day 0 with PBS (< LOD of 1 log₁₀Geq/mg) similarly no or low liver viral loads were detected in A129 mice administered with Stamaril® reference vaccine (GMT = 0.8 at day 6, < LOD at day 11).

15 After immunization with the vYF pMSL candidates, no significant superiority of liver viral load was observed compared to the liver viral load induced after immunization with the Stamaril® control (all p-values > 0.1 for TV2212, TV2232, TV2241, TV3111, TV3112 and TV4221 at day 6, no statistical analysis performed on day 11 due to the high number of non-responders < LOD).

20 **c) In Brain – Figure 5**

Results are expressed in log₁₀ Geq/mg of organ. Individual viral load as well as geometric mean titers and standard deviations calculated for each group and time points are depicted in **Figure 5**.

As expected, no brain viral load was detected at day 11 in A129 mice
25 administered at day 0 with PBS (< LOD of 1 log₁₀Geq/mg) whereas brain viral loads were detected in A129 mice administered with Stamaril® reference vaccine (GMT = 0.6 at day 6, 3.7 at day 11).

After immunization with the vYF pMSL candidates, no significant superiority of brain viral load was observed compared to the brain viral load induced after
30 immunization with the Stamaril® control (p-values > 0.06 for TV2212 and TV2232). Additionally TV2241, TV3111, TV3112 and TV4221 induced significantly lower brain viral load at day 11 than Stamaril® control (p-values ≤ 0.003).

d) In Spleen – Figure 6

Results are expressed in $\log_{10}$ Geq/mg of organ. Individual viral load as well as geometric mean titers and standard deviations calculated for each group and time points are depicted in **Figure 6**.

5 As expected, no spleen viral load was detected at day 11 in A129 mice administered at day 0 with PBS ($< \text{LOD}$ of 1 $\log_{10}$ Geq/mg) whereas spleen viral loads were detected in A129 mice administered with Stamaril® reference vaccine (GMT = 4.1 at day 6, 2 at day 11).

10 After immunization with the vYF pMSL candidates, no significant superiority of spleen viral load was observed compared to the spleen viral load induced after immunization with the Stamaril® control (all p-values > 0.09 for TV2212, TV2232, TV2241, TV3111, TV3112 and TV4221 whatever the time point).

3.2.4/ Survival

15 In order to calculate survival rate for each group (for groups A, B, D, F, H, J, L and N), the number of surviving mice were recorded daily for 11 days after sub-cutaneous immunization with 4 $\log_{10}\text{CCID}_{50}/\text{dose}$ of Stamaril® or one of the 6 vYF pMSL candidates.

20 As depicted in the Kaplan Meir curves (**Figure 7**), 100% (6 mice out of 6) of mice survived all along the study course when administered with either PBS buffer, Stamaril® or one of the five vYF strains TV2212, TV2241, TV3111, TV3112 and TV4221.

At the opposite, only 80% of mice survived in group F administered with the TV2232 strain coming from the Stamaril® lineage as on day 10 one mouse was euthanized for ethical reasons (see 3.2.1 above).

25

EXAMPLE 4 – Immunogenicity of the vYF strain candidates in a hamster model

The immunogenicity of the 6 vYF pMSL candidates in the Hamster model was evaluated and compared to the Stamaril® reference vaccine.

30

4.1/ Methods

4.1.1/ Group Definition

Fifteen 5-6 weeks old female Golden Syrian hamsters were included in each group and 2 doses, *i.e.* a low suboptimal dose of $2.5 \log_{10} \text{CCID}_{50}/\text{dose}$ and a high dose of $5.5 \log_{10} \text{CCID}_{50}/\text{dose}$, were administered for each of the 6 pMSL candidates.

For the Stamaril® reference, only 10 hamsters per group were included for the 2 tested doses described above.

In total 200 female Golden Syrian Hamsters were randomly allocated to one of the 14 following groups (group A to N) described in Table 6 below (no adjuvant; subcutaneous administration route; 200 µl at D0 and D26).

Table 6: Group definition

Group	Hamster number per group	Product under test	
		Active substance	
		Name	Dose
A	10	Stamaril®	$2.5 \log \text{PFU}$
B	10		$5.5 \log \text{PFU}$
C	15	TV2212	$2.5 \log_{10} \text{CCID}_{50}$
D	15		$5.5 \log_{10} \text{CCID}_{50}$
E	15	TV2232	$2.5 \log_{10} \text{CCID}_{50}$
F	15		$5.5 \log_{10} \text{CCID}_{50}$
G	15	TV2241	$2.5 \log_{10} \text{CCID}_{50}$
H	15		$5.5 \log_{10} \text{CCID}_{50}$
I	15	TV3111	$2.5 \log_{10} \text{CCID}_{50}$
J	15		$5.5 \log_{10} \text{CCID}_{50}$
K	15	TV3112	$2.5 \log_{10} \text{CCID}_{50}$
L	15		$5.5 \log_{10} \text{CCID}_{50}$
M	15	TV4221	$2.5 \log_{10} \text{CCID}_{50}$
N	15		$5.5 \log_{10} \text{CCID}_{50}$

10

4.1.2/ Study Schedule

The study schedule is summarized in **Figure 8**.

The planning of interventions and the intervention details are described in Table 7 below.

Table 7: Study Schedule

Day	Group Number	Number of Animals	Interventions	Specific Characteristics
D0	A to N	200	Bleeding Immunization (first injection)	Chemical anaesthesia (IP route) Intermediate blood sampling Immunization SC route
D26	A to N	200	Bleeding Immunization (second injection)	Chemical anaesthesia (IP route) Intermediate blood sampling Immunization SC route

D41	A to N	200	Bleeding	Chemical anaesthesia (IP route) Intermediate blood sampling
D55	A to N	200	Bleeding	Chemical anaesthesia (IP route) Final blood sampling Euthanasia

4.1.3/ Biological Sampling and seroneutralization assays

a) Biological Sampling

Intermediate blood samples were taken under anaesthesia from the retro-orbital sinus (ROS) at D0, D26 and D41 from all the animals. The final blood sampling was taken
5 under anaesthesia *via* an intracardiac puncture. The anaesthesia was performed by Imalgène (150 mg/kg) and Rompun (10 mg/kg) administered under a volume of 200 µl by intraperitoneal route.

Blood was collected in vials containing clot activator and serum separator (BD Microtainer SST). After a night at +4°C or 2h at 37°C, the blood was centrifugated at 2000
10 x g during 10 minutes and the serum collected and stored at -20°C until analysis.

b) Seroneutralization assays

The functional neutralizing antibodies present in the serum of the immunized animals were titrated at D0, D26 and D41 from the first injection.

Briefly, the heat-inactivated sera were serially 2-fold diluted in IMDM + 4%
15 foetal calf serum (FCS) starting from 1:5. YF-17D Stamaril® virus grown on Vero cells was diluted in order to obtain 4000 µPFU/mL in IMDM and incubated 90 minutes with 2-fold diluted serum samples (v/v). The virus/serum mixture was then added to Vero cells in 96-well plates and incubated for 45 +/- 2 hours. After incubation, cells were fixed with 85% acetone before immunostaining. Plates were blocked with PBS + 0.05% Tween 20 +
20 2.5% skim milk and incubated first with an anti-flavivirus monoclonal antibody 4G2, and second with a goat anti-mouse IgG HRP conjugate. Finally, plates were stained with the Trueblue™ chromogen. Plaques were counted with Viruscope reader from Microvision™.

The final seroneutralizing antibody titer is calculated using the least square method and correspond to the inverse of the dilution demonstrating a neutralization of 50%
25 of virus plaques. The LOD of the assay was 10, corresponding to the first reciprocal dilution in the final volume.

For calculation of the mean values per group, an arbitrary titer of 5 was assigned (half of the LOD) to all titers below 10.

4.2/ Results – Seroneutralization

The neutralizing activity against the Yellow Fever 17D vaccine strain on Vero cells was monitored by seroneutralization assays in individual serum samples collected from all animals at baseline (D0), four weeks after one immunization (D26) and two weeks after two immunizations (D41). Geometric mean titers (GMT) as well as individual
5 neutralizing titers and the 95% confidence interval (CI) are depicted in **Figure 9** and **Figure 10**.

As expected, no or low neutralizing antibody titers (≤ 40) were detected in naïve hamsters at baseline (D0) with group GMT ≤ 12 , whatever the pMSL vYF candidates or the Stamaril® reference. A responder threshold was defined at 20.87 (1.32
10 $\log_{10}$) by the statistical analysis of all the individual data obtained at D0 (superior tolerance interval with proportion of 0.99 and risk alpha of 5%).

Regarding the response kinetics, one month after 1 immunization (D26; **Figure 9**), a marked increased neutralizing response was observed for all the immunized groups with at least a 10- up to an 850-fold increase of neutralizing GMT compared to D0
15 baseline. Two weeks after the second immunization (D41; **Figure 10**), neutralizing GMTs were further increased for all groups with a 0.8- to 7.5-fold increase of neutralizing GMT compared to D26.

The neutralizing antibody response induced by Stamaril® reference was significantly lower (p-values= 0.007 and 0.023 at D26 and D41, respectively) for the 2.5
20 $\log_{10}$ CCID₅₀ dose (GMT 281 and 544 at D26 and D41, respectively) than for the 5.5 $\log_{10}$ CCID₅₀ dose (GMT 5061 and 11714 at D26 and D41, respectively). Of note, 100% of the hamsters from group administered with 5.5 $\log_{10}$ CCID₅₀ dose were defined as responder (>20.87 threshold) as soon as the first immunization (at D26) whereas only 60% and 70% of the hamsters from group administered with 2.5 $\log_{10}$ CCID₅₀ dose were found responder
25 at D26 and D41, respectively.

For the vYF pMSL candidates from the Stamaril® lineage TV2212, TV2232 and TV2241, no significant difference was observed between the two tested doses (all p-values >0.07 whatever the vYF pMSL candidates and the time point) with GMT ranging from 144 to 505 at D26 and from 115 to 1159 at D41 for 2.5 $\log_{10}$ CCID₅₀ dose compared
30 to GMT ranging from 115 to 373 at D26 and from 465 to 955 at D41 for 5.5 $\log_{10}$ CCID₅₀. None of the vYF pMSL candidates from the Stamaril® lineage was able to induce a sustained neutralizing antibody response in all the immunized animal whatever the tested

dose and whatever the immunization schedule (after 1 or 2 immunization). The percentage of responder hamsters was found to range from 53% to 93% after one immunization and from 43% to 93% after two immunizations with TV2212, TV2232 and TV2241, whatever the dose.

5 For the vYF pMSL candidates from the YF-VAX® lineage TV3111, TV3112 and TV4221, no significant difference was observed between the two tested doses (all p-values > 0.06 whatever the vYF strains and the time point) except for TV3111 for which the 2.5 log₁₀ CCID₅₀ dose induced significantly higher neutralizing antibody titers than 5.5 log₁₀ CCID₅₀ (p-value=0.04 and 0.003 at D26 and D41, respectively). For 2.5 log₁₀ CCID₅₀ dose, the induced GMTs were high and ranging from 3939 to 8898 at D26 and from 3771 to 13674 at D41 whereas for 5.5 log₁₀ CCID₅₀ dose, GMTs ranged from 2071 to 5145 at D26 and from 1821 to 6421 at D41. The vYF pMSL candidates from the YF-VAX® lineage were able to induce a sustained neutralizing antibody response in most of the immunized animal after 1 immunization (93% of responder for 2.5 log₁₀ CCID₅₀ dose of TV3111 and 100% responders for all the other vYF pMSL candidates from the YF-VAX® lineage whatever the tested dose). After two immunizations all vYF pMSL candidates from the YF-VAX® lineage were able to induce a sustained neutralizing antibody response in 100% of the immunized hamster, whatever the dose.

20 With respect to the comparison of each of the vYF pMSL candidates to the Stamaril® reference, the neutralizing responses induced by 2.5 log₁₀ CCID₅₀ dose of the vYF pMSL candidates issued from the YF-VAX® lineage TV3111, TV3112 and TV4221 were significantly non inferior to those obtained with Stamaril® reference vaccine (p-values ≤ 0.010 and ≤ 0.047 at D26 and D41, respectively). No significant non inferiority was shown for the vYF strains issued from the Stamaril® lineage (all p-values ≥ 0.25, whatever the dose and the time point) neither for the vYF strains issued from the YF-VAX® lineage administered at 5.5 log₁₀ CCID₅₀ (p-values ≥ 0.49, whatever the time point).

EXAMPLE 5 – Toxicity and immunogenicity of the vYF TV3112 strain in a monkey model

30 A preliminary toxicity study and an immunogenicity study were conducted in nonhuman primates (NHP). The non-human primates, and particularly the rhesus macaques or the cynomolgus macaques, are conventionally used to evaluate the safety and

the infectivity, as measured by viremia, and the immunogenicity of vaccine candidates against flaviruses (dengue, yellow fever ...). In the context of yellow fever, monkeys are natural hosts; the virus was first isolated in monkeys and it is in this model that the attenuation of vaccine strains was evaluated. Since the 2000's, "small animal" models have
5 been described and can be used to evaluate certain properties of candidate vaccines as performed for the vYF pMSL candidate selection. These models (hamster, mouse A129) however have limitations and macaque remains to this day the most predictive gold standard model compared to humans and is widely described in the literature, e.g. Julander (2016), Mason *et al.* (1973), Monath *et al.* (2010) and Moulin *et al.* (2013). In addition,
10 this model is recommended in the regulatory guidelines.

5.1/ Methods

5.1.1/ Group Definition and objectives

Three groups of nine 2 years old male cynomolgus monkeys (*Macaca fascicularis*) imported from Mauritius were immunized by SC route with 500 µL of
15 Stamaril® (one human dose corresponding to 4.2 log₁₀CCID₅₀/dose), one dose of YF-VAX® (6.2 log₁₀CCID₅₀) or 4.2 log₁₀CCID₅₀ of vYF TV3112 WSL candidate.

As primary readouts, the vaccine candidate was compared to each of the reference vaccines, for evaluation of i) vaccine safety, ii) its ability to induce YF specific viremia and viral load in organs: liver, spleen, kidney, lymph node and brain (assessed by
20 viral RNA quantification by YF-NS5 qRT-PCR, as described in Mantel *et al.* (2008)) and iii) induction of yellow fever specific seroneutralizing antibody responses (assessed by µPRNT₅₀ assay) defined as the correlate of protection.

As secondary readouts, in order to identify other potential biomarkers of vaccine performance, different parameters were monitored. These analyses addressed: i)
25 the persistence of the antibody response, and ii) the B and T cellular immune response including memory responses.

5.1.2/ Anaesthesia

For the immunizations and at certain occasions (e.g. blood samplings combined with other manipulations during the acclimatization period or if monkeys are refractory to
30 blood samplings without anaesthesia), a mild anaesthesia was performed. Ketamine (Imalgene 1000, MERIAL®) at 10 mg/kg was injected intramuscularly in the thigh.

5.1.3/ Monitoring

All animals were weighed at D-29 and D0, observed daily for clinical signs up to D7 and their individual body temperature (transponder system) was recorded at D-17 and during viremia expected period at D0, D3, D4, D5, D6 and D7. Hematology, biochemistry and blood viremia were assessed in all monkeys at D1, D3 and D7. Three monkeys of each group were then euthanized and their organs sampled at D7 for histopathology and viral load in organs assessments. The 6 remaining monkeys in each group were further weighed at D27, D60, D90, D122, D153 and D181 and their individual body temperature was further recorded during viremia expected period at D10 and D14. The 6 remaining monkeys in each group were also observed daily for clinical signs up to D221. Blood samples for seroneutralization, T cells and memory B cells assays were collected from the 6 remaining monkeys in each group at D27, D60, D90, D122, D153, D181 and D221.

5.1.4/ Testing methods

YF-specific seroneutralizing antibodies were titrated using the μ PRNT50 method on VERO cells. Briefly, the heat-inactivated sera were serially 2-fold diluted in IMDM (THERMOFISHER SCIENTIFIC) + 4% FCS starting from 1:5. YF-17D Stamaril® virus grown on Vero cells was diluted in order to obtain 4000 μ PFU/mL in IMDM and incubated 90 minutes with 2-fold diluted serum samples (v/v). The virus/serum mixture was then added to Vero cells in 96-well plates and incubated for 45 +/- 2 hours. After incubation, cells were fixed with 85% acetone before immunostaining. Plates were blocked with PBS + 0.05% Tween 20 + 2.5% skim milk and incubated first with an anti-flavivirus monoclonal antibody 4G2, and second with a goat anti-mouse IgG HRP conjugate. Finally, plates were stained with the Trueblue™ chromogen. Plaques were counted with Viruscope reader from Microvision™. The final titer is calculated using the least square method and corresponds to the reciprocal of the dilution demonstrating a neutralization of 50% of the plaques.

The LOD of the μ PRNT50 assay was about 20 μ PRNT50 when the first dilution of serum tested was 1:10. For calculation of the mean value per group, an arbitrary value of half of the LOD was assigned to all samples below the LOD, *i.e.* 10 μ PRNT50.

Memory cellular responses were measured by ELISPOT. The fluorescent-linked immunospot (FLUOROSPOT) is used for detecting and enumerating individual

memory B cells secreting antibodies irrespective of antigen specificity (total IgM or total IgG).

On D0 Frozen PBMC were thawed in RPMI medium (THERMOFISHER SCIENTIFIC®) supplemented with 10% FBS and 100µg/mL of DNase and incubated for
5 1 hour at 37°C; 5% CO₂. After 1 hour, cells were diluted at 1 million cells/mL and stimulated by incubation for 4 days at 37°C; 5% CO₂ in RPMI 10% FBS supplemented with rIL2 (10µg/mL).

On D3, the membrane of 96-well FluoroSpot microplates equipped with a low-fluorescent PVDF membrane (MERCK Millipore®) was pre-wetted for 1 minute with 35
10 µL of 35% ethanol. Each well was washed twice with 200 µL of PBS 1X. Microplates were then coated with YF-17D infected Vero cell lysate (SANOFI PASTEUR®) at dilution 1:80 or a mix of monoclonal antibodies specific for monkeys IgG and IgM at a dilution of 15µg/mL, and were incubated overnight at 4°C.

On D4, plates were washed with PBS and then blocked at least 2h at 37°C with
15 RPMI 10% FBS. After plates washing, 2×10^5 or 4×10^5 of stimulated PBMC were added in the wells coated with YF-17D infected Vero cell lysate. A range dilution of stimulated cells (5×10^3 to $6,2 \times 10^2$) was added to the wells coated with anti-IgG and anti-IgM antibodies.

After 5 hours, the plates were washed 3 times with PBS 1X and stored à 4°C
20 for the night.

On D7, the plates were washed 6 times with PBS 1X-BSA 0.5% (150 µL/well). After the washing step, 100 µL/well of the anti-monkey IgM-FITC and IgG-CY3 antibodies were added respectively at a dilution of 1/500 in PBS1X-BSA 0.5% for 2 hours at room temperature, in the dark. The plates were washed again 6 times with PBS 1X-BSA
25 0.5% (150µL/well). The plates were stored at 5°C±3°C in the dark until reading.

Each spot, corresponding to an antibody secreting cell, was enumerated with an automatic FLUOROSPOT plate reader (Microvision™). Results were expressed as number of ACS secreting cell per 10⁶ cells.

T cell responses were determined by IFN-γ IL-2 responses Dual FluoroSpot
30 (FS-2122-10 Monkey IFN-γ/IL-2 FluoroSpot kit from Mabtech®) on isolated PBMC.

Briefly, FluoroSpot PVDF membrane equipped microplates were pre-treated with 35% ethanol, washed, and coated overnight by incubation with monoclonal antibodies

against monkey IFN- γ (clone GZ-4, Mabtech®) and against monkey IL-2 (clones IL2M-I/249, Mabtech®) at a concentration of 15 $\mu\text{g/mL}$ in sterile phosphate-buffered saline (PBS) at 4°C. Plates were washed 3 times with PBS and then blocked by incubation for 2 hours at 37°C with RPMI 1640 medium (Gibco®) supplemented with 10% FCS. PBMC (4×10^5) were added to each well with 0.1 $\mu\text{g/mL}$ of monoclonal antibody CD28-A (Mabtech®). YF-Env and YF-NS3 peptide pools (15-mers overlapping peptides covering YF-Env and YF-NS3 amino acid sequences) were added to a final concentration of each peptide in the culture medium of 1 $\mu\text{g/mL}$. Anti-CD3 mAb (Mabtech®) was used as positive control at 2.5 $\mu\text{g/mL}$. Plates were incubated for 24 hours at 37°C in an atmosphere containing 5% CO₂. After incubation, plates were washed 6 times with PBS. FITC anti-IFN- γ antibody (clone 7-B6-1-FS-FITC, Mabtech®) and biotinylated anti-IL-2 antibody (IL2- biotin MT8G10, Mabtech®) were added at a concentration of 1:200 and 1 $\mu\text{g/mL}$, respectively, in 0.5% BSA in PBS; the plates were incubated 2 hours at 37°C. After 3 washes with PBS, incubation was performed with anti-FITC-490 (1:200, Mabtech®) and Streptavidin SA-550 (1:200, Mabtech®) diluted in 0.5% BSA in PBS for 1 hour at room temperature and washed 6 times with PBS. The plates were stored at 5°C $\pm$ 3°C in the dark until reading. Fluorescent spots, corresponding to an IFN- γ or IL-2 secreting cell (IFN- γ SC or IL5 SC) as well as polyfunctional T cells secreting both IFN- γ and IL-2 cytokines, were enumerated with an automatic FLUOROSPOT plate reader (Microvision™). Results were expressed as number of IFN- γ or IL-2 secreting cell per 10⁶ PBMCs.

YF vaccine viremia and viral load in organs were monitored by YF-NS5 qRT-PCR (as described in Mantel *et al.* (2008)).

5.2/ Results

The correlate of protection for live-attenuated yellow fever vaccines is defined in the WHO TRS 978, Annex 5, as the induction of measurable neutralizing antibody in a previously seronegative individual, *e.g.* as a PRNT titer > limit of detection. Neutralizing antibodies far above the pre-established protective threshold (LOD = 20) were detected in all monkeys as soon as D14 and during at least 9 months (see **Figure 11**). Neutralizing antibody titers were not significantly different from the titers detected after immunization with the current vaccines.

This long-lasting neutralizing antibody response was also supported by a sustained B memory cell frequency monitored in peripheral blood from day 14 up to day

221 after vYF TV3112 vaccination (see **Figure 12** and **Figure 13**). These data show that both IgM (**Figure 12**) and IgG (**Figure 13**) memory B cells developed as soon as day 14 post-vaccination and lasted during the study period for at least 221 days. With respect to vYF TV3112, the kinetics and the percentage of induced memory B cells was similar to the profile of memory B cells induced by both reference vaccines Stamaril® and YF-VAX®.

Moreover, a specific Th1 cellular response (IFN- γ and IL-2 secreting cells) to YF-ENV and YF-NS3 was induced after vYF TV3112 vaccination and was similar to the cellular response observed after vaccination with Stamaril® or YF-VAX® (see **Figure 14**).

This study also demonstrated the conserved safety profile of vYF TV3112, as compared to the control vaccines: no clinical signs, no body weight loss, no variation of temperature, no haematology (white and red blood cells; neutrophils; lymphocytes; monocytes; eosinophils; basophils; reticulocytes; platelets; hemoglobin; hematocrit; mean corpuscular volume; mean corpuscular hemoglobin) or biochemical (alkaline phosphatase; alanine transferase; aspartate transferase; gamma glutamyl-transferase; C-reactive protein; bile acids; total bilirubin; albumin; blood urea nitrogen; creatinine) disorders (no statistical differences with Stamaril® and YF-VAX®, *via* PLS-DA statistical analysis), no or very low viremia ($<4 \log_{10}$ Geq/mL in 1 of the 9 monkeys), no or very low viral RNA detected in Yellow Fever-target organs (100 to 10 000 fold lower than viral load observed after wild-type Asibi infection) (see **Figure 15**), no vaccine related histopathological findings in Yellow Fever-target organs.

EXAMPLE 6 – Protection induced by the vYF TV3112 strain against a lethal challenge in a macaque model

The objective was to evaluate protection against yellow-fever virus challenge in macaques immunized with vYF TV3112 vaccine candidate.

6.1/ Methods

6.1.1/ Animals

Nine months after immunization with Stamaril®, YF-VAX® or vYF TV3112 vaccine candidate, the 6 monkeys remaining at D221 from each of the three animal groups that were studied in the example 5 above were challenged against Yellow Fever with Asibi

virulent strain to evaluate vaccine efficacy. Another group of 6 naïve control monkeys was also challenged.

6.1.2/ YFV and buffers

The challenge was conducted with Yellow Fever virus strain Asibi (YFV) from University of Texas Medical Branch (UTMB). YFV (lot 19455, infectious titer $7.7 \text{ Log}_{10} \text{ CCID}_{50}/\text{mL}$ on VERO cells) was diluted in NaCl+HSA buffer (NaCl 0.4% + Human Serum Albumin (HSA) 2.5%). Each animal was challenged subcutaneously in the up right back site with 10^3 CCID_{50} of YFV in 1mL of NaCl+HSA buffer.

6.1.3/ Monitoring

Animals were followed for 28 days after Asibi challenge. The animals were observed daily for food consumption and behaviour. Rectal temperature and body weight were recorded at each sampling time point. Blood sampling was performed as described in Table 8 below.

Table 8: Time schedule

Days post-infection	-8	0	2	3	4	5	7	10	14	28
Challenge		X								
Euthanasia										X
Observations ¹	X	X	X	X	X	X	X	X	X	X
Local scoring	X	X	X	X	X	X	X	X	X	X
Blood for haematology ²	X		X	X	X	X	X	X	X	X
Blood for plasma	X		X	X	X	X	X	X	X	X
Serum for Neut. ³ and Antibody binding	X						X		X	X
Serum for biochemistry ⁴	X		X	X	X	X	X	X	X	X

¹ Clinical observation, body weight, rectal temperature. ²White & red blood cells; neutrophils; lymphocytes; monocytes; eosinophils; basophils; reticulocytes; platelets; hemoglobin; hematocrit; mean corpuscular volume; mean corpuscular hemoglobin. ³ Neutralization assay. ⁴ Alkaline phosphatase; alanine transferase; aspartate transferase; gamma glutamyl-transferase; C-reactive protein; bile acids; total bilirubin; albumin; blood urea nitrogen; creatinine.

6.1.4/ In-life observations

Animals were observed 7 days a week. At each time of bleeding, clinical examination was performed as described in Table 9 below.

Table 9: Chart of the animal observations

Mortality	Daily
Overt signs of disease such as diarrhoea, loss of appetite and lethargy	Daily
Body weight	At sampling day ¹
Body temperature (rectal)	At sampling day ¹
Food and water consumption	Daily evaluation

¹ See Table 8.

5

6.2/ Results

All the vaccinated monkeys were protected from the challenge effects: viremia (only low viremia, *i.e.* $<3.6 \log_{10} \text{GEq/mL}$ in 2/6 monkeys for only one day, measured by YF-NS5 qRT-PCR as described in Mantel *et al.* (2008)), haematological disorders, blood biochemistry disorders and death.

10

In this study, 3 out of the 6 NHP in the non-vaccinated control group survived to the challenge but all the 6 control NHP presented viremia ($> 8 \log_{10} \text{GEq/mL}$), lymphopenia, thrombocytopenia and blood biochemistry disorders with high increase of the level of transaminases, CRP, bilirubin and bile acids.

Accordingly, the vYF TV3112 vaccine candidate was able to protect cynomolgus monkeys, one of the best predictive animal models for yellow fever vaccines, from wild-type Asibi infection, as were the currently available vaccines Stamaril® and YF-VAX®.

EXAMPLE 7 – Sequence analysis

RNA viruses naturally present high genetic variability levels that are responsible for the quasi-species intrinsic nature of these viruses. Even if the error rate of the Yellow Fever polymerase is described as low for a RNA virus, the polymerase error rate is about 10^{-6} substitution per genome per infectious cycle.

The well-defined virus production process is set-up so as to limit this phenomenon to a strict minimum by always keeping the same virus growth conditions. But statistically, virus quasi-species are continuously produced every time the virus replicates in a cell and whenever a variant brings a growth advantage to the virus, it will be conserved and amplified on the long term, progressively replacing the initial population.

25

In addition, as the new virus growth system will move from egg to Vero cell culture, some adaptation mutation are expected to probably take place. In particular, several mutations in NS4B were described in different Flavivirus models as positive adaptation of the virus to grow in Vero cells (Blaney et al., 2003; Tang et al., 2005; 5 Beasley et al. 2013).

Moreover, the current seed were never cloned, so a mixture of quasi-species co-exists in the current vaccine strains. Reference sequences will be first established by high-throughput sequencing of the genomes of YF-VAX® and Stamaril® vaccines, and then the new pMSL candidate genomes will be compared to them.

10 As the new pMSL candidates are obtained after 2 cloning steps they represent homogeneous viral populations.

7.3/ Methods

7.3.1/ Principles

The sequencing of the Yellow Fever virus is carried out after extraction and 15 purification of the viral RNA.

The RNA is then retrotranscribed into complementary DNA and then the genome is completely amplified by PCR using specific primers. PCR products are then used to form a library thanks to the Nextera® XT DNA sample preparation kit (Illumina, Inc.). The formation of the library takes place in several steps. First, the amplicons are 20 assembled in an equimolar manner. Then they are fragmented using transposomes (Tagmentase). Transposomes cut DNA and add adapters. Then a step of amplification by PCR is carried out thanks to primers complementary to adapters. This step allows the addition, on both sides of the fragments, of indexes (used for tagging the sample) and hitch the fragments to the sequencing support. Finally, the library is purified using Agencourt® 25 beads (AMPure® XP, Beckman Coultern Genomics, Inc.) and sequenced using the MiSeq sequencer (Illumina®, Inc.).

Once the sequences obtained, the analysis is then carried out with the analysis module "Quality-based variant detection (legacy)" of the CLC Genomics Workbench software (QIAGEN®).

30 7.3.2) RNA extraction

The viral RNA was extracted from 140 µl of viral suspension at a minimum concentration of 10⁸ Geq/mL (quantification by YF-NS5 qRT-PCR) with the Qiampr Viral kit

(QIAGEN®) according to the supplier's recommendations. Purified viral RNA purified was eluted in 140 µl of nuclease-free water.

7.3.3) RT-PCR

First, a specific retrotranscription (RT) step of the RNA into cDNA was carried out using the three antisense primers, intended to overlap the genome of the yellow fever virus. Then a PCR amplification was performed using the three primer pairs described in Table 10.

Table 10: Primers sequences (MWG®)

SEQ ID NO.	Sequence (5'→3')	Nb of nt	Size of the amplicon	Position on genome
9	GCTAGGCAATAAACACATTGGA	23	4146	49-4195
10	TTCCTGGGATACTCCTTCGC	21		
11	ATCAAATACCATCTTGCCCTC	22	4009	3940 -7949
12	AGTAAATCCTTTGACCCCACT	21		
13	GGCTTACCGCAATGCACT	18	4235	6553-10788
14	CAGAGAACCACTCCGGTC	18		

Three mixes containing one of the three antisense primers (**SEQ ID NO. 10**; **SEQ ID NO. 12** or **SEQ ID NO. 14**) were prepared (see Table 11 below).

Table 11: Mix for one sample

Mix of reverse primers (10 µM)	1 µL
dNTP (10 mM)	1 µL
Nuclease-free water	5 µL
Extracted RNA	5 µL

The samples were heated in the thermocycler for 5 minutes at + 65°C, and a heat shock was immediately carried out by incubating the tubes for 3 minutes in the ice.

A mix was then prepared as described in Table 12 below.

Table 12: Mix for one viral RNA sample (4 RT reactions):

RT buffer	16 µL
DTT (0.1 M)	4 µL
RNAse OUT (LIFE TECHNOLOGIES®)	2 µL
Superscript III (LIFE TECHNOLOGIES®)	2 µL

Nuclease-free water	8 µL
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To each of the three RNA/primers tubes, 12 µl of this mix were added and the reverse transcription run was performed as described in Table 13 follows:

Table 13: RT program

Step	Time (min)	Temperature (°C)
Reverse transcription step	60 min	+ 50°C
Inactivation of the enzyme	5 min	+ 85°C
Hold	-	+ 10°C

1 µl of RNase H are added in each of the tubes and the tubes were incubated for 20 minutes at 37°C in a thermocycler.

From the obtained cDNA, the amplification was performed into three amplicons by PCR.

Three PCR mixes were prepared as described in Table 14 below containing one of the three pairs of primers (**SEQ ID NO. 9** and **SEQ ID NO. 10**; **SEQ ID NO. 11** and **SEQ ID NO. 12**; **SEQ ID NO. 13** and **SEQ ID NO. 14**).

Table 14: PCR mix for one sample

5X Phusion HF Buffer (BioLabs®)	5 µL
dNTPs 10 mM	1 µL
Forward primer 10 µM	1 µL
Reverse primer 10 µM	1 µL
Nuclease-free water	12.5 µL
Phusion HF DNA polymerase (BioLabs®)	0.5 µL

20 µl of each mix were added to the 5 µl of corresponding cDNA.

The PCR program was as described in Table 15 below.

Table 15: PCR program

Step	Time (sec or min)	Temperature (°C)
Initial denaturation	30 sec	+98°C
35 cycles		
- Denaturation	10 sec	+98°C
- Hybridization of primers	20 sec	+58°C
- Elongation	4 min	+72°C

Final elongation	5 min	+72°C
Hold	-	+10°C

7.3.5) Analysis and purification of the amplicons

All amplicons were analysed on 1.2% agarose gel in order to check the quality of amplification. The amplicons were purified manually using the QIAQuick® PCR purification kit (QIAGEN®) according to the supplier's recommendations.

5 7.3.6) Formation of a library with the Nextera® XT kit (Illumina, Inc.)

Purified amplicons were quantified with the Qubit® 2.0 Fluorometer (LIFE TECHNOLOGIES®) using the Qubit® dsDNA HS Assay kit, according to the supplier's recommendations.

Following the assay, the amplicons were serially diluted in nuclease-free water in order to obtain a final concentration of 0.2 ng/μL. Then, for each sample, the three amplicons were mixed to obtain a single concentrated PCR pool at 0.6 ng/μL.

The PCR program was as described in Table 16 below.

Table 16: PCR program

Step	Time (sec or min)	Temperature (°C)
Activation of the DNA polymerase	3 min	+72°C
Initial denaturation	30 sec	+95°C
12 cycles		
- Denaturation	10 sec	+95°C
- Hybridization of primers	30 sec	+55°C
- Elongation	30 sec	+72°C
Final elongation	5 min	+72°C
Hold	-	+10°C

The amplicons were purified and calibrated by the mean of the Agencourt® AMPure® XP kit (BECKMAN COULTER®) according to the supplier's recommendations. The library was stable at -20°C for a week.

7.3.7) Analysis of the library

Quantification of the library was performed with the Qubit® 2.0 Fluorometer (LIFE TECHNOLOGIES®) using the Qubit® dsDNA HS Assay kit, according to the supplier's recommendations.

7.3.8) Sequencing of the library

The library was sequenced by a MiSeq system (ILLUMINA®), according to the supplier's recommendations. The sequences were analysed by the ILLUMINA® Sequencing Analysis Viewer (Illumina, Inc.), according to the supplier's recommendations.

- 5 The analysis of the generated sequences was performed with the CLC Genomics Workbench 7.5.2 software (QIAGEN®), according to the supplier's recommendations.

7.4/ Results

7.4.1) Reference sequences for YF-VAX® and Stamaril® vaccines

The reference sequence of YF-VAX® vaccine was represented as **SEQ ID**

- 10 **NO. 2.** The reference sequence of Stamaril® vaccine was found as **SEQ ID NO. 3.**

7.4.1.1) Stamaril®-derived pMSLs

pMSL candidate genomes (passage n°8) were sequenced and compared to their parent strain genome. The table 17 below provides the result of the high throughput sequencing for the three strains from the Stamaril® lineage.

- 15 **Table 17:** Sequencing result of strains from the Stamaril® lineage

Strain	#nt ¹	Ref nt (Stamaril®) ²	Seq nt ³	Ref codon (Stamaril®)	Seq codon	Freq (%)	Annotation ⁴	AA change
TV2212	2524	C	U	GAC	GAU	100%	NS1-24	-
TV2232	5590	U	G	GUU	GUG	100%	NS3-340	-
	5695	C	U	GUC	GUU	100%	NS3-375	-
	7766	U	C	UUG	CUG	100%	NS5-47	-
	8404	C	U	GAC	GAU	100%	NS5-256	-
	6379	A	G	GAA	GAG	99%	NS3-603	-
TV2241	2524	C	U	GAC	GAU	100%	NS1-24	-

¹ Nucleotide position from the 5' first nucleotide. ² Nucleotide of the Stamaril® reference genome. ³ Mutated nucleotide as compared to the corresponding nucleotide from the Stamaril® reference genome. ⁴ Corresponding YFV protein and corresponding amino acid position in the protein.

- 20 TV2241 and TV2212 present a single mutation compared to the Stamaril® parent strain used as the reference (nucleotide 2524 located in the NS1 coding region, silent at the amino acid level). TV2232 shows a different profile, having five mutations in NS3 and NS5, all silent.

7.4.1.2) YF-VAX®-derived pMSLs

The table 18 below provides the result of the high throughput sequencing for the three pMSL candidates (passage n°8) from the YF-VAX® lineage.

Table 18: Sequencing results of YF-VAX®-derived strains

Strain	#nt ¹	Ref nt (YF- VAX®) ²	Seq nt ³	Ref codon (YF- VAX®)	Seq codon	Freq (%)	Annotation ⁴	AA change ⁵
TV3111	2411	G	U	GUA	UUA	100%	E-480	Val480Leu
	3701	A	G	AUG	GUG	100%	NS2a-65	Met65Val
	6496	A	G	AAA	AAG	100%	NS4a-19	-
TV3112	3701	A	G	ATG	GUG	100%	NS2a-65	Met65Val
	2411	G	U	GUA	UUA	100%	E-480	Val480Leu
	6496	A	G	AAA	AAG	100%	NS4a-19	-
	1408	A	U	GUA	GUU	100%	E-145	-
TV4221			No variant identified					

¹ Nucleotide position from the 5' first nucleotide. ² Nucleotide of the YF-VAX® reference genome. ³ Mutated nucleotide as compared to the corresponding nucleotide from the YF-VAX® reference genome. ⁴ Corresponding YFV protein and corresponding amino acid position in the protein. ⁵ Mutated amino acid and position in the protein as compared to the corresponding amino acid from the YF-VAX® reference.

TV4221 is identical to the reference sequence of YF-VAX® vaccine strain.

TV3111 has 3 mutations at position 2411 (E-480, Val to Leu), 3701 (NS2a-65, Met to Val) and 6496 (NS4a-19, silent).

TV3112 has the same mutations as TV3111, plus one additional mutation at position 1408 (E-145, silent).

TV3112 and TV3111 strains comprise an envelope protein represented by **SEQ ID NO 15** (with a leucine residue at position 480). **SEQ ID NO 16** (with a valine residue at position 65) is the sequence of the NS2a protein from TV3112 and TV3111 strains. **SEQ ID NO 17** (with a G nucleotide at position 57) is the RNA sequence coding for the NS4a protein from TV3112 strain. **SEQ ID NO 18** (with a U nucleotide at position 435) is the RNA sequence coding for the envelope protein from TV3112 strain.

It is well-known to the skilled person that the role of the genome is to be the support of information and that the proteins through their function have a role in the virus phenotype. Silent mutations have no impact on the function of the proteins. Accordingly, TV3112 and TV3111 strains can be described as live-attenuated yellow fever virus strains comprising a nucleic acid molecule encoding:

(i) an envelope protein comprising a mutation at position 480 which results in an amino acid change from valine to leucine, and

(ii) a NS2a protein comprising a mutation at position 65 which results in an amino acid change from methionine to valine. Or TV3112 and TV3111 strains can be described as live-attenuated yellow fever virus strains comprising a nucleic acid molecule encoding:

(i) an envelope protein which comprises a leucine residue at the position within the protein that corresponds to position 480 of SEQ ID NO. 15 ; and

(ii) an NS2a protein which comprises a valine residue at the position within the protein that corresponds to position 65 of SEQ ID NO. 16.

7.4.2) YF-VAX®-derived TV3112 strain, at the MSL and WSL stages

The consensus sequence of the TV3112 MSL remained identical to that of its pMSL parent (TV3112 pMSL). The consensus sequence of TV3112 WSL remained identical to its MSL parent (TV3112 MSL). TV3112 strain is genetically stable and keeps in its consensus sequence the mutations at nucleotide positions 1408, 2411, 3701 and 6496, from pMSL to WSL stages.

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