CLAIMS

1. A process for preparing an attenuated tetravalent dengue vaccine comprising:

(i) amplifying Vero cells in culture with low passage of passage 123 to produce Master and Working banks of Vero cells, wherein the Vero cells are adapted for growth in serum-free medium, are grown through successive subcultures in 225 cm^2 tissue culture (TC) flasks using serum-free medium and trypsin of non-animal origin and stabilized with 5% DMSO in order to detach the cells and then amplifying the Vero cells in a system for multilayer cell culture later;

(ii) infecting Vero cells from the Master or Working bank with attenuated dengue virus serotypes 1, 2, 3 and 4 from a Virus Master Seed (~~M~~VMS) or Virus Working Seed (VW~~V~~S) bank of each virus, wherein the Vero cells suspension are independently infected with attenuated dengue virus serotypes 1, 2, 3, and 4 in separate cultures with serum free medium under stirring in order to obtain the culture that will be used to prepare ~~the~~ dengue virus bulks ~~(BVS)~~ wherein the multiplicity-of-infection (MOI) for DENV1 and DENV4 is from 0.01 to 0.03, the MOI for DENV2 is from 0.02 to 0.04 and the MOI for DENV3 is from 0.05 to 0.08 and wherein ~~a~~the ~~M~~VMS ~~may prepared by or~~and the ~~W~~VWS ~~is~~ are prepared by infecting the Vero Cells from ~~the~~a Master or Working~~—~~ bank, respectively, in a 225 cm^2 tissue culture flasks or in a multilayer system for cell culture;

(iii) incubating the 225 cm^2 TC-flasks or multilayer system for cell culture containing the Vero cells infected with each dengue virus at 36.5°C (± 1°C) in an incubator for 10 to 20 days;

(iv) harvesting the supernatants of each culture by means of successive harvestings of supernatants of infected cultures, with replacement of culture medium;

(v) filtering each dengue virus suspension from step (iv) through a membrane with 0.2µm of porosity and storing the filtered dengue virus at -80°C (± 5°C);

(vi) preparing dengue virus bulks of the serotypes 1, 2, 3 and 4 by concentrating the harvests virus suspension, purifying the concentrate by tangential filtration and filtering it in membrane 0.2 µm;

(vii) formulating four monovalent vaccines one of each dengue serotype;

(viii) formulating tetravalent vaccine by mixing the monovalent vaccines;

(ix) filling vials with the tetravalent vaccine;

(x) lyophilizing the tetravalent vaccine in the vials;

(xi) sealing the lyophilized tetravalent vaccine in the vials; and

(xii) storing the lyophilized and sealed product at 2-8°C, thereby preparing an attenuated tetravalent dengue vaccine.

2. The process according to claim 1, wherein the supernatant is harvested in step (iv), an equal volume of serum free medium is added to the culture, and steps (iii) and (iv) are repeated.

3. The process according to claim 2, wherein steps (iii) and (iv) are repeated one to ~~about~~ seven times.

4. The process according to claim 1, wherein the dengue virus strains used are rDEN1Δ30-1545 (SEQ ID NO:1); rDEN2/4Δ30(ME)-1495,7163 (SEQ ID NO:2) ; rDEN3Δ30/31-7164 (SEQ ID NO:3), and rDEN4Δ30-7132,7163,8308 (SEQ ID NO:4).

5. A process for preparing an attenuated tetravalent dengue vaccine comprising:

(i) amplifying Vero cells with low passage (passage 123) in culture to produce Master and Working banks of Vero cells, wherein the Vero cells are adapted for growth in serum-free medium, are grown through successive subcultures in 225 cm^2 tissue culture flasks using serum-free medium with trypsin of non-animal origin and stabilized with 5% DMSO in order to detach the cells and then amplifying the Vero cells in a system for multilayer cell culture later;

(ii) infecting Vero cells from the Master or Working bank with attenuated dengue virus serotypes 1, 2, 3 and 4 from a Virus Master Seed (~~M~~VMS) or Virus Working Seed bank (~~W~~VWS) of each virus, wherein the Vero cells suspension are independently infected with attenuated dengue virus serotypes 1, 2, 3, and 4 in separate cultures with serum free medium under stirring in order to obtain the culture that will be used to prepare ~~the~~ dengue virus bulks~~virus bulks (BVS)~~ wherein the MOI for DENV1 and DENV4 is from 0.01 to 0.03, the MOI for DENV2 is from 0.02 to 0.04 and the MOI for DENV3 is from 0.05 to 0.08 and wherein ~~a~~the MVS ~~may be prepared or~~and the WVS ~~is~~ are prepared by infecting the Vero Cells from ~~the~~a Master or Working bank, respectively, in a 225 cm^2 tissue culture flasks or in a multilayer cell system;

(iii) incubating the Vero cells infected with each dengue virus obtained at step (ii) above at 36.5°C (± 1°C) for 10 to 20 days (10 to 15 days for DENV1 and DENV4, 12 to 17 for DENV2 and 13 to 20 days for DENV3) in a tissue culture flask or multilayer system for cell culture;

(iv) harvesting the supernatants of each culture by

means of successive harvestings of the supernatants of infected cultures, with replacement of culture medium;

(v) filtering each dengue virus suspension from step (iv) through a membrane with 0.2µm of porosity and storing the filtered dengue virus at -80°C (± 5°C);

(vi) preparing dengue virus bulks of the serotypes 1, 2, 3 and 4 by concentrating the harvests virus suspension, purifying the concentrate by tangential filtration and filtering it in membrane 0.2 µm;

(vii) formulating monovalent vaccines with dengue bulks; and

(viii) formulating tetravalent vaccine by mixing the monovalent vaccines.

6. The process according to claim 5 wherein the Vero cell line used is ATCC CCL-81.4.

7. The process according to claim 5, wherein the supernatant is harvested in step (iv), an equal volume of serum free medium is added to the culture, and steps (iii) and (iv) are repeated.

8. The process according to claim 7, wherein steps (iii) and (iv) are repeated one to ~~about~~ seven times.

9. The process according to claim 5, wherein the dengue virus strains used are rDEN1Δ30-1545 (SEQ ID NO:1) ; rDEN2/4Δ30(ME)-1495,7163 (SEQ ID NO:2) ; rDEN3Δ30/31-7164 (SEQ ID NO:3) , and rDEN4Δ30-7132,7163,8308 (SEQ ID NO:4).

~~10. The process according to claim 9, wherein the multiplicity-of-infection (MOI) of dengue virus strains for each dengue serotype is: 0.01 to 0.03 for DENV 1 and 4, 0.02 to 0.04 for DENV 2, and 0.05 to 0.08 for DENV3.~~

1~~1~~0. The process according to claim 5, wherein the monovalent vaccines are mixed in the same ratio of volume to

obtain the tetravalent dengue vaccine 1,2,3,4 ~~(attenuated)~~

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1~~2~~1. The process according to any one of claims 5 to 1~~1~~0, that further comprises the steps of filling vials with the tetravalent vaccine; lyophilizing the tetravalent vaccine in the vials; sealing the lyophilized tetravalent vaccine in the vials;
and storing the lyophilized and sealed product at 2-8°C.

1~~3~~2. The process according to claim 1~~2~~1, wherein the parameters used in the lyophilizing process are: freezing (-30 to -50°C), vacuum (20 to 100µbar), primary drying from -30 to -50°C (36 to 42h), and -5 to -10°C (18 to 24h) secondary drying 25 to 29°C (8 to 15h).

~~14. A process for preparing a tetravalent dengue vaccine for administration to a subject that comprises the step of reconstituting the sealed and lyophilized tetravalent dengue vaccine from any one of claims 1 to 4 or 12 to 13 in a composition comprising 0.2M sodium phosphate monobasic dihydrate, 0.2M sodium phosphate dibasic heptahydrate, and water.~~

1~~5~~3. A process for preparing a tetravalent dengue vaccine for administration to a subject that comprises the step of reconstituting the sealed and lyophilized tetravalent dengue vaccine from claim 4 ~~, 9~~ or 1~~2~~1 to 1~~3~~2 when dependent on claim ~~4 or~~ 9 in a composition comprising 0.2M sodium phosphate monobasic dihydrate, 0.2M sodium phosphate dibasic heptahydrate, and water.

~~16. The process according to claim 14 or 15, wherein the sealed and lyophilized vaccine is reconstituted in 5 milliliters of the composition.~~

~~17. A tetravalent dengue vaccine kit that comprises an~~

~~attenuated tetravalent dengue vaccine that is produced by the process as described in any one of claims 1 to 16, a reconstitution composition comprising 0.2M sodium phosphate monobasic dihydrate, 0.2M sodium phosphate dibasic heptahydrate and water.~~

~~18. A tetravalent dengue vaccine kit that comprises an attenuated tetravalent dengue vaccine that is produced by the process as described in any one of claims 4, 9; 12 to 13 when dependent on claim 4 or 9; or 14 to 15, a reconstitution composition comprising 0.2M sodium phosphate monobasic dihydrate, 0.2M sodium phosphate dibasic heptahydrate and water.~~