WHAT IS CLAIMED IS:

1. A cold-adapted temperature sensitive Enterovirus 71 strain.

2. The cold-adapted temperature sensitive Enterovirus 71 strain of claim 1, wherein the strain is EV71:TLLβP20.

3. The cold-adapted temperature sensitive Enterovirus 71 strain of claim 1, wherein the strain is EV71:TLLαP20.

4. A composition comprising the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3.

5. The composition of claim 4 which further comprises a pharmaceutically acceptable carrier.

6. The composition of claim 4 or 5 which further comprises an adjuvant.

7. The composition of any one of claims 4 to 7 which is a vaccine.

8. A cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for use in eliciting a protective immune response in a subject.

9. A cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for use in preventing a subject from becoming afflicted with an Enterovirus 71-associated disease.

10. A cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for use in delaying the onset of or slowing the rate of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject.

11. Use of cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for the manufacture of a medicament for eliciting a protective immune response in a subject.

12. Use of cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for the manufacture of a medicament for preventing a subject from becoming afflicted with an Enterovirus 71-associated disease.

13. Use of cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for the manufacture of a medicament for delaying the onset of or slowing the rate of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject.

14. A method of eliciting a protective immune response in a subject comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or the composition of any one of claim 4 to 8.

15. The method of claim 14, wherein the subject has been exposed to wild-type Enterovirus 71.

16. A method of preventing a subject from becoming afflicted with an Enterovirus 71-associated disease comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or the composition of any one of claim 4 to 8.

17. The method of claim 16, wherein the subject has been exposed to wild-type Enterovirus 71.

18. A method of delaying the onset of or slowing the rate of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of the cold-adapted

temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or the composition of any one of claim 4 to 8.

19. The method of any one of claims 14 to 18, wherein the subject is a human subject.

20. A kit for immunization of a subject with of a cold-adapted temperature sensitive Enterovirus 71 strain comprising the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claims 4 to 8, a pharmaceutically acceptable carrier, and an instructional material for the use thereof.

21. The kit of claim 20 which further comprises an applicator.

22. A method of producing a cold-adapted temperature sensitive virus comprising the following steps:

(i) preparing a reference stock of parental wild-type virus;

(ii) incubating a culture of cells infected with the reference stock of parental wild-type virus at higher multiplicity of infection (MOI) and an incubation temperature of about 34° C to about 36° C, preferably about 34° C, for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE;

(iii) incubating a culture of cells infected with the resultant virus of the previous step at higher MOI and an incubation temperature of about 1° C to about 3° C lower than in the previous step for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE; and

(iv) repeating step (iii) in a systematic stepwise manner of incremental lower incubation temperature until the targeted temperature chosen for optimal replication of the virus is achieved.

23. The method of claim 22, wherein the reference stock of parental wild-type virus is prepared by incubating a culture of cells infected with a wild-type virus at a temperature of about 36° C to about 38° C, preferably about 37° C, for one or two passages until a full cytopathic effect (CPE) is obtained.

24. The method of claim 22 or 23, wherein the virus is a member of the Picornaviridae family, preferably the Enterovirus genus.

25. The method of claim 24, wherein the virus is Enterovirus 71 or coxsackievirus A16.

26. The method of any of claims 22-25, wherein the targeted temperature is about 26° C to about 29° C, preferably about 28° C.

27. The method of any of claims 22-26, wherein the virus is passaged in each step of the process by obtaining a clarified culture supernatant containing the virus and passing this supernatant into each successive fresh new culture flask of cells as soon as the virus obtains a full cytopathic effect (CPE).

28. The method of any of claims 22-27, wherein the higher MOI is 20.

29. The method of claim 28, wherein a new culture flask containing freshly confluent monolayer Vero cells is inoculated with virus at the MOI of 20 for three more passages after the virus causes a full CPE.

30. The method of any of claims 22-29, wherein the lower MOI is 5 to 10.

31. The method of claim 30, wherein a new culture flask containing freshly confluent monolayer Vero cells is inoculated with virus at the MOI of 5 to 10 for least three more passages after the virus causes a full CPE.

32. The method of claim 31, wherein step (iii) is initiated or repeated after said at least three more passages at the MOI of 5 to 10.

33. The method of any one of claims 27 to 32, wherein the full CPE occurs within 3 days after inoculation.

34. The method of any of claims 22-33, wherein the cells are Vero cells.

35. The method of any of claims 22-34, wherein the Vero cells are cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with fetal calf serum (FCS).

36. The method of claim 35, wherein the medium is supplemented with 1% FCS.

~~44~~ 39

AMENDED CLAIMS
received by the International Bureau on 22 October 2013 (22.10.13)

1. A cold-adapted temperature sensitive Enterovirus 71 strain.

2. The cold-adapted temperature sensitive Enterovirus 71 strain of claim 1, wherein the strain is EV71:TLLβP20.

3. The cold-adapted temperature sensitive Enterovirus 71 strain of claim 1, wherein the strain is EV71:TLLαP20.

4. A composition comprising the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3.

5. The composition of claim 4 which further comprises a pharmaceutically acceptable carrier.

6. The composition of claim 4 or 5 which further comprises an adjuvant.

7. The composition of any one of claims 4 to 7 which is a vaccine.

8. A cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for use in eliciting a protective immune response in a subject.

9. A cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for use in preventing a subject from becoming afflicted with an Enterovirus 71-associated disease.

10. A cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for use in delaying the onset of or slowing the rate of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject.

11. Use of cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for the manufacture of a medicament for eliciting a protective immune response in a subject.

12. Use of cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for the manufacture of a medicament for preventing a subject from becoming afflicted with an Enterovirus 71-associated disease.

13. Use of cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claim 4 to 8 for the manufacture of a medicament for delaying the onset of or slowing the rate of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject.

14. A method of eliciting a protective immune response in a subject comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or the composition of any one of claim 4 to 8.

15. The method of claim 14, wherein the subject has been exposed to wild-type Enterovirus 71.

16. A method of preventing a subject from becoming afflicted with an Enterovirus 71-associated disease comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or the composition of any one of claim 4 to 8.

17. The method of claim 16, wherein the subject has been exposed to wild-type Enterovirus 71.

18. A method of delaying the onset of or slowing the rate of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of the cold-adapted

temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or the composition of any one of claim 4 to 8.

19. The method of any one of claims 14 to 18, wherein the subject is a human subject.

20. A kit for immunization of a subject with of a cold-adapted temperature sensitive Enterovirus 71 strain comprising the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or a composition of any one of claims 4 to 8, a pharmaceutically acceptable carrier, and an instructional material for the use thereof.

21. The kit of claim 20 which further comprises an applicator.

22. A method of producing a cold-adapted temperature sensitive virus comprising the following steps:

(i) preparing a reference stock of parental wild-type virus;

(ii) incubating a culture of cells infected with the reference stock of parental wild-type virus at higher multiplicity of infection (MOI) and an incubation temperature of about 34° C to about 36° C, preferably about 34° C, for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE;

(iii) incubating a culture of cells infected with the resultant virus of the previous step at higher MOI and an incubation temperature of about 1° C to about 3° C lower than in the previous step for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE; and

(iv) repeating step (iii) in a systematic stepwise manner of incremental lower incubation temperature until the targeted temperature chosen for optimal replication of the virus is achieved.

23. The method of claim 22, wherein the reference stock of parental wild-type virus is prepared by incubating a culture of cells infected with a wild-type virus at a temperature of about 36° C to about 38° C, preferably about 37° C, for one or two passages until a full cytopathic effect (CPE) is obtained.

24. The method of claim 22 or 23, wherein the virus is a member of the Picornaviridae family, preferably the Enterovirus genus.

25. The method of claim 24, wherein the virus is Enterovirus 71 or coxsackievirus A16.

26. The method of any of claims 22-25, wherein the targeted temperature is about 26° C to about 29° C, preferably about 28° C.

27. The method of any of claims 22-26, wherein the virus is passaged in each step of the process by obtaining a clarified culture supernatant containing the virus and passing this supernatant into each successive fresh new culture flask of cells as soon as the virus obtains a full cytopathic effect (CPE).

28. The method of any of claims 22-27, wherein the higher MOI is 20.

29. The method of claim 28, wherein a new culture flask containing freshly confluent monolayer Vero cells is inoculated with virus at the MOI of 20 for three more passages after the virus causes a full CPE.

30. The method of any of claims 22-29, wherein the lower MOI is 5 to 10.

31. The method of claim 30, wherein a new culture flask containing freshly confluent monolayer Vero cells is inoculated with virus at the MOI of 5 to 10 for least three more passages after the virus causes a full CPE.

32. The method of claim 31, wherein step (iii) is initiated or repeated after said at least three more passages at the MOI of 5 to 10.

33. The method of any one of claims 27 to 32, wherein the full CPE occurs within 3 days after inoculation.

34. The method of any of claims 22-33, wherein the cells are Vero cells.

35. The method of any of claims 22-34, wherein the Vero cells are cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with fetal calf serum (FCS).

36. The method of claim 35, wherein the medium is supplemented with 1% FCS.

37. A method of making a vaccine comprising using the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3.

38. A method of making a vaccine comprising using a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:6 or SEQ ID NO:7.

39. Use of the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 for vaccine development.

40. Use of a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:6 or SEQ ID NO:7 for vaccine development.

41. A cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 for use in vaccine development.

42. A nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:6 or SEQ ID NO:7 for use in vaccine development.

43. Use of the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3.

44. Use of a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:6 or SEQ ID NO:7.

WHAT IS CLAIMED IS:

1. A cold-adapted temperature sensitive Enterovirus 71 strain which is the strain EV71:TLLβP20 as deposited with the American Type Culture Collection under Accession No. PTA-13285 or the strain EV71:TLLαP20 as deposited with the American Type Culture Collection under Accession No. PTA-13284.

~~2. The cold-adapted temperature sensitive Enterovirus 71 strain of claim 1, wherein the strain is EV71:TLLβP20.~~

~~3. The cold-adapted temperature sensitive Enterovirus 71 strain of claim 1, wherein the strain is EV71:TLLαP20.~~

24. A composition comprising the cold-adapted temperature sensitive Enterovirus 71 strain of ~~any one of~~ claims 1 ~~to 3~~.

35. The composition of claim 24 which further comprises a pharmaceutically acceptable carrier.

46. The composition of claim 24 or 35 which further comprises an adjuvant.

57. The composition of any one of claims 24 to 47 which is a vaccine.

68. A cold-adapted temperature sensitive Enterovirus 71 strain of ~~any one of~~ claims 1 ~~to 3~~ or a composition of any one of claims 24 to 58 for use in eliciting a protective immune response in a subject.

79. A cold-adapted temperature sensitive Enterovirus 71 strain of ~~any one of~~ claims 1 ~~to 3~~ or a composition of any one of claims 24 to 58 for use in preventing a subject from becoming afflicted with an Enterovirus 71-associated disease.

810. A cold-adapted temperature sensitive Enterovirus 71 strain of ~~any one of~~ claims 1 ~~to 3~~ or a composition of any one of claims 24 to 58 for use in delaying the onset of or slowing the rate of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject.

9~~11~~. Use of cold-adapted temperature sensitive Enterovirus 71 strain of ~~any one of~~ claims 1 ~~to 3~~ or a composition of any one of claims 2~~4~~ to 5~~8~~ for the manufacture of a medicament for eliciting a protective immune response in a subject against an Enterovirus 71-associated disease.

10~~12~~. Use of cold-adapted temperature sensitive Enterovirus 71 strain of ~~any one of~~ claims 1 ~~to 3~~ or a composition of any one of claims 2~~4~~ to 5~~8~~ for the manufacture of a medicament for preventing a subject from becoming afflicted with an Enterovirus 71-associated disease.

11~~13~~. Use of cold-adapted temperature sensitive Enterovirus 71 strain of any ~~one of~~ claims 1 ~~to 3~~ or a composition of any one of claims 2~~4~~ to 5~~8~~ for the manufacture of a medicament for delaying the onset of or slowing the rate of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject.

12. The Enterovirus strain according to claim 1 or the composition according to any one of claims 2 to 5 for eliciting a protective immune response in a subject against Enterovirus 71.

13. The Enterovirus strain or composition of claim 12, wherein the subject has been exposed to wild-type Enterovirus 71.

14. The Enterovirus strain according to claim 1 or the composition according to any one of claims 2 to 5 for preventing a subject from becoming afflicted with an Enterovirus 71-associated disease.

15. The Enterovirus strain or composition of claim 14, wherein the subject has been exposed to wild-type Enterovirus 71.

16. The Enterovirus strain according to claim 1 or the composition according to any one of claims 2 to 5 for slowing the onset of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject.

17. The Enterovirus strain or composition of any one of claims 12 to 16, wherein the subject is a human subject.

~~14. A method of eliciting a protective immune response in a subject comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of the cold-adapted~~

~~temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or the composition of any one of claim 4 to 8.~~

~~15. The method of claim 14, wherein the subject has been exposed to wild type Enterovirus 71.~~

~~16. A method of preventing a subject from becoming afflicted with an Enterovirus 71 associated disease comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of the cold adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or the composition of any one of claim 4 to 8.~~

~~17. The method of claim 16, wherein the subject has been exposed to wild type Enterovirus 71.~~

~~18. A method of delaying the onset of or slowing the rate of an Enterovirus 71 associated disease in an Enterovirus 71 infected subject comprising administering to the subject a prophylactically, therapeutically or immunologically effect amount of the cold adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3 or the composition of any one of claim 4 to 8.~~

~~19. The method of any one of claims 14 to 18, wherein the subject is a human subject.~~

18~~20~~. A kit for immunization of a subject with of a cold-adapted temperature sensitive Enterovirus 71 strain comprising the cold-adapted temperature sensitive Enterovirus 71 strain of ~~any one of~~ claims 1 ~~to 3~~ or a composition of any one of claims 24 to 5~~8~~, a pharmaceutically acceptable carrier, and an instructional material for the use thereof.

19~~21~~. The kit of claim 18~~20~~ which further comprises an applicator.

~~22. A method of producing a cold adapted temperature sensitive virus comprising the following steps:~~

~~(i) preparing a reference stock of parental wild type virus;~~

~~(ii) incubating a culture of cells infected with the reference stock of parental wild type virus at higher multiplicity of infection (MOI) and an incubation temperature of about 34° C to about 36° C, preferably about 34° C, for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE;~~

~~(iii) incubating a culture of cells infected with the resultant virus of the previous step at higher MOI and an incubation temperature of about 1° C to about 3° C lower than in the previous step for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE; and~~

~~(iv) repeating step (iii) in a systematic stepwise manner of incremental lower incubation temperature until the targeted temperature chosen for optimal replication of the virus is achieved.~~

~~23. The method of claim 22, wherein the reference stock of parental wild-type virus is prepared by incubating a culture of cells infected with a wild-type virus at a temperature of about 36° C to about 38° C, preferably about 37° C, for one or two passages until a full cytopathic effect (CPE) is obtained.~~

~~24. The method of claim 22 or 23, wherein the virus is a member of the Picornaviridae family, preferably the Enterovirus genus.~~

~~25. The method of claim 24, wherein the virus is Enterovirus 71 or coxsackievirus A16.~~

~~26. The method of any of claims 22-25, wherein the targeted temperature is about 26° C to about 29° C, preferably about 28° C.~~

~~27. The method of any of claims 22-26, wherein the virus is passaged in each step of the process by obtaining a clarified culture supernatant containing the virus and passing this supernatant into each successive fresh new culture flask of cells as soon as the virus obtains a full cytopathic effect (CPE).~~

~~28. The method of any of claims 22-27, wherein the higher MOI is 20.~~

~~29. The method of claim 28, wherein a new culture flask containing freshly confluent monolayer Vero cells is inoculated with virus at the MOI of 20 for three more passages after the virus causes a full CPE.~~

~~30. The method of any of claims 22-29, wherein the lower MOI is 5 to 10.~~

~~31. The method of claim 30, wherein a new culture flask containing freshly confluent monolayer Vero cells is inoculated with virus at the MOI of 5 to 10 for least three more passages after the virus causes a full CPE.~~

~~32. The method of claim 31, wherein step (iii) is initiated or repeated after said at least three more passages at the MOI of 5 to 10.~~

~~33. The method of any one of claims 27 to 32, wherein the full CPE occurs within 3 days after inoculation.~~

~~34. The method of any of claims 22 33, wherein the cells are Vero cells.~~

~~35. The method of any of claims 22 34, wherein the Vero cells are cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with fetal calf serum (FCS).~~

~~36. The method of claim 35, wherein the medium is supplemented with 1% FCS.~~

20~~37~~. A method of making a vaccine comprising using the cold-adapted temperature sensitive Enterovirus 71 strain of ~~any one of~~ claims 1 ~~to 3~~.

~~38. A method of making a vaccine comprising using a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:6 or SEQ ID NO:7.~~

21~~39~~. Use of the cold-adapted temperature sensitive Enterovirus 71 strain of ~~any one of~~ claims 1 ~~to 3~~ for vaccine development.

~~40. Use of a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:6 or SEQ ID NO:7 for vaccine development.~~

22~~41~~. A cold-adapted temperature sensitive Enterovirus 71 strain of ~~any one of~~ claims 1 ~~to 3~~ for use in vaccine development.

~~42. A nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:6 or SEQ ID NO:7 for use in vaccine development.~~

~~43. Use of the cold-adapted temperature sensitive Enterovirus 71 strain of any one of claims 1 to 3.~~

~~44. Use of a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:6 or SEQ ID NO:7.~~

23. A strain according to claim 1 or a composition of any one of claims 2 to 5 for use as a medicament.

24. Use of the strain according to claim 1 or a composition of any one of claims 2 to 5 for the manufacture of a medicament for the treatment of hand foot and mouth disease.