Methods and Compositions for Producing a Virus

CLAIMS

1. A method for generating a recombinant adenovirus comprising a nucleotide sequence encoding a heterologous gene of interest for use as a vaccine comprising the steps of:

(i) inserting the heterologous gene of interest into the adenovirus genome by recombining terminal protein complexed adenovirus genomic DNA (TPC-Ad gDNA) with a polynucleotide comprising a nucleotide sequence encoding the gene of interest and having 5' and 3' ends that are homologous to the insertion site sequence of the adenovirus genomic DNA in an *in vitro* recombination reaction,

(ii) transfecting cells growing in individual vessels with a dilution of the *in vitro* recombination reaction mixture from (i) such that a number of such individual vessels contain a single cell that is infected by a recombinant adenovirus comprising the nucleotide sequence encoding the heterologous gene of interest, and

(iii) identifying those individual vessels in which a single cell has been infected by the recombinant adenovirus comprising the nucleotide sequence encoding the heterologous gene of interest.

2. A method according to claim 1, wherein the TPC-Ad gDNA comprises serotype-matched terminal protein and adenovirus genome.

3. A method according to claim 1 or claim 2, wherein the gene of interest codes for a single epitope, a string of epitopes, a segment of an antigen or a complete antigen protein.

4. A method according to any previous claim, wherein the polynucleotide is a synthetic DNA molecule, a purified DNA restriction fragment or a polymerase chain reaction (PCR) product.

5. A method according to any previous claim, wherein the polynucleotide has between 5 and 50bp at its 5' end and between 5 and 50bp at its 3' end that are homologous to the insertion site sequence of the adenovirus genomic DNA.

6. A method according to claim 5, wherein the polynucleotide has between 10 and 20bp at its 5' end and between 10 and 20bp at its 3' end that are homologous to the insertion site sequence of the adenovirus genomic DNA.

7. A method according to claim 6, wherein the polynucleotide has 15bp at its 5' end and 15bp at its 3' end that are homologous to the insertion site sequence of the adenovirus genomic DNA.

8. A method according to any previous claim, wherein the insertion site sequence of the adenovirus genomic DNA is located within the E1 locus.

9. A method according to any previous claim, wherein the TPC-Ad gDNA is digested at a unique restriction site within the insertion site sequence of the adenovirus genomic DNA that is flanked at its 5' end by the long tetracycline-regulated CMV promoter that drives expression of the gene of interest and at its 3' end by the bovine growth hormone polyadenylation sequence.

10. A method according to any previous claim, wherein the *in vitro* recombination reaction comprises 40ng digested TPC-Ad gDNA and 44fmol 3' and 5' ends of synthetic DNA encoding the gene of interest.

11. A method according to any previous claim, wherein cells to be transfected are seeded in individual vessels at a density of 3.75×10^5 cells.ml^{-1} one day before transfection.

12. A method according to any previous claim, wherein cells are transfected while growing at approximately 80% confluence in individual vessels.

13. A method according to any previous claim, wherein the cells being transfected stably express the tetracycline repressor.

14. A method according to any previous claim, wherein the *in vitro* recombination reaction mixture is diluted in transfection medium and divided equally so as to transfect cells growing in 60 individual vessels.

15. A method according to any previous claim, wherein transfected cells are frozen and thawed to release cell-associated virus and presence of recombinant adenovirus is identified by quantitative PCR (qPCR) using cell lysate from each well of transfected cells and a set of primers and a probe designed to bind to the left end of the genome downstream of the adenoviral inverted terminal repeat (ITR) and upstream of the insertion site of the gene of interest in a non-coding region.

16. A method according to any previous claim, wherein the adenovirus genome is derived from a simian adenovirus.

17. A method according to claim 16, wherein the simian adenovirus is ChAdOx1 or ChAdOx2 or ChAd3 or ChAd63.

18. A method according to any one of claims 1 to 15, wherein the adenovirus genome is derived from a human adenovirus.

19. A method according to claim 18, wherein the human adenovirus is not a serotype 5 human adenovirus.

20. A method according to any previous claim, wherein the individual vessels are separate wells in a multiwell plate.

21. A method according to any previous claim, wherein the TPC-Ad gDNA is

provided from a stock of TPC-Ad gDNA material that has undergone and passed requisite quality control assays allowing use in good manufacturing practice (GMP) biomanufacture.

22. A recombinant adenovirus produced by the method according to any previous claim.

23. A composition that comprises an adenoviral genome in which the E1 gene is replaced by an expression cassette comprising a DNA sequence encoding a fluorescent marker protein flanked by a first pair of unique restriction sites not present anywhere else in the adenoviral genome for use in a method of any previous claim.

24. A composition according to claim 23, wherein the adenoviral genome is derived from a simian adenovirus.

25. A composition according to claim 24, wherein the simian adenovirus is ChAdOx1 or ChAdOx2 or ChAd3 or ChAd63.

26. A composition according to claim 23, wherein the adenovirus genome is derived from a human adenovirus.

27. A composition according to claim 26, wherein the human adenovirus is not a serotype 5 human adenovirus.

28. A composition according to any one of claims 23 to 27, wherein the fluorescent marker protein is green fluorescent protein (GFP).

29. A composition according to any one of claims 23 to 28, wherein the first pair of unique restriction sites are selected from PsiI, AsiSi or RsrII sites.

30. A composition according to any one of claims 23 to 29, wherein the expression cassette further comprises the long tetracycline-regulated CMV promoter 5' to the DNA sequence encoding the fluorescent marker protein and the bovine growth hormone polyadenylation sequence located 3' to the DNA sequence encoding the fluorescent marker protein, wherein the first pair of unique restriction sites are located between the long tetracycline-regulated CMV promoter and the DNA sequence encoding the fluorescent marker protein and between the DNA sequence encoding the fluorescent marker protein and the bovine growth hormone polyadenylation sequence.

31. A composition according to claim 30, wherein the expression cassette further comprises a second pair of unique restriction sites that are different to the first pair of unique restriction sites and are located 5' to the long tetracycline-regulated CMV promoter and 3' to the bovine growth hormone polyadenylation sequence.

32. A composition according to claim 31, wherein second pair of unique restriction sites are selected from PsiI, AsiSi or RsrII sites.

33. A composition according to any one of claims 23 to 32, wherein the adenoviral genome is further engineered to comprise an additional unique restriction site at the S15/E4 locus.

34. A composition according to claim 33, wherein the additional unique restriction site is selected from PsiI, AsiSi or RsrII sites.

35. A composition according to any one of claims 23 to 34, wherein the adenoviral genome is complexed with autologous terminal protein.

36. A composition according to any one of claims 23 to 34, wherein the adenoviral genome is complexed with heterologous terminal protein.

37. A composition according to any one of claims 23 to 36, wherein the adenoviral vector lacks Gateway recombination sequences.

38. A composition according to any one of claims 23 to 37 that has undergone and passed requisite quality control assays allowing use in GMP biomanufacture.

39. A recombinant adenoviral vector immunogen comprising any of the compositions of claims 23 to 37 which expresses a pathogen or tumour epitope or antigen to which an immune response is generated in a mammal.

CLAIMS

1. A method for generating a recombinant adenovirus comprising a nucleotide sequence encoding a heterologous gene of interest for use as a vaccine comprising the steps of:

(i) inserting the heterologous gene of interest into the adenovirus genome by recombining terminal protein complexed adenovirus genomic DNA (TPC-Ad gDNA) with a polynucleotide comprising a nucleotide sequence encoding the gene of interest and having 5' and 3' ends that are homologous to the insertion site sequence of the adenovirus genomic DNA in an *in vitro* recombination reaction,

(ii) transfecting cells growing in individual vessels with a dilution of the *in vitro* recombination reaction mixture from (i) such that a number of such individual vessels contain a single cell that is infected by a recombinant adenovirus comprising the nucleotide sequence encoding the heterologous gene of interest, and

(iii) identifying those individual vessels in which a single cell has been infected by the recombinant adenovirus comprising the nucleotide sequence encoding the heterologous gene of interest.

2. A method according to Claim 1, in which the TPC-Ad gDNA comprises serotype-matched terminal protein and adenovirus genome.

3. A method according to Claim 1 or 2, in which the gene of interest codes for a single epitope, a string of epitopes, a segment of an antigen or a complete antigen protein.

4. A method according to Claim 1, 2 or 3, in which the polynucleotide has between 5 and 50bp at its 5' end and between 5 and 50bp at its 3' end that are homologous to the insertion site sequence of the adenovirus genomic DNA, or in which the polynucleotide has between 10 and 20bp at its 5' end and between 10 and 20bp at its 3' end that are homologous to the insertion site sequence of the adenovirus genomic DNA, or in which the polynucleotide has 15bp at its 5' end and 15bp at its 3' end that are homologous to the insertion site sequence of the adenovirus genomic DNA.

5. A method according to any preceding claim, in which the insertion site sequence of the adenovirus genomic DNA is located within the E1 locus.

6. A method according to any preceding claim, in which the TPC-Ad gDNA is digested at a unique restriction site within the insertion site sequence of the adenovirus genomic DNA that is flanked at its 5' end by the long tetracycline-regulated CMV promoter that drives expression of the gene of interest and at its 3' end by the bovine growth hormone polyadenylation sequence.

7. A method according to any preceding claim, in which the *in vitro* recombination reaction comprises 40ng digested TPC-Ad gDNA and 44fmol 3' and 5' ends of synthetic DNA encoding the gene of interest.

8. A method according to any preceding claim, in which cells to be transfected are seeded in individual vessels at a density of 3.75×10^5 cells.ml^{-1} one day before transfection.

9. A method according to any preceding claim, in which cells are transfected while growing at approximately 80% confluence in individual vessels.

10. A method according to any preceding claim, in which the *in vitro* recombination reaction mixture is diluted in transfection medium and divided equally so as to transfect cells growing in 60 individual vessels.

11. A method according to any preceding claim, in which transfected cells are frozen and thawed to release cell-associated virus and presence of recombinant adenovirus is identified by quantitative PCR (qPCR) using cell lysate from each well of transfected cells and a set of primers and a probe designed to bind to the left end of the genome downstream of the adenoviral inverted terminal repeat (ITR) and upstream of the insertion site of the gene of interest in a non-coding region.

12. A method according to any preceding claim, in which the adenovirus genome is derived from a simian adenovirus.

13. A method according to Claim 12, in which the simian adenovirus is ChAdOx1 or ChAdOx2 or ChAd3 or ChAd63.

14. A method according to any of Claims 1 to 11, in which the adenovirus genome is derived from a human adenovirus.

15. A recombinant adenovirus produced by the method according to any preceding claim.