CLAIMS

We claim:

1. A method of purifying a recombinant AAV (rAAV) vector, the method comprising:
loading a solution comprising the rAAV vector on an affinity chromatography stationary phase;
applying a pre-elution wash solution to the stationary phase, wherein the pre-elution wash solution comprises 15% to 25% ethanol and a buffering agent, and has a pH of 5 to 6;
eluting the rAAV vector from the stationary phase with an elution buffer, wherein the elution buffer comprises a salt, an amino acid and a buffering agent and has a pH of 2 to 4, to produce an affinity eluate.

2. The method of claim 1, wherein the affinity chromatography stationary phase is in a column.

3. The method of any one of claims 1-2, wherein the elution buffer comprises 5 mM to 150 mM of the salt, optionally wherein the salt is magnesium chloride.

4. The method of any one of claims 1-3, wherein the elution buffer comprises 50 mM to 150 mM of the amino acid, optionally wherein the amino acid is glycine.

5. The method of any one of claims 1-4, wherein the elution buffer comprises 75 mM to 250 mM of the buffering agent, optionally wherein the buffering agent is sodium acetate.

6. The method of any one of claims 1-5, wherein the elution buffer has a pH of 2.5 to 3.5.

7. The method of any one of claims 1-6, wherein the elution buffer comprises 50 mM to 150 mM of glycine, 10 mM to 100 mM of $MgCl_2$, 50 mM to 200 mM of sodium acetate, and a pH of 2.5 to 3.5, optionally wherein the elution buffer has a conductivity of 5 mS/cm to 40 mS/cm or 20 mS/cm to 35 mS/cm.

8. The method of any one of claims 1-7, wherein 2 column volumes (CV) to 10 CV or 4.5 CV to 5.5 CV of the elution buffer is applied to the stationary phase.

9. The method of any one of claims 1-8, wherein the elution buffer i) elutes the rAAV vector from the stationary phase, ii) does not elute residual impurities from the stationary phase; iii) does not result in precipitation of the affinity eluate; iv) maximizes a % vg recovery; v) does not interfere with binding of the rAAV vector to an anion exchange chromatography (AEX) stationary phase; vi) does not contain a trivalent anion; vii) does not contain citrate ions, or a

combination thereof.

10. The method of any one of claims 1-9, wherein 0.1 CV to 10 CV of the affinity eluate is collected from the stationary phase.

11. The method of any one of claims 1-10, wherein the solution comprising the rAAV vector comprises host cell protein and host cell DNA.

12. The method of any one of claims 1-11, wherein the solution comprising the rAAV vector is loaded onto the stationary phase to achieve a challenge of 1 x 10^{12} viral genomes (vg)/mL stationary phase to 1.5 x 10^{14} vg/mL stationary phase.

13. The method of any one of claims 1-12, wherein the rAAV vector comprises a capsid protein from an AAV serotype.

14. The method of claim 13, wherein the rAAV vector comprises a capsid protein from a AAV serotype selected from the group consisting of AAV1, AAV2, AAV3 (including AAV3A and AAV3B), AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV12, AAVrh8, AAVrh10, AAVrh39, AAVrh43, AAVrh74, AAVrh32.22, AAV1.1, AAV2.5, AAV6.1, AAV6.2, AAV6.3.1, AAV9.45, AAVShH10, HSC15/17, RHM4-1, RHM15-1, RHM15-2, RHM15-3/RHM15-5, RHM15-4, RHM15-6, AAVhu.26, AAV2i8, AAV29G, AAV2, AAV8G9, AAV-LK03, AAV2-TT, AAV2-TT-S312N, AAV3B-S312N, AAVavian, AAVbat, AAVbovine, AAVcanine, AAVequine, AAVprimate, AAVnon-primate, AAVovine, AAVmuscovy duck, AAVporcine4, AAVporcine5, AAVsnake NP4, NP22, NP66, AAVDJ, AAVDJ/8, AAVDJ/9, AAVHSC1, AAVHSC2, AAVHSC3, AAVHSC4, AAVHSC5, AAVHSC6, AAVHSC7, AAVHSC8, AAVHSC9, AAVHSC10, AAVHSC11, AAVHSC12, AAVHSC13, AAVHSC14, AAVHSC15, AAVv66, AAVv33, AAVv37, AAVv40, AAVv67, AAVv70, AAVv72, AAVv84, AAVv86, AAVv87 and AAVv90.

15. The method of any one of claims 1-14, wherein the stationary phase comprises a macromolecule that binds an AAV capsid.

16. The method of any one of claims 1-15, wherein the pre-elution wash solution comprises 10 mM to 500 mM of the buffering agent, optionally wherein the buffering agent is sodium acetate.

17. The method of any one of claims 1-16, wherein the pre-elution wash solution comprises 15% to 25% of ethanol, 100 mM to 200 mM of sodium acetate, and has a pH of 5 to 6, optionally wherein 1 CV to 10 CV or 4.5 to 5.5 CV of the pre-elution wash solution is applied to

the stationary phase.

18. The method of any one of claims 1-17, wherein the pre-elution wash i) removes bound impurities from the stationary phase; ii) maintains rAAV-stationary phase ligand binding; iii) has a reduced pH to improve removal of proteins other than rAAV proteins, such as host cell proteins, or a combination thereof.

19. The method of any one of claims 1-18, further comprising equilibration of the stationary phase i) prior to loading the solution comprising the rAAV vector on the stationary phase, ii) after loading the solution comprising the rAAV vector on the stationary phase, iii) prior to application of a pre-elution wash, iv) after application of a pre-elution wash v) prior to eluting the rAAV vector from the stationary phase with an elution buffer, vi) after eluting the rAAV vector from the stationary phase with an elution buffer, vii) prior to contacting the stationary phase with a first regeneration buffer, or a combination thereof.

20. The method of claim 19, wherein equilibration comprises application of a buffer solution to the stationary phase and removal of all or a portion of the buffer solution from the stationary phase.

21. The method of claim 20, wherein the buffer solution comprises Tris or sodium acetate.

22. The method of claim 19 or 20, wherein the buffer solution comprises about 100 mM Tris at about pH 7.5 or about 153 mM sodium acetate at about pH 5.6.

23. The method of any one of claims 1-22, further comprising contacting the stationary phase with a first regeneration buffer after obtaining at least a portion of the affinity eluate.

24. The method of claim 23, wherein the first regeneration buffer comprises 0.05 N to 1.5 N of an acid, optionally wherein the acid is phosphoric acid.

25. The method of claim 23 or 24, wherein the first regeneration buffer comprises 0.10 N to 0.15 N phosphoric acid, and a pH of 1.5 to 2.5.

26. The method of any one of claims 23-25, wherein the stationary phase is contacted with 1 CV to 10 CV or 4.5 CV to 5.5 CV of the first regeneration buffer.

27. The method of any one of claims 23-26, wherein the method further comprises loading another solution comprising an rAAV vector on the affinity chromatography stationary phase

after removing at least a portion of the first regeneration buffer.

28. The method of any one of claims 23-27, wherein the method further comprises applying a second amount of the pre-elution wash solution on the affinity chromatography stationary phase after removing at least a portion of the first regeneration buffer.

29. The method of any one of claims 23-28, wherein a number of purification cycles that can be run on the stationary phase that is contacted with the first regeneration buffer is 8 or more;

wherein a number of purification cycles that can be run on the stationary phase that is contacted with the first regeneration buffer is 10 or more;

wherein a % vg recovery of an eluate from a last purification cycle after contacting the stationary phase with the first regeneration buffer is not decreased more than 10% as compared to a % vg recovery of an eluate from a first purification cycle before the stationary phase is contacted with the first regeneration buffer;

wherein an amount of unbound rAAV vector in a flow through of a last purification cycle after contacting the stationary phase with the first regeneration buffer is not increased more than 10% as compared to an amount of unbound rAAV vector in a flow through of a first purification cycle before the stationary phase is contacted with the first regeneration buffer;

wherein a column pressure of a last purification cycle after contacting the stationary phase with the first regeneration buffer is not higher than 0.4 MPa;

wherein a % purity of an eluate from a last purification cycle after contacting the stationary phase with the first regeneration buffer is not decreased more than 10% as compared to a % purity of an eluate from a first purification cycle before the stationary phase is contacted with the first regeneration buffer;

wherein an amount of HCP of an eluate from a last purification cycle after contacting the stationary phase with the first regeneration buffer is not increased more than 10% as compared to an amount of HCP of an eluate from a first purification cycle before the stationary phase is contacted with the first regeneration buffer;

wherein an amount of HCDNA of an eluate from a last purification cycle after contacting the stationary phase with the first regeneration buffer is not increased more than 10% as compared to an amount of HCDNA of an eluate from a first purification cycle before the stationary phase is contacted with the first regeneration buffer;

wherein an average A260/A280 of an eluate from a last purification cycle after contacting the stationary phase with the first regeneration buffer is not decreased more than 10% as compared to an average A260/A280 of an eluate from a first purification cycle before the stationary phase is contacted with the first regeneration buffer, or a combination thereof.

30. The method of any one of claims 23-29, wherein a number of purifications cycles that

can be run on the stationary phase that is contacted with the first regeneration buffer is increased as compared to a stationary phase that is contacted with a buffer comprising guanidine HCl.

31. The method of any one of claims 23-30, further comprising contacting the stationary phase with a second regeneration buffer after contacting the stationary phase with the first regeneration buffer, wherein the second regeneration buffer is different than the first regeneration buffer.

32. The method of claim 31, wherein the second regeneration buffer comprises 0.1% to 5% of a detergent, optionally wherein the detergent is sarkosyl.

33. The method of claim 31 or 32, wherein the second regeneration buffer comprises 50 mM to 150 mM of a buffering agent, optionally wherein the buffering agent is Tris.

34. The method of any one of claims 31-33, wherein the second regeneration buffer comprises 0.1% to 1.5% of sarkosyl, 50 mM to 150 mM of Tris, and a pH of 7 to 8, optionally wherein the stationary phase is contacted with 1 CV to 10 CV or 4.5 CV to 5.5 CV of the second regeneration buffer.

35. The method of any one of claims 31-34, wherein the method further comprises loading another solution comprising a rAAV vector on the affinity chromatography stationary phase after removing at least a portion of the second regeneration buffer.

36. The method of any one of claims 31-35, wherein the method further comprises applying a second amount of the pre-elution wash solution on the affinity chromatography stationary phase after removing at least a portion of the second regeneration buffer.

37. A method of purifying a rAAV vector, the method comprising:
loading a solution comprising the rAAV vector on an affinity chromatography stationary phase in a column;
applying a pre-elution wash solution comprising 15% to 25% ethanol, 100 mM to 200 mM sodium acetate, and a pH of 5 to 6 to the stationary phase; and
eluting the rAAV vector from the stationary phase with an elution buffer comprising 50 mM to 150 mM glycine, 10 mM to 100 mM $MgCl_2$, 50 mM to 250 mM sodium acetate, and a pH of 2.5 to 3.5 to produce an affinity eluate containing the rAAV vector.

38. The method of claim 37, wherein 1 CV to 10 CV or 4.5 CV to 5.5 CV of the pre-elution

wash solution is applied to the stationary phase.

39. The method of claim 37 or 38, wherein the pre-elution wash has a reduced pH to improve removal of a protein other than a rAAV protein from the stationary phase, wherein the pre-elution wash maintains rAAV-stationary phase ligand binding or both and optionally wherein the proteins other than rAAV protein is a host cell protein.

40. The method of any one of claims 37-39, wherein the elution buffer does not result in precipitation of an affinity eluate, wherein the elution buffer does not interfere with binding of the rAAV vector to an anion exchange chromatography (AEX) stationary phase or both.

41. The method of any one of claims 37-40, wherein a % vg recovery in the affinity eluate is 50% to 100%, optionally as measured by qPCR; wherein a vg per mL of the affinity eluate is about 1.0 x 10^{12} vg/mL to about 1.0 x 10^{14} vg/mL, optionally as measured by qPCR; wherein a % purity of the affinity eluate is about 95% to about 100% capsid protein of total protein, optionally as measured by SEC or reverse phase HPLC, non-reducing, or a combination thereof.

42. The method of any one of claims 37-41, further comprising contacting the stationary phase with a first regeneration buffer after obtaining at least a portion of the affinity eluate.

43. The method of any one of claims 37-42, further comprising contacting the stationary phase with a second regeneration buffer after the first regeneration buffer, wherein the second regeneration buffer is different than the first regeneration buffer.

~~44. A method of regenerating an affinity chromatography stationary phase, the method comprising: contacting the stationary phase with a first regeneration buffer comprising an acid and a pH of 1 to 4, wherein impurities are removed from the stationary phase.~~

~~45. The method of claim 44, wherein the first regeneration buffer comprises 0.05 N to 1.5 N of the acid, optionally wherein the acid is phosphoric acid or acetic acid.~~

~~46. The method of claim 44 or 45, wherein the first regeneration buffer comprises 0.10 N to 0.15 N phosphoric acid, and a pH of 1.5 to 2.5.~~

~~47. The method of any one of claims 44-46, wherein 1 CV to 10 CV or 4.5 CV to 5.5 CV of the first regeneration buffer is applied to the stationary phase, wherein the stationary phase is contacted with about half of the volume of a first regeneration buffer, followed by a hold period~~

of about 45 minutes, followed by contacting the stationary phase with a second half of the volume of the first regeneration buffer.

48. The method of any one of claims 44-47, wherein the method further comprises loading a solution comprising an rAAV vector on the affinity chromatography stationary phase after removing at least a portion of the first regeneration buffer.

49. The method of any one of claims 44-48, wherein the method further comprises applying a pre-elution wash solution on the affinity chromatography stationary phase after removing at least a portion of the first regeneration buffer.

50. The method of any one of claims 44-49, further comprising contacting the stationary phase with a second regeneration buffer after the first regeneration buffer, wherein the second regeneration buffer is different than the first regeneration buffer.

51. The method of claim 50, wherein the second regeneration buffer comprises a detergent and a buffering agent.

52. The method of claim 50 or 51, wherein the second regeneration buffer comprises 0.1% to 5% of the detergent, optionally wherein the detergent is sarkosyl.

53. The method of any one of claims 50-52, wherein the second regeneration buffer comprises 50 mM to 150 mM of the buffering agent, optionally wherein the buffering agent is Tris.

54. The method of any one of claims 50-53, wherein the second regeneration buffer comprises 0.1% to 1.5% of sarkosyl, 50 mM to 150 mM of Tris, and a pH of 7 to 8.

55. The method of any one of claims 50-54, wherein 1 CV to 10 CV or 4.5 CV to 5.5 CV of the second regeneration buffer is applied to the stationary phase.

56. The method of any one of claims 50-55, wherein the method further comprises loading a solution comprising an rAAV vector on the affinity chromatography stationary phase after removing at least a portion of the second regeneration buffer.

57. The method of any one of claims 50-56, wherein the method further comprises applying a pre-elution wash solution on the affinity chromatography stationary phase after removing at least a portion of the second regeneration buffer.