

WHAT IS CLAIMED IS:

1. A method of producing a cell comprising edits at two or more target loci:
 - (a) combining two or more guide RNAs (gRNAs) capable of directing CRISPR/Cas9-mediated indel formation at respective target loci with Cas9 protein to form a ribonucleoprotein complex (RNP);
 - (b) serially transfecting a population of cells with the RNP until at least about ~~10~~50% indel formation is achieved at each target locus; and
 - (c) isolating a cell comprising edits at two or more target loci by single cell cloning of the cell from the population of serially transfected cells.
2. The method of claim 1, wherein the gRNA is an sgRNA.
3. The method of claim 1, wherein the gRNA comprises a crRNA and a tracrRNA.
4. The method of claim 3, wherein the crRNA is an XT-gRNA.
- ~~5. The method of any of claims 1-4, wherein the population of cells is serially transfected with the RNP until at least about 20% indel formation is achieved at each target locus.~~
- ~~6. The method of any of claims 1-4, wherein the population of cells is serially transfected with the RNP until at least about 30% indel formation is achieved at each target locus.~~
- ~~7. The method of any of claims 1-4, wherein the population of cells is serially transfected with the RNP until at least about 40% indel formation is achieved at each target locus.~~
- ~~8. The method of any of claims 1-4, wherein the population of cells is serially transfected with the RNP until at least about 50% indel formation is achieved at each target locus.~~
- ~~9.~~5. The method of any of claims 1-4, wherein the population of cells is serially transfected with the RNP until at least about 60% indel formation is achieved at each target locus.

~~10.6.~~ The method of claim 1, wherein the ratio of moles of RNP to number of transfected cells is between about 0.1 pmol per 10^6 cells to about 5 pmol per 10^6 cells

~~11.7.~~ The method of claim 1, wherein the ratio of moles of RNP to number of transfected cells is about 0.15 pmol per 10^6 cells.

~~12.8.~~ The method of claim 1, wherein the ratio of moles of RNP to number of transfected cells is about 0.17 pmol per 10^6 cells.

~~13.9.~~ The method of claim 1, wherein the ratio of moles of RNP to number of transfected cells is about 0.2 pmol per 10^6 cells.

~~14.10.~~ The method of claim 1, wherein the ratio of moles of RNP to number of transfected cells is about 1 pmol per 10^6 cells.

~~15.11.~~ The method of claim 1, wherein the ratio of moles of RNP to number of transfected cells is about 2 pmol per 10^6 cells.

~~16.12.~~ The method of claim 1, wherein the ratio of moles of RNP to number of transfected cells is about 3 pmol per 10^6 cells.

~~17.13.~~ The method of claim 1, wherein three or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about ~~10~~50% indel formation is achieved at each target locus.

~~18.14.~~ The method of claim 1, wherein four or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about ~~10~~50% indel formation is achieved at each target locus.

~~19.15.~~ The method of claim 1, wherein five or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a

population of cells until at least about ~~40~~50% indel formation is achieved at each target locus.

~~20~~16. The method of claim 1, wherein six or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about ~~40~~50% indel formation is achieved at each target locus.

~~21~~17. The method of claim 1, wherein seven or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about ~~40~~50% indel formation is achieved at each target locus.

~~22~~18. The method of claim 1, wherein eight or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about ~~40~~50% indel formation is achieved at each target locus.

~~23~~19. The method of claim 1, wherein nine or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about ~~40~~50% indel formation is achieved at each target locus.

~~24~~20. The method of claim 1, wherein ten or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about ~~40~~50% indel formation is achieved at each target locus.

~~25. The method of any one of claims 17-24 wherein the RNPs are serially transfecting into a population of cells until at least about 20% indel formation is achieved at each target locus.~~

~~26. The method of any one of claims 17-24 wherein the RNPs are serially transfecting into a population of cells until at least about 20% indel formation is achieved at each target locus.~~

~~27. The method of any one of claims 17-24 wherein the RNPs are serially transfecting into a population of cells until at least about 30% indel formation is achieved at each target locus.~~

~~28. The method of any one of claims 17-24 wherein the RNPs are serially transfecting into a population of cells until at least about 40% indel formation is achieved at each target locus.~~

~~29. The method of any one of claims 17-24 wherein the RNPs are serially transfecting into a population of cells until at least about 50% indel formation is achieved at each target locus.~~

30-21. The method of any one of claims ~~17-24~~ 13-20 wherein the RNPs are serially transfecting into a population of cells until at least about 60% indel formation is achieved at each target locus.

31-22. The method of any one of claims 1-~~30-21~~, wherein the cell is a T cell, an NK cell, a B cell, a dendritic cell, a CHO cell, a COS-7 cell; an HEK 293 cell, a BHK cells, a TM4 cell, a CV1 cell; a VERO-76 cell; a HELA cells; or an MDCK cell.

32-23. The method of claim 1, wherein the two or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are identified via a efficiency screen comprising:

- (a) transfecting a population of cells with a population of RNPs, where each RNP comprises a gRNA capable of directing CRISPR/Cas9-mediated indel formation at a target locus; and
- (b) sequencing the target loci to identify gRNAs based on their efficiency in directing CRISPR/Cas9-mediated indel formation.

33-24. The method of claim ~~32-23~~, wherein the sequencing is performed using Sanger sequencing.

~~34. A cell composition, wherein the cell comprises edits at two or more target loci, wherein the edits are the result of:~~

- ~~(a) combining two or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci with Cas9 protein to form an RNP;~~
- ~~(b) serially transfecting a population of cells with the RNP until at least about 10% indel formation is achieved at each target locus; and~~
- ~~(c) isolating the cell comprising edits at two or more target loci by single cell cloning of the cell from the population of serially transfected cells~~

~~35. A host cell composition, wherein the host cell comprises:~~

- ~~(a) a nucleic acid encoding a non-endogenous polypeptide of interest; and~~
- ~~(b) edits at two more target loci, wherein the edits are the result of:~~
 - ~~i. combining two or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci with Cas9 protein to form an RNP;~~
 - ~~ii. serially transfecting a population of cells with the RNP until at least about 10% indel formation is achieved at each target locus; and~~
 - ~~iii. isolating the host cell comprising edits at two or more target loci by single cell cloning of the host cell from the population of serially transfected cells.~~

~~36. The cell composition of claim 34 or the host cell composition of claim 35, wherein the gRNA is an sgRNA.~~

~~37. The cell composition of claim 34 or the host cell composition of claim 35, wherein the gRNA comprises a crRNA and a tracrRNA.~~

~~38. The cell composition or host cell composition of claim 37, wherein the crRNA is an XT-gRNA.~~

~~39. The cell composition of claim 34 or host cell composition of claim 35, wherein the population of cells is serially transfected with the RNP until at least about 20% indel formation is achieved at each target locus.~~

~~40. The cell composition of claim 34 or host cell composition of claim 35, wherein the population of cells is serially transfected with the RNP until at least about 30% indel formation is achieved at each target locus.~~

- ~~41. The cell composition of claim 34 or host cell composition of claim 35, wherein the population of cells is serially transfected with the RNP until at least about 40% indel formation is achieved at each target locus.~~
- ~~42. The cell composition of claim 34 or host cell composition of claim 35, wherein the population of cells is serially transfected with the RNP until at least about 50% indel formation is achieved at each target locus.~~
- ~~43. The cell composition of claim 34 or host cell composition of claim 35, wherein the population of cells is serially transfected with the RNP until at least about 60% indel formation is achieved at each target locus.~~
- ~~44. The cell composition of claim 34 or host cell composition of claim 35, wherein the ratio of moles of RNP to number of transfected cells is between about 0.1 pmol per 10^6 cells to about 5 pmol per 10^6 cells~~
- ~~45. The cell composition of claim 34 or host cell composition of claim 35, wherein the ratio of moles of RNP to number of transfected cells is about 0.15 pmol per 10^6 cells.~~
- ~~46. The cell composition of claim 34 or host cell composition of claim 35, wherein the ratio of moles of RNP to number of transfected cells is about 0.17 pmol per 10^6 cells.~~
- ~~47. The cell composition of claim 34 or host cell composition of claim 35, wherein the ratio of moles of RNP to number of transfected cells is about 0.2 pmol per 10^6 cells.~~
- ~~48. The cell composition of claim 34 or host cell composition of claim 35, wherein the ratio of moles of RNP to number of transfected cells is about 1 pmol per 10^6 cells.~~
- ~~49. The cell composition of claim 34 or host cell composition of claim 35, wherein the ratio of moles of RNP to number of transfected cells is about 2 pmol per 10^6 cells.~~
- ~~50. The cell composition of claim 34 or host cell composition of claim 35, wherein the ratio of moles of RNP to number of transfected cells is about 3 pmol per 10^6 cells.~~
- ~~51. The cell composition of claim 34 or host cell composition of claim 35, wherein three or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs~~

~~are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.~~

~~52. The cell composition of claim 34 or host cell composition of claim 35, wherein four or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.~~

~~53. The cell composition of claim 34 or host cell composition of claim 35, wherein five or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.~~

~~54. The cell composition of claim 34 or host cell composition of claim 35, wherein six or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.~~

~~55. The cell composition of claim 34 or host cell composition of claim 35, wherein seven or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.~~

~~56. The cell composition of claim 34 or host cell composition of claim 35, wherein eight or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.~~

~~57. The cell composition of claim 34 or host cell composition of claim 35, wherein nine or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs~~

~~are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.~~

~~58. The cell composition of claim 34 or host cell composition of claim 35, wherein ten or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.~~

~~59. The cell composition or host cell composition of claim any one of claims 51-58, wherein the RNPs are serially transfecting into a population of cells until at least about 20% indel formation is achieved at each target locus.~~

~~60. The cell composition or host cell composition of claim any one of claims 51-58, wherein the RNPs are serially transfecting into a population of cells until at least about 20% indel formation is achieved at each target locus.~~

~~61. The cell composition or host cell composition of claim any one of claims 51-58, wherein the RNPs are serially transfecting into a population of cells until at least about 30% indel formation is achieved at each target locus.~~

~~62. The cell composition or host cell composition of claim any one of claims 51-58, wherein the RNPs are serially transfecting into a population of cells until at least about 40% indel formation is achieved at each target locus.~~

~~63. The cell composition or host cell composition of claim any one of claims 51-58, wherein the RNPs are serially transfecting into a population of cells until at least about 50% indel formation is achieved at each target locus.~~

~~64. The cell composition or host cell composition of claim any one of claims 51-58, wherein the RNPs are serially transfecting into a population of cells until at least about 60% indel formation is achieved at each target locus.~~

~~65. The cell composition or host cell composition of claim any one of claims 34-58, wherein the cell is a T cell, an NK cell, a B cell, a dendritic cell, a CHO cell, a COS-7 cell; an HEK 293 cell, a BHK cells, a TM4 cell, a CV1 cell; a VERO 76 cell; a HELA cells; or an MDCK cell.~~

~~66. The cell composition of claim 34 or host cell composition of claim 35, wherein the two or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are identified via an efficiency screen comprising:~~

- ~~(a) transfecting a population of cells with a population of RNPs, where each RNP comprises a gRNA capable of directing CRISPR/Cas9-mediated indel formation at a target locus; and~~
- ~~(b) sequencing the target loci to identify gRNAs based on their efficiency in directing CRISPR/Cas9-mediated indel formation.~~

~~67. The composition or host cell composition of claim 49, wherein the sequencing is performed using Sanger sequencing.~~

~~68. A method producing a polypeptide of interest comprising:~~

- ~~(a) culturing a host cell composition comprising:~~
 - ~~i. a nucleic acid encoding a non-endogenous polypeptide of interest;~~
 - ~~and~~
 - ~~ii. edits at two or more target loci, wherein the edits are the result of:~~
 - ~~1. combining two or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci with Cas9 protein to form an RNP;~~
 - ~~2. serially transfecting a population of cells with the RNP until about 10% indel formation is achieved at each target locus;~~
 - ~~and~~
 - ~~3. isolating the host cell comprising edits at two or more target loci by single cell cloning of the host cell from the population of serially transfected cells; and~~
- ~~(b) isolating the polypeptide of interest expressed by the cultured host cell.~~

~~69. The method of claim 68, wherein the gRNA is an sgRNA.~~

~~70. The method of claim 68, wherein the gRNA comprises a crRNA and a tracrRNA.~~

~~71. The method of claim 70, wherein the crRNA is an XT gRNA.~~

- ~~72. The method of any of claims 68-71, wherein the population of cells is serially transfected with the RNP until at least about 20% indel formation is achieved at each target locus.~~
- ~~73. The method of any of claims 68-71, wherein the population of cells is serially transfected with the RNP until at least about 30% indel formation is achieved at each target locus.~~
- ~~74. The method of any of claims 68-71, wherein the population of cells is serially transfected with the RNP until at least about 40% indel formation is achieved at each target locus.~~
- ~~75. The method of any of claims 68-71, wherein the population of cells is serially transfected with the RNP until at least about 50% indel formation is achieved at each target locus.~~
- ~~76. The method of any of claims 68-71, wherein the population of cells is serially transfected with the RNP until at least about 60% indel formation is achieved at each target locus.~~
- ~~77. The method of claim 68, wherein the ratio of moles of RNP to number of transfected cells is between about 0.1 pmol per 10^6 cells to about 5 pmol per 10^6 cells.~~
- ~~78. The method of claim 68, wherein the ratio of moles of RNP to number of transfected cells is about 0.15 pmol per 10^6 cells.~~
- ~~79. The method of claim 68, wherein the ratio of moles of RNP to number of transfected cells is about 0.17 pmol per 10^6 cells.~~
- ~~80. The method of claim 68, wherein the ratio of moles of RNP to number of transfected cells is about 0.2 pmol per 10^6 cells.~~
- ~~81. The method of claim 68, wherein the ratio of moles of RNP to number of transfected cells is about 1 pmol per 10^6 cells.~~
- ~~82. The method of claim 68, wherein the ratio of moles of RNP to number of transfected cells is about 2 pmol per 10^6 cells.~~

83. The method of claim 68, wherein the ratio of moles of RNP to number of transfected cells is about 3 pmol per 10^6 cells.
84. The method of claim 68, wherein three or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.
85. The method of claim 68, wherein four or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.
86. The method of claim 68, wherein five or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.
87. The method of claim 68, wherein six or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.
88. The method of claim 68, wherein seven or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.
89. The method of claim 68, wherein eight or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a

population of cells until at least about 10% indel formation is achieved at each target locus.

90. The method of claim 68, wherein nine or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.

91. The method of claim 68, wherein ten or more gRNAs capable of directing CRISPR/Cas9 mediated indel formation at respective target loci are combined with Cas9 protein to produce RNPs and the RNPs are serially transfecting into a population of cells until at least about 10% indel formation is achieved at each target locus.

92. The method of any one of claims 84-91, wherein the RNPs are serially transfecting into a population of cells until at least about 20% indel formation is achieved at each target locus.

93. The method of any one of claims 84-91, wherein the RNPs are serially transfecting into a population of cells until at least about 20% indel formation is achieved at each target locus.

94. The method of any one of claims 84-91, wherein the RNPs are serially transfecting into a population of cells until at least about 30% indel formation is achieved at each target locus.

95. The method of any one of claims 84-91, wherein the RNPs are serially transfecting into a population of cells until at least about 40% indel formation is achieved at each target locus.

96. The method of any one of claims 84-91, wherein the RNPs are serially transfecting into a population of cells until at least about 50% indel formation is achieved at each target locus.

97. The method of any one of claims 84-91, wherein the RNPs are serially transfecting into a population of cells until at least about 60% indel formation is achieved at each target locus.

- ~~98. The method of any one of claims 68-97, wherein the cell is a T cell, an NK cell, a B cell, a dendritic cell, a CHO cell, a COS-7 cell; an HEK-293 cell, a BHK cells, a TM4 cell, a CV1 cell; a VERO-76 cell; a HELA cells; or an MDCK cell.~~
- ~~99. The method of claim 68, wherein the two or more gRNAs capable of directing CRISPR/Cas9-mediated indel formation at respective target loci are identified via a efficiency screen comprising:~~
- ~~(a) transfecting a population of cells with a population of RNPs, where each RNP comprises a gRNA capable of directing CRISPR/Cas9-mediated indel formation at a target locus; and~~
 - ~~(b) sequencing the target loci to identify gRNAs based on their efficiency in directing CRISPR/Cas9-mediated indel formation.~~
- ~~100. The method of claim 99, wherein the sequencing is performed using Sanger sequencing.~~
- ~~101. The method of any of claims 68-100, wherein the method comprises purifying the product of interest, harvesting the product of interest, and/or formulating the product of interest.~~
- ~~102. The method of any of claims 68-100, wherein the cell is a mammalian cell.~~
- ~~103. The method of claim 102, wherein the mammalian cell is a CHO cell.~~
- ~~104. The method of any of claims 68-100, wherein polypeptide of interest comprises an antibody or an antigen-binding fragment thereof.~~
- ~~105. The method of claim 104, wherein the antibody is a multispecific antibody or an antigen-binding fragment thereof.~~
- ~~106. The method of claim 104, wherein the antibody consists of a single heavy chain sequence and a single light chain sequence or antigen-binding fragments thereof.~~
- ~~107. The method of claim 104, wherein the antibody is a chimeric antibody, a human antibody or a humanized antibody.~~
- ~~108. The method of claim 104, wherein the antibody is a monoclonal antibody.~~

- ~~109. The host cell composition of claim 35, wherein polypeptide of interest comprises an antibody or an antigen binding fragment thereof.~~
- ~~110. The host cell composition of claim 109, wherein the antibody is a multispecific antibody or an antigen binding fragment thereof.~~
- ~~111. The host cell composition of claim 109, wherein the antibody consists of a single heavy chain sequence and a single light chain sequence or antigen binding fragments thereof.~~
- ~~112. The host cell composition of claim 109, wherein the antibody is a chimeric antibody, a human antibody or a humanized antibody.~~
- ~~113. The host cell composition of claim 109, wherein the antibody is a monoclonal antibody.~~