

Claims

- [Claim 1] A composition for cleaving target DNA in eukaryotic cells or organisms comprising a guide RNA specific for target DNA or DNA that encodes the guide RNA, and Cas protein-encoding nucleic acid or Cas protein.
- [Claim 2] The composition of claim 1, wherein the target DNA is an endogenous target DNA.
- [Claim 3] The composition of claim 1, wherein the guide RNA is a dualRNA comprising a crRNA and a tracrRNA.
- [Claim 4] The composition of claim 1, wherein the guide RNA is a single-chain guide RNA (sgRNA).
- [Claim 5] The composition of claim 4, wherein the single-chain guide RNA comprises portion of a crRNA and a tracrRNA.
- [Claim 6] The composition of claim 1, wherein the guide RNA further comprises one or more additional nucleotides at the 5' end of the single-chain guide RNA or the crRNA of the dualRNA.
- [Claim 7] The composition of claim 1, wherein the guide RNA further comprises 2 additional guanine nucleotides at the 5' end of the single-chain guide RNA or the crRNA of the dualRNA.
- [Claim 8] The composition of claim 1, which induces targeted mutagenesis in eukaryotic cells or organisms.
- [Claim 9] The composition of claim 1, for use in the genotyping of a genome in the eukaryotic cells or organisms *in vitro*.
- [Claim 10] The composition of claim 1, wherein the guide RNA and the Cas protein function as a pair, and wherein the pair comprises two guide RNAs which induce two nicks on different strands.
- [Claim 11] The composition of claim 1, wherein the guide RNA is in the form of an isolated RNA, or is encoded in a vector, wherein the vector is a viral vector, plasmid vector, or agrobacterium vector.
- [Claim 12] The composition of claim 1, comprising a guide RNA specific for the target DNA and Cas9 protein.
- [Claim 13] The composition of claim 1, for cleaving the target DNA in eukaryotic cells or organisms *ex vivo* or *in vivo*.
- [Claim 14] The composition of claim 1, wherein the Cas protein-encoding nucleic acid or the Cas protein is derived from the genus *Streptococcus*.
- [Claim 15] The composition of claim 14, wherein the genus *Streptococcus* is *Streptococcus pyogenes*.

- [Claim 16] The composition of claim 15, wherein the Cas protein recognizes the NGG trinucleotide.
- [Claim 17] The composition of claim 1, wherein the Cas protein is Cas9 protein or variant thereof.
- [Claim 18] The composition of claim 1, wherein the Cas protein is linked to a protein transduction domain.
- [Claim 19] The composition of claim 17, wherein the variant of Cas9 protein is a mutant form of Cas9 in which the catalytic aspartate residue is changed to any other amino acid.
- [Claim 20] The composition of claim 19, wherein the amino acid is an alanine.
- [Claim 21] The composition of claim 1, wherein the Cas protein-encoding nucleic acid comprises a nucleotide sequence of SEQ ID No. 1 or a nucleotide sequence having at least 50% homology to SEQ ID NO. 1.
- [Claim 22] Use of a composition of any one of claims 1 to 21 for targeted mutagenesis in eukaryotic cells or organisms.
- [Claim 23] A kit for cleaving a target DNA in eukaryotic cells or organisms comprising the composition according to any one of claims 1 to 21.
- [Claim 24] A method for preparing a eukaryotic cell or organism comprising Cas protein and a guide RNA comprising a step of co-transfecting or serial-transfecting the eukaryotic cell or organism with a Cas protein-encoding nucleic acid or Cas protein, and a guide RNA or DNA that encodes the guide RNA.
- [Claim 25] The method of claim 24, wherein the guide RNA is a dualRNA comprising a crRNA and a tracrRNA.
- [Claim 26] The method of claim 24, wherein the guide RNA is a single-chain guide RNA.
- [Claim 27] The method of claim 26, wherein the single-chain guide RNA comprises portion of a crRNA and a tracrRNA.
- [Claim 28] The method of claim 24, wherein the guide RNA further comprises one or more additional nucleotides at the 5' end of the single-chain guide RNA or the crRNA of the dualRNA.
- [Claim 29] The method of claim 24, wherein the guide RNA further comprises 2 additional guanine nucleotides at the 5' end of the single-chain guide RNA or the crRNA of the dualRNA.
- [Claim 30] The method of claim 24, wherein the eukaryotic cell or organism is co-transfected or serial-transfected with a Cas9 protein and a guide RNA.
- [Claim 31] The method of claim 24, wherein the serial-transfection is performed by transfection with Cas protein-encoding nucleic acid first, followed

- by second transfection with naked guide RNA.
- [Claim 32] The method of claim 24, wherein the Cas protein is Cas9 protein or variant thereof.
- [Claim 33] The method of claim 24, wherein the Cas protein-encoding nucleic acid or the Cas protein is derived from the genus *Streptococcus*.
- [Claim 34] The method of claim 33, wherein the genus *Streptococcus* is *Streptococcus pyogenes*.
- [Claim 35] The method of claim 32, wherein the variant of Cas9 protein is a mutant form of Cas9 in which the catalytic aspartate residue is changed to any other amino acid.
- [Claim 36] The method of claim 35, wherein the amino acid is an alanine.
- [Claim 37] The method of claim 24, wherein the guide RNA and Cas protein function as a pair, and wherein the pair comprises two guide RNAs which induce two nicks on different DNA strands.
- [Claim 38] The method of claim 37, wherein the two nicks are separated by at least 100 bps.
- [Claim 39] The method of claim 24, wherein the transfection is performed by the method selected from the group consisting of microinjection, electroporation, DEAE-dextran treatment, lipofection, nanoparticle-mediated transfection, protein transduction domain mediated transduction, virus-mediated gene delivery, and PEG-mediated transfection in protoplast.
- [Claim 40] A eukaryotic cell or organism comprising Cas protein and a guide RNA prepared by the method according to any one of claims 24 to 39.
- [Claim 41] A method for cleaving a target DNA in eukaryotic cells or organisms comprising a step of transfecting the eukaryotic cells or organisms comprising a target DNA with the composition according to any one of claims 1 to 21.
- [Claim 42] The method of claim 41, wherein the eukaryotic organisms are mammals or plants.
- [Claim 43] The method of claim 41, wherein the guide RNA and Cas protein function as a pair, and wherein the pair comprises two guide RNAs which induce two nicks on different DNA strands.
- [Claim 44] The method of claim 43, wherein the two nicks are separated by at least 100 bps.
- [Claim 45] The method of claim 41, wherein the transfection is co-transfection or serial-transfection.
- [Claim 46] The method of claim 45, wherein the serial-transfection is performed by transfection with Cas protein-encoding nucleic acid first, followed

- by second transfection with naked guide RNA.
- [Claim 47] The method of claim 41, further comprising a step of analysing the pattern of a cleavage, wherein the pattern indicates the detection of mutation or variation in the genome.
- [Claim 48] An embryo comprising a genome edited by the composition according to any one of claims 1 to 21.
- [Claim 49] A genome-modified animal obtained by transferring the embryo of claim 48 into the oviducts.
- [Claim 50] A plant regenerated from the genome-modified protoplasts prepared by the method of any one of claims 24 to 39.
- [Claim 51] A method of preparing a genome-modified animal comprising a step of introducing the composition according to any one of claims 1 to 21 into an embryo of an animal; and a step of transferring the embryo into an oviduct of pseudopregnant foster mother to produce a genome-modified animal.
- [Claim 52] A composition for genotyping mutations or variations in an isolated biological sample, comprising a guide RNA specific for the target DNA sequence Cas protein.
- [Claim 53] The composition of claim 52, wherein the mutations or variations are induced by engineered nucleases in cells.
- [Claim 54] The composition of claim 52, wherein the mutations or variations are a naturally-occurring mutations or variations.
- [Claim 55] A composition for genotyping nucleic acid sequences in pathogenic microorganisms in an isolated biological sample, comprising a guide RNA specific for the target DNA sequence and Cas protein.
- [Claim 56] A kit for genotyping mutations or variations in an isolated biological sample, comprising the composition of any one of claims 52 to 55.
- [Claim 57] A method of genotyping mutations or variations in an isolated biological sample, using the composition of any one of claims 52 to 55.

Claims

1. A composition comprising a Type II Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system, said CRISPR/Cas system comprising (i) a nucleic acid encoding a Cas9 polypeptide comprising a nuclear localization sequence, and (ii) a nucleic acid encoding a guide RNA that hybridizes to a target nucleic acid, wherein the guide RNA is a chimeric guide RNA comprising a CRISPR RNA (crRNA) portion fused to a trans activating crRNA (tracrRNA) portion.
2. The composition of claim 1, wherein said Cas9 polypeptide is a *Streptococcus* Cas9 polypeptide.
3. The composition of claim 2, wherein said Cas9 polypeptide is a *Streptococcus pyogenes* Cas9 polypeptide.
4. The composition of any one of claims 1-3, wherein said nucleic acid encoding a Cas9 polypeptide is codon-optimized for expression in eukaryotic cells.
5. The composition of claim 4, wherein said nucleic acid encoding a Cas9 polypeptide is codon-optimized for expression in mammalian cells.
6. The composition of any one of claims 1-5, wherein said nuclear localization sequence is located at the C terminus of the Cas9 polypeptide.
7. The composition of any one of claims 1-6, wherein the target nucleic acid is an endogenous target nucleic acid.
8. The composition of any one of claims 1-7, wherein the guide RNA is in the form of a vector.
9. The composition of any one of claims 1-8, wherein the guide RNA comprises one or more additional nucleotides 5' to the crRNA portion.
10. The composition of claim 9, wherein the guide RNA comprises two nucleic acid residues at the 5' end and there are no additional nucleic acid residues between the two nucleic acid residues and the crRNA portion.
11. The composition of claim 10, wherein the two nucleotide residues at the 5' end are both guanine residues.
12. A composition comprising a Type II Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system, said CRISPR/Cas system comprising (i) a nucleic acid encoding a Cas9 polypeptide, wherein said nucleic acid comprises SEQ

ID NO:1, and (ii) a nucleic acid encoding a guide RNA that hybridizes to a target nucleic acid, wherein the guide RNA is a chimeric guide RNA comprising a CRISPR RNA (crRNA) portion fused to a trans activating crRNA (tracrRNA) portion.

13. The composition of claim 12, wherein said nucleic acid encoding a Cas9 polypeptide comprises a nuclear localization sequence

14. The composition of claim 12 or 13, wherein said nucleic acid encoding a Cas9 polypeptide is codon-optimized for expression in eukaryotic cells.

15. The composition of claim 14, wherein said nucleic acid encoding a Cas9 polypeptide is codon-optimized for expression in mammalian cells.

16. The composition of any one of claims 13-15, wherein said nuclear localization sequence is located at the C terminus of the Cas9 polypeptide.

17. The composition of any one of claims 12-16, wherein the target nucleic acid is an endogenous target nucleic acid.

18. The composition of any one of claims 12-17, wherein the guide RNA is in the form of a vector.

19. The composition of any one of claims 12-18, wherein the guide RNA comprises one or more additional nucleotides 5' to the crRNA portion.

20. The composition of claim 19, wherein the guide RNA comprises two nucleic acid residues at the 5' end and there are no additional nucleic acid residues between the two nucleic acid residues and the crRNA portion.

21. The composition of claim 20, wherein the two nucleotide residues at the 5' end are both guanine residues.

22. An *in vitro* method of introducing a site-specific, double-stranded break at a target nucleic acid sequence in a eukaryotic cell, the method comprising introducing into the eukaryotic cell a Type II Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system, wherein the CRISPR/Cas system comprises:

a) a nucleic acid encoding a Cas9 polypeptide comprising a nuclear localization signal, and

b) a nucleic acid encoding a guide RNA that hybridizes to the target nucleic acid, wherein the guide RNA is a chimeric guide RNA comprising a CRISPR RNA (crRNA) portion fused to a trans activating crRNA (tracrRNA) portion, wherein

the target nucleic acid sequence comprises a first strand that binds to the crRNA portion and a second strand having a trinucleotide protospacer adjacent motif (PAM),

and wherein the Cas9 polypeptide and the guide RNA form a Cas9/RNA complex in the eukaryotic cell, whereby a site-specific, double stranded break at the target nucleic acid sequence is introduced.

23. The method of claim 22, wherein the Cas9 polypeptide is a *Streptococcus* Cas9 polypeptide.

24. The method of claim 23, wherein the Cas9 polypeptide is a *Streptococcus pyogenes* Cas9 polypeptide.

25. The composition of claim 22 or 23, wherein said nucleic acid encoding a Cas9 polypeptide is codon-optimized for expression in eukaryotic cells.

26. The method of claim 25, wherein the nucleic acid encoding the Cas 9 polypeptide is codon-optimized for expression in mammalian cells.

27. The method of any one of claims 22-26, wherein the nuclear localization signal is located at the C terminus of the Cas9 polypeptide.

28. The method of any one of claims 22-27, wherein the eukaryotic cell is a mammalian cell.

29. The method of claim 28, wherein the mammalian cell is a human cell.

30. The method of any one of claims 22-29, wherein the target nucleic acid sequence is a genomic sequence located at its endogenous site in the genome of the eukaryotic cell.

31. The method of any one of claims 22-30, wherein the nucleic acid encoding the guide RNA is a vector.

32. The method of any one of claims 22-30, wherein the nucleic acid encoding the guide RNA is in vitro transcribed RNA.

33. The method of any one of claims 22-30, wherein the guide RNA comprises one or more additional nucleotides 5' to the crRNA portion.

34. The method of claim 33, wherein the guide RNA comprises two nucleic acid residues at the 5' end and there are no additional nucleic acid residues between the two nucleic acid residues and the crRNA portion.

35. The method of claim 34, wherein the two nucleotide residues at the 5' end are both guanine residues.
36. The method of any one of claims 22-35, wherein the nucleic acid encoding the Cas9 polypeptide is introduced into the eukaryotic cell before introducing the nucleic acid encoding the guide RNA into the eukaryotic cell.
37. A method of introducing a site-specific, double-stranded break at a target nucleic acid sequence in a non-human eukaryotic cell, the method comprising introducing into the eukaryotic cell a Type II Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system, wherein the CRISPR/Cas system comprises:
- a) a nucleic acid encoding a Cas9 polypeptide comprising a nuclear localization signal, and
 - b) a nucleic acid encoding a guide RNA that hybridizes to the target nucleic acid, wherein the guide RNA is a chimeric guide RNA comprising a CRISPR RNA (crRNA) portion fused to a trans activating crRNA (tracrRNA) portion, wherein the target nucleic acid sequence comprises a first strand that binds to the crRNA portion and a second strand having a trinucleotide protospacer adjacent motif (PAM),
- and wherein the Cas9 polypeptide and the guide RNA form a Cas9/RNA complex in the eukaryotic cell, whereby a site-specific, double stranded break at the target nucleic acid sequence is introduced.
38. The method of claim 37, wherein the Cas9 polypeptide is a *Streptococcus* Cas9 polypeptide.
39. The method of claim 38, wherein the Cas9 polypeptide is a *Streptococcus pyogenes* Cas9 polypeptide.
40. The composition of claim 37 or 38, wherein said nucleic acid encoding a Cas9 polypeptide is codon-optimized for expression in eukaryotic cells.
41. The method of claim 40, wherein the nucleic acid encoding the Cas 9 polypeptide is codon-optimized for expression in mammalian cells.
42. The method of any one of claims 37-41, wherein the nuclear localization signal is located at the C terminus of the Cas9 polypeptide.
43. The method of any one of claims 37-42, wherein the eukaryotic cell is a

mammalian cell.

44. The method of any one of claims 37-43, wherein the target nucleic acid sequence is a genomic sequence located at its endogenous site in the genome of the eukaryotic cell.
45. The method of any one of claims 37-44, wherein the nucleic acid encoding the guide RNA is a vector.
46. The method of any one of claims 37-44, wherein the nucleic acid encoding the guide RNA is in vitro transcribed RNA.
47. The method of any one of claims 37-46, wherein the guide RNA comprises one or more additional nucleotides 5' to the crRNA portion.
48. The method of claim 47, wherein the guide RNA comprises two nucleic acid residues at the 5' end and there are no additional nucleic acid residues between the two nucleic acid residues and the crRNA portion.
49. The method of claim 48, wherein the two nucleotide residues at the 5' end are both guanine residues.
50. The method of any one of claims 37-49, wherein the nucleic acid encoding the Cas9 polypeptide is introduced into the eukaryotic cell before introducing the nucleic acid encoding the guide RNA into the eukaryotic cell.
51. A method of preparing a genome-modified animal comprising a step of introducing the composition according to any one of claims 1 to 21 into an embryo of an animal; and a step of transferring the embryo into an oviduct of pseudopregnant foster mother to produce a genome-modified animal.
52. A composition for genotyping mutations or variations in an isolated biological sample, comprising a guide RNA specific for the target DNA sequence Cas protein.
53. The composition of claim 52, wherein the mutations or variations are induced by engineered nucleases in cells.
54. The composition of claim 52, wherein the mutations or variations are a naturally-occurring mutations or variations.
55. A composition for genotyping nucleic acid sequences in pathogenic microorganisms in an isolated biological sample, comprising a guide RNA specific for the target DNA sequence and Cas protein.

56. A kit for genotyping mutations or variations in an isolated biological sample, comprising the composition of any one of claims 52 to 55.
57. A method of genotyping mutations or variations in an isolated biological sample, using the composition of any one of claims 52 to 55.