Claims:

1. A method of making multiple alterations to target DNA in a cell expressing an enzyme that forms a co-localization complex with RNA complementary to the target DNA and that cleaves the target DNA in a site specific manner comprising

(a) introducing into the cell a first foreign nucleic acid encoding one or more RNAs complementary to the target DNA and which guide the enzyme to the target DNA, wherein the one or more RNAs and the enzyme are members of a co-localization complex for the target DNA,

introducing into the cell a second foreign nucleic acid encoding one or more donor nucleic acid sequences,

wherein the one or more RNAs and the one or more donor nucleic acid sequences are expressed,

wherein the one or more RNAs and the enzyme co-localize to the target DNA, the enzyme cleaves the target DNA and the donor nucleic acid is inserted into the target DNA to produce altered DNA in the cell, and

repeating step (a) multiple times to produce multiple alterations to the DNA in the cell.

2. The method of claim 1 wherein the enzyme is an RNA-guided DNA binding protein.

3. The method of claim 1 wherein the enzyme is Cas9.

4. The method of claim 1 wherein the cell is a eukaryotic cell.

5. The method of claim 1 wherein the cell is a yeast cell, a plant cell or an animal cell.

6. The method of claim 1 wherein the RNA is between about 10 to about 500 nucleotides.

7. The method of claim 1 wherein the RNA is between about 20 to about 100 nucleotides.

8. The method of claim 1 wherein the one or more RNAs is a guide RNA.

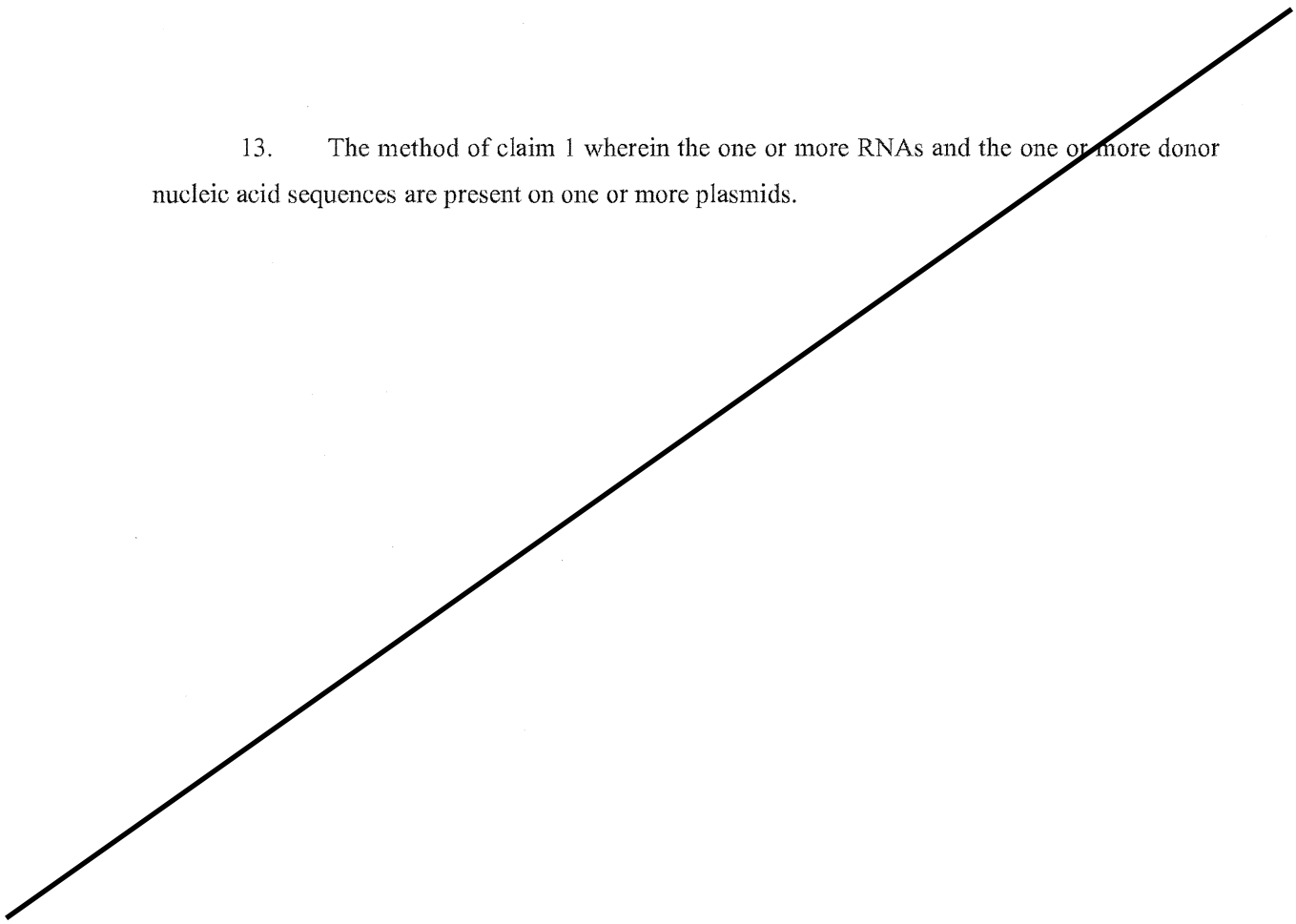
9. The method of claim 1 wherein the one or more RNAs is a tracrRNA-crRNA fusion.

10. The method of claim 1 wherein the DNA is genomic DNA, mitochondrial DNA, viral DNA, or exogenous DNA.

11. The method of claim 1 wherein the one or more donor nucleic acid sequences are inserted by recombination.

12. The method of claim 1 wherein the one or more donor nucleic acid sequences are inserted by homologous recombination.

13. The method of claim 1 wherein the one or more RNAs and the one or more donor nucleic acid sequences are present on one or more plasmids.



Claims:

1. A method of making multiple alterations to target DNA in a eukaryotic cell constitutively expressing a Cas9 enzyme that forms a co-localization complex with a guide RNA complementary to the target DNA and that cleaves the target DNA in a site specific manner comprising

(a) introducing into the cell consitutively expressing the Cas9 enzyme a plurality of guide RNAs complementary to different sites of the target DNA, wherein each of the plurality of guide RNAs is a tracrRNA-crRNA fusion, wherein each of the plurality of guide RNAs and the Cas9 enzyme are members of co-localization complexes for the target DNA, wherein the plurality of guide RNAs is introduced without also introducing a foreign nucleic acid encoding the Cas9 enzyme,

introducing into the cell constitutively expressing the Cas9 enzyme a plurality of donor nucleic acid sequences,

wherein at least one of the plurality of guide RNAs and the Cas9 enzyme co-localize to a site of the target DNA, the Cas9 enzyme cleaves the target DNA and one of the plurality of donor nucleic acid sequences is inserted into the target DNA at the site of cleavage to produce altered DNA in the cell, and

(b) repeating step (a) to produce multiple alterations to the DNA in the cell.

2. The method of claim 1 wherein the eukaryotic cell is a yeast cell, a plant cell or an animal cell.

3. The method of claim 1 wherein each of the plurality of guide RNAs is about 100 nucleotides.

4. The method of claim 1 wherein the DNA is genomic DNA, mitochondrial DNA, viral DNA, or exogenous DNA.

5. The method of claim 1 wherein the one of the plurality of donor nucleic acid sequences is inserted by recombination.

6. The method of claim 1 wherein the one of the plurality of donor nucleic acid sequences is inserted by homologous recombination.

7. The method of claim 1 wherein a nucleic acid encoding one or more of each of the plurality of guide RNAs and each of the plurality of donor nucleic acid sequences are present on one or more plasmids.

8. The method of claim 1 wherein the plurality of donor nucleic acid sequences includes homology sequences or arms flanking the site of cleavage.

9. The method of claim 1 wherein each of the plurality of donor nucleic acids includes a sequence to remove the site of cleavage.