CLAIMS

1. A method for introducing a scarless targeted genetic modification in a preexisting targeting vector, comprising:

(a) performing bacterial homologous recombination between the preexisting targeting vector and a modification cassette in a population of bacterial cells,

wherein the modification cassette comprises the targeted genetic modification and comprises an insert nucleic acid flanked by a 5' homology arm corresponding to a 5' target sequence in the preexisting targeting vector and a 3' homology arm corresponding to a 3' target sequence in the preexisting vector, wherein the insert nucleic acid comprises from 5' to 3':

(i) a first repeat sequence;

(ii) a first target site for a first nuclease agent;

(iii) a selection cassette;

(iv) a second target site for a second nuclease agent; and

(v) a second repeat sequence identical to the first repeat sequence;

(b) selecting bacterial cells comprising a modified targeting vector comprising the selection cassette;

(c) cleaving the first target site in the modified targeting vector with the first nuclease agent and cleaving the second target site in the modified targeting vector with the second nuclease agent to remove the selection cassette and expose the first repeat sequence and the second repeat sequence in the modified targeting vector; and

(d) assembling the exposed first repeat sequence with the exposed second repeat sequence in an intramolecular *in vitro* assembly reaction to generate the targeting vector comprising the scarless targeted genetic modification,

wherein neither the first target site for the first nuclease agent nor the second target site for the second nuclease agent are present and only a single copy of the repeat sequence is present in the targeting vector comprising the scarless targeted genetic modification.

2. The method of claim 1, wherein:

(I) (a) the repeat sequence is identical to a sequence in the preexisting targeting vector, or

(b) the targeted genetic modification comprises an insertion, and the repeat sequence is identical to the 5' end or the 3' end of the insertion; and/or

(II) the repeat sequence is at least 20 nucleotides in length, optionally wherein the repeat sequence is between 20 nucleotides and 100 nucleotides in length.

3. The method of claim 1 or 2, wherein:

(I) the modification cassette is a linear, double-stranded nucleic acid; and/or

(II) the modification cassette is from 1 kb to 15 kb in length.

4. The method of any preceding claim, wherein the 5' homology arm and the 3' homology arm are each at least 35 nucleotides in length, or wherein the 5' homology arm and the 3' homology arm are each between 35 nucleotides and 500 nucleotides in length.

5. The method of any preceding claim, wherein the first nuclease agent and/or the second nuclease agent is a rare-cutting nuclease agent.

6. The method of any preceding claim, wherein:

(I) the first target site and/or the second target site is not present in the preexisting targeting vector; and/or

(II) the first target site is identical to the second target site, and the first nuclease agent is identical to the second nuclease agent.

7. The method of any preceding claim, wherein:

(I) the first nuclease agent and/or the second nuclease agent comprises a rare-cutting restriction enzyme, optionally wherein the rare-cutting restriction enzyme is NotI, XmaIII, SstII, SalI, NruI, NheI, Nb.BbvCI, BbvCI, AscI, AsiSI, FseI, PacI, PmeI, SbfI, SgrAI, SwaI, BspQI, SapI, SfiI, CspCI, AbsI, CciNI, FspAI, MauBI, MreI, MssI, PalAI, RgaI, RigI, SdaI, SfaAI, SgfI, SgrDI, SgsI, SmiI, SrfI, Sse2321, Sse83871, LguI, PciSI, AarI, AjuI, AloI, BarI, PpiI, or PsrI; or

(II) the first nuclease agent and/or the second nuclease agent is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) protein and a guide RNA (gRNA), a zinc finger nuclease (ZFN), a Transcription Activator-Like Effector Nuclease (TALEN), or an engineered meganuclease,

optionally wherein the first nuclease agent and/or the second nuclease agent is the Cas protein and the gRNA, wherein the Cas protein is a Cas9 protein, and wherein the gRNA comprises a CRISPR RNA (crRNA) and a trans-activating CRIPSR RNA (tracrRNA).

8. The method of any preceding claim, wherein the targeted genetic modification comprises:

(I) a modification in the 5' homology arm or the 3' homology arm, or

(II) a modification in the insert nucleic acid.

9. The method of any preceding claim, wherein the targeted genetic modification comprises a point mutation, a deletion, an insertion, a replacement, or a combination thereof.

10. The method of any preceding claim, wherein the selection cassette imparts resistance to an antibiotic,

optionally wherein the selection cassette imparts resistance to ampicillin, chloramphenicol, tetracycline, kanamycin, spectinomycin, streptomycin, carbenicillin, bleomycin, erythromycin, or polymyxin B.

11. The method of any preceding claim, wherein the preexisting targeting vector is a large targeting vector at least 10 kb in length, or wherein the preexisting targeting vector is at least 100 kb in length.

12. The method of any preceding claim, wherein the preexisting targeting vector comprises a second selection cassette,

optionally wherein the second selection cassette imparts resistance to an antibiotic,

optionally wherein the selection cassette in the modification cassette and the second selection cassette in the preexisting targeting vector each imparts resistance to a different antibiotic, and/or

optionally wherein the second selection cassette allows for selection in both bacterial and mammalian cells.

13. The method of any preceding claim, wherein step (c) occurs *in vitro*.

14. The method of any preceding claim, wherein step (d) comprises:

(i) contacting the modified targeting vector with an exonuclease to expose complementary sequences between the first repeat sequence and the second repeat sequence;

(ii) annealing the exposed complementary sequences;

(iii) extending the 3' ends of the annealed complementary sequences; and

(iv) ligating the annealed complementary sequence, and

optionally wherein step (d) comprises incubating the modified targeting vector with an exonuclease, a DNA polymerase, and a DNA ligase.

15. The method of any preceding claim, further comprising:

(e) treating the targeting vector with the first nuclease agent and the second nuclease agent following the *in vitro* assembly in step (d) to verify that neither the first target site for the first nuclease agent nor the second target site for the second nuclease agent are present.

16. A method for introducing a scarless targeted genetic modification in a preexisting targeting vector, comprising:

(a) performing bacterial homologous recombination between the preexisting targeting vector and a deletion cassette in a population of bacterial cells,

wherein the deletion cassette comprises an insert nucleic acid flanked by a 5' homology arm corresponding to a 5' target sequence in the preexisting targeting vector and a 3' homology arm corresponding to a 3' target sequence in the preexisting vector, wherein the 5' target sequence and the 3' target sequence flank a region of the preexisting targeting vector into which the targeted genetic modification is to be introduced, and wherein the insert nucleic acid comprises from 5' to 3':

(i) a first target site for a first nuclease agent;

(ii) a selection cassette; and

(iii) a second target site for a second nuclease agent;

(b) selecting bacterial cells comprising a modified targeting vector comprising the selection cassette;

(c) cleaving the first target site in the modified targeting vector with the first nuclease agent and cleaving the second target site in the modified targeting vector with the second nuclease agent to remove the selection cassette and expose an upstream end sequence and a downstream end sequence in the modified targeting vector; and

(d) assembling in an *in vitro* assembly reaction the cleaved targeting vector with a modification cassette comprising the targeted genetic modification flanked by an upstream end sequence overlapping the upstream end sequence in the modified targeting vector and a downstream end sequence overlapping the downstream end sequence in the modified targeting vector to generate the targeting vector comprising the scarless targeted genetic modification,

wherein neither the first target site for the first nuclease agent nor the second target site for the second nuclease agent are present in the targeting vector comprising the scarless targeted genetic modification.

17. The method of claim 16, wherein:

(I) the deletion cassette is from 1 kb to 15 kb in length, and/or

(II) the deletion cassette is a linear, double-stranded nucleic acid.

18. The method of claim 16 or 17, wherein the 5' homology arm and the 3' homology arm are each at least 35 nucleotides in length, or wherein the 5' homology arm and the 3' homology arm are each between 35 nucleotides and 500 nucleotides in length.

19. The method of any one of claims 16-18, wherein the first nuclease agent and/or the second nuclease agent is a rare-cutting nuclease agent.

20. The method of any one of claims 16-19, wherein:

(I) the first target site and/or the second target site is not present in the preexisting targeting vector; and/or

(II) the first target site is identical to the second target site, and the first nuclease agent is identical to the second nuclease agent.

21. The method of any one of claims 16-20, wherein:

(I) the first nuclease agent and/or the second nuclease agent comprises a rare-cutting restriction enzyme, optionally wherein the rare-cutting restriction enzyme is NotI,

XmaIII, SstII, SalI, NruI, NheI, Nb.BbvCI, BbvCI, AscI, AsiSI, FseI, PacI, PmeI, SbfI, SgrAI, SwaI, BspQI, SapI, SfiI, CspCI, AbsI, CciNI, FspAI, MauBI, MreI, MssI, PalAI, RgaI, RigI, SdaI, SfaAI, SgfI, SgrDI, SgsI, SmiI, SrfI, Sse2321, Sse83871, LguI, PciSI, AarI, AjuI, AloI, BarI, PpiI, or PsrI; or

(II) the first nuclease agent and/or the second nuclease agent is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) protein and a guide RNA (gRNA), a zinc finger nuclease (ZFN), a Transcription Activator-Like Effector Nuclease (TALEN), or an engineered meganuclease,

optionally wherein the first nuclease agent and/or the second nuclease agent is the Cas protein and the gRNA, wherein the Cas protein is a Cas9 protein, and wherein the gRNA comprises a CRISPR RNA (crRNA) and a trans-activating CRIPSR RNA (tracrRNA).

22. The method of any one of claims 16-21, wherein the selection cassette imparts resistance to an antibiotic, optionally wherein the selection cassette imparts resistance to ampicillin, chloramphenicol, tetracycline, kanamycin, spectinomycin, streptomycin, carbenicillin, bleomycin, erythromycin, or polymyxin B.

23. The method of any one of claims 16-22, wherein the preexisting targeting vector is a large targeting vector at least 10 kb in length, or wherein the preexisting targeting vector is at least 100 kb in length.

24. The method of any one of claims 16-23, wherein the preexisting targeting vector comprises a second selection cassette,

optionally wherein the second selection cassette imparts resistance to an antibiotic,

optionally wherein the selection cassette in the deletion cassette and the second selection cassette in the preexisting targeting vector each imparts resistance to a different antibiotic, and/or

optionally wherein the second selection cassette allows for selection in both bacterial and mammalian cells.

25. The method of any one of claims 16-24, wherein the length of the overlap between the upstream end sequence in the modification cassette and the upstream end sequence

in the modified targeting vector and/or the length of the overlap between the downstream end sequence in the modification cassette and the downstream end sequence in the modified targeting vector is at least 20 nucleotides in length,

or wherein the length of the overlap between the upstream end sequence in the modification cassette and the upstream end sequence in the modified targeting vector and/or the length of the overlap between the downstream end sequence in the modification cassette and the downstream end sequence in the modified targeting vector is between 20 and 100 nucleotides in length.

26. The method of any one of claims 16-25, wherein step (c) occurs *in vitro*.

27. The method of any one of claims 16-26, wherein step (d) comprises:

(i) contacting the cleaved targeting vector and the modification cassette with an exonuclease to expose complementary sequences between the end sequences in the modified targeting vector and the end sequences in the modification cassette;

(ii) annealing the exposed complementary sequences;

(iii) extending the 3' ends of the annealed complementary sequences; and

(iv) ligating the annealed complementary sequence, and

optionally wherein step (d) comprises incubating the cleaved targeting vector and the modification cassette with an exonuclease, a DNA polymerase, and a DNA ligase.

28. The method of any one of claims 16-27, wherein:

(I) the modification cassette is a linear, double-stranded nucleic acid;

(II) the modification cassette is at least 200 nucleotides in length, optionally wherein the modification cassette is at least 10 kb in length; and/or

(III) the modification cassette modification cassette is a size that cannot be directly synthesized or generated by polymerase chain reaction.

29. The method of any one of claims 16-28, wherein the targeted genetic modification comprises a point mutation, a deletion, an insertion, a replacement, or a combination thereof.

30. The method of any one of claims 16-29, further comprising:

(e) treating the targeting vector with the first nuclease agent and the second nuclease agent following the *in vitro* assembly in step (d) to verify that neither the first target site for the first nuclease agent nor the second target site for the second nuclease agent are present.