

THAT WHICH IS CLAIMED

1. A method for serial modification of a target locus in a cell, comprising:

(a) providing the cell comprising the target locus, wherein the target locus

5 comprises a first selection cassette comprising: (1) a nucleic acid encoding a first selection marker; and (2) a first nuclease recognition site for a first nuclease agent, wherein the first nuclease recognition site is located in a coding region of the first selection marker or any non-protein coding region of the first selection marker;

(b) introducing into the cell:

10 (i) the first nuclease agent, wherein the first nuclease agent induces a nick or a double-strand break at the first nuclease recognition site, thereby disrupting expression or activity of the first selection marker; and

(ii) a first targeting vector comprising a first insert polynucleotide flanked by a first homology arm corresponding to a first target site located in the target locus and
15 a second homology arm corresponding to a second target site located in the target locus, wherein the first insert polynucleotide comprises a second selection cassette comprising: (1) a nucleic acid encoding a second selection marker, wherein the first selection marker and the second selection marker are different; and (2) a second nuclease recognition site for a second nuclease agent, wherein the second nuclease recognition site is located in a coding region of the second
20 selection marker or any non-protein-coding region of the second selection marker; and

(c) identifying a modified cell comprising the first insert polynucleotide at the target locus, wherein the modified cell has the activity of the second selection marker but does not have the activity of the first selection marker;

(d) introducing into the modified cell:

25 (i) the second nuclease agent, wherein the second nuclease agent induces a nick or a double-strand break at the second nuclease recognition site, thereby disrupting expression or activity of the second selection marker; and

(ii) a second targeting vector comprising a second insert polynucleotide flanked by a third homology arm corresponding to a third target site located in
30 the target locus and a fourth homology arm corresponding to a fourth target site located in the target locus, wherein the second insert polynucleotide comprises a third selection cassette

comprising: (1) a nucleic acid encoding a third selection marker, wherein the first selection marker and the third selection marker are identical; and (2) a third nuclease recognition site for a third nuclease agent, wherein the third nuclease recognition site is identical to the first nuclease recognition site and different from the second nuclease recognition site, and the first nuclease agent and the third nuclease agent are identical to one another and are different from the second nuclease agent; and

(e) identifying at least one cell comprising the second insert polynucleotide integrated at the target locus.

2. The method of claim 1, wherein:

(I) the identifying step (c) comprises:

(i) culturing the cell under conditions that allow identification of cells that do not have the activity of the first selection marker; or

(ii) identifying at least one cell comprising the first insert polynucleotide integrated at the first and second target sites; and/or

(II) the identifying step (e) comprises:

(i) culturing the cell under conditions that allow identification of cells that do not have the activity of the second selection marker; or

(ii) identifying at least one cell comprising the second insert polynucleotide integrated at the third and fourth target sites.

3. The method of claim 1 or 2, wherein the identifying step (c) is carried out via a modification of allele (MOA) assay, and/or the identifying step (e) is carried out via a MOA assay.

4. The method of any one of claims 1-3, wherein the selection cassette in the modified cell in step (c) is flanked by the third target site and the fourth target site.

5. The method of any one of claims 1-4, wherein the first, the second, or the third selection marker imparts resistance to an antibiotic,

optionally wherein the antibiotic comprises G418, hygromycin, blasticidin, neomycin, or puromycin, and

optionally wherein the first and third selection markers impart resistance to

neomycin and the second selection marker imparts resistance to hygromycin, or the first and third selection markers impart resistance to hygromycin and the second selection marker imparts resistance to neomycin.

6. The method of any one of claims 1-4, wherein the first, the second, or the third selection marker is operably linked to an inducible promoter, and expression of the selection marker is toxic to the cell,

optionally wherein the first, the second, or the third selection marker comprises hypoxanthine-guanine phosphoribosyltransferase (HGPRT) or thymidine kinase of Herpes simplex virus (HSV-TK).

7. The method of any one of claims 1-6, wherein the combined use of the first targeting vector with the first nuclease agent results in an increased targeting efficiency compared to the use of the first targeting vector alone,

optionally wherein the targeting efficiency of the first targeting vector is increased at least two-fold compared to the use of the first targeting vector alone.

8. The method of any one of claims 1-7, wherein the first nuclease agent, the second nuclease agent, or the third nuclease agent comprises an expression construct comprising a nucleic acid sequence encoding the nuclease agent, wherein the nucleic acid sequence encoding the nuclease agent is operably linked to a promoter active in the cell, or wherein the first nuclease agent, the second nuclease agent, or the third nuclease agent comprises an mRNA encoding the nuclease agent.

9. The method of any one of claims 1-8, wherein the first nuclease agent, the second nuclease agent, or the third nuclease agent is:

- (a) a zinc finger nuclease (ZFN);
- (b) a Transcription Activator-Like Effector Nuclease (TALEN);
- (c) a meganuclease; or
- (d) a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) protein and a guide RNA (gRNA).

10. The method of claim 9, wherein the first nuclease agent, the second

nuclease agent, or the third nuclease agent is the zinc finger nuclease (ZFN), and the first nuclease recognition site, the second nuclease recognition site, or the third nuclease recognition site comprises any one of SEQ ID NOS: 9-12.

11. The method of claim 9, wherein the first nuclease agent, the second
5 nuclease agent, or the third nuclease agent is the Cas protein and the gRNA, wherein the Cas protein is Cas9, and wherein the gRNA comprises:

(a) a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)
RNA (crRNA) that targets the first, the second, or the third recognition site, wherein the first, the
second, or the third recognition site is immediately flanked by a Protospacer Adjacent Motif
10 (PAM) sequence; and

(b) a trans-activating CRISPR RNA (tracrRNA),
optionally wherein the target locus comprises the nucleotide sequence of SEQ ID
NO: 1.

12. The method of claim 11, wherein the gRNA comprises any one of SEQ ID
15 NOS: 13-20.

13. The method of claim 11 or 12, wherein the gRNA comprises SEQ ID NO:
2, 3, 4, 5, 6, 7, or 8,
optionally wherein the guide RNA comprises SEQ ID NO: 3 or 7.

14. The method of claim 10, wherein the first selection marker imparts
20 resistance to neomycin, the first nuclease agent is a zinc finger nuclease, and the first nuclease
recognition site comprises SEQ ID NO: 9 or 10, and wherein the second selection marker
imparts resistance to hygromycin, the second nuclease agent is a zinc finger nuclease, and the
second nuclease recognition site comprises SEQ ID NO: 11 or 12.

15. The method of any one of claims 11-13, wherein the first selection marker
25 imparts resistance to neomycin, and the first nuclease agent is a Cas9 protein and a guide RNA
comprising any one of SEQ ID NOS: 13-16, and wherein the second selection marker imparts
resistance to hygromycin, and the second nuclease agent is a Cas9 protein and a guide RNA
comprising any one of SEQ ID NOS: 17-20,

optionally wherein the first selection marker imparts resistance to neomycin, and the first nuclease agent is the Cas9 protein and the guide RNA comprising SEQ ID NO: 13, and wherein the second selection marker imparts resistance to hygromycin, and the second nuclease agent is the Cas9 protein and the guide RNA comprising SEQ ID NO: 17.

5 16. The method of any one of claims 1-15, wherein the first nuclease recognition site is located in an intron, an exon, a promoter, a promoter regulatory region, or an enhancer region of the first selection marker, and the second nuclease recognition site is located in an intron, an exon, a promoter, a promoter regulatory region, or an enhancer region of the second selection marker.

10 17. The method of any one of claims 1-16, wherein:
 (a) the first target site and the second target site are immediately adjacent to the first nuclease recognition site;

 (b) the first target site and the second target site are about 10 nucleotides to about 14 kb from the first nuclease recognition site;

15 (c) the third target site and the fourth target site are immediately adjacent to the second nuclease recognition site; or

 (d) the third target site and the fourth target site are about 10 nucleotides to about 14 kb from the second nuclease recognition site.

 18. The method of any one of claims 1-17, wherein:

20 (a) the sum total of the first homology arm and the second homology arm is at least about 10 kb, and/or the sum total of the third homology arm and the fourth homology arm is at least about 10 kb; and/or

 (b) the first insert polynucleotide ranges from about 5 kb to about 300 kb in length, and/or the second insert polynucleotide ranges from about 5 kb to about 300 kb in length.

25 19. The method of any one of claims 1-18, wherein the first insert polynucleotide further comprises a first polynucleotide of interest and/or the second insert polynucleotide comprises a second polynucleotide of interest,

 optionally wherein the first polynucleotide of interest and/or the second polynucleotide of interest comprises a human polynucleotide.

20. The method of claim 19, wherein the first polynucleotide of interest and/or the second polynucleotide of interest comprises a polynucleotide encoding a region of a T cell receptor,

optionally wherein the T cell receptor is a T cell receptor alpha, and

5 optionally wherein the first polynucleotide of interest and/or the second polynucleotide of interest comprises at least one variable region gene segment and/or a joining region gene segment of the T cell receptor alpha locus.

21. The method of claim 19, wherein the first polynucleotide of interest and/or the second polynucleotide of interest comprises a genomic nucleic acid sequence that encodes a
10 human immunoglobulin heavy chain variable region amino acid sequence,

optionally wherein the first polynucleotide of interest and/or the second polynucleotide of interest comprises an unrearranged human immunoglobulin heavy chain variable region nucleic acid sequence operably linked to a non-human immunoglobulin heavy chain constant region nucleic acid sequence.

15 22. The method of claim 19, wherein the first polynucleotide of interest and/or the second polynucleotide of interest comprises a genomic nucleic acid sequence that encodes a human immunoglobulin light chain variable region amino acid sequence,

optionally wherein the genomic nucleic acid sequence comprises an unrearranged human λ and/or κ light chain variable region nucleic acid sequence or wherein the genomic
20 nucleic acid sequence comprises a rearranged human λ and/or κ light chain variable region nucleic acid sequence.

23. The method of claim 19, wherein the first polynucleotide of interest and/or the second polynucleotide of interest comprises at least one disease allele.

24. The method of any one of claims 1-23, wherein:

25 (I) the first insert polynucleotide comprises:

(i) a first polynucleotide of interest comprising a nucleic acid sequence that is homologous or orthologous to a nucleic acid sequence in the genome of the cell;
or

(ii) a first polynucleotide of interest comprising an exogenous nucleic

acid sequence; and/or

(II) the second insert polynucleotide comprises:

(i) a second polynucleotide of interest that is homologous or orthologous to a nucleic acid sequence in the genome of the cell; or

5 (ii) a second polynucleotide of interest comprising an exogenous nucleic acid sequence.

25. The method of any one of claims 1-24, wherein:

(I) integration of the first insert polynucleotide into the target locus results in a knockout, a knock-in, a point mutation, a domain swap, an exon swap, an intron swap, a regulatory sequence swap, a gene swap, or a combination thereof; and/or

10 (II) integration of the second insert polynucleotide into the target locus results in a knockout, a knock-in, a point mutation, a domain swap, an exon swap, an intron swap, a regulatory sequence swap, a gene swap, or a combination thereof.

26. The method of any one of claims 1-25, wherein the target locus is in the genome of the cell or is located in a vector in the cell.

27. The method of claim 26, wherein the target locus comprises an immunoglobulin locus.

28. The method of claim 26, wherein the target genomic locus comprises a T cell receptor locus,

20 optionally wherein the T cell receptor locus is a T cell receptor alpha locus.

29. The method of any one of claims 1-28, wherein the cell is a eukaryotic cell, optionally wherein the cell is a mammalian cell, optionally wherein the cell is:

(a) a non-human mammalian cell;

(b) a pluripotent cell;

25 (c) a human induced pluripotent stem cell;

(d) a human fibroblast;

(e) a non-human embryonic stem (ES) cell;

(f) a mouse embryonic stem (ES) cell or a rat embryonic stem (ES) cell;

- (g) a hematopoietic stem cell; or
- (h) a neuronal stem cell.
- (i) a rodent cell.

30. The method of claim 29, wherein the cell is the mouse ES cell or the rat

5 ES cell.