

CLAIMS

1. A method for targeted integration of a donor vector into a pre-defined genomic location of an isolated eukaryotic cell, said method comprising:
 - i) Providing an isolated eukaryotic cell comprising a pre-defined genomic location, which pre-defined location comprises:
 - a. a nucleic acid sequence I1 comprising a recognition site for a first DNA enzyme;
 - b. a nucleic acid sequence E1 comprising a recognition site for a second DNA enzyme; and
 - c. a promotor nucleic acid sequence P1;
 - ii) Providing a donor vector comprising:
 - a. a nucleic acid sequence I2;
 - b. a nucleic acid sequence of interest;
 - c. a nucleic acid sequence E2 comprising a recognition site for said second DNA enzyme;
 - d. a nucleic acid sequence encoding a first selection marker;
 - e. optionally an expression cassette encoding a second selection marker;
 - iii) Contacting the donor vector with the cell in the presence of a first DNA enzyme, wherein the presence of the first DNA enzyme enables recombination between the nucleic acid sequence I2 of the donor vector and the nucleic acid sequence I1 present in the pre-defined genomic location of the cell;
 - iv) Selecting a cell having the donor vector integrated at the pre-defined genomic location by detecting the expression of the first selection marker in the cell, wherein the expression of the first selection marker is activated by the promotor nucleic acid sequence P1 at the pre-defined genomic location; and
 - v) Isolating the cell selected in the preceding step.
2. The method of the preceding claim, further comprising a step vi), comprising excising a nucleic acid sequence flanked by the nucleic acid sequences E1 and E2 from the pre-defined genomic location of the cell isolated in step v) of claim 1 in the presence of a second DNA enzyme, wherein the presence of the second DNA enzyme enables recombination between the nucleic acid sequences E1 and E2, wherein the presence of a nucleic acid sequence flanked by the nucleic acid sequences E1 and E2 in said cell is indicative of a stable integration of the donor vector into the pre-defined genomic location of the cell.
3. The method of claim 2, wherein step vi) forms part of step iii), or is performed after step iii), such as after step iv) or after step v).

4. The method of claim 3, wherein step vi) is performed before step v).
5. The method of claim 4, wherein the donor vector of step ii) further comprises e) an expression cassette encoding a second selection marker and wherein the cell isolated in step v) additionally has been selected based on its non-expression of the second selection marker, wherein expression of the second selection marker signals that a donor vector has been integrated at a different location than the predefined genomic location of a cell.
6. The method of claim 3, wherein step vi) is performed after step v), and the method further comprises a step vii), performed after step vi), comprising isolating a cell in which the nucleic acid sequence being flanked by the nucleic acid sequences E1 and E2 has been excised from the pre-defined genomic location of the cell isolated in step vi).
7. The method of claim 6, wherein the donor vector of step ii) further comprises e) an expression cassette encoding a second selection marker, and wherein the cell isolated in step vii) has been selected based on its non-expression of the second selection marker, wherein expression of the second selection marker signals that a donor vector has been integrated at a different location than the predefined genomic location of a cell.
8. The method of any one of the preceding claims, wherein the nucleic acid sequence of interest of the donor vector of step ii) comprises at least one expression cassette comprising a gene encoding a protein of interest.
9. The method of claim 8 when dependent on any one of claims 2-7, wherein the excised nucleic acid sequence lacks the at least one expression cassette containing a gene encoding a protein of interest.
10. The method of any one of the preceding claims, wherein the donor vector of step ii) further comprises:
 - f. a nucleic acid sequence I3 comprising a recognition site for a first DNA enzyme; and
 - g. a promotor nucleic acid sequence P2.
11. The method of claim 10 when dependent on claim 9, further comprising performing a sequential targeted integration of n additional donor vectors into the pre-defined genomic location of the eukaryotic cell, wherein n is an integer ≥ 1 ;
the method comprising:
 - (A) Integrating a first additional donor vector into the pre-defined genomic location of the cell, comprising:
 - VIII. Providing the cell isolated in step v) of claim 5 or isolated in step vii) of claim 6 or 7;

- IX. Providing a donor vector comprising:
- A. a nucleic acid sequence I4, which in the presence of said first DNA enzyme is capable of recombining with the corresponding nucleic acid sequence I3 present in the cell provided in the preceding step;
 - B. a nucleic acid sequence of interest;
 - C. a nucleic acid sequence E2;
 - D. a nucleic acid sequence encoding a first selection marker; and
 - E. optionally an expression cassette encoding a second selection marker;
 - F. optionally a nucleic acid sequence I1 comprising a recognition site for a first DNA enzyme and a promotor nucleic acid sequence P1;
- X. Introducing the donor vector of step II) into the cell in the presence of a first DNA enzyme, wherein the presence of the first DNA enzyme enables recombination between the nucleic acid sequence I4 of the donor vector and the nucleic acid sequence I3 present in the pre-defined genomic location of the cell;
- XI. Selecting a cell having the donor vector integrated at the pre-defined genomic location by detecting the expression of the first selection marker in the cell, wherein the expression of the first selection marker is activated by the promotor nucleic acid sequence P2 at the pre-defined genomic location of the cell;
- XII. Isolating the cell selected in the preceding step;
- XIII. Excising a nucleic acid sequence flanked by the nucleic acid sequences E1 and E2 from the pre-defined genomic location of the cell isolated in step V in the presence of a second DNA enzyme, wherein the presence of the second DNA enzyme enables recombination between the nucleic acid sequences E1 and E2, wherein the presence of a nucleic acid sequence flanked by the nucleic acid sequences E1 and E2 in said cell is indicative of a stable integration of the donor vector into the pre-defined genomic location of the cell;
- XIV. Isolating a cell in which the nucleic acid sequence being flanked by the sequences E1 and E2 has been excised from said pre-defined genomic location of the cell isolated in step V;
- (B) Provided that n is larger than the number of additional donor vectors integrated at the pre-defined genomic location of the cell isolated in the preceding step, integrating

an additional donor vector into the pre-defined genomic location of the cell, comprising:

III. Providing the cell isolated in the preceding step, which cell comprises the nucleic acid sequence I1 integrated at the pre-defined genomic location;

5 IV. Performing step ii) of claim 1 or 10, steps iii)-iv) of claim 1, step v) of claim 1 or 5, step vi) of any one of claims 2-4 or 6, and step vii) of claim 6 or 7;

(C) Provided that n is larger than the number of additional donor vectors integrated at the pre-defined genomic location of the cell isolated in the preceding step, integrating an additional donor vector into the pre-defined genomic location of the cell, comprising:

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III. Providing the cell obtained by performing the preceding step (B), which cell comprises the nucleic acid sequence I3 integrated at the pre-defined genomic location;

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IV. Repeating steps (A)II to (A)IX and step (B) until the cell has n additional donor vectors integrated at the pre-defined genomic location.

12. The method of any one of claims 1-8, wherein:

- the cell of step i) of claim 1 comprises n pre-defined genomic locations, each of which comprises a nucleic acid sequence I, I₁ to I_{1n}, respectively, wherein the I₁ to I_{1n} are different from each other;

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- step ii) of claim 1 comprises providing 1 to n donor vectors, each of the 1 to n donor vectors comprising a nucleic acid sequence I₂₁ to I₂_n, respectively, which is capable of recombining with the corresponding I₁₁ to I₁_n nucleic acid sequence of said donor vector in the presence of said first DNA enzyme, and each of the 1 to n donor vectors comprising a first selection marker, SM₁ to SM_n, wherein the SM₁ to SM_n are different from each other;

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- step iv) of claim 1 comprises introducing the 1 to n donor vectors into the cell;

- step v) of claim 1 comprises selecting a cell having each of the 1 to n donor vectors integrated at its corresponding pre-defined genomic location by detecting each of the different first selection markers, SM₁ to SM_n, in the cell;

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wherein n is an integer ≥ 2 .

13. The method of claim 8, wherein the donor vector of step ii) of claim 1 further comprises:

f. a nucleic acid sequence I3 comprising a recognition site for a first DNA enzyme; and;

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g. a promotor nucleic acid sequence P2, and

wherein the excised sequence comprises the expression cassette containing a gene encoding protein of interest.

14. The method of any one of the preceding claims, wherein said first DNA enzyme is a recombinase.
15. The method of claim 14, wherein:
- 5 (a) I1 comprises two recombinase recognition site variants I1a and I1b; and
- (b) I2 comprises two recombinase recognition site variants I2a and I2b; and
- (c) I1a is capable of recombination with I2a and I1b is capable of recombination with I2b in the presence of said first DNA enzyme.
16. The method of claim 15, wherein I1a is identical to I2a and I1b is identical to I2b.
17. The method of claim 16, wherein I1a, I1b, I2a and I2b are selected from *loxP*, *rox* or *FRT* or variants thereof, respectively, and wherein the first DNA enzyme is selected from the group consisting of a Cre recombinase, a Dre recombinase and a FLP recombinase, respectively.
- 10 18. The method of claim 14, wherein:
- (a) I1 comprises a single recombinase recognition site; and
- 15 (b) I2 comprises a single recombinase recognition site; and
- (c) I1 and I2 are capable of recombination in the presence of said first DNA enzyme.
19. The method of claim 18, wherein the recombinase recognition site comprised by I1 differs in sequence from the recombinase recognition site comprised by I2.
20. The method of claim 19, wherein said recombinase recognition sites are selected from *attB*, *attP* or a variant thereof.
- 20 21. The method of any of claims 18-20, wherein said recombinase is a PhiC31 or Bxb1 recombinase or a mutant thereof.
22. The method of any one of claim 1-13, wherein said first DNA enzyme is a gene editing nuclease.
- 25 23. The method of claim 22, wherein:
- (a) I1 comprises a cut site for said gene editing nuclease and two sequence regions LHA1 and RHA1; and
- (b) I2 comprises two sequence regions LHA2 and RHA2 homologous to LHA1 and LHA2; and (c) I1 and I2 are capable of recombination in the presence of said first
- 30 DNA enzyme.
24. The method of any of claims 22-23, wherein said gene editing enzyme is selected from the group consisting of (i) zinc finger nucleases (ZFNs); (ii) homing endonucleases, such as meganucleases; (iii) TALENS and (iv) DNA or RNA guided nucleases, such as CRISPR/Cas 9.
- 35 25. The method of any one of the preceding claims, wherein the nucleic acid sequences E1 and E2 are identical recombinase recognition sites, such as *loxP*, *rox* or *FRT* or variants thereof, respectively, provided that E1 and E2 are different from I1 and I2.

26. The method of claims 24-25, wherein said second DNA enzyme is selected from the group consisting of a Cre recombinase, a Dre recombinase and a FLP recombinase, provided that the first DNA enzyme is not a Cre recombinase, a Dre recombinase or a FLP recombinase.
- 5 27. The method of any one of the preceding claims, wherein the promotor nucleic acid sequence P1 and/or P2 when integrated at said pre-defined genomic location is functionally fused to the 5'-part of a split intron.
28. The method of any one of the preceding claims, wherein said excised nucleic acid sequence comprise;
- 10 (a) Said nucleic acid sequence encoding a first selection marker;
(b) Said promotor nucleic acid sequence P1 or P2; and/or
(c) Said expression cassette encoding a second selection marker.
29. The method of any one of the preceding claims, wherein said first selection marker is selected from the groups of (i) fluorescent proteins and (ii) heterologous cell surface
- 15 markers.
30. The method of any one of the preceding claims, wherein the first DNA enzyme is provided in the form of a plasmid, mRNA or a purified protein, optionally wherein said first DNA enzyme is encoded by and expressed from said donor vector.
31. The method of any one of the preceding claims, wherein the second DNA enzyme is
- 20 provided in the form of a plasmid, mRNA or a purified protein.
32. The method of any one of claims 1-5 or 8-30, wherein said donor vector of step ii) further comprises an expression cassette encoding a second DNA enzyme, the expression of said second DNA enzyme being activated when said donor vector has been integrated into a pre-defined genomic location of a cell of step i).
- 25 33. The method of any one of the preceding claims, wherein the first DNA enzyme is expressed from an expression cassette encoding said first DNA enzyme present in the pre-defined genomic location of a cell of step i).
34. The method of any one of the preceding claims, wherein said eukaryotic cell is selected from the group consisting of a yeast cell, a filamentous fungus cell, a plant
- 30 cell, an insect cell or a mammalian cell.
35. An isolated eukaryotic cell obtainable by the method of any one of the preceding claims.
36. A method for producing a recombinant protein, said method comprising:
- 35 i) obtaining an isolated eukaryotic cell comprising one or more nucleic acid sequences of interest integrated at a pre-defined genomic location by performing the method of any one of claims 1 to 34, wherein at least one nucleic acid sequence of interest comprises at least one expression cassette comprising a gene encoding a protein of

interest;

- ii) in said cell of step i), producing a protein encoded by the gene of interest; and
- iii) isolating the protein of step ii).