

[Patent Literature]

[0003]

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[~~Summary of Invention~~]

[Technical Problem]

[0004]

Conventional genome engineering techniques fail to efficiently engineer two or more alleles at the same time by HDR. Furthermore, these techniques have a limitation in the size of a genomic region that can be engineered by HDR, and find difficulty in efficiently performing the large-scale engineering of the genome (e.g., 10 kbp).

[0005]

Accordingly, an object of the present ~~invention~~  
disclosure is to provide a genome engineering method and a genome engineering kit which can efficiently engineer two or more alleles and are capable of engineering a relatively large region.

[Solution to Problem]

[0005a]

In one aspect, the present disclosure provides an *in vitro* method for preparing a cell in which two or more alleles in the chromosomal genome are engineered, comprising the steps of:

(a) introducing the following (i) and (ii) to a cell comprising two or more alleles to introduce a selective marker gene to each of the two or more alleles:

(i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule capable of targeting a target region in the two or more alleles in the chromosomal genome and cleaving the target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(ii) two or more donor DNAs for selective markers, each of which has an upstream homology arm having a nucleotide sequence that can be homologously recombined with an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence that can be homologously recombined with a downstream nucleotide sequence of the target region, and comprises a nucleotide sequence of the selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs for selective markers respectively having distinguishably different selective marker genes, wherein the selective marker gene is unique to each type of donor DNA for the selective marker, and the number of types of the donor DNAs for selective markers is equal to or more than the number of the alleles that are subject to genome engineering; and

(b) after the step (a), selecting a cell having the distinguishably different unique selective marker genes,

which are respectively introduced in the two or more alleles by respective homologous recombination of the two or more alleles with different types of donor DNAs for selective markers, and expressing all the distinguishably different selective marker genes thus introduced (step for positive selection).

[0005b]

In another aspect, the present disclosure provides an *in vitro* method for engineering two or more alleles in the chromosomal genome, comprising the steps of:

(a) introducing the following (i) and (ii) to a cell comprising two or more alleles to introduce a selective marker gene to each of the two or more alleles:

(i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule capable of targeting a target region in the two or more alleles in the chromosomal genome and cleaving the target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(ii) two or more donor DNAs for selective markers, each of which has an upstream homology arm having a nucleotide sequence that can be homologously recombined with an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence that can be homologously recombined with a downstream nucleotide sequence of the target region, and

comprises a nucleotide sequence of the selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs for selective markers respectively having distinguishably different selective marker genes, wherein the selective marker gene is unique to each type of donor DNA for the selective marker, and the number of types of the donor DNAs for selective markers is equal to or more than the number of the alleles that are subject to genome engineering; and

(b) after the step (a), selecting a cell having the distinguishably different unique selective marker genes, which are respectively introduced in the two or more alleles by respective homologous recombination of the two or more alleles with different types of donor DNAs for selective markers, and expressing all the distinguishably different selective marker genes thus introduced (step for positive selection).

[0005c]

In yet another aspect, the present disclosure provides a genome engineering kit for engineering two or more alleles in the chromosomal genome, comprising the following (i) and (ii): (i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule capable of targeting a target region in the chromosomal genome and cleaving the target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(ii) two or more donor DNAs for selective markers, each of which has an upstream homology arm having a nucleotide sequence that can be homologously recombined with an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence that can be homologously recombined with a downstream nucleotide sequence of the target region, and comprises a nucleotide sequence of a selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs for selective markers respectively having distinguishably different selective marker genes, wherein the selective marker gene is unique to each type of donor DNA for the selective marker, and the number of types of the donor DNAs for selective markers is equal to or more than the number of the alleles that are subject to genome engineering.

[0005d]

In yet another aspect, the present disclosure provides a cell having two or more alleles in the chromosomal genome in relation to a target region, wherein the respective target regions of the two or more alleles are deleted, and the upstream and downstream sequences of the target region are seamlessly linked without insertion, substitution and deletion of a base.

[0006]

As one example, the present ~~invention~~ disclosure includes the following ~~aspects~~ examples.

[Claim 1]

An in vitro method for preparing a cell in which two or more alleles in the chromosomal genome are engineered, comprising the steps of:

(a) introducing the following (i) and (ii) to a cell comprising two or more alleles to introduce a selective marker gene to each of the two or more alleles:

(i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule capable of targeting a target region in the two or more alleles in the chromosomal genome and cleaving the target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(ii) two or more donor DNAs for selective markers, each of which has an upstream homology arm having a nucleotide sequence that can be homologously recombined with an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence that can be homologously recombined with a downstream nucleotide sequence of the target region, and comprises a nucleotide sequence of the selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs for selective markers respectively having distinguishably different selective marker genes, wherein the selective marker gene is unique to each type of donor DNA for the selective marker, and the number of types of the donor DNAs for

selective markers is equal to or more than the number of the alleles that are subject to genome engineering; and (b) after the step (a), selecting a cell having the distinguishably different unique selective marker genes, which are respectively introduced in the two or more alleles by respective homologous recombination of the two or more alleles with different types of donor DNAs for selective markers, and expressing all the distinguishably different selective marker genes thus introduced (step for positive selection).

[Claim 2]

An in vitro method for engineering two or more alleles in the chromosomal genome, comprising the steps of:

(a) introducing the following (i) and (ii) to a cell comprising two or more alleles to introduce a selective marker gene to each of the two or more alleles:

(i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule capable of targeting a target region in the two or more alleles in the chromosomal genome and cleaving the target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(ii) two or more donor DNAs for selective markers, each of which has an upstream homology arm having a nucleotide sequence that can be homologously recombined with an upstream nucleotide sequence of the target region

and a downstream homology arm having a nucleotide sequence that can be homologously recombined with a downstream nucleotide sequence of the target region, and comprises a nucleotide sequence of the selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs for selective markers respectively having distinguishably different selective marker genes, wherein the selective marker gene is unique to each type of donor DNA for the selective marker, and the number of types of the donor DNAs for selective markers is equal to or more than the number of the alleles that are subject to genome engineering; and (b) after the step (a), selecting a cell having the distinguishably different unique selective marker genes, which are respectively introduced in the two or more alleles by respective homologous recombination of the two or more alleles with different types of donor DNAs for selective markers, and expressing all the distinguishably different selective marker genes thus introduced (step for positive selection).

[Claim 3]

The method according to claim 1 or 2, wherein the target region has a length of 5 kbp or more, or 8 kbp or more.

~~{Claim 4}~~

~~— The method according to claim 3, wherein the target region has a length of 8 kbp or more.~~

[Claim ~~54~~4]

The method according to any one of claims 1 to ~~43~~3, wherein each of the two or more donor DNAs for selective markers has a selective marker gene for positive selection, a marker gene for negative selection, and a target sequence between the upstream homology arm and the downstream homology arm, wherein in the case of using the selective marker gene both for positive selection and for negative selection, another selective marker gene for negative selection is optionally absent,

the method further comprising the steps of:

(c) after the step (b), introducing the following (iii) and (iv) to the selected cell to introduce a donor DNA for recombination to each of the two or more alleles:

(iii) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule capable of targeting the target sequence and cleaving the target sequence, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(iv) a donor DNA for recombination comprising a desired nucleotide sequence, the donor DNA for recombination having an upstream homology arm having a nucleotide sequence that can be homologously recombined with an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence that can be homologously recombined with a downstream nucleotide sequence of the target region; and

(d) after the step (c), selecting a cell not expressing the marker gene for negative selection (step for negative selection).

~~[Claim 6]~~

~~— The method according to claim 3, wherein~~

~~— each of the two or more donor DNAs for selective markers has a selective marker gene for positive selection, a marker gene for negative selection, and a target sequence between the upstream homology arm and the downstream homology arm, wherein in the case of using the selective marker gene both for positive selection and for negative selection, another selective marker gene for negative selection is optionally absent,~~

~~— the method further comprising the steps of:~~

~~(c) after the step (b), introducing the following (iii) and (iv) to the selected cell to introduce a donor DNA for recombination to each of the two or more alleles:~~

~~— (iii) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule capable of targeting the target sequence and cleaving the target sequence, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and~~

~~— (iv) a donor DNA for recombination comprising a desired nucleotide sequence, the donor DNA for recombination having an upstream homology arm having a nucleotide sequence that can be homologously recombined with an upstream nucleotide sequence of the target region~~

~~and a downstream homology arm having a nucleotide sequence that can be homologously recombined with a downstream nucleotide sequence of the target region; and (d) after the step (c), selecting a cell not expressing the marker gene for negative selection (step for negative selection).~~

~~{Claim 7}~~

~~— The method according to claim 4, wherein~~

~~— each of the two or more donor DNAs for selective markers has a selective marker gene for positive selection, a marker gene for negative selection, and a target sequence between the upstream homology arm and the downstream homology arm, wherein in the case of using the selective marker gene both for positive selection and for negative selection, another selective marker gene for negative selection is optionally absent,~~

~~— the method further comprising the steps of:~~

~~(c) after the step (b), introducing the following (iii) and (iv) to the selected cell to introduce a donor DNA for recombination to each of the two or more alleles:~~

~~— (iii) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule capable of targeting the target sequence and cleaving the target sequence, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and~~

~~— (iv) a donor DNA for recombination comprising a desired nucleotide sequence, the donor DNA for~~

~~recombination having an upstream homology arm having a nucleotide sequence that can be homologously recombined with an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence that can be homologously recombined with a downstream nucleotide sequence of the target region; and (d) after the step (c), selecting a cell not expressing the marker gene for negative selection (step for negative selection).~~

[Claim ~~8~~5]

The method according to any one of claims ~~5 to 7~~ 4, wherein the region between the upstream homology arm and the downstream homology arm of the donor DNA for recombination has a length of 5 kbp or more, or wherein the region between the upstream homology arm and the downstream homology arm of the donor DNA for recombination has a length of 8 kbp or more.

~~[Claim 9]~~

~~— The method according to claim 6 or 7, wherein the region between the upstream homology arm and the downstream homology arm of the donor DNA for recombination has a length of 5 kbp or more.~~

~~[Claim 10]~~

~~— The method according to claim 7, wherein the region between the upstream homology arm and the downstream homology arm of the donor DNA for recombination has a length of 5 kbp or more.~~

~~{Claim 11}~~

~~—The method according to claim 8, wherein the region between the upstream homology arm and the downstream homology arm of the donor DNA for recombination has a length of 8 kbp or more.~~

[Claim 126]

A genome engineering kit for engineering two or more alleles in the chromosomal genome, comprising the following (i) and (ii):

(i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule capable of targeting a target region in the chromosomal genome and cleaving the target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and  
(ii) two or more donor DNAs for selective markers, each of which has an upstream homology arm having a nucleotide sequence that can be homologously recombined with an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence that can be homologously recombined with a downstream nucleotide sequence of the target region, and comprises a nucleotide sequence of a selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs for selective markers respectively having distinguishably different selective marker genes, wherein the selective marker gene is unique to each type of donor DNA for the selective marker, and

the number of types of the donor DNAs for selective markers is equal to or more than the number of the alleles that are subject to genome engineering.

[Claim ~~13~~7]

The kit according to claim ~~12~~6, wherein the target region has a length of 5 kbp or more, or 8 kbp or more.

~~{Claim 14}~~

~~— The kit according to claim 13, wherein the target region has a length of 8 kbp or more.~~

[Claim ~~15~~8]

The kit according to any one of claims ~~12-6~~6 to ~~14~~7, further comprising a donor DNA for recombination.

[Claim ~~16~~9]

The kit according to any one of claims ~~12-6~~6 to ~~15~~8, wherein a region between an upstream homology arm and a downstream homology arm of the donor DNA for recombination has a length of 5 kbp or more, or 8 kbp or more.

~~{Claim 17}~~

~~— The kit according to any one of claims 12 to 16, wherein a region between an upstream homology arm and a downstream homology arm of the donor DNA for recombination has a length of 8 kbp or more.~~

[Claim ~~18~~10]

The kit according to claim ~~12~~6, wherein the target region has a length of 5 kbp or more, and a region between an upstream homology arm and a downstream

homology arm of the donor DNA for recombination has a length of 5 kbp or more.

~~{Claim 19}~~

~~— The kit according to claim 18, or wherein the target region has a length of 8 kbp or more, and a region between an upstream homology arm and a downstream homology arm of the donor DNA for recombination has a length of 8 kbp or more.~~

[Claim ~~20~~11]

The method according to claim ~~5~~4, wherein the donor DNA for recombination has no nucleotide sequence in the region between the upstream homology arm and the downstream homology arm of the donor DNA for recombination, and the upstream and downstream sequences of the target region are seamlessly linked, without insertion, substitution and deletion of a base, in the thus-engineered two or more alleles in the chromosomal genome.

~~{Claim 21}~~

~~— The method according to claim 6 or 7, wherein the donor DNA for recombination has no nucleotide sequence in the region between the upstream homology arm and the downstream homology arm of the donor DNA for recombination, and the upstream and downstream sequences of the target region are seamlessly linked, without insertion, substitution and deletion of a base, in the~~

~~thus-engineered two or more alleles in the chromosomal genome.~~

[Claim ~~22~~12]

The method according to claim 3, wherein a target sequence of site-specific recombinase is absent in the thus-engineered two or more alleles in the chromosomal genome.

~~[Claim 23]~~

~~— The method according to claim 4, wherein a target sequence of site-specific recombinase is absent in the thus-engineered two or more alleles in the chromosomal genome.~~

[Claim ~~24~~13]

The method according to claim ~~5~~4, wherein a target sequence of site-specific recombinase is absent in the thus-engineered two or more alleles in the chromosomal genome.

~~[Claim 25]~~

~~— The method according to claim 6 or 7, wherein a target sequence of site-specific recombinase is absent in the thus-engineered two or more alleles in the chromosomal genome.~~

[Claim ~~26~~14]

The method according to any one of claims 1 to ~~11~~5 and ~~20~~11 to ~~25~~13, wherein in the step (b), single-cell cloning is not performed in a process up to the selection

of the cell in which the two or more alleles are engineered.

[Claim ~~27~~15]

A cell having two or more alleles in the chromosomal genome in relation to a target region, wherein the respective target regions of the two or more alleles are deleted, and the upstream and downstream sequences of the target region are seamlessly linked without insertion, substitution and deletion of a base.

[Claim ~~28~~16]

The cell according to claim ~~27~~15, wherein the target region has a length of 5 kbp or more.

[Claim ~~29~~17]

The cell according to claim ~~27-15~~ or ~~28~~16, wherein the cell has no target sequence of site-specific recombinase in the genome.

~~[Claim 30]~~

~~—The method according to claim 6 or 7, wherein the region between the upstream homology arm and the downstream homology arm of the donor DNA for recombination has a length of 8 kbp or more.~~

[Claim ~~31~~18]

The kit according to claim ~~15~~8, wherein the donor DNA for recombination has no nucleotide sequence in the region between the upstream homology arm and the downstream homology arm of the donor DNA for recombination.