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(54) PROCÉDE SERVANT À CREER DES SEQUENCES DE
POLYNUCLEOTIDES ET DE POLYPEPTIDES
(54) METHOD FOR CREATING POLYNUCLEOTIDE AND
POLYPEPTIDE SEQUENCES

(57) L’invention concerne des procédés servant à faire évoluer un polynucléotide vers l’acquisition d’une propriété souhaitée. Ces procédés consistent en l’incubation d’une population de variantes parentales de polynucléotides dans des conditions capables de générer des polynucléotides renaturés comprenant des hétéroduplex. On expose ensuite ces hétéroduplex à un système de réparation d’ADN cellulaire afin de convertir ces hétéroduplex en variantes parentales de polynucléotides ou en variantes de recombinaison de polynucléotides. On effectue ensuite le criblage des polynucléotides obtenus ou leur sélection afin de rechercher la propriété souhaitée.

(57) The invention provides methods for evolving a polynucleotide toward acquisition of a desired property. Such methods entail incubating a population of parental polynucleotide variants under conditions to generate annealed polynucleotides comprising heteroduplexes. The heteroduplexes are then exposed to a cellular DNA repair system to convert the heteroduplexes to parental polynucleotide variants or recombinant polynucleotide variants. The resulting polynucleotides are then screened or selected for the desired property.
METHOD FOR CREATING POLYNUCLEOTIDE AND POLYPEPTIDE SEQUENCES

The invention provides methods for evolving a polynucleotide toward acquisition of a desired property. Such methods entail incubating a population of parental polynucleotide variants under conditions to generate annealed polynucleotides comprising heteroduplexes. The heteroduplexes are then exposed to a cellular DNA repair system to convert the heteroduplexes to parental polynucleotide variants or recombined polynucleotide variants. The resulting polynucleotides are then screened or selected for the desired property.
WHAT IS CLAIMED IS:

1. A method for evolving a polynucleotide toward acquisition of a desired property, comprising
   (a) incubating a population of parental polynucleotide variants under conditions to generate annealed polynucleotides comprising heteroduplexes;
   (b) exposing the heteroduplexes to a cellular DNA repair system to convert the heteroduplexes to parental polynucleotide variants or recombined polynucleotide variants;
   (c) screening or selecting the recombined polynucleotide variants for the desired property.

2. The method of claim 1, wherein the heteroduplexes are exposed to the cellular DNA repair system in vitro.

3. The method of claim 2, wherein the cellular DNA repair system comprises cellular extracts.

4. The method of claim 1, further comprising introducing the heteroduplexes into cells, whereby the heteroduplexes are exposed to the DNA repair system of the cells in vivo.

5. The method of claim 4, wherein the annealed polynucleotides further comprise homoduplexes and the introducing step selects for transformed cells comprising the heteroduplexes relative to transformed cells comprising homoduplexes.

6. The method of claim 4, wherein a first polynucleotide variant is provided as a component of a first vector, and a second polynucleotide variant is provided as a component of a second vector, and the method further comprises converting the first and second vectors to linearized forms in which the first and second polynucleotide variants occur at opposite ends, whereby in the incubating step single-stranded forms of the first linearized vector reanneal with each other to form linear first vector, single-stranded forms of the second linearized vector reanneal with each other to form linear second vector, and single-stranded linearized forms of the first and second vectors anneal with each to form a circular heteroduplex bearing a nick in each strand, and the
introducing step selects for transformed cells comprising the circular heteroduplexes relative to the linear first and second vector.

7. The method of claim 6, wherein the first and second vectors are converted to linearized forms by PCR.

8. The method of claim 6, wherein the first and second vectors are converted to linearized forms by digestion with first and second restriction enzymes.

9. The method of claim 1, wherein the population of polynucleotide variants are provided in double stranded form, and the method further comprising converting the double stranded polynucleotides to single stranded polynucleotides before the annealing step.

10. The method of claim 1, wherein the converting step comprises: conducting asymmetric amplification of the first and second double stranded polynucleotide variants to amplify a first strand of the first polynucleotide variant, and a second strand of the second polynucleotide variant, whereby the first and second strands anneal in the incubating step to form a heteroduplex.

11. The method of claim 10, wherein the first and second double-stranded polynucleotides variants are provided in vector-free form, and the method further comprises incorporating the heteroduplex into a vector.

12. The method of claim 4 wherein the population of polynucleotides comprises first and second polynucleotides provided in double stranded form, and the method further comprises incorporating the first and second polynucleotides as components of first and second vectors, whereby the first and second polynucleotides occupy opposite ends of the first and second vectors, whereby in the incubating step single-stranded forms of the first linearized vector reanneal with each other to form linear first vector, single-stranded forms of the second linearized vector reanneal with each other to form linear second vector, and single-stranded linearized forms of the first and second vectors anneal with each to form a circular heteroduplex bearing a nick in each
strand, and the introducing step selects for transformed cells comprises the
1 circular heteroduplexes relative to the linear first and second vector.

13. The method of claim 4, further comprising sealing nicks in the
2 heteroduplexes to form covalently-closed circular heteroduplexes before the introducing
3 step.

14. The method of claim 11, wherein the first and second
2 polynucleotides are obtained from chromosomal DNA..

15. The method of claim 1, further comprising repeating steps (a)-(c)
2 whereby the incubating step in a subsequent cycle is performed on recombinant variants
3 from a previous cycle.

16. The method of claim 1, wherein the polynucleotide variants encode
2 a polypeptide.

17. The method of claim 1, wherein the population of polynucleotide
2 variants comprises at least 20 variants.

18. The method of claim 1, wherein the population of polynucleotide
2 variants are at least 10 kb in length.

19. The method of claim 1, wherein the population of polynucleotide
2 variants comprises natural variants.

20. The method of claim 1, wherein the population of polynucleotides
2 comprises variants generated by mutagenic PCR.

21. The method of claim 1, wherein the population of polynucleotide
2 variants comprises variants generated by site directed mutagenesis.

22. The method of claim 1, wherein the cells are bacterial cells.

23. The method of claim 1, further comprising at least partially
2 demethylating the population of variant polynucleotides.
24. The method of claim 23, whether the at least partially
demethylating step is performed by PCR amplification of the population of variant
polynucleotides.

25. The method of claim 23, wherein the at least partially
demethylating step is performed by amplification of the population of variant
polynucleotides in host cells.

26. The method of claim 25, wherein the host cells are defective in a
gene encoding a methylase enzyme.

27. The method of claim 1, wherein the population of variant
polynucleotide variants comprises at least 5 polynucleotides having at least 90% sequence
identity with one another.

28. The method of claim 1, further comprising isolating a screened
recombinant variant.

29. The method of claim 28, further comprising expressing a screened
recombinant variant to produce a recombinant protein.

30. The method of claim 29 further comprising formulating the
recombinant protein with a carrier to form a pharmaceutical composition.

31. The method of claim 1, wherein the polynucleotide variants encode
enzymes selected from the group consisting of proteases, lipases, amylases, cutinases,
cellulases, amylases, oxidases, peroxidases and phytases.

32. The method of claim 1, wherein the polynucleotide variants encode
a polypeptide selected from the group consisting of insulin, ACTH, glucagon,
somatostatin, somatotropin, thymosin, parathyroid hormone, pigmentary hormones,
somatomedin, erthropoietin, luteinizing hormone, chorionic gonadotropin, hyperthalnic
releasing factors, antidiuretic hormones, thyroid stimulating hormone, relaxin, interferon,
thrombopoietin (TPO), and prolactin.
33. The method of claim 1, wherein the polynucleotide variants encode a plurality of enzymes forming a metabolic pathway.

34. The method of claim 1, wherein the polynucleotide variants are in concatemeric form.
PCR (P1/P2~100)

target

Duplex Formation

Digestion

Ligation

Transformation

TRANSFORMANTS

Fig. 3
Fig. 4
Fig. 12
Fig. 13B
Fig. 13C
2881 CCACAGCTCGACACCGGCACCGGACACCTGGTCTGGCGCTTGCAAGCGCCCGGAGCC
563 RMHGAEELARDDDLVALCRRQP

2941 GACCGCGAACCGCCCTCGAACGGCGCAATCCTGCAGCTCCTCAACCCGCGACCTGGCTTCGC
583 TATASNGAIVDTrTaAcatals

3001 CCGCTTCGTACAGGGTCCACGGACACCTGGTCTGGCTAAGCGGTGGTACGGGTGGAACCTGGGACCCG
603 RFDERADLDSDRGAHFLTFEF

3061 CCTCCGGCGGGCCTGCATTACAGGGTCTCGACCGACCGGCGGGCCCTGGCTTCGC
623 LAGGIRFADTFEVTDPPVRTP

3121 CCGCGCTCTGAAACCCACACCGATCCCGGCGGCTAGCGGACGCGGCTCCTCGCGACCGGCGCTCAGAG
643 RLNNTDPDPRVTALADAVQR

3181 GCTCCGGCGGATCCCCCTCGACGACGCGAAGCTGGGGAACATGCCAACCGACAGCCCGGGCGCA
663 LAGIPLDAKLGDIDTDSRGE

3241 ACACCACATCCCATCCCCAGGGTTGCGCCGGCGGAAGCCAGGCACCTCCAGATCAGCTCAGGGCA
683 RRIPINHGGRGEGATFTNVITK

3301 CCCCCGCTCGACCCGGGCGGTGGGAATACCCCGCAGGTCTCGACCGGAACATGTCGTGGATTGCG
703 PLVPGVGVPQVHPHGTSFVM

3361 GTGACACCTCCGGCGCCACGGCGGGCGTCTGGGACCGACGCTGACTGCGACATCCAGGCGACCTGGG
723 VELPGPHGPSGRQILTYAQST

3421 GAACCGAACTCACCACTGGTACTCGCGACGAGCCAGGTCTCCTACTCGCGGAAGGGCGTTGGGA
743 NPNSPFWYADQTVLYRSRKGDW

3481 CACCATCAAGTACCCAGGCGACGATCCGCGCGCCACCGGAACTCCGCGGCTACCAGGTT
763 TIKYTEAAQAADPNNLVRVR

3541 GGACACACGGGACCTGACACCGCGCGGGCGCTCGCGCGGGCGCGAGGGCC

Fig. 13D
Fig. 13E

783 A Q R G R

3601 CCGATCGTCCTTGCATCGCCCCGGTGCCCGGGGCTGGGCTGACCCGCGGGGCGCCGGTGCG
3661 CGCCCGCGTCGGCGCGCAGCGCTGCGAGAGCGCGCCGCGCGCGCGCGGCGAGGT
3721 TTGTGAAACATAGCTAGCCGCAGGGTCCCGGTCTGAGGATCGCCGGCAGAGTGCACGCTCGG
3781 CATCCGTGACACATGCGGGCGCGGGGCGTATCGCGCCGACCGATATACGTCGCGG
3841 TCAGACGTGCGCGCGAGAACCGGTGCGGGGAGTCAGGTTGCGTCCTGCGGCGAC
3901 CCTCGACCAATCGTCCGACCACGGCCGGCGGCGGCTCCGAGAAAACTGCAAGCGGGCCGACACCGGGGCG
3961 GCCGGCGCGTCGGGGCGCGAGAACCTGACGCGCTGCGGCAGGCTGGCCTGGTGCCCGGA
4021 AACTCGCGGGSCAGCGAG
Cloned pBE3-1 and pBE3-2

Amplification & Demethylation

Plasmid Prep

Restriction with enzyme Bam HI

Restriction with enzyme Nde I

Demethylated target molecules

Bam HI

Bam HI

Nde I

Nde I

Heteroduplex formation

Heteroduplex pool (shown only insert with parts of vector)

Transformatton & Repair

Recombinitants (shown only the inserts)

Screening

Recombinitants (shown only the inserts)

Fig. 14
Fig. 15
![Thermostability index (Ar/Ai) vs Clones graph showing N181D+N218S type, N181D or N218S type, and Wild type]

**Fig. 16**