

Claims

1. An *in vitro* method of joining a set of two or more double-stranded (ds) or single-stranded (ss) DNA molecules, wherein adjacent DNA molecules to be joined contain overlapping sequences at their termini, said method comprising

contacting *in vitro* the two or more DNA molecules in a single vessel with a mixture of components comprising:

(a) an isolated ~~non-thermostable~~ 5' to 3' ~~exonuclease that lacks 3'~~ exonuclease activity;

~~(b) a crowding agent,~~

~~(c) an isolated thermostable non-strand-displacing DNA polymerase with 3' exonuclease activity, or a mixture of said DNA polymerase with a second DNA polymerase that lacks 3' exonuclease activity,~~

~~(d) an isolated thermostable ligase,~~

~~(e) a mixture of dNTPs, and~~

~~(f) a suitable buffer,~~

under isothermal conditions at a temperature of 45-60°C and in amounts that are effective for joining the two or more DNA molecules to form a first assembled dsDNA molecule in a one-step concerted reaction in said single vessel.

2. The method of claim 1, wherein the exonuclease of (a) is a T5 exonuclease ~~and said contacting is under isothermal conditions.~~

3. The method of claim 1, further comprising a crowding agent.

~~4. The method of claim 1, wherein the crowding agent of (b) is selected from PEG, and/or dextran. the non-strand-displacing DNA polymerase of (c) is Phusion™ DNA polymerase or VENT® DNA polymerase, and/or the ligase of (d) is Taq ligase.~~

5. The method of claim 3, wherein the crowding agent is PEG.

6. The method of claim 2, wherein the ligase of (c) is Taq ligase.

7. The method of claim 1, wherein the ligase of (c) is Taq ligase.

48. The method of claim 1, wherein the conditions are also suitable for digesting any unpaired, non-homologous, single-stranded DNAs following the joining reaction.

59. The method of claim 48, wherein at least some of the DNA molecules to be joined comprise, at one terminus, a sequence that is non-homologous to any of the DNA molecules of interest, and wherein the non-homologous sequences comprise one or more binding regions for PCR primers, and/or regions of homology to vector sequences, and/or recognition sites for one or more restriction enzymes.

~~6. The method of claim 5, wherein the non-homologous sequences comprise one or more binding regions for PCR primers, and/or regions of homology to vector sequences, and/or recognition sites for one or more restriction enzymes.~~

710. The method of ~~any of claims 1-6~~, further comprising repeating the method to join a second set of two or more DNA molecules to one another to obtain a second assembled DNA molecule, and then joining the first and the second assembled DNA molecules to obtain a third assembled ds DNA molecule.

11. The method of claim 1, wherein the DNA polymerase has 3' exonuclease activity.

12. The method of claim 1, wherein the DNA polymerase and the ligase are thermostable.

13. The method of claim 1, wherein the isothermal conditions occur at a temperature of 50°C.

~~814. A kit for a one step *in vitro* reaction to join a set of two or more double stranded (ds) or single stranded (ss) DNA molecules, wherein adjacent DNA molecules to be joined contain overlapping sequences at their termini, comprising in a single vessel~~

- ~~(a) The method of claim 3, an isolated non-thermostable wherein the 5' to 3' exonuclease that lacks 3' exonuclease activity;~~
- ~~(b) — a crowding agent,~~
- ~~(c) — an isolated thermostable non-strand displacing DNA polymerase with 3' exonuclease activity, or a mixture of said DNA polymerase with a second DNA polymerase that lacks 3' exonuclease activity,~~
- ~~(d) — an isolated thermostable ligase,~~
~~in amounts such that when said two or more DNA molecules are added to the kit, in the presence of a suitable buffer solution and dNTPs, and incubated under isothermal conditions, the two or more DNA molecules are assembled in a concerted reaction.~~

~~915. The kit/method of claim 814, comprising~~

- ~~(a) wherein the exonuclease of (a) is a T5 exonuclease;~~

~~16. (b) The method of claim 15, wherein the crowding agent is selected from PEG and/or dextran;~~

- ~~(c) — Phusion™ DNA polymerase, and~~
- ~~(d) — Taq ligase.~~

~~17. The method of claim 16, wherein the crowding agent is PEG.~~

~~10. — A method of modifying the properties of a whole nucleic acid molecule, said method comprising:~~

- ~~(a) — representationally dividing the nucleic acid sequence of said whole nucleic acid molecule into a multiplicity of portions along its length thereby identifying the sequences of partial nucleic molecules;~~
- ~~(b) — providing, for at least 3 of said partial nucleic molecules, a multiplicity of variants of each partial nucleic acid molecule;~~
- ~~(c) — combinatorially assembling *in vitro* said variants along with any partial nucleic acid molecules which are not varied, wherein the partial nucleic acid molecules or variants~~

thereof contain overlapping sequences at their termini whereby assembly of the partial nucleic acid molecules and variants thereof in the mixture would result in assembly of a multiplicity of variants of the whole nucleic acid molecule; and

(d) — expressing the variants of the whole nucleic acid molecule to determine any modified properties of said variants of said whole nucleic acid molecule;

wherein the assembling of step (c) is performed by the method of claim 1.

11. — An *in vitro* method of joining a set of two or more double-stranded (ds) or single-stranded (ss) DNA molecules, wherein adjacent DNA molecules to be joined contain overlapping sequences at their termini, said method comprising contacting *in vitro* the two or more DNA molecules in a single vessel with

(a) — an isolated non-thermostable 3' to 5' exonuclease active in the presence of dNTPs,

(b) — a crowding agent,

(c) — an isolated heat-activated DNA polymerase,

(d) — an isolated thermostable ligase,

(e) — a mixture of dNTPs, and

(f) — a suitable buffer,

under conditions that are effective for joining the two or more DNA molecules to form a first assembled dsDNA molecule in a one-step thermocycled reaction.

12. — The method of claim 11, wherein the exonuclease of (a) is Exonuclease III.

13. — The method of claim 11, wherein polymerase of (c) is heat-activated by the removal of an inactivating moiety combined with the polymerase in a heat-sensitive manner.

14. — The method of claim 11, wherein

the crowding agent of (b) is PEG, and/or

the DNA polymerase of (c) is AMPLITAQ GOLD[®], and/or

the ligase of (d) is Taq ligase.

15. — The method of any of claims 11–14, further comprising repeating the method to join a second set of two or more DNA molecules to one another to obtain a second assembled

DNA molecule, and then joining the first and the second assembled DNA molecules to obtain a third assembled ds-DNA molecule.

16. — A kit for a one-step *in vitro* reaction to join a set of two or more double-stranded (ds) or single-stranded (ss) DNA molecules, wherein adjacent DNA molecules to be joined contain overlapping sequences at their termini, comprising in a single vessel

(a) — an isolated non-thermostable 3' to 5' exonuclease active in the presence of dNTPs;

(b) — a crowding agent;

(c) — an isolated heat-activated DNA polymerase;

(d) — an isolated thermostable ligase;

(e) — a mixture of dNTPs, and

(f) — a suitable buffer;

in amounts such that when said two or more DNA molecules are added to the kit, in the presence of a suitable buffer solution and dNTPs, and incubated under thermocycled conditions, the two or more DNA molecules are assembled in a concerted reaction.

17. — The kit of claim 16, comprising

(a) — Exonuclease III;

(b) — PEG;

(c) — AMPLITAQ GOLD[®] DNA polymerase, and

(d) — Taq ligase.

18. — A method of modifying the properties of a whole nucleic acid molecule, said method comprising:

(a) — representationally dividing the nucleic acid sequence of said whole nucleic acid molecule into a multiplicity of portions along its length thereby identifying the sequences of partial nucleic molecules;

(b) — providing, for at least 3 of said partial nucleic molecules, a multiplicity of variants of each partial nucleic acid molecule;

(c) — combinatorially assembling *in vitro* said variants along with any partial nucleic acid molecules which are not varied, wherein the partial nucleic acid molecules or variants thereof contain overlapping sequences at their termini whereby assembly of the partial nucleic

acid molecules and variants thereof in the mixture would result in assembly of a multiplicity of variants of the whole nucleic acid molecule; and

(d) — expressing the variants of the whole nucleic acid molecule to determine any modified properties of said variants of said whole nucleic acid molecule;

wherein the assembling of step (c) is performed by the method of claim 11.

19. — A method of modifying the properties of a whole nucleic acid molecule, said method comprising:

(a) — representationally dividing the nucleic acid sequence of said whole nucleic acid molecule into at least 5 portions along its length thereby identifying the sequences of partial nucleic molecules;

(b) — providing, for at least 3 of said partial nucleic molecules, a multiplicity of variants of the partial nucleic acid molecule;

(c) — combinatorially assembling *in vitro* said variants along with any partial nucleic acid molecules which are not varied, wherein the partial nucleic acid molecules or variants thereof contain overlapping sequences at their termini whereby assembly of the partial nucleic acid molecules and variants thereof in the mixture would result in assembly of a multiplicity of variants of the whole nucleic acid molecule; and

(d) — expressing the variants of the whole nucleic acid molecule to determine any modified properties of the variants of said whole nucleic acid molecule.

20. — The method of claim 19, wherein the variants of the partial nucleic acid molecule provide degenerate forms of the codon for one or more amino acids encoded by the partial nucleic acid molecules; or

wherein the variants of the partial nucleic acid molecule provide a multiplicity of nucleic acid control sequences affecting transcription or translation of the whole nucleic acid molecule; or

wherein the variants of the partial nucleic acid molecule provide a multiplicity of regions encoding domains or motifs of peptides or proteins encoded by the whole nucleic acid molecule; or

wherein the peptides or proteins encoded by said partial nucleic acid molecules function together in a metabolic pathway.

21. ~~A method of modifying the properties of a whole nucleic acid molecule, said method comprising:~~

~~(a) — representationally dividing the nucleic acid sequence of said whole nucleic acid molecule into a multiplicity of portions along its length thereby identifying the sequences of partial nucleic molecules;~~

~~(b) — providing, for at least 3 of said partial nucleic molecules, a multiplicity of variants of the partial nucleic acid molecule;~~

~~(c) — combinatorially assembling *in vitro* said variants along with any partial nucleic acid molecules which are not varied, wherein the partial nucleic acid molecules or variants thereof contain overlapping sequences at their termini whereby assembly of the partial nucleic acid molecules and variants thereof in the mixture would result in assembly of a multiplicity of variants of the whole nucleic acid molecule; and~~

~~(d) — expressing the variants of the whole nucleic acid molecule to determine any modified properties of the variants of said whole nucleic acid molecule;~~

~~wherein the variants of the partial nucleic acid molecule provide degenerate forms of the codon for one or more amino acids encoded by the partial nucleic acid molecules, or~~

~~wherein the variants of the partial nucleic acid molecule provide a multiplicity of nucleic acid control sequences affecting transcription or translation of the whole nucleic acid molecule, or~~

~~wherein the variants of the partial nucleic acid molecule provide a multiplicity of regions encoding domains or motifs of peptides or proteins encoded by the whole nucleic acid molecule, or~~

~~wherein peptides or proteins encoded by said partial nucleic acid molecules function together in a metabolic pathway.~~

22. ~~The method of claim 19, 20 or 21, wherein the assembly in step (c) is performed~~

~~(1) — by a method comprising:~~

~~—— (a) — contacting said variants along with any partial nucleic acid molecules which are not varied with a non-processive 5' exonuclease; and with~~

~~—— (b) — a single stranded DNA binding protein (SSB) which accelerates nucleic acid annealing; and with~~

~~—— (c) — a non-strand-displacing DNA polymerase; and with~~

~~——(d)—— a ligase,~~

~~under conditions effective to join the variants and partial nucleic acid molecules that are not varied so as to result in assembly of a multiplicity of variants of the whole nucleic acid molecule; or~~

~~(2)—— by *in vivo* assembly.~~

~~23.—— The method of any of claims 19-21, further comprising assembling two or more whole nucleic acid molecules prepared according to said methods *in vitro* or *in vivo*.~~

~~24.—— A library of variant DNA molecules prepared by the method of any of claims 10 or 18-21.~~

~~25.—— A modified DNA molecule prepared by the method of any of claims 10 or 18-21.~~

~~26.—— The modified DNA of claim 25 which comprises at least one optimized codon; and/or~~

~~at least one optimized control sequence; and/or~~

~~at least one nucleotide sequence that encodes an optimized protein; and/or~~

~~at least one sequence that encodes an optimized pathway.~~