

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 5' to 3' exonuclease;
- (b) an isolated DNA polymerase,
- (c) an isolated ligase,
- (d) a mixture of dNTPs, and
- (e) a suitable buffer,

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

- 2. The method of claim 1, wherein the exonuclease of (a) is a T5 exonuclease.
- 3. The method of claim 1, further comprising a crowding agent.
- 4. The method of claim 3, wherein the crowding agent is selected from PEG and/or dextran.
- 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.
- 8. The method of claim 1, wherein the conditions are also suitable for digesting any unpaired, non-homologous, single-stranded DNAs following the joining reaction.

9. The method of claim 8, 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.

10. The method of claim 1, 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.

14. The method of claim 3, wherein the 5' to 3' exonuclease lacks 3' exonuclease activity.

15. The method of claim 14, wherein the exonuclease of (a) is a T5 exonuclease.

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

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