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 consisting essentially of:

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

(b) a crowding agent selected from the group consisting of: polyethylene glycol and dextran,

(c) an isolated thermostable non-strand-displacing DNA polymerase with 3' exonuclease activity,

(d) an isolated thermostable ligase,

(e) a mixture of dNTPs, and

(f) a suitable buffer,

under isothermal conditions 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 and said contacting is under isothermal conditions.

3. The method of claim 1, wherein

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

the ligase of (d) is Taq ligase.

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

5. The method of claim 4, 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.

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.

7. 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.