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JESSBERGER R ET AL: "A mammalian protein complex that repairs double-strand breaks and deletions by recombination" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BIRMINGHAM,, US, vol. 268, no. 20, 15 July 1993 (1993-07-15), pages 15070-15079, XP002088396 ISSN: 0021-9258
LAI YING-TA ET AL: "Repair of double-strand breaks by incorporation of a molecule of homologous DNA" MOLECULAR MICROBIOLOGY, vol. 36, no. 2, April 2000 (2000-04), pages 437-446, XP002414373 ISSN: 0950-382X cited in the application
ZHOU MEIXIA ET AL: "An improved ligase-free method for directional subcloning of PCR amplified DNA" NUCLEIC ACIDS RESEARCH, vol. 23, no. 6, 1995, pages 1089-1090, XP002414374 ISSN: 0305-1048

DESCRIPTION

FIELD OF THE INVENTION

[0001] This invention relates, *e.g.*, to *in vitro* methods for joining (recombining) double stranded DNA molecules via a region of homology. In one embodiment, a plurality of DNA molecules are joined into a longer DNA molecule in a predefined order and orientation.

BACKGROUND INFORMATION

[0002] Homologous recombination of linear double stranded DNA has long been known to be crucial for the repair of double stranded DNA breaks. In most organisms, the initiation of homologous recombination requires the action of an exodeoxyribonuclease. The single stranded DNA fragment generated can then pair with homologous sequence on other DNA molecules to complete the recombination. Although homologous recombination has been intensely studied, the mechanism involved is still not fully understood. The most efficient homologous recombination system has been discovered in *Deinococcus radiodurans*, which can survive 15,000 Gy of ionizing radiation, while doses below 10 Gy are lethal to almost all other organisms (Daly et al. (1996) J. of Bacteriology 178, 4461-4471). However, due to the complexity of the *D.radiodurans* genome, it is extremely difficult to pinpoint the proteins involved in the homologous recombination process.

[0003] Homologous recombination has also been demonstrated in the enterobacteria phage T7 system, the efficiency of which can be more than 50% (Lai et al. (1998) J. of Bacteriology 180, 6193-6202). T7 phage contain only 56 genes which encode 59 proteins, and therefore would be a more suitable system to isolate proteins involved in homologous recombination. In the T7 genome, genes that are involved in similar functions are normally clustered together. It has been reported that the early genes from gene 1.3 ligase to gene 6 exonuclease may be important in recombination (Lee et al. (1983) J. of Virology 48, 647-653; Lai et al. (1998), *supra*; Lai et al. (2000) Molecular Microbiology 36, 437-466; Yu et al (2001) J. of Bacteriology 183, 1862-1869). However, it is not known whether host proteins are also important in this process.

[0004] A paper by Jessberger et al, (The Journal of Biological Chemistry, 1993, 268:15070-150799) discloses an assay in which DNA double strand breaks and deletions are repaired by recombination. In this assay the activity of a purified complex (RC-1) is tested that contains the enzymatic activities of a DNA polymerase, a DNA ligase, and a 5' to 3' exonuclease. Notably all of the processes that were described in this document require restriction enzymes and that it is essential that the DNA was digested with such enzymes.

[0005] Patent Application WO 01/66775A2 (University of Guelph) describes a DNA joining

method involving the use of a DNA polymerase having a 5' to 3' exonuclease activity, and does not require a ligation step. This patent application discloses a method of joining linear DNA molecules using specific DNA polymerases. The method depends on the provision of nucleic acid molecules having overhanging ends for complementarity between the two DNA sequences to be joined. Those overhanging ends can be generated by restriction enzymes or by chemically synthesising the sequences to have overhanging ends but must be provided in order to generate complementary nucleotides for binding.

[0006] US Patent No. 6,150,111 (Trustees of the University of Pennsylvania) describes a method for recombining and cloning nucleic acids without the use of a restriction enzyme or DNA ligase. The patent provides a method of recombining nucleic acid molecules utilising exonucleases such as lambda exonuclease but this method requires that the exonuclease reaction be terminated prior to beginning the DNA polymerase reaction in order to prevent the exonuclease reaction from proceeding too far and ruining the joining method. The exonuclease reaction can be controlled by stopping it at a set time interval, whether by lowering the temperature or by adjusting the pH.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007]

Figure 1 is a cartoon illustrating a joining reaction of the invention. The joined molecules are shown as linear molecules; however, it is to be understood that the ends of the linear molecule are preferably joined to form a circle, and/or are joined to a linearized vector, to form a circle.

Figure 2 is a cartoon illustrating a method for adding overlapping sequences by PCR amplification. The joined molecules are shown as linear molecules; however, it is to be understood that the ends of the linear molecule are preferably joined to form a circle, and/or are joined to a linearized vector, to form a circle.

Figure 3 is a cartoon illustrating a method for inserting a fragment into a vector.

Figure 4 is a cartoon illustrating a method for adding overlapping sequences by PCR amplification.

Figure 5 shows the results of an incubating four DNA fragments by a method of the invention, for 20 minutes or 45 minutes.

Figure 6 shows the results of an incubating four DNA fragments by a method of the invention, for 60 minutes.

DESCRIPTION OF THE INVENTION

[0008] The invention is set out in the claims.

[0009] The present inventors have identified four T7 gene products (or substitutes therefor) that are sufficient to constitute an *in vitro* system for recombining DNAs via a region of homology. The method allows, e.g., for the joining of DNA molecules of interest to one another in a predefined order and orientation, without the use of restriction enzymes.

[0010] The present invention relates, e.g., to an *in vitro* method, using isolated protein reagents (proteins), for joining two double stranded (ds) DNA molecules of interest, comprising: providing a first dsDNA molecule and a second dsDNA molecule which share a region of sequence identity at an end on each DNA molecule and contacting the two DNA molecules simultaneously with

1. (a) a purified non-processive 5' exonuclease;
2. (b) a purified single stranded DNA binding protein (SSB) which accelerates nucleic acid annealing;
3. (c) a purified non strand-displacing DNA polymerase wherein the 5' exonuclease and the DNA polymerase are different entities; and
4. (d) a purified ligase,

under conditions whereby a 3' single-stranded overhang is generated in each molecule by the exonuclease without the use of a restriction enzyme; the two single-stranded overhangs anneal to form a gapped molecule; the gaps are filled in by the polymerase; and nicks are sealed by the ligase, thereby joining the molecules and forming an intact (un-nicked) double stranded DNA molecule, in which a single copy of the region of sequence identity is retained, wherein none of the enzymatic reactions is actively terminated prior to beginning another of the reactions. In this method, the 5' exonuclease generates 3' single stranded overhangs in both DNA molecules which comprise the region of sequence identity; the two single stranded overhangs anneal to form a gapped molecule; the DNA polymerase fills in the gaps; and the ligase seals the nicks. The method is illustrated schematically in Figure 1.

[0011] The "joining" of two DNA molecules so that a single copy of the region of sequence identity is retained is sometimes referred to herein as "recombination" of the two DNA molecules. In the method of the invention, the four proteins (a) through (d) are each isolated (e.g., purified); cell extracts are not employed. The four proteins act together in a concerted fashion; the individual enzymatic reactions are not actively terminated (e.g., by an experimenter or investigator) before a subsequent reaction begins. In some embodiments, formation of a double stranded DNA molecule results in the molecule being relatively withdrawn or inert from the reactions. Conditions which are effective for joining the two DNA molecules allow for the net assembly of DNA molecules, rather than the degradation of the DNA molecules by the exonuclease. That is, the gaps formed by digestion by the 5' exonuclease are filled in by the polymerase substantially immediately after they are formed. This is accomplished by contacting the DNA molecules with a substantially lower amount of 5'

exonuclease activity than the amount of DNA polymerase activity.

[0012] The method can be used to join more than two DNA molecules. To accomplish this, the DNA molecules to be joined are designed such that, for each pair of DNA molecules to be joined, the distal region of one DNA molecule comprises a region of sequence identity with the proximal region of the other DNA molecule. To facilitate the joining of the DNA molecules in a predetermined orientation and order, each set of distal and proximal regions of sequence identity is selected (designed) to be unique (to be different from the regions of sequence identity of the other pairs of DNA molecules). The method allows a number of DNA molecules to be joined with a single operation (e.g. in a single tube). See Figure 1 for a schematic representation of such predetermined joining.

[0013] Advantages of the method of the invention include the ability to perform the joining (recombination) reactions under well-defined conditions, using well-characterized, isolated (e.g. purified) proteins (e.g. enzymes). This allows the joining reactions to be controlled and reproducible. In the method of the invention, the joining process is not subject to competing reactions brought about by other enzymes in the reaction mixture, such as exonucleases and endonucleases which can be present in cell extracts. The method allows one to recombine regions of sequence identity (homologous regions) that are less than about 150 base pairs in length. This is in contrast, e.g., to recombination systems using cell lysates rather than isolated enzymes; in systems using cell lysates, efficient joining does not occur with overlaps of less than about 150 bp. The ability to join DNA molecules in a defined order and orientation allows, for example, for the cloning of a fragment of interest into a linearized vector in a defined orientation; or for the assembly of component DNA portions of a longer sequence of interest (such as the assembly of component parts of a synthetic gene or genome; or the assembly and cloning of sub-fragments of a DNA which are too large to clone using a PCR amplification step). The method allows one to join and/or clone DNA molecules of interest without having to rely on the presence of restriction enzyme recognition sites at the ends of the fragments to be joined. The *in vitro* procedure also allows one to assemble DNAs that are unstable and thus would be difficult to clone by a method requiring transformation into and replication in a bacterium. If desired, DNAs assembled by a method of the invention can then be amplified *in vitro* (e.g., by rolling circle amplification or PCR), again without having to passage the DNA through a bacterium.

[0014] One aspect of the invention is an *in vitro* joining method as above, wherein the 5' exonuclease is the phage T7 gene 6 product, RedA of lambda phage, or RecE of Rac prophage; the SSB is the phage T7 gene 2.5 product, the *E. coli* recA protein, RedB of lambda phage, or RecT of Rac prophage; the DNA polymerase is the phage T7 gene 5 product, phage T4 DNA polymerase, or *E. coli* pol I; and/or the ligase is the phage T7 gene 1.3 product, phage T4 DNA ligase, or *E. coli* DNA ligase.

[0015] The description describes an *in vitro* method, using isolated protein reagents, for joining two double stranded (ds) DNA molecules of interest, wherein the distal region of the first DNA molecule and the proximal region of the second DNA molecule share a region of

sequence identity, comprising generating 3' single stranded (ss) overhangs at both ends of the DNA molecules; annealing the single stranded overhangs in the presence of a ssDNA binding protein; filling in the gaps formed, and sealing the nicks.

[0016] The description describes a kit for the *in vitro* joining of a plurality of dsDNA molecules comprising, in separate containers,

1. (a) a mixture of the isolated proteins
 1. (i) a single stranded DNA binding protein (SSB) which accelerates nucleic acid annealing (e.g., the T7 gene 2.5 product, the *E. coli* recA protein, RedB of lambda phage, or Rec T of Rac prophage);
 2. (ii) a non strand-displacing DNA polymerase (e.g. the phage T7 gene 5 product, phage T4 DNA polymerase, or *E. coli* pol 1); and
 3. (iii) a DNA ligase (e.g. the phage T7 gene 1.3 product, phage T4 DNA ligase, or *E. coli* DNA ligase),wherein the ratios of activities of (i), (ii) and (iii) are effective, when in the presence of a non-processive 5' exonuclease, to achieve *in vitro* joining of the dsDNA molecules, and
2. (b) an isolated non-processive 5' exonuclease (e.g. the phage T7 gene 6 product, RedA of lambda phage, or RecE of Rac prophage).

[0017] Another aspect of the invention is a composition comprising

1. (a) an isolated non-processive 5' exonuclease (e.g. the phage T7 gene 6 product, RedA of lambda phage, or RecE of Rac prophage);
2. (b) a single stranded DNA binding protein (SSB) which accelerates nucleic acid annealing (e.g., the T7 gene 2.5 product, the *E. coli* recA protein, RedB of lambda phage, or Rec T of Rac prophage);
3. (c) a non strand-displacing DNA polymerase (e.g. the phage T7 gene 5 product, phage T4 DNA polymerase, or *E. coli* pol 1); and
4. (d) a DNA ligase (e.g. the phage T7 gene 1.3 product, phage T4 DNA ligase, or *E. coli* DNA ligase).

Such a composition can be present, for example, in a reaction mixture in which a plurality of DNA molecules are being joined by a method of the invention.

[0018] As used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. For example, "an" isolated exonuclease, as used above, includes two or more exonuclease molecules, which can be the same or different.

[0019] The term, an "isolated" protein, as used herein, means that the protein is removed from its original environment (e.g., the natural environment if it is naturally occurring), and isolated or separated from at least one other component with which it is naturally associated.

For example, a naturally-occurring protein present in its natural living host (e.g. a bacteriophage protein present in a bacterium that has been infected with the phage) is not isolated, but the same protein, separated from some or all of the coexisting materials in the natural system, is isolated. Such proteins can be part of a composition or reaction mixture, and still be isolated in that such composition or reaction mixture is not part of its natural environment. The term "an isolated protein," as used herein, can include 1, 2, 3, 4 or more copies of the protein, *i.e.*, the protein can be in the form of a monomer, or it can be in the form of a multimer, such as dimer, trimer, tetramer or the like, depending on the particular protein under consideration. In some embodiments, the protein is purified. Methods for purifying the proteins of the invention are conventional. In some embodiments, the protein is substantially purified or is purified to homogeneity. By "substantially purified" is meant that the protein is separated and is essentially free from other proteins, *i.e.*, the protein is the primary and active constituent. The purified protein can then be contacted with the DNAs to be joined, where it then acts in concert with other proteins to achieve the joining. The proteins can be contacted with (combined with) the DNAs in any order; for example, the proteins can be added to a reaction mixture comprising the DNAs, or the DNAs can be added to a reaction mixture comprising the proteins. Proteins used in the methods of the invention can be in the form of "active fragments," rather than the full-length proteins, provided that the fragments retain the activities (enzymatic activities or binding activities) required to achieve the joining. One of skill in the art will recognize how to generate such active fragments.

[0020] Any non-processive 5'→3' double strand specific exodeoxyribonuclease may be used in the methods of the invention. The terms "5' exonuclease" or "exonuclease" are sometimes used herein to refer to a 5'→3' exodeoxyribonuclease. A "non-processive" exonuclease, as used herein, is an exonuclease that degrades a limited number (*e.g.*, only a few) nucleotides during each DNA binding event. Among other properties which are desirable for the 5' exonuclease are that it lacks 3' exonuclease activity, it is double strand DNA specific, it generates 5' phosphate ends, and it initiates degradation from both 5'-phosphorylated and unphosphorylated ends. Suitable 5' exonucleases will be evident to the skilled worker. Among the preferred 5' exonucleases are the phage T7 gene 6 product, RedA of lambda phage (lambda exonuclease), RecE of Rac prophage, or any of a variety of 5'→3' exonucleases that are involved in homologous recombination reactions. Methods for preparing the T7 gene 6 product and optimal reaction conditions for using it are conventional. See, *e.g.*, Kerr et al. (1972) *The Journal of Biological Chemistry* 247, 305-310. Methods for preparing and using the other noted exonucleases are conventional; and many are available from commercial sources, such as USB Corporation, 26111 Miles Road, Cleveland, Ohio 44128, or New England Biolabs, Inc. (NEB), 240 County Road, Ipswich, MA 01938-2723.

[0021] Without wishing to be bound by any particular mechanism of action, it is suggested that a single stranded DNA binding protein (SSB) used in a method of the invention protects the single stranded overhangs generated by the 5' exonuclease, as well as facilitating the rapid annealing of the homologous single stranded regions. Any SSB which accelerates nucleic acid annealing may be used in a method of the invention. An SSB which "accelerates nucleic acid annealing," as used herein, is an SSB which accelerates nucleic acid binding by a factor of

greater than about 500 fold, compared to the binding in the absence of the SSB. See, e.g., USP 5,534,407. Among other properties which are desirable for the SSB are that it binds single stranded DNA (ssDNA) more tightly than double stranded DNA (dsDNA), and that it interacts with both the exonuclease and the DNA polymerase. Suitable SSBs will be evident to the skilled worker. Among the preferred SSBs are the T7 gene 2.5 product, the *E. coli* RecA protein, RedB of lambda phage, and RecT of Rac prophage. Methods for preparing the T7 protein and optimal reaction conditions for using it are conventional. See, e.g., Rezende et al. (2002) *Journal of Biological Chemistry* 277, 50643-53 and Yu *et al.* (2001), *supra*. Methods for preparing and using the other SSBs are conventional; and many are available commercially, e.g. from USB or NEB, as noted above. In yet a further embodiment, polyethylene glycol ("PEG") may be used to enhance the annealing process.

[0022] Any non strand-displacing DNA polymerase may be used in the methods of the invention to fill in the gaps left by the 5' exonuclease digestion. The term "polymerase" is sometimes used herein to refer to a DNA polymerase. A "non strand-displacing DNA polymerase," as used herein, is a DNA polymerase that terminates synthesis of DNA when it encounters DNA strands which lie in its path as it proceeds to copy a dsDNA molecule, or degrades the encountered DNA strands as it proceeds while currently with filling in the gap thus created, thereby generating a "moving nick." Among the other properties which are desirable for the non strand-displacing DNA polymerase are that it synthesizes DNA faster than the exonuclease in the reaction mixture degrades it. Suitable non strand-displacing DNA polymerases will be evident to the skilled worker. Among the preferred enzymes are the T7 gene 5 product, T4 DNA polymerase, and *E. coli* Pol I. Methods for preparing and using the above-noted DNA polymerases are conventional; and many are available commercially, e.g. from USB or NEB, as noted above.

[0023] Any DNA ligase can be used in the methods of the invention. The term "ligase" is sometimes used herein to refer to a DNA ligase. Suitable DNA ligases include, e.g., the T7 gene 1.3 product, T4 DNA ligase, *E. coli* DNA ligase and Taq Ligase. Methods for their preparation and optimal reaction conditions are conventional. Alternatively, they can be purchased from commercial sources, such as USB or NEB, as noted above. In a preferred embodiment, substantially all of the nicks (e.g., all of the nicks) are sealed during the reaction procedure, in order to prevent degradation by the exonuclease. However, in one embodiment, joined DNA which still comprises some nicks is transformed into a bacterium, such as *E. coli*, and the nicks are sealed by the bacterial machinery.

[0024] The four proteins used in the methods of the invention (the exonuclease, SSB, polymerase and ligase) may be contacted with the DNA molecules to be joined (e.g., added to a reaction mixture comprising a solution containing suitable salts, buffers, ATP, deoxynucleotides, etc. plus the DNA molecules) simultaneously. In one embodiment, the four proteins are added substantially simultaneously. For example, a mixture of the four proteins in suitable ratios can be added to the reaction mixture with a single pipetting operation.

[0025] In another embodiment of the invention, the DNAs are added to a reaction mixture

comprising a solution containing suitable salts, buffers, ATP, deoxynucleotides, etc. and the four proteins. The description describes how the DNAs are added to a reaction mixture comprising a solution containing suitable salts, buffers, ATP, deoxynucleotides, etc. and a subset of the four proteins, and the remaining proteins are then added, in any order or in any combination (*e.g.* the exonuclease is added last; and preceding the addition of the exonuclease, the SSB, polymerase and ligase are added sequentially, in any order, or two of the proteins are added substantially simultaneously, and the other protein is added before or after those two proteins).

[0026] In the methods of the invention, a plurality of DNA molecules are contacted with the four proteins under conditions "effective" to join the DNA molecules to form a substantially intact (preferably having no nicks) double stranded DNA molecule, in which a single copy of the region of sequence identity is retained. An important factor in achieving the joining is that the amount of 5' exonuclease activity should be substantially lower than the amount of DNA polymerase activity, so that the net assembly of DNA molecules is greater than the degradation of DNA molecules by the exonuclease. That is, the gaps formed by digestion by the 5' exonuclease are filled in by the polymerase substantially immediately after they are formed, and the intact (un-nicked) reaction product is "fixed" by the ligation reaction. Suitable amounts of activities include: exonuclease activity between about 0.1 and about 50 U/mL (unit defined by USB); DNA polymerase between about 10 and about 30 U/mL (unit defined by USB); SSB between about 0.1 and about 1 μ M; and ligase between about 0.1 and about 1 μ M. Lower amounts of polymerase would likely not be able to catch up with the exonuclease, and higher amounts would likely degrade the 3' overhang generated by exonuclease, resulting in overlaps being digested before annealing can occur. Lower amounts of SSB would likely not allow annealing to occur rapidly enough, and higher amounts would likely stimulate exonuclease processivity, also resulting in polymerase cannot catch up. See Example I for some typical ratios that can be used.

[0027] Reaction conditions (such as the presence of salts, buffers, ATP, dNTPs, etc. and the times and temperature of incubation) are conventional and can be optimized readily by one of skill in the art. Preferably, the incubation temperature is about 25°C to about 45°C, and the reaction is carried out for about 1-1.5 hours at 37°C, or for about 2-3 hours at 30°C. Typical reaction conditions are presented in Example I.

[0028] Because a non-strand displacing DNA polymerase used in the methods of the invention must elongate in the 5' direction from a primer molecule, the method cannot tolerate a free 5' end (*e.g.* at the 5' end of the most 5' DNA to be joined). Because no primer is available in such a molecule to be extended, such a molecule would be digested by the exonuclease and the resulting gap could not be filled in by a polymerase. In one embodiment, the 5' ends of the terminal DNA fragments that are joined are blocked so that 5' exonuclease cannot digest them. The blocking agent is preferably reversible, so that the joined DNA molecule can eventually be joined into a vector. Suitable blocking agents will be evident to the skilled worker. These include, *e.g.*, phosphorothioate bonds, 5' spacer molecules, Locked Nucleic Acid (LNA) etc. In another embodiment of the invention, the fragments are selected

(designed) so that the two terminal fragments join to one another to form a circle. In another embodiment, the joined fragments are designed so that they become integrated into a vector which is also present in the reaction mixture.

[0029] DNA molecules of any length can be joined by methods of the invention, and from two to an essentially unlimited upper level of DNA molecules can be joined. In general, at least about 10 fragments can be joined. The number of fragments which can be joined depends, *e.g.*, on the length of the overlaps and the lengths of the fragments. For example, with fragments of greater than about 3 kb, having overhangs of about 150 to about 200 bp, the number of fragments that can be joined is substantially unlimited.

[0030] As noted above, in embodiments of the invention in which no blocker is used, the joined DNA molecules preferably form a circle and/or become ligated into a vector to form a circle. The lower size limit for a dsDNA to circularize is about 200 base pairs. Therefore, the total length of the joined fragments (including, in some cases, the length of the vector) is preferably at least about 200 bp in length. There is no upper size limit, and joined DNAs of a few hundred kilobase pairs, or larger, can be generated by a method of the invention. Although the rate at which the circles can form may be reduced for very long molecules, that does not prevent the circle from forming and reaching a steady state in which the rate of filling in gaps is greater than the rate of exonuclease digestion, once all of the nicks have been sealed. Example 1 illustrates joining/recombination reactions in which four DNA molecules, of 2.2kb, 1.5kb, 1.55kb and 1.2 kb, are joined.

[0031] In methods of the invention, the distal region of one of a pair of dsDNA molecules to be joined shares a region of sequence identity with the proximal region of the other dsDNA molecule. The term "distal" as used herein refers to the 3' end of a first DNA molecule of a pair to be joined (the 5'-most DNA molecule), and the term "proximal" refers to the 5' end of the second DNA molecule of the pair. The regions of identity are sometimes referred to herein as "overlaps" or "regions of overlap." Figure 1 shows a schematic representation of the distal and proximal regions of DNA molecules to be joined. The region of sequence identity should be sufficiently long to allow the recombination to occur. The length can vary from a minimum of about 15 base pairs (bp) to a maximum of about 300 bp or more. In general, it is preferable that the length of the overlap is not greater than about 1/10 the length of the fragment to be recombined; otherwise there may not be sufficient time for annealing and gap filling. For the joining of 2 or 3 fragments, about 20-30 bp overlap may be sufficient. For more than 10 fragments, a preferred overlap is about 150 bp to about 300 bp. If longer overlaps are used, the T7 endonuclease may also be required to debranch the joint molecules. In one embodiment, the region of sequence identity is of a length that allows it to be generated readily by synthetic methods, *e.g.* about 40 bp (*e.g.*, about 35 to about 45 bp).

[0032] In a preferred embodiment, when a plurality of DNA molecules are to be joined, for each pair of DNA molecules to be joined, the distal region of one of the DNA molecules of the pair is designed to share a region of sequence identity with the proximal region of the other DNA molecule of the pair, and the distal and proximal regions of sequence identity for each

pair of DNA molecules are designed to be unique (to be different from the regions of sequence identity of the other pairs of DNA molecules). When the overlapping regions of identity are designed in this manner, the orientation and order of the DNA molecules in the joined molecule can be predetermined. A number of DNA molecules (for example, 4 or 6 molecules) can thus be incubated together in a single reaction mixture (in a single vessel or container) with the four proteins of the invention, and be joined into a longer DNA molecule in which the individual DNAs are arranged in any desired order and orientation.

[0033] The regions of sequence identity present in the proximal and distal regions of the DNAs to be joined can be generated by any of a variety of methods.

[0034] For example, in one embodiment of the invention, synthetically prepared fragments of a gene or genome of interest (*e.g.*, about 5 kb in length) are optionally amplified (*e.g.* by PCR or by a rolling circle mechanism) and are joined by a method of the invention in the order and orientation in which they are located in the gene or genome. This procedure allows the preparation of a synthetic gene or genome. In this method, the first DNA fragment (*e.g.* in the 5' most portion of the gene or genome) is synthesized so that the region at its 3' end (the distal end) contains a sequence (*e.g.* about 40 bp) that is identical to the sequence at the 5' end (the proximal end) of the DNA fragment to which it is to be joined. The second DNA fragment, in turn, is synthesized so that it has, at its distal end, a sequence which is identical to the sequence at the proximal end of the third DNA fragment, and so on.

[0035] In other embodiments of the invention, the regions of identity are introduced by PCR amplification.

[0036] In one such method, as illustrated in Figure 3, a fragment of interest is inserted into a vector. For example, a plasmid vector can be linearized with a restriction enzyme, generating a sequence A (*e.g.* having 40 bp) to the left of the restriction enzyme cut and a sequence B (*e.g.* having 40 bp) to the right of the restriction enzyme cut. The fragment to be cloned into the vector is PCR amplified, using PCR primers which will introduce sequence A at the left end of the fragment, and sequence B at the right end of the fragment. The regions of sequence identity (in this example, each having 40 bp) allow the fragment to be joined to the vector in a desired orientation, to form a circular molecule. Alternatively, particularly when it is desirable to avoid errors which might be introduced into an insert during PCR amplification, the *vector* can be PCR amplified in order to introduce at the ends of a cloning site sequences which overlap sequences at the ends of the insert. This methods described above allow for the directional cloning of any insert of interest, without having to rely on the presence of, or introduction of, restriction enzyme sites on the insert.

[0037] In another such method, as illustrated in Figure 2, a plurality of DNA fragments are joined to one another. In this embodiment, the regions of sequence identity are introduced into the fragments by PCR amplification, using suitable primers. For each DNA fragment to be joined to another fragment, a sequence is introduced to the 3' (distal) end of the first fragment which overlaps with the sequence at the 5' (proximal) end of the fragment to which it is to be

joined. PCR primers are used in which the regions of sequence identity (*e.g.* 40 nt) lie 5' to a PCR primer (*e.g.* having 20 nt). As shown in Figure 2, after a suitable number of rounds of PCR amplification, DNA fragments are produced in which defined regions of sequence identity are present at the ends of the fragments. The resulting fragments can then be joined in a predetermined order and orientation by a method of the invention. A variant of this method is shown in Figure 4. In this method, starting with two representative fragments having no regions of sequence identity at their ends, sequences are added by PCR amplification by primers having only 40 nt (instead of 60 nt); the resulting regions of sequence identity are 40 bp in length. In Figures 1-3, the joined molecules are shown as linear molecules. As discussed above, the fragments at either end of a linear molecule are preferably joined to form a circle, and/or are joined to a linearized vector, to form a circle.

[0038] If desired, a vector can be present in the joining reaction, so that the joined fragments are introduced into the vector. The efficiency of joining a large number of fragments (*e.g.*, 6 or 8 fragments) into a vector by a method of the invention is more efficient than when using a method which employs compatible restriction enzyme sites. To increase the efficiency even further, the DNAs from a joining reaction can be separated by size (*e.g.* by gel electrophoresis or a sizing column); and a DNA molecule of the desired size (having the correct number of joined fragments) can be isolated and introduced into a vector by a method of the invention.

[0039] In one embodiment, joined fragments and/or fragments inserted into vectors are introduced into a host cell, such as a bacterial or eukaryotic cell (*e.g.* by transfection or transformation). Alternatively, the reaction mixture comprising the joined DNA molecules can be introduced into a host cell; only those DNAs which have recombined to form circular molecules can survive in the host cell. In another embodiment, the joined fragments and/or fragments inserted into vectors are used directly, without further passage through a cell, such as a bacterial cell.

[0040] Molecular biology methods of the invention can be carried out using conventional procedures. See, *e.g.*, discussions in Sambrook, et al. (1989), *Molecular Cloning, a Laboratory Manual*, Cold Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Ausubel et al. (1995). *Current Protocols in Molecular Biology*, N.Y., John Wiley & Sons; Davis et al. (1986), *Basic Methods in Molecular Biology*, Elsevier Sciences Publishing, Inc., New York; Hames et al. (1985), *Nucleic Acid Hybridization*, IL Press; Dracopoli et al. (current edition) *Current Protocols in Human Genetics*, John Wiley & Sons, Inc.; and Coligan et al. (current edition) *Current Protocols in Protein Science*, John Wiley & Sons, Inc.

[0041] A variety of other uses for the inventive method will be evident to the skilled worker. In particular, the inventive method can be substituted for any method in which restriction enzyme digests are used to generate compatible sequences for joining DNA molecules. In one embodiment of the invention, DNA molecules that are too large to be amplified by PCR can be cloned by joining sub-fragments by a method of the invention and then inserting them into a suitable vector. An *in vitro* recombination system of the invention (*e.g.*, the four proteins of the invention, in a suitable ratio) can be used to recombine any homologous DNAs of interest, *e.g.*

to repair double stranded DNA breaks or gaps, etc. Another application of the method is to introduce a mutation into a DNA. In this method, a mutation is introduced into both the upper and lower strand PCR primers, so the amplified fragments are 100% mutant; then the fragments are joined by the method of the invention.

[0042] The disclosed methods can be used to join any nucleic acid molecules of interest. The nucleic acid molecules can come from any source, including a cellular or tissue nucleic acid sample, cloned fragments or subclones thereof, chemically synthesized nucleic acids, genomic nucleic acid samples, cDNAs, nucleic acid molecules obtained from nucleic acid libraries, etc. The DNAs can be radioactively labeled or can comprise binding entities, such as a biotinylated nucleotides, which can aid in the purification of the joined DNAs. If desired, the DNA molecules to be joined, or primers for adding overlapping regions of sequence identity, can be prepared synthetically. Conventional synthesis techniques include using phosphoroamidite solid-phase chemistry to join nucleotides by phosphodiester linkages. Chemistry for joining nucleotides by phosphorothioate linkages or different linkages, such as methylphosphonate linkages, can also be used. For example, the cyanoethyl phosphoramidite method can be used, employing a Milligen or Beckman System 1 Plus DNA synthesizer (for example, Model 8700 automated synthesizer of Milligen-Bioscience, Burlington, MA or ABI Model 380B). Synthetic methods useful for making DNA molecules are also described by Ikuta et al. (1984) *Ann Rev. Biochem.* 53, 323-356, (phosphotriester and phosphite-triester methods), and Narang et al. (1980) *Methods Enzymol.* 65, 610-620 (phosphotriester method). DNAs prepared by methods as above are available from commercial sources, such as Integrated DNA Technologies (IDT), Coralville, IA.

[0043] Methods of the invention can be carried out in a high throughput fashion, using automated (e.g. robotic) systems, which allow many DNA joining reactions to be carried out simultaneously.

[0044] The description describes how any combination of the materials useful in the disclosed methods can be packaged together as a kit for performing any of the disclosed methods. For example, the four proteins: a non-processive 5' exonuclease (e.g., the phage T7 gene 6 product); a single stranded DNA binding protein (SSB) which accelerates nucleic acid annealing (e.g., the T7 gene 2.5 product or the *E. coli* recA protein); a non strand-displacing DNA polymerase (e.g., the T7 gene 5 product, T4 DNA polymerase, and *E. coli* pol I); and a ligase (e.g., T4 DNA ligase or *E. coli* DNA ligase) can be packaged individually or in various combinations. The four proteins may be packaged together in a single container (such as an Eppendorf tube). The exonuclease may be packaged separately, so that this enzyme can be added last to a reaction mixture containing the DNA molecules to be joined and the other three proteins. The SSB, polymerase and ligase may be packaged together in suitable ratios so that an aliquot can be removed and added to a reaction mixture containing DNAs so that, following the addition of an exonuclease, DNA joining takes place. If desired, the protein reagents can be packaged in single use form, suitable for carrying one set of DNA joining reactions.

[0045] Optionally, kits may comprise instructions for performing the method. Other optional elements of a kit include suitable buffers, packaging materials, etc. The protein reagents of the kit may be in containers in which they are stable, e.g., in lyophilized form or as stabilized liquids. Preferably, the proteins are stored as solutions in 50% glycerol.

[0046] DNAs used in methods of the invention can have one or more modified nucleotides. For example, they may contain one or more modifications to either the base, sugar, or phosphate moieties. Modifications to the base moiety would include natural and synthetic modifications of A, C, G, and T as well as different purine or pyrimidine bases, such as uracil-5-yl, hypoxanthin-9-yl (I), and 2-aminoadenin-9-yl. A modified base includes but is not limited to 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Additional base modifications can be found for example in U.S. Pat. No. 3,687,808, Englisch et al. (1991) *Angewandte Chemie*, International Edition 30, 613, and Sanghvi, Y. S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S. T. and Lebleu, B. ed., CRC Press, 1993. Certain nucleotide analogs, such as 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine can increase the stability of duplex

formation. Base modifications often can be combined with for example a sugar modification, such as 2'-O-methoxyethyl, to achieve unique properties such as increased duplex stability. There are numerous United States patents such as U.S. Pat. Nos. 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, which detail and describe a range of base modifications.

[0047] Nucleotide analogs can also include modifications of the sugar moiety. Modifications to the sugar moiety would include natural modifications of the ribose and deoxyribose as well as synthetic modifications. Sugar modifications include but are not limited to the following modifications at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C1 to C10, alkyl or C2 to C10 alkenyl and alkynyl. 2' sugar modifications also include but are not limited to $--O[(CH_2)_nO]_m CH_3$, $--O(CH_2)_nOCH_3$, $-O(CH_2)_nNH_2$, $-O(CH_2)_nCH_3$, $--O(CH_2)_n--ONH_2$, and $--O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10.

[0048] Other modifications at the 2' position include but are not limited to: Cl to C10 lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, CF, Br, CN, CF₃, OCF₃, SOCH₃, SO₂, CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl,

aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Similar modifications may also be made at other positions on the sugar, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Modified sugars would also include those that contain modifications at the bridging ring oxygen, such as CH₂ and S. Nucleotide sugar analogs may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. There are numerous United States patents that teach the preparation of such modified sugar structures such as U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920.

[0049] Nucleotide analogs can also be modified at the phosphate moiety. Modified phosphate moieties include but are not limited to those that can be modified so that the linkage between two

nucleotides contains a phosphorothioate, chiral phosphorothioate, phosphorodithioate, phosphotriester, aminoalkylphosphotriester, methyl and other alkyl phosphonates including 3'-alkylene phosphonate and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkyl-phosphonates, thionoalkylphosphotriesters, and boranophosphates. It is understood that these phosphate or modified phosphate linkages between two nucleotides can be through a 3'-5' linkage or a 2'-5' linkage, and the linkage can contain inverted polarity such as 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included. Numerous United States patents teach how to make and use nucleotides containing modified phosphates and include but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

[0050] It is understood that nucleotide analogs need only contain a single modification, but may also contain multiple modifications within one of the moieties or between different moieties.

[0051] Nucleotide substitutes are nucleotides or nucleotide analogs that have had the phosphate moiety and/or sugar moieties replaced. Nucleotide substitutes include molecules having similar functional properties to nucleotides, but which do not contain a phosphate moiety, such as peptide nucleic acid (PNA). Nucleotide substitutes include molecules that will recognize and hybridize to complementary nucleic acids in a Watson-Crick or Hoogsteen manner, but which are linked together through a moiety other than a phosphate moiety. Nucleotide substitutes are able to conform to a double helix type structure when interacting with the appropriate target nucleic acid.

[0052] Substitutes for the phosphate can be for example, short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts. Numerous United States patents disclose how to make and use these types of phosphate replacements and include but are not limited to U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

[0053] It is also understood in a nucleotide substitute that both the sugar and the phosphate moieties of the nucleotide can be replaced, by for example an amide type linkage (aminoethylglycine) (PNA). U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262 teach how to make and use PNA molecules. See also Nielsen et al. (1991) Science 254, 1497-1500.

[0054] DNA molecules of the invention can be made up of different types of nucleotides or the same type of nucleotides. For example, one or more of the nucleotides in a primer can be ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides; about 10% to about 50% of the nucleotides can be ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides; about 50% or more of the nucleotides can be ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides; or all of the nucleotides are ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides. The nucleotides can be comprised of bases (that is, the base portion of the nucleotide) and can comprise different types of bases. For example, one or more of the bases can be universal bases, such as 3-nitropyrrole or 5-nitroindole; about 10% to about 50% of the bases can be universal bases; about 50% or more of the bases can be universal bases; or all of the bases can be universal bases.

[0055] In the foregoing and in the following example, all temperatures are set forth in uncorrected degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

EXAMPLES

Example I

[0056] Four DNA molecules, having lengths of 2.2 kb, 1.5 kb, 1.55 kb and 1.2 kb, were incubated according to a method of the invention, under the conditions noted in Figures 5 and 6. The reaction mixes were subjected to gel electrophoresis, along with molecular weight markers. When the samples were incubated for 45 minutes or 60 minutes, a major band of about 6.3 kb formed. This is the size expected for a joined product containing one copy of each of the DNAs.

Example II

[0057] 4 enzymes (T7 5' -exonuclease, Taq polymerase, VENT polymerase, and Taq ligase) plus two or more overlapping DNA fragments which can form a circular DNA molecule when recombined, are recombined in a single reaction mixture such that the final product is a circular molecule containing the recombined fragments.

[0058] T7 5'-exonuclease is used to chew-back the 5'-ends of the duplex DNA fragments, thus exposing the overlapping regions. This enzyme has no activity on 3'-ends. It acts at free ends and at nicks in the DNA. Once the overlaps are exposed, they can anneal to form joints that can be repaired because the 3' -ends of the annealed regions can be extended by the Taq polymerase, which prior to the exposure and annealing of the overlaps was inactive in the reaction mixture. The purpose of the VENT polymerase, which is in very low amount, is to remove any single 3'nucleotide additions that are produced by the Taq polymerase on the DNA fragments ends prior to the action of the T7 exonuclease. When the extending 3' ends catch up to the 5'-ends, the Taq ligase completes the repair by ligating the 5' -3' nick. The repaired joint is then resistant to further reaction by the enzymes. When the fragments are joined into a complete circle, that product is resistant to further reaction and is the desired end product of the reaction.

[0059] Annealing of the overlapping ends is accelerated by carrying out the reaction at elevated temperature (e.g. 45 to 60°C) in the presence of 5% PEG 8000 in the reaction buffer.

[0060] For an efficient reaction the enzymes must be balanced in amount so that the T7 degradation is somewhat slower than the rate of annealing, polymerization, and ligation. Taq ligase should be in large excess so that repair is completed as soon as the polymerization fills in the gaps created when the overlapping ends anneal with each other.

[0061] A suitable buffer for the reaction contains 20mM Tris acetate, 50mM potassium acetate, 10mM magnesium chloride, 5mM DTT, 25ug per ml BSA, 5% PEG-8000, 200uM dNTP' s, 1mM NAD, 0.1% Triton X-100, adjusted to pH 7.9.

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- [WO0166775A2 \[0005\]](#)
- [US6150111A \[0006\]](#)
- [US5534407A \[0021\]](#)
- [US3687808A \[0046\] \[0049\]](#)
- [US4845205A \[0046\]](#)
- [US5130302A \[0046\]](#)
- [US5134066A \[0046\]](#)
- [US5175273A \[0046\]](#)
- [US5367066A \[0046\]](#)
- [US5432272A \[0046\]](#)
- [US5457187A \[0046\]](#)
- [US5459255A \[0046\]](#)
- [US5484908A \[0046\]](#)
- [US5502177A \[0046\]](#)
- [US5525711A \[0046\]](#)
- [US5552540A \[0046\]](#)
- [US5587469A \[0046\]](#)
- [US5594121A \[0046\]](#)
- [US5596091A \[0046\]](#)
- [US5614617A \[0046\]](#)
- [US5681941A \[0046\]](#)
- [US4981957A \[0048\]](#)
- [US5118800A \[0048\]](#)
- [US5319080A \[0048\]](#)
- [US5359044A \[0048\]](#)
- [US5393878A \[0048\]](#)
- [US5446137A \[0048\]](#)
- [US5466786A \[0048\]](#)
- [US5514785A \[0048\]](#)
- [US5519134A \[0048\]](#)
- [US5567811A \[0048\]](#)
- [US5576427A \[0048\]](#)
- [US5591722A \[0048\]](#)
- [US5597909A \[0048\]](#)
- [US5610300A \[0048\]](#)

- [US5627053A](#) [0048]
- [US5639873A](#) [0048]
- [US5646265A](#) [0048]
- [US5658873A](#) [0048]
- [US5670633A](#) [0048]
- [US5700920A](#) [0048]
- [US4469863A](#) [0049]
- [US4476301A](#) [0049]
- [US5023243A](#) [0049]
- [US5177196A](#) [0049]
- [US5188897A](#) [0049]
- [US5264423A](#) [0049]
- [US5276019A](#) [0049]
- [US5278302A](#) [0049]
- [US5286717A](#) [0049]
- [US5321131A](#) [0049]
- [US5399676A](#) [0049]
- [US5405939A](#) [0049]
- [US5453495A](#) [0049]
- [US5455233A](#) [0049]
- [US5466677A](#) [0049] [0052]
- [US5476925A](#) [0049]
- [US5519126A](#) [0049]
- [US5536821A](#) [0049]
- [US5541306A](#) [0049]
- [US5550111A](#) [0049]
- [US5563253A](#) [0049]
- [US5571799A](#) [0049]
- [US5587361A](#) [0049]
- [US5625050A](#) [0049]
- [US5034506A](#) [0052]
- [US5166315A](#) [0052]
- [US5185444A](#) [0052]
- [US5214134A](#) [0052]
- [US5216141A](#) [0052]
- [US5235033A](#) [0052]
- [US5264562A](#) [0052]
- [US5264564A](#) [0052]
- [US5405938A](#) [0052]
- [US5434257A](#) [0052]
- [US5470967A](#) [0052]
- [US5489677A](#) [0052]
- [US5541307A](#) [0052]
- [US5561225A](#) [0052]

- [US5596086A](#) [0052]
- [US5602240A](#) [0052] [0052]
- [US5610289A](#) [0052] [0052]
- [US5608046A](#) [0052]
- [US5618704A](#) [0052]
- [US5623070A](#) [0052]
- [US5663312A](#) [0052]
- [US5633360A](#) [0052]
- [US5677437A](#) [0052]
- [US5677439A](#) [0052]
- [US5539082A](#) [0053]
- [US5714331A](#) [0053]
- [US5719262A](#) [0053]

Non-patent literature cited in the description

- DALY et al. J. of Bacteriology, 1996, vol. 178, 4461-4471 [0002]
- LAI et al. J. of Bacteriology, 1998, vol. 180, 6193-6202 [0003]
- LEE et al. J. of Virology, 1983, vol. 48, 647-653 [0003]
- LAI et al. Molecular Microbiology, 2000, vol. 36, 437-466 [0003]
- YU et al. J. of Bacteriology, 2001, vol. 183, 1862-1869 [0003]
- JESSBERGER et al. Journal of Biological Chemistry, 1993, vol. 268, 15070-150799 [0004]
- KERR et al. Journal of Biological Chemistry, 1972, vol. 247, 305-310 [0020]
- REZENDE et al. Journal of Biological Chemistry, 2002, vol. 277, 50643-53 [0021]
- SAMBROOK et al. Molecular Cloning, a Laboratory Manual Cold Harbor Laboratory Press 19890000 [0040]
- AUSUBEL et al. Current Protocols in Molecular Biology John Wiley & Sons 19950000 [0040]
- DAVIS et al. Basic Methods in Molecular Biology Elsevier Sciences Publishing, Inc. 19860000 [0040]
- HAMES et al. Nucleic Acid Hybridization Press 19850000 [0040]
- DRACOPOLI et al. Current Protocols in Human Genetics John Wiley & Sons, Inc. [0040]
- COLIGAN et al. Current Protocols in Protein Science John Wiley & Sons, Inc. [0040]
- IKUTA et al. Ann Rev. Biochem., 1984, vol. 53, 323-356 [0042]
- NARANG et al. Methods Enzymol., 1980, vol. 65, 610-620 [0042]
- ENGLISCH et al. Angewandte Chemie, International Edition, 1991, vol. 30, 613- [0046]
- SANGHVI, Y. S. Antisense Research and Applications CRC Press 19930000289-302 [0046]
- NIELSEN et al. Science, 1991, vol. 254, 1497-1500 [0053]

PATENTKRAV

1. *In vitro* fremgangsmåde ved anvendelse af isolerede proteiner til sammenføjning af to dobbeltstrengede (ds) DNA-molekyler af interesse, omfattende:

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tilvejebringelse af et første dsDNA-molekyle og et andet dsDNA-molekyle der deler en sekvensidentitetsregion ved en ende på hvert DNA-molekyle; og

bringe de to DNA-molekyler i kontakt samtidigt med:

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- (a) en oprenset ikke-processiv 5'-exonuklease;
- (b) et oprenset enkeltstrengt DNA-bindingsprotein (SSB), som accelererer nukleinsyreannealing;
- (c) en oprenset ikke-strengs-displacerende DNA-polymerase, hvor 5'-exonukleasen og DNA-polymerasen er forskellige enheder; og
- (d) en oprenset ligase under betingelser hvorved:

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et 3'-enkeltstrengt overhæng er genereret i hvert molekyle af exonukleasen uden anvendelse af et restriktionenzym;

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de to enkeltstrengede overhænger annealer for at danne et hullet molekyle;

hullerne er udfyldt af polymerasen;

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og

brudene er forsejlet af ligasen, derved sammenføjes molekylerne og danner et intakt dobbeltstrengt DNA-molekyle, hvor en enkelt kopi af sekvensidentitetsregionen er bevaret, hvor ingen af de enzymatiske reaktioner er aktivt afsluttet før begyndelsen af en anden af reaktionerne.

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2. Fremgangsmåde ifølge krav 1, hvor exonukleaseaktiviteten er, i det væ-

sentlige, lavere end DNA-polymeraseaktiviteten, således at hullerne er udfyldt af polymerasen, i det væsentlige, umiddelbart som de dannes.

3. Fremgangsmåde ifølge krav 1 eller krav 2, hvor

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(a) den ikke-processive 5'-exonuklease er gen 6 produktet fra phag-T7, RedA fra lambda-phag eller RecE fra Rac-prophag;

(b) SSB'en er gen 2,5 produktet fra phag-T7, *E. coli* recA-proteinet, RedB fra lambda-phag eller RecT fra Rac-prophag;

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(c) DNA-polymerasen er gen 5 produktet fra phag-T7, DNA-polymerasen fra phag-T4 eller *E. coli* pol I; og/eller

(d) ligasen er gen 1,3 produktet fra phag-T7, DNA-ligasen fra phag-T4 eller *E. coli*-DNA-ligasen.

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4. Fremgangsmåde ifølge et hvilket som helst af kravene 1-3, hvor et eller flere af DNA-molekylerne der skal sammenføjes er genereret syntetisk.

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5. Fremgangsmåde ifølge et hvilket som helst af kravene 1-4, hvor sekvensidentitetsregionen er mindre end 150 basepar i længden, eventuelt omkring 40 bp.

25

6. Fremgangsmåde ifølge et hvilket som helst af kravene 1-5, yderligere omfattende sammenføjning af ekstra DNA-molekyler til de to DNA-molekyler, hvor for hvert par af ekstra DNA-molekyler, der skal sammenføjes, den distale region af et af DNA-molekylerne i parret deler en sekvensidentitetsregion med den proksimale region af det andet DNA-molekyle i parret.

30

7. Fremgangsmåde ifølge krav 6, hvor sekvensidentitetsregionen er unik for hvert par af DNA-molekyler.

8. Fremgangsmåde ifølge krav 6 eller krav 7, hvor mindst omkring ti DNA-molekyler er sammenføjet med hinanden i en enkelt reaktionsbeholder.

9. In vitro-fremgangsmåde til sammenføjning af mere end to dobbeltstrengede DNA-molekyler i en defineret orientering og orden, omfattende

5 (a) udvælgelse af mere end to DNA-molekyler, således at; for hvert par af molekyler der skal sammenføjes, deler molekylerne en sekvensidentitetsregion ved en ende af hvert DNA-molekyle, hvor hver sekvensidentitetsregion er unik for hvert par af DNA-molekyler der skal sammenføjes; og

10 (b) bringe DNA-molekylerne i kontakt i en reaktionsblanding i en enkelt reaktionsbeholder samtidig med

(i) en oprenset ikke-processiv 5'-exonuklease;

(ii) et oprenset enkeltstrengt DNA-bindingsprotein (SSB), som accelererer nukleinsyreannealing;

15 (iii) en oprenset ikke-strengs-displacerende DNA-polymerase, hvor 5'-exonukleasen og DNA-polymerasen er forskellige enheder; og

(iv) en oprenset ligase under betingelser hvorved:

20 et 3'-enkeltstrengt overhæng er genereret i hvert molekyle af exonukleasen uden anvendelse af et restriktionsenzym;

de enkeltstrengede overhænger annealer for at danne et hullet molekyle;

25 hullerne er udfyldt af polymerasen;

og

30 brudene er forsejlet af ligasen derved sammenføjes mere end to dsDNA-molekyler for at danne et intakt duplex-DNA-molekyle hvor sekvensidentitetsregionen er bevaret, hvor ingen af de enzymatiske reaktioner er aktivt afsluttet før begyndelsen af en anden af reaktionerne.

10. Fremgangsmåde ifølge et hvilket som helst af kravene 1-9, hvor de 3'-enkelstrengede overhæng indeholder sekvensidentitetsregionen.
- 5 11. Fremgangsmåde ifølge et hvilket som helst af kravene 1-10, hvor de fire proteiner fungerer på en samordnet måde.
12. Fremgangsmåde ifølge krav 9, hvor et eller flere af de dsDNA-molekyler, der skal sammenføjes er genereret syntetisk.
- 10 13. Fremgangsmåde ifølge krav 1 eller krav 12, hvor de dsDNA-molekyler er genereret syntetisk og omfatter tilstødende dele af et gen eller genom af interesse; de dsDNA-molekyler er syntetiseret for at omfatte overlappende sekvensidentitetsregioner ved deres ender; og sammenføjningsreaktionen sammenføjer de dsDNA-molekyler til at danne dele eller hele det syntetiske
- 15 gen eller genom.
14. Fremgangsmåde ifølge et hvilket som helst af kravene 1-13, hvor sekvensidentitetsregionerne er tilføjet til de dsDNA-molekyler, der skal sammenføjes med PCR-amplifikation.
- 20 15. Fremgangsmåde ifølge et hvilket som helst af kravene 1-14, yderligere omfattende udsættelse af de sammenføjede DNA-molekyler for en størrelsesprocedure, isolering af DNA-molekyler med en ønsket længde og indføring af de isolerede DNA-molekyler i en celle af interesse.
- 25 16. Fremgangsmåde ifølge et hvilket som helst af kravene 1-4 eller 9-15, hvor mere end de to dsDNA-molekyler omfatter mindst 10 dsDNA-molekyler og for hvert par af DNA-molekyler der er sammenføjet, er sekvensidentitetsregionen mellem 150 og 300 basepar i længden.
- 30 17. Fremgangsmåde ifølge et hvilket som helst af kravene 1-16, som er en fremgangsmåde til indsættelse af et DNA-fragment af interesse i en lineariseret vektor til at danne et cirkulært molekyle, yderligere omfattende tilføjelse af sekvenser ved PCR-amplifikation til hver ende af det intakte dobbeltstrengede

DNA-molekyle, der er identiske med sekvenser på hver ende af den lineariserede vektor.

DRAWINGS

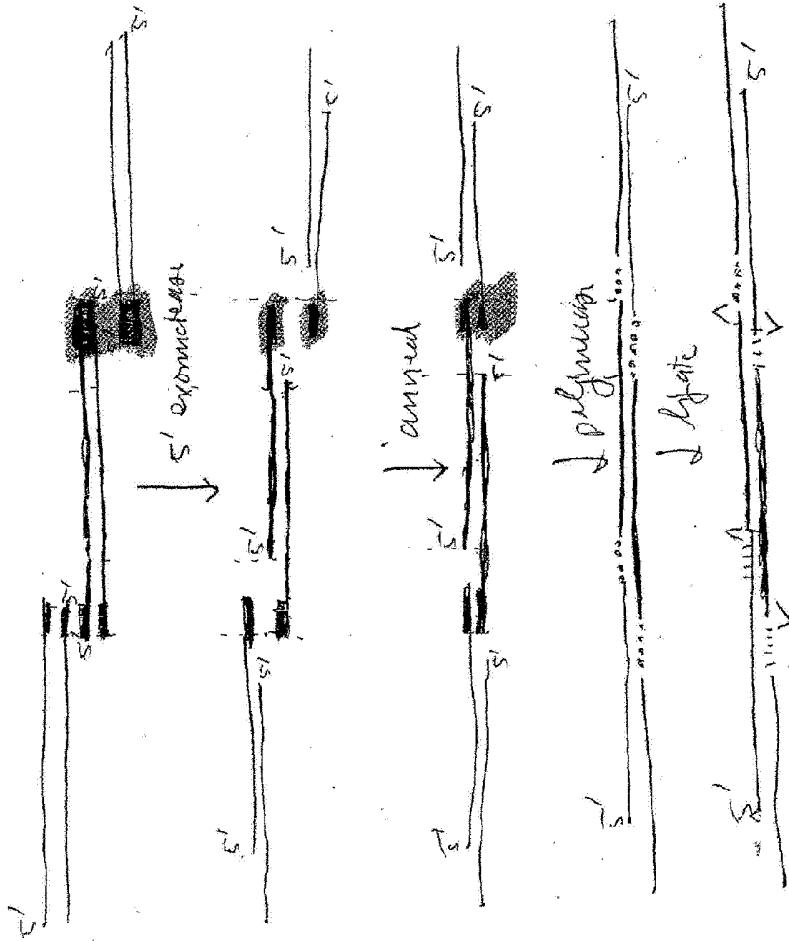
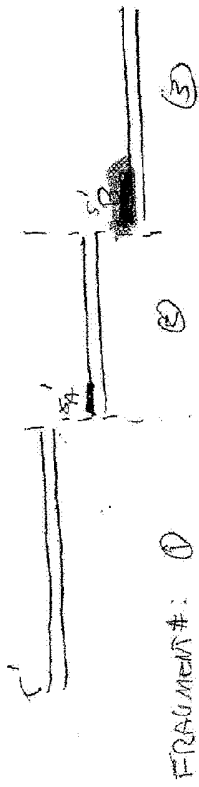
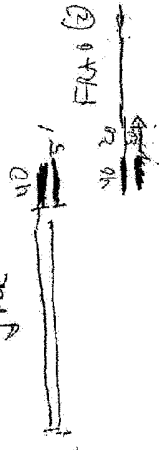
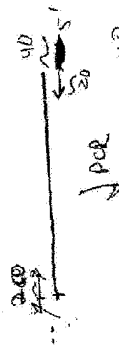


FIG. 1



FRAG 1



SHARP IN
LIGHTS NO. F161



FIG 2

FRAG 1 is per amplified 5' no
to add blue SEQ at 3' end

FRAG 2 is per amplified 5' no
to add red SEQ at 3' end

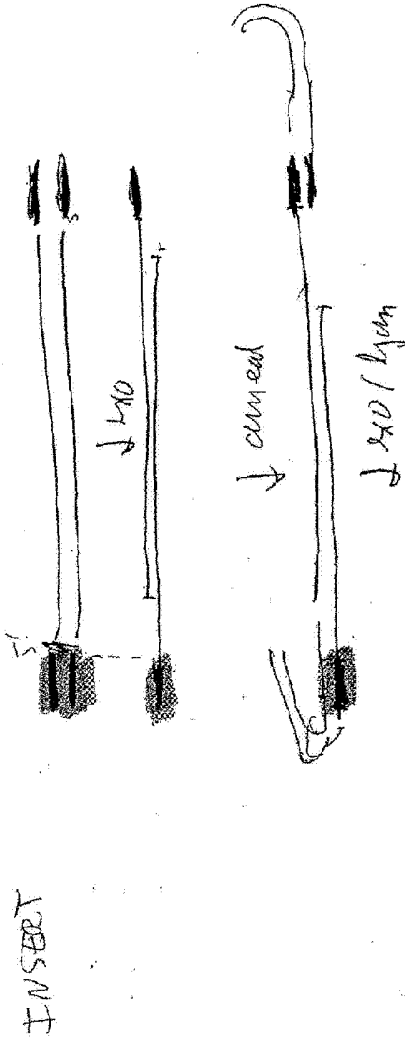
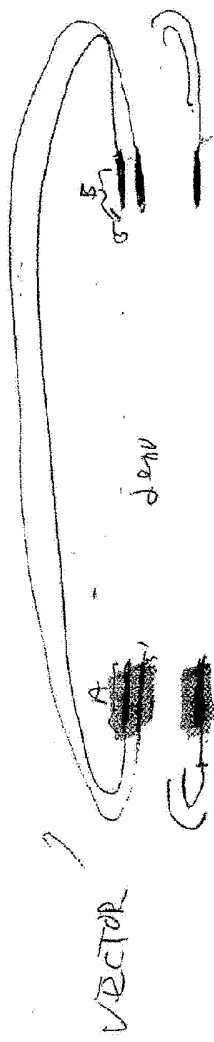
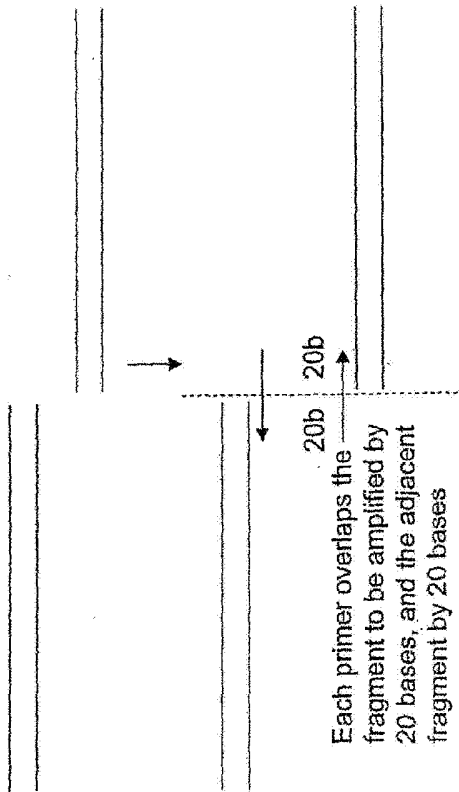


FIG. 3

Fragments to be amplified, no overlaps



Now both fragments overlap by 40bp

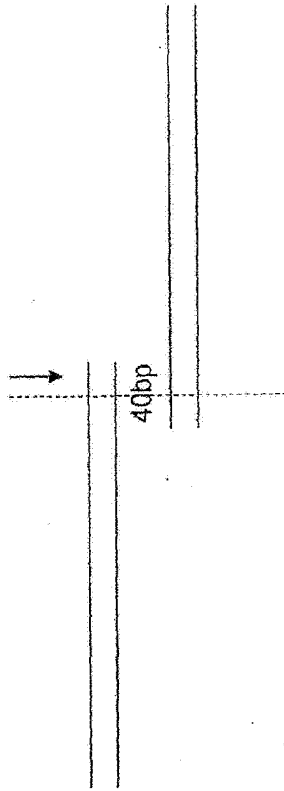


FIG. 4

4 fragments

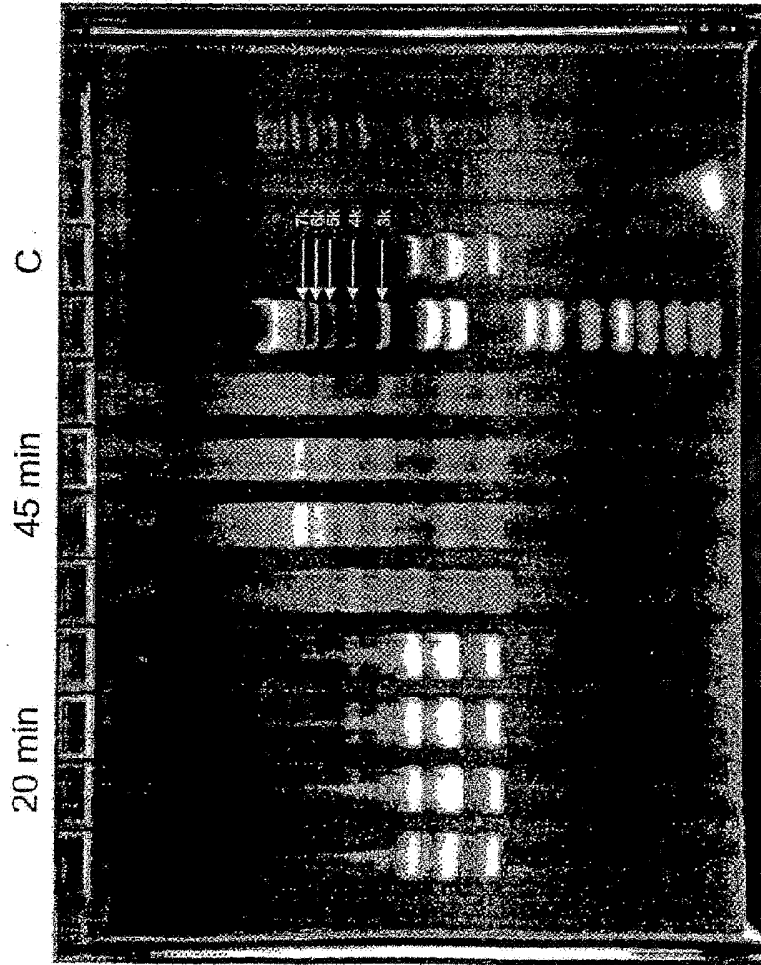


Figure 1: Lanes 1-4 are incubated for 20 min, 5-8 for 45 min at 30°C. each lane starts with 6 µg total DNA (4 fragment of 2.2kb, 1.5kb, 1.55kb and 1.2kb), 30 U/ml T7 DNA polymerase, 4000 U/ml T4 DNA ligase. Lanes 1-4 and 5-8 contain 20, 2, 4, 0.4 U/ml exonuclease and 1, 1.5, 2, 2 µM T7 ssb, respectively.

FIG. 5

M 1 2 3 4 5

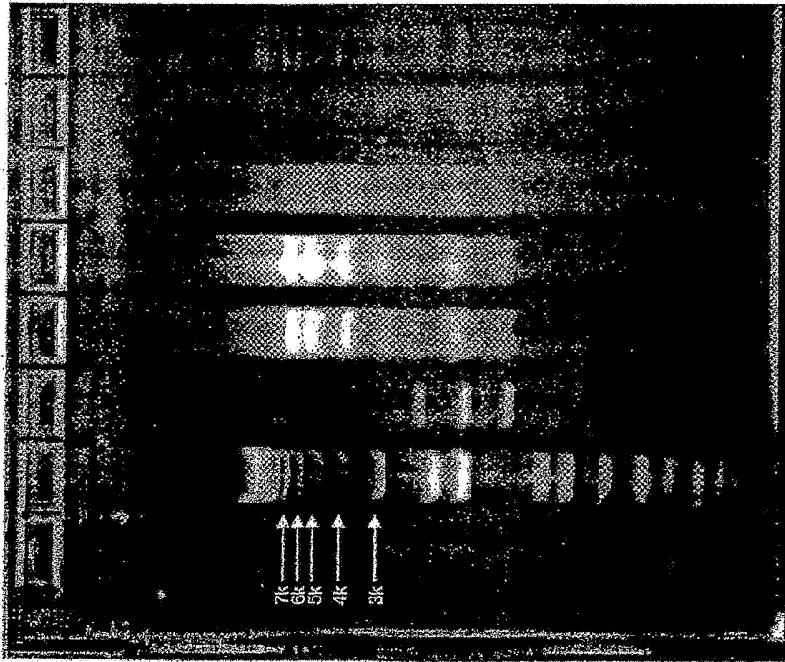


Fig 2. Conditions similar to lane 2 and 6 in Fig 1. Lane 1 is control DNA only, lane 2 and 3 are duplicates incubated at 30 °C for 60 minutes. Lane 4 is without ligase, lane 5 is without ssb.

FIG. 6