DESCRIPTION

Mutant PCNA

TECHNICAL FIELD

[0001] The present invention relates to a DNA replication factor, and more particularly relates to a proliferating cell nuclear antigen (PCNA) which has excellent function to assist DNA replication.

BACKGROUND ART

[0002] Gene amplification technology and PCR method

A technology to amplify nucleic acids such as DNA and RNA has been widely disseminated in both basic research and industrial application along with development of gene engineering technology. At an early stage, in order to obtain a particular target nucleic acid sequence, the sequence were in general prepared by cutting out the necessary sequence with restriction enzymes from nucleic acids amplified in yeast or *Escherichia coli* cells. Subsequently, it became possible to amplify a target particular nucleic acid region *in vitro* by the development of PCR by Mullis et al and dissemination thereof. For the PCR method, various experimental and industrial applications have been developed in parallel with active commercialization and distribution of specific apparatuses and related reagents. Thus the PCR method became the substantial standard method for amplifying the gene. As the research for the DNA replication has advanced, various technologies whose principals are different from that of PCR have been designed and developed, but they are less common in terms of operationality, cost and quality, and no technology which sweeps aside the PCR method has appeared.

[0003] Points for evaluating PCR method

The principle of the PCR method is thought to be based on minimally mimicking the intracellular DNA replication. That is, the principle is based on (1) dissociation of a target nucleic acid template by thermal denaturation, (2) pairing of the target sequence with a pair of complementary primers and (3) extension of the primer complementary to the template by DNA polymerase. The objective nucleic acid sequence is exponentially amplified by repeating these reactions continuously. In a general protocol of PCR, several kb of the target sequence in the template in nano gram order is often amplified to obtain a product in μg order by the reaction for about two hours.

[0004]

The PCR method has technical restrictions, which representatively include the following 4 points: (1) fidelity to the template (performance to amplify the sequence precisely corresponding to the template), (2) extendibility (performance to amplify the longer sequence), (3) efficiency to amplyfy the target sequence, and (4) reaction specificity. These are also often addressed as the points which evaluate PCR. Reagents and kits for PCR have been being developed for the purpose of overcoming these restrictions.

[0005] Improvement of PCR

As DNA polymerase for PCR, DNA polymerase derived from a thermophilic bacterium *Thermus·aquaticus* (Taq DNA polymerase) used to be generally used in the initial period (Non-patent Document 1). Based on this enzyme, subsequently various enzymes and reagents/kits for PCR have been developed and distributed. Most of them have an improved feature on any of the aforementioned technical restrictions, and the reagents/kits having such

characteristics have been distributed by various manufacturers. Some examples of the improved PCR methods are shown below.

[0006] As an example of enhancing the fidelity to the template, DNA polymerase with high fidelity is generally used for the PCR method. Taq DNA polymerase is a PolI type DNA polymerase having only 5' --> 3' polymerase activity and having no 3' --> 5' exonuclease activity. On the contrary, DNA polymerase derived from an ultrathermophilic archaebacterium, *Pyrococcus furiosus* is an α type DNA polymerase which has both the 5' --> 3' polymerase activity and the 3' --> 5' exonuclease activity. The 3' --> 5' exonuclease activity works as a proof reading activity. Thus, when this DNA polymerase is used for PCR, the fidelity to the template upon amplification is strikingly enhanced compared to amplification with Taq DNA polymerase having no 3' --> 5' exonuclease activity (Non-Patent Document 2).

[0007] Examples of commercially available products among such DNA polymerases may include Pyrobest DNA polymerase (TAKARA BIO INC.), Pfu DNA polymerase (Stratagene), KOD DNA polymerase (Toyobo Co., Ltd.), DeepVent DNA polymerase (New England Biolabs (NEB)), Vent DNA polymerase (NEB) and Pwo DNA polymerase (Roche Diagnostics).

[0008] Barns et al. has reported that when the α type DNA polymerase having the proof reading function and the PolI type DNA polymerase having no proof reading function were mixed at an appropriate ratio and used for PCR, the length of the extendable target sequence was increased and the amplification efficiency was also enhanced (Non-patent Document 3, Patent Document 1). Examples of the commercially available products of such mixed DNA polymerases may include TaKaRa EX Taq DNA polymerase

(TAKARA BIO INC.) and Taq Plus Long (Stratagene).

[0009] As one example of the strategy directing to enhancement of the reaction specificity of PCR, a hot start method has been known. Non-specificity in PCR is often caused by non-specific annealing of the primer to the template DNA. To prevent this phenomenon, the hot start method is effective in which the PCR is started immediately after completely mixing a PCR reaction solution at high temperature. Several methods for the hot start method have been reported. In a current mainstream, DNA polymerase is complexed with its specific antibody and inactivated at low temperature, and the DNA polymerase is activated under a high temperature condition upon hot start to initiate the PCR reaction. It has been reported that the specificity of the PCR reaction is enhanced by this method (Patent Document 2). Products associated with this hot start method are also distributed by gene engineering manufacturers, and generally utilized.

[0010] The aforementioned methods for improving the PCR method all have come into practical use, commercialized and generally used, but all have both advantages and disadvantages, and do not satisfy all the points for improvement in the PCR method discussed in the above. For example, the α type DNA polymerase with high fidelity is often inferior to the PolI type DNA polymerase in terms of extendibility. As to the PCR method using the mixture of the α type DNA polymerase and the PolI type DNA polymerase, the fidelity thereof is inferior to that of DNA polymerase with high fidelity. Thus it is desired to develop DNA polymerase or a DNA amplification system which is excellent in all of the points.

[0011] DNA replication process

Generally, it is required for initiation of the DNA

replication that a double strand structure is unpaired at an origin of the replication. DNA helicase is required for that process. A single strand DNA binding protein is bound to the unpaired DNA to stabilize the single strand. Furthermore, primase works on each chain to synthesize the primer. Subsequently, a replication factor C (RFC) recognizes the primer and binds thereto for inducing a proliferation cell nuclear antigen (PCNA) on the DNA chain. PCNA serves as a clump to fasten the DNA polymerase on the DNA chain. And DNA polymerase complexed with PCNA synthesizes a new chain. In a process of continuous synthesis, the long new chain is synthesized in accordance with the aforementioned manner. In a process of discontinuous synthesis, RNA primer attached to each Okazaki fragment is decomposed with nuclease whereby it is replaced with the DNA chain. Subsequently the fragments are connected with DNA ligase, to complete one new chain (Non-patent Documents 4 and 5).

[0012] Pfu-PCNA and RFC

It has been reported that *Pyrococcus·furiosus* PCNA (hereinafter represented by "Pfu-PCNA" or "PfuPCNA") has a molecular weight of 28.0 kDa, forms a homotrimer in a similar manner to PCNA in eukaryotic organisms and works by interacting with polymerase (Non-Patent Document 6). Meanwhile, *Pyrococcus·furiosus* RFC (hereinafter represented by "Pfu-RFC" or "PfuRFC") has a structure constituted by subunits RFCS and RFCL. The open reading frame for Pfu-RFCS encodes one intein and mature Pfu-RFCS has the molecular weight of 37.4 kDa. Pfu-RFCL has the molecular weight of 55.3 kDa. It has been reported that an addition of Pfu-RFC and Pfu-PCNA remarkably promoted the DNA extension activity of Pfu DNA polymerase in a primer

extension analysis (Non-patent Document 7).

[0013] Structure and nature of Pfu-PCNA

A crystal structure analysis of Pfu-PCNA was performed (Non-patent Document 8). According to that analysis, a Pfu-PCNA trimer is formed with hydrogen bonds between main chain anti-parallel β strands, βI1 and βD2, of two subunits (T108-K178, T110-E176, R112-E174). Further, an intermolecular ion pair network consisting of acidic and basic amino acid side chains is involved in keeping the trimer structure. There is a report on investigation for two mutants, PfuPCNA (D143A) and PfuPCNA (D143A/D147A), obtained by substituting D143 and D147 with alanine, which is a neutral amino acid, among residues involved in the ion pair network (R82, K84 and R109 in an N terminal region and E139, D143 and D147 in a C terminal region) (Non-patent Document 9). In this report, it has been described that PfuPCNA (D143A) and PfuPCNA (D143A/D147A) are eluted in gel filtration at positions corresponding to monomers, and that the crystal is obtained as not the trimers but V-shaped dimers. The report also describes additional results of measuring the activity in the primer extension analysis. In the report, it is concluded that PfuPCNA (D143A/D147A) did not exhibit a DNA synthesis promoting activity both in the cases of PCNA alone and in combination with RFC, whereas PfuPCNA (D143A) exhibited the DNA synthesis promoting activity regardless of the presence or absence of RFC and, in the case of PCNA alone, showed better result than a wild type PCNA.

[0014] KOD-PCNA and RFC

KOD-PCNA (hereinafter represented by "KOD-PCNA" or "KODPCNA") and KOD-RFC (hereinafter represented by "KOD-RFC" or "KODRFC") are PCNA and RFC obtained from *Thermococcus·kodakaraensis* KOD-1 strain.

[0015] KOD-PCNA has been reported in Non-patent Document 12. According to this report, KOD-PCNA has 249 residues and its theoretical molecular weight is 28.2 kDa. Pfu-PCNA reported previously also has 249 residues, and 84.3% of amino acid residues in both amino acid sequences are identical. KOD-PCNA as well as Pfu-PCNA keeps all of the conservative regions characteristic for PCNA. Although the crystal structure analysis of KOD-PCNA is not performed, it has been described that it is highly likely that KOD-PCNA forms the homotrimer in the same form as in Pfu-PCNA assumed on the basis of its high homology to Pfu-PCNA.

[0016] KOD-RFC has been reported in Non-patent Document 10. According to the report, KOD-RFC takes the same subunit structure consisting of RFCL and RFCS as the other RFCs. KOD-RFCL has the molecular weight of 57.2 kDa. The RFCS gene encodes one intein in the open reading frame, and mature KOD-RFCS has the molecular weight of 37.2 kDa.

[0017] The above two documents have also reported the effects when KOD-PCNA and KOD-RFC were added to the DNA synthesis system with KOD-DNA polymerase. In the reports, it has been described that KOD-PCNA alone and KOD-PCNA in combination with KOD-RFC promoted the extension activity in the primer extension experiment, and the "sensitivity" was enhanced when KOD-PCNA was added in the PCR reaction system.

[0018] Patent Document 1: US Patent No. 5,436,149

Patent Document 2: US Patent No. 5,338,671

Nonpatent Document 1: Saiki, R. K., Gelfand, D.H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G.T., Mullis, K. B. and Erlich, H. A., Science, 239, 487-491 (1988)

Nonpatent Document 2: Cline, JC Braman, and HH Hogrefe Nucl. Acids Res., 24, 3546-3551 (1996)

Nonpatent Document 3: Barnes, W. M., Proc. Natl. Acad. Sci., 91, 2216-2220 (1994)

Nonpatent Document 4: Waga, S. and Stillman, B. Annu. Rev. Biochem., 67, 721-751 (1998)

Nonpatent Document 5: Kornberg, A. and Baker, T.A., DNA replication, 2nd ed. W.H. Freeman, New York. (1992)

Nonpatent Document 6: Cann et al., J. Bacteriol., 181, 6591-6599 (1999)

Nonpatent Document 7: Cann et al., J. Bacteriol., 183, 2614-2623 (2001)

Nonpatent Document 8: Matsumiya et al., Protein Sci., 10, 17-23 (2001)

Nonpatent Document 9: Matsumiya et al., Protein Sci., 12, 823-831 (2003)

Nonpatent Document 10: Kitabayashi et al. Biosci Biotechnol Biochem., Nov; 67(11): 2373-2380 (2003)

Nonpatent Document 11: Takagi et al., Appl. Environ. Microbiol., Nov;63(11): 4504-4510 (1997)

Nonpatent Document 12: Kitabayashi et al., Biosci. Biotechnol. Biochem., Oct;66(10): 2194-2200 (2002)

DISCLOSURE OF INVENTION

PROBLEM TO BE SOLVED BY THE INVENTION

[0019] Under such a circumstance, it is an object to construct a DNA amplification system which is excellent in general versatility and is used easily.

MEANS FOR SOLVING PROBLEM

[0020] The present inventors have attempted to establish a novel DNA amplification system which overcomes shortcomings in the existing PCR technology by reconstructing an intracellular DNA replication system *in vitro* using a variety of factors utilized in the aforementioned DNA replication process (hereinafter also represented by an " accessory protein or "AP") and DNA

polymerase. In particular, the present inventors focused on DNA polymerase derived from an ultrathermophilic archaebacterium, *Pyrococcus furiosus*, which has the 3' --> 5' exonuclease activity and the high fidelity to the template, and PCNA and RFC which are highly likely to directly interact with DNA polymerase among various factors (accessory proteins) in the intracellular DNA replication system, and advanced the extensive study.

[0021] As a result, the present inventors have obtained findings that

a PCNA which can promote a DNA extension reaction with well-balanced extendibility and the fidelity without depending on the presence or absence of RFC can be obtained by preparing PCNA so as to include a site which causes, when an N terminal region of the PCNA monomer and a C terminal region of another PCNA monomer act as an interface to form a multimeric complex, an intermolecular interaction of the monomers in an interface region of the monomers, said PCNA monomer further comprising amino acid residues in said site causing mutual charge repulsion, and thereby have completed the present invention. The present invention thus provides the following PCNA and other inventions.

[0022]

(1) A mutant PCNA monomer comprising a site which causes, when an N terminal region of the PCNA monomer and a C terminal region of another PCNA monomer act as an interface to form a multimeric complex, an intermolecular interaction of the monomers in an interface region of the monomers, said PCNA monomer further comprising amino acid residues in said site causing mutual charge repulsion;

said monomer itself or the multimeric complex thereof having an activity to promote DNA replication.

(2) The mutant PCNA monomer according to (1) wherein said PCNA monomer has an amino acid sequence obtained by substituting an amino acid residue at least at one position selected from the group consisting of positions 82, 84, 109, 139, 143 and 147 in an amino acid sequence according to SEQ ID NO:2 or 32 with another amino acid residue, and

wherein amino acid residues are composed so that one or more amino acid residues selected from the group (i) and one or more amino acid residues selected from the group (ii) cause the mutual charge repulsion:

(i) a group of amino acid residues at positions 82, 84 and 109, and

(ii) a group of amino acid residues at positions 139, 143 and 147.

(3) The mutant PCNA monomer according to (2) comprising mutations of one or several amino acid residues selected from the group consisting of an addition, an insertion, a substitution and a deletion at position(s) other than the positions 82, 84, 109, 139, 143 and 147.

(4) The mutant PCNA monomer according to (3) having a sequence obtained by substituting the amino acid residue at position 73 with leucine in an amino acid sequence according to SEQ ID NO:2 or 32.

(5) The mutant PCNA monomer according to any one of (2) to (4) wherein one or more amino acids selected from said group (i) and one or more amino acids selected from said group (ii) are both acidic amino acids or both basic amino acids.

(6) The mutant PCNA monomer according to any one of (2) to

(5) having the sequence obtained by substituting the amino acid residue at position 143 with arginine in the amino acid sequence according to SEQ ID NO:2 or 32.

(7) A polynucleotide encoding an amino acid sequence of the PCNA monomer according to any one of (1) to (6).

(8) A transformant in which the polynucleotide according to (7) has been introduced.

(9) A method for producing a mutant PCNA wherein the transformant according to (8) is cultured in a medium and a PCNA monomer and/or a multimeric complex composed of said monomer is accumulated in said transformant and/or the medium.

(10) A reagent for DNA replication comprising the PCNA monomer according to any one of (1) to (6) and/or a multimeric complex composed of said monomer.

(11) A kit for DNA replication comprising the reagent according to (10).

(12) The kit for DNA replication according to (11) further comprising a reagent for PCR.

(13) A method for replicating DNA wherein DNA synthesis is performed in the presence of the PCNA monomer according to any one of (1) to (6) and/or a multimeric complex composed of said monomer, and DNA polymerase.

(14) The method for replicating the DNA according to (13) wherein said DNA synthesis is PCR.

[0023] The present invention has specified the amino acid sequence in the interface region of PCNA monomers so that the DNA replication reaction can be further promoted. The PCNA of the present invention has applicability to many DNA polymerases derived from different bacterial hosts and the high versatility. The PCNA of the present invention can exert an extremely unique performance compared with conventional ones in terms of exerting the excellent DNA extension promoting activity even when RFC is not used in combination.

EFFECT OF THE INVENTION

[0024] According to the present invention, a DNA replication promoting factor which promotes a DNA extension reaction and has the high versatility is provided. According to the present invention, it is possible to perform the DNA extension reaction which is excellent in various properties such as extendibility and reaction rate.

BRIEF DESCRIPTION OF DRAWINGS

[0025] FIG. 1 is a view schematically showing a trimer of PCNA.

FIG. 2 is a view showing one example of an intermolecular interaction site of monomers formed in a mutual interface region which is a joint of the monomers when PCNA monomers form a multimeric complex.

FIG. 3 is a view showing a flowchart for preparing an expression vector for a PCNA or RFC gene.

FIG. 4 is a view showing an expression plasmid for the PCNA gene.

FIG. 5 is a view showing a flowchart for preparing PCNA (protein).

FIG. 6 is a view showing a result of polyacrylamide gel electrophoresis of a PCNA protein preparation.

FIG. 7 is a view showing an expression plasmid for RFCL.

FIG. 8 is a view showing an expression plasmid for RFCSm.

FIG. 9 is a view showing a flowchart for preparing RFC (protein).

FIG. 10 is a view showing a result of polyacrylamide gel electrophoresis of a RFC protein preparation.

FIG. 11 is a view showing effects of adding various PCNA mutants and RFC to Pyrobest (case of 2 kb amplification).

FIG. 12 is a view showing effects of adding various PCNA mutants and RFC to Pyrobest (case of 8.4 kb amplification).

FIG. 13 is a view showing effects of adding various PCNA mutants and RFC to Pyrobest (case of 15.8 kb amplification).

FIG. 14 is a view showing effects of adding PCNA13 to Pyrobest for each extension time when an amplified length was 2 kb.

FIG. 15 is a view showing effects of adding PCNA13 to Pyrobest for each extension time when the amplified length was 8.4.

FIG. 16 is a view showing effects of adding PCNA13 to Pyrobest for each extension time when the amplified length was 15.8.

FIG. 17 is a view showing effects of adding PCNA13 to ExTaq for each extension time when the amplified length was 2 kb.

FIG. 18 is a view showing effects of adding PCNA13 to ExTaq for each extension time when the amplified length was 8.4 kb.

FIG. 19 is a view showing effects of adding PCNA13 to ExTaq for each extension time when the amplified length was 15.8 kb.

FIG. 20 is a view showing effects of adding PCNA13 to

Vent DNA polymerase for each extension time when the amplified length was 2 kb.

FIG. 21 is a view showing effects of adding PCNA13 to Vent DNA polymerase for each extension time when the amplified length was 8.4 kb.

FIG. 22 is a view showing effects of adding PCNA13 to Deep Vent DNA polymerase for each extension time when the amplified length was 2 kb.

FIG. 23 is a view showing effects of adding PCNA13 to Deep Vent DNA polymerase for each extension time when the amplified length was 8.4 kb.

FIG. 24 is a view showing effects of adding PCNA13 to Pfu Turbo DNA polymerase.

FIG. 25 is a view showing effects of adding PCNA13 to KOD DNA polymerase.

FIG. 26 is a view showing effects of adding PCNA13 to Pwo DNA polymerase.

FIG. 27 is a view showing a flowchart for preparing an expression vector for a KOD-PCNA gene.

FIG. 28 is a view showing the expression plasmid for the KOD-PCNA gene.

FIG. 29 is a view showing a flowchart for preparing a KOD-PCNA protein.

FIG. 30 is a view showing a result of polyacrylamide gel electrophoresis of a KOD-PCNA protein preparation.

FIG. 31 is a view showing a flowchart for preparing an expression vector for a KOD-RFCL gene.

FIG. 32 is a view showing the expression plasmid for the KOD-RFCL gene.

FIG. 33 is a view showing a flowchart for preparing an expression vector for a KOD-RFCSm (mature RFCS) gene.

FIG. 34 is a view showing the expression plasmid for the KOD-RFCS gene.

FIG. 35 is a view showing a flowchart for preparing a KOD-RFC protein.

FIG. 36 is a view showing a result of polyacrylamide gel electrophoresis of a KOD-RFC protein preparation.

FIG. 37 is a view showing effects of adding a KOD-PCNA mutant and KOD-RFC to KOD DNA polymerase.

FIG. 38 is a view showing effects of adding the KOD-PCNA mutant and KOD-RFC to Pyrobest.

FIG. 39 is a view showing a flowchart for measuring fidelity to a PCR template.

FIG. 40 is a view showing a result of polyacrylamide gel electrophoresis of a PCNA protein preparation.

FIG. 41 is a view showing effects of adding various PCNA mutants and RFC to Pyrobest (in the case of 2 kb amplification).

FIG. 42 is a view showing effects of adding various PCNA mutants and RFC to Pyrobest (in the case of 8.4 kb amplification).

FIG. 43 is a view showing effects of adding various PCNA mutants and RFC to Pyrobest (in the case of 15.8 kb amplification).

FIG. 44 is a view showing effects of adding various PCNA mutants and RFC to Pyrobest (in the case of 2 kb amplification).

FIG. 45 is a view showing effects of adding various PCNA mutants and RFC to Pyrobest (in the case of 8.4 kb amplification).

FIG. 46 is a view showing effects of adding various PCNA mutants and RFC to Pyrobest (in the case of 15.8 kb amplification).

EXPLANATIONS OF LETTERS OR NUMERALS

[0026] 1 PCNA trimer

10a, 10b and 10c PCNA monomer
20 Joint of PCNA monomers
PCNA gene PCNA gene ORF
T7 promoter: T7 promoter
rbs Ribosome binding site
T7 terminator: T7 terminator
Amp Ampicillin resistant gene
Ori Origin of replication
RFCL gene RFCL gene ORF
RFCSm gene Mature RFCS gene ORF
Kan Kanamycin resistant gene

BEST MODE FOR CARRYING OUT THE INVENTION

[0027] The present invention will be described in more detail below with referring to embodiments of the present invention. Upon carrying out biochemical or gene engineering techniques in the present invention, descriptions in various experimental manuals, e.g., Molecular Cloning: A LABORATORY MANUAL, 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001); *Shin-Idenshi Kogaku Handbook* (New Genetic Engineering Handbook; edited by Masami Muramatsu, Yodosha, Experimental Medicine, Supplementary Volume, 3rd edition, 1999); *Tanpakushitsu Jikken no Susumekata* (How to Proceed with Experiments on Proteins; edited by Masato Okada and Kaori Miyazaki, Yodosha, 1st edition, 1998); *Tanpakushitsu Jikken Note* (Notebook for Experiments on Proteins; edited by Masato Okada and Kaori Miyazaki, Yodosha, 2nd edition, 1999); *Tanpakushitsu Jikken Handbook* (Handbook for Experiments on Proteins; edited by Tadaomi Takenawa, Experimental Medicine, Supplementary Volume, 1st edition, Aug. 15, 2003); and *PCR Jikken Note* (Notebook for Experiments for PCR; edited by Taketoshi Taniguchi, Yodosha,

1st edition, 1997) are available as references.

[0028] For base sequences, amino acid sequences and individual components thereof, symbols simplified by alphabetical representation are sometimes used herein, and the representation in all cases comply with common practice in the molecular biological and gene engineering fields. To simply show the mutation in the amino acid sequence herein, for example, the representation such as "D143A" is used. "D143A" indicates that aspartic acid at position 143 was substituted with alanine, i.e., indicates the type of the amino acid residue before the substitution, its position and the type of the amino acid residue after the substitution. Unless otherwise specified, each sequence ID number corresponds to SEQ ID NO described in Sequence Listing.

[0029] 1. PCNA in the present invention

In the PCNA monomer of the present invention, the amino acid residue at a certain location in the interface region which contributes to the multimeric formation is specified. That is, in the PCNA monomer of the present invention, the site at which the intermolecular interaction is formed in the interface region is composed of the amino acid residues which generate a mutual charge repulsion between the monomers. Here the "intermolecular interaction" refers to the physical, chemical or electric interaction which occurs between the molecules, and examples thereof may include actions caused by a molecular structure such as a conformation and a steric structure, as well as various intermolecular actions such as ion bonds, hydrogen bonds and a hydrophobic interaction. When the PCNA monomers form a multimeric complex, the multimeric complex is formed by the intermolecular interaction, e.g., mutual attraction or joining of the interface regions of

the monomers. In one preferable embodiment, the amino acid residues are composed so that the amino acid residues which form intermolecular ion pairs or an ion pair network are mutually repulsed in terms of electrical charge. The PCNA monomer of the present invention has an activity to promote the DNA replication. The activity is excerted by the monomer itself or the multimeric complex thereof. The "mutant" when referred to as the "mutant PCNA" herein means comprising the amino acid sequence different from that of conventionally known PCNA, and does not distinguish whether the mutant is artificially mutated or naturally occurs.

[0030] The multimeric complex of PCNA which is one of the factors involved in the DNA replication reaction is formed by joining of the monomers on an interface of an N terminal region of one monomer and a C terminal region of another monomer. The N terminal region herein means the portion from the center to the N terminus and the C terminal region herein means the portion from the center to the C terminus when the protein is regarded as one chain. In eukaryotic cells and archaebacteria, PCNA forms the trimer in many cases. A trimer model of PCNA was illustrated in FIG. 1. As shown in FIG. 1, the PCNA monomer 10a, 10b or 10c is joined with another monomer in each terminal region to form the multimeric complex 1 having a ring structure. At a joint 20, the terminal region of the monomers makes the interface, and the intermolecular interaction which attracts the monomers is formed inside thereof. A model of the intermolecular interaction in *Pyrococcus·furiosus* PCNA is shown in FIG. 2. In FIG. 2, PCNA having the amino acid sequence described in SEQ ID NO:2 (amino acid sequence of wild type Pfu-PCNA) is described. The amino acid residues contained in the N terminal region of one monomer 10a and the amino acid

residues contained in the C terminal region of another monomer 10c form an intermolecular pair. As shown in FIG. 2, it is conceivable that the amino acid residue group at positions 139, 143 and 147 and the amino acid residue group at positions 82, 84 and 109 in the amino acid sequence described in SEQ ID NO:2 form the network of mutual affection.

[0031] On the contrary, in the present invention, the interface is constituted so that at least some amino acid residues at the site at which the intermolecular interaction occurs are positively repulsed mutually. "Mutual charge repulsion" herein refers to the mutual repulsion by the charges of the amino acid residues in the interface region. Therefore, examples of such a combination may include an combination where the amino acid residues contained in the interface region are both composed of positively charged molecules and an combination where the amino acid residues contained in the interface region are both composed of negatively charged molecules. More specifically, it is preferable that combination consists of amino acid residues both of which are acidic amino acids or both of which are basic amino acids. The acidic amino acid may include aspartic acid (abbreviated as Asp or D) and glutamic acid (abbreviated as Glu or E). The basic amino acid may include lysine (abbreviated as Lys or K), arginine (abbreviated as Arg or R) and histidine (abbreviated as His or H).

[0032] Therefore, on the basis of the PCNA having the amino acid sequence described in SEQ ID NOS: 2 or 32, the PCNA of the present invention is obtainable by altering the amino acid residue at least at one position selected from the group consisting of positions 82, 84, 109, 139, 143 and 147 in the amino acid sequence described in SEQ ID NOS: 2

or 32. As a preferable embodiment of the present invention, an embodiment composed of the amino acid residues where one or more amino acid residues selected from the following group (i) and one or more amino acid residues selected from the following group (ii) cause the mutual charge repulsion is exemplified.

(i) Amino acid residue group at positions 82, 84 and 109; and

(ii) amino acid residue group at positions 139, 143 and 147.

More specifically, it is suitable that the combination of one or more amino acid residues selected from the aforementioned (i) and one or more amino acid residues selected from the aforementioned (ii) are both the acidic amino acids or are both the basic amino acids. That is, the preferable embodiments may include an embodiment in which at least one amino acid residue selected from the group (i) and at least one amino acid residue selected from the group (ii) are both the acidic amino acids selected from the group consisting of aspartic acid and glutamic acid, and an embodiment in which at least one amino acid residue selected from the group (i) and at least one amino acid residue selected from the group (ii) are both the basic amino acids selected from the group consisting of lysine, arginine and histidine.

[0033] The activity to promote the DNA replication (DNA replication promoting activity) means that the DNA replication is promoted compared with the replication with the original wild type PCNA. Specifically, it is more preferable that the DNA replication promoting activity is higher than that of the PCNA monomer having the amino acid sequence described in SEQ ID NOS:2 and 32 or the multimeric complex thereof. As another specific indicator, it is preferable to have the DNA replication promoting activity

which increases the activity to an equivalent extent and up to 10 times or more compared with the activity of Taq polymerase in terms of extendibility and reaction rate when the extendibility and the reaction rate are measured according to the method of measuring the DNA replication activity shown in the Examples mentioned below.

[0034] The PCNA of the present invention includes a PCNA which is substantially identical to the PCNA having the aforementioned mutation-containing amino acid sequence. Specifically, the PCNA of the present invention includes a mutant PCNA having the preferable DNA replication promoting activity as described above and comprising mutations of one or several amino acid residues selected from the group consisting of an addition, an insertion, a substitution and a deletion in the range of not inhibiting the effect of the present invention at positions other than the positions 82, 84, 109, 139, 143 and 147. "Several amino acids" are specifically 2 to 50, preferably 2 to 30, more preferably 2 to 10 and particularly preferably 2 to 5 amino acids. In such a protein, the amino acid residues corresponding to the positions selected from the group (i) and the group (ii) in the amino acid sequence described in SEQ ID NOS:2 and 32 are constituted to mutually cause the charge repulsion. That is, other mutations are acceptable in the amino acid residues other than the predetermined amino acid residues according to the positions of the group (i) and/or the group (ii), provided that the activity is not remarkably impaired. Such a mutation may include a so-called conservative substitution of the amino acid residue. When the mutation e.g., the deletion, the insertion or the addition is introduced into the position other than the positions 82, 84, 109, 139, 143 and 147, the number of the amino acid residues after introducing the mutation may be

different from the number of the amino acid residues before introducing the mutation. However, insofar as the position corresponds to the position shown in the aforementioned (i) and (ii) which locate in the interface and mutually give the intermolecular interaction for forming the multimeric complex, the PCNA having the shifted number is included in the PCNA of the present invention. Among the aforementioned mutant PCNA, particularly those containing only the substitution of the amino acid residue at position 73 with leucine or those containing the other mutation in addition thereto are preferable because they can easily prepared and purified.

[0035] An action mechanism whereby the PCNA of the present invention can promote the DNA replication reaction is not exactly clear. However, it is speculated that the ring structure formed by the multimeric complex becomes in a form such that the complex is dissociated upon temperature elevation, whereby the DNA replication reaction is smoothly repeated and consequently the DNA replication reaction is further promoted. Conventionally, the idea which has been widely accepted was that PCNA monomers joined firmly to form the multimeric complex would exert a role as a stable clamp and working as the DNA replication promoting factor. However, in this point, the present invention provides findings different from conventional one. That is, without concurrent use of RFC, too firm bonds in the multimeric complex are not good for the PCNA activity. In particular upon repetition of the DNA replication such as PCR, when the ring structure is weakened so that the complex is dissociated upon temperature elevation, the PCNA exerts higher promoting activity than that the wild type PCNA exerts in combination with RFC.

[0036] A specific example of the preferable PCNA of the

present invention may include the PCNA monomer having the sequence (D143R) in which aspartic acid at position 143 has been substituted with arginine. The amino acid residue at position 143 belongs to the group (ii). In this case, the amino acid residues at positions 82, 84 and 109 belonging to the group (i) are arginine, lysine and arginine, respectively, and the intermolecular interaction may be in a state where the interface regions are mutually repulsed more strongly than usual. The PCNA in this form has well-balanced extendibility and reaction rate in the DNA replication reaction and exerts a particularly excellent ancillary action.

[0037] The present invention also provides a polynucleotide encoding the PCNA of the present invention. The amino acid is deduced from the base sequence by a codon table, and due to degeneracy of the codons, one amino acid sequence may be encoded by a plurality of base sequences. For example, the amino acid sequences described in SEQ ID NOS:2 and 32 are encoded by the base sequences described in SEQ ID NOS:1 and 31. Therefore, an embodiment of the polynucleotide of the present invention may be the following polynucleotide (a).

(a) Polynucleotide having a base sequence encoding an amino acid sequence in which one or more amino acid residues selected from the group (i) and one or more amino acid residues selected from the group (ii) in the amino acid sequence described in SEQ ID NOS:2 and 32 have been substituted to mutually cause the charge repulsion.

[0038] The polynucleotide having the base sequence such as (a) may be easily prepared by modifying the base sequence at the positions corresponding to the amino acid residues at the predetermined positions in the base sequence described in SEQ ID NOS:1 and 31. Conversion of

the base sequence based on the type of the amino acid may be easily performed based on the codon table. The polynucleotide referred to herein may include both DNA and RNA, a double strand and a single strand, and may also include chimeric molecules of DNA and RNA and hybrids of DNA and RNA.

[0039] The following polynucleotide (b) may also be included in the polynucleotide of the present invention.

(b) Polynucleotide which hybridizes with the polynucleotide having the base sequence complementary to the base sequence of the aforementioned polynucleotide (a) under a stringent condition; which has the base sequence encoding the amino acid sequence combining the amino acid residues so that one or more amino acid residues selected from the group (i) and one or more amino acid residues selected from the group (ii) mutually cause the charge repulsion; and which encodes the PCNA having the aforementioned DNA replication promoting activity.

[0040] A probe which may be used for obtaining the gene to be hybridized may be prepared by standard methods based on the base sequence described in SEQ ID NO:1 or 31. The objective polynucleotide may also be isolated by using the probe to pick up the polynucleotide to be hybridized therewith according to the standard method. For example, the probe may be prepared by amplifying the base sequence cloned in a plasmid or a phage vector, and cutting out and extracting the base sequence for use as the probe with restriction enzymes. A site to be cut out may be regulated depending on the objective DNA.

[0041] The "stringent condition" refers to the condition where a so-called specific hybrid is formed whereas no non-specific hybrid is not formed. Examples thereof may be conditions where hybridization occurs at salts

concentrations equivalent to 1 x SSC and 0.1% SDS at 60°C and preferably 0.1 x SSC and 0.1% SDS at 60°C, which is a washing condition of an ordinary Southern hybridization. As the polynucleotide to be hybridized under the "stringent condition", it is suitable that the aforementioned polynucleotide (b) has preferably 50% or more, more preferably 80% or more, still more preferably 90% or more and still more preferably 95% or more homology to the polynucleotide (a). As the method for calculating the homology, for example BLAST, FASTA, Clustal W and the like may be used.

[0042] The protein comprising the amino acid sequence obtained by translating the base sequence of the aforementioned polynucleotide (b) has the DNA replication promoting activity. More specifically, this protein has the same DNA replication promoting activity as the activity of the aforementioned protein of the present invention.

[0043] The polynucleotide of the present invention can be incorporated into an appropriate vector and used as a recombinant polynucleotide. The recombinant polynucleotide herein refers to a hybrid molecule obtained by ligating two or more polynucleotides. The preferable embodiment of the recombinant polynucleotide of the present invention may include an expression vector. Various forms of the expression vectors have been already known and some of them are commercially available. The embodiment of the recombinant polynucleotide may be appropriately selected depending on the intended use, and the publicly known expression vectors such as commercially available expression vectors can be used. Specific examples related to the expression vectors are shown below in a non-limited manner.

[0044] As the vector, plasmids derived from *Escherichia*

coli (e.g., pBR322, pBR325, pUC12, pUC13, commercially available pBT Vector and pTRG Vector(Stratagene)); plasmids derived from yeast (e.g., YEp24, YCp50); bacteriophages such as λ phage; animal viruses such as retrovirus, vaccinia virus and baculovirus; plasmids suitably used for *Bacillus subtilis* (e.g., pUB110, pTP5, pC194); as well as pA1-11, pXT1, pRc/CMV, pRc/RSV, pcDNAI/Neo and the like are used.

[0045] Any promoters may be used as long as they are suitably associated with host cells for the gene expression. For example, when the host cell is derived from genus *Escherichia*, a trp promoter, a lac promoter, a recA promoter, a λPL promoter, an lpp promoter and a T7 promoter may be used. When the host cell is derived from genus *Bacillus*, an SPO1 promoter, an SPO2 promoter and a penP promoter may be used. When the host cell is yeast, a PHO5 promoter, a PGK promoter, a GAP promoter and an ADH promoter may be used. When the host cell is an insect cell, a polyhedrin promoter and a P10 promoter may be used. When an animal cell is used as the host cell, an SRα promoter, an SV40 promoter, an HIV/LTR promoter, a CMV (cytomegalovirus) promoter and an HSV-TK promoter may be used.

[0046] It is preferable in terms of easy recombination manipulation that the expression vector has multicloning sites. In addition to the aforementioned, a selection marker, an enhancer, a splicing signal, a poly A addition signal, an SV40 replication origin (hereinafter sometimes abbreviated as SV40ori) and a terminator can be incorporated into the expression vector if desired. Examples of the selection marker may include an ampicillin resistant gene (also referred to as a carbenicillin

resistant gene, sometimes abbreviated as Amp), a kanamycin resistant gene (sometimes abbreviated as Kam), a tetracycline resistant gene (sometimes abbreviated as Tet), a dihydrofolic acid reducing enzyme (sometimes abbreviated as dhfr) gene (methotrexate [MTX] resistant) and a neomycin resistant gene (sometimes abbreviated as Neo, G418 resistant). If necessary, a signal sequence adapted for the host cell is added to the N terminal side of a random oligonucleotide or a cassette of the present invention. When the host cell is derived from genus *Escherichia*, a PhoA signal sequence and an OmpA signal sequence can be utilized. When the host cell is derived from genus *Bacillus*, an α-amylase signal sequence and a subtilisin signal sequence can be utilized. When the host cell is yeast, an MFα signal sequence and an SUC2 signal sequence can be utilized. When the host cell is an animal cell, an insulin signal sequence, an α-interferon signal sequence and an Ras farnesylation signal sequence can be utilized.

[0047] A transformant which expresses the DNA polymerase of the present invention can be produced by preparing the expression vector containing the polynucleotide of the present invention as discussed above and then introducing this into the host cell.

[0048] Examples of the host cells may include bacterial cells derived from *streptococci*, *staphylococci*, *Escherichia coli*, *Streptomyces* and *Bacillus subtilis*; fungal cells derived from yeast and *Aspergillus*; insect cells such as Drosophila S2 and Sf9(*Spodoptera* Sf9); animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK293, Bows cells, melanoma cells and hematopoietic cells as well as plant cells.

[0049] The expression vector can be introduced into the

host cell by the method described in many standard experimental manuals such as Davis et al., BASIC METHODS IN MOLECULAR BIOLOGY(1986); Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001). More specifically, calcium phosphate transfection, the transfection mediated through DEAE-dextran, microinjection, the transfection mediated through cation lipid, electroporation, transduction, biolistics introduction or infection is available.

[0050] The transformant may be cultured by controlling conditions depending on the type of the host. Many types of the hosts are available, and some specific examples of the conditions are as follows. For example, when a transformant derived from *Escherichia* or *Bacillus* as the host is cultured, the medium used for culturing may be a liquid medium or an agar medium, to which carbon sources, nitrogen sources, inorganic matters and others required for the growth of the transformant may be added. Examples of the carbon sources may include glucose, dextrin, soluble starch and sucrose. Examples of the nitrogen sources may include inorganic or organic substances such as ammonium salts, nitrate salts, corn steep liquor, peptone, casein, meat extracts, soybean cakes and potato extracts. Examples of the inorganic salts may include calcium chloride, sodium dihydrogen phosphate and magnesium chloride. Yeast extracts, vitamins and growth promoting factors may also be added. A pH value of the medium is desirably about 5 to 8. As the suitable medium when the *Escherichia* cells are cultured, specifically LB medium containing yeast extract, trypton and the salt (NaCl) is exemplified. In order to make the promoter work efficiently as needed, an inducer such as isopropyl 1-thio-β-galactoside (IPTG) may be added

thereto. When the host is *Escherichia*, culturing is typically performed at about 15 to 43°C for about 3 to 24 hours, and if necessary ventilation and stirring may be performed. When the host is *Bacillus*, culturing is typically performed at about 30 to 40°C for about 6 to 24 hours, and if necessary ventilation and stirring may be performed.

[0051] When the transformant derived from the yeast as the host is cultured, examples of the medium therefor may include a Burkholder minimum medium and an SD medium containing 0.5% casamino acids. It is preferable that the pH value in the medium is adjusted to about 5 to 8. Culturing is typically performed at about 20 to 35°C for about 24 to 72 hours, and if necessary ventilation and stirring may be performed.

[0052] When the transformant derived from the insect cell or the insect as the host is cultured, those obtained by appropriately adding an additive such as 10% inactivated bovine serum to Grace's Insect Medium (Grace, T. C. C., Nature, 195, 788(1962) may be used as the medium. It is preferable that the pH value in the medium is adjusted to about 6.2 to 6.4. Culturing is typically performed at about 27°C for about 3 to 5 days, and if necessary ventilation and stirring may be performed.

[0053] When the transformant derived from the animal cell as the host is cultured, a MEM medium, a DMEM medium, an RPMI1640 medium (The Journal of the American Medical Association, 199: 519 (1967)) and a 199 medium (Proceeding of the Society for the Biological Medicine, 73: 1(1950)) containing about 5 to 20% fetal calf serum may be used. It is preferable that the pH value is adjusted to about 6 to 8. Culturing is typically performed at about 30 to 40°C for

about 15 to 60 hours, and if necessary ventilation and stirring may be performed. If necessary, a concentration of CO_2 is controlled.

[0054] The protein of the present invention produced by the transformant can be purified and isolated if necessary by the standard methods for protein purification. As discussed above, the PCNA of the present invention can be obtained using the transformant.

[0055] 2. Method for DNA replication using PCNA of the present invention

The method for DNA replication of the present invention is characterized in that DNA is synthesized in the presence of the PCNA monomer and/or the multimeric complex composed of the PCNA monomers with DNA polymerase. The method for synthesizing the DNA may be PCR, a primer extension, a nick translation, and the synthesis of the first strand cDNA by reverse transcriptase.

[0056] A preferable example of the method for DNA replication of the present invention may be the DNA amplification by PCR. In the PCR, the DNA replication using the primers and the template DNA is repeated to amplify the DNA exponentially. Thus, it is desirable that the PCNA serves as a function of a clamp for DNA polymerase, and the PCNA is rapidly removed from the template after DNA polymerase is stabilized on the template or after the predetermined region is amplified. It is speculated that the PCNA of the present invention has such properties.

[0057] The PCNA of the present invention is well-compatible with various DNA polymerases and thus has high versatility. In general, DNA polymerases are excellent in either one of extendibility and fidelity, and thus has both advantages and disadvantages. However, by combining with the PCNA of the present invention, it is possible to

enhance the extendibility without reducing the fidelity. Thus, the DNA replication activity can be augmented with reinforcing the shortcoming of DNA polymerase. Examples of the DNA polymerase which is applicable to the method for DNA replication of the present invention may include most DNA polymerases mainly used at present, e.g., Pyrobest DNA Polymerase (TAKARA BIO INC.), TaKaRa EX Taq(TAKARA BIO INC.), Vent DNA Polymerase (NEW ENGLAND BioLabs), Deep VentR DNA Polymerase (New England Biolabs), Pfu Turbo DNA Polymerase(Stratagene), KOD DNA Polymerase(Toyobo Co., Ltd.) and Pwo DNA Polymerase (Roche Diagnostics). The PCNA of the present invention is particularly effective for enhancing the extendibility, and is particularly useful when used in combination with α-type DNA polymerase which is superior in fidelity but inferior in extendibility.

[0058] In the method for DNA replication of the present invention, the PCNA of the present invention requires no RFC, and thus, RFC need not be added to a reaction system. No RFC preparation is commercially available in general, and preparation thereof is a burden. Even if the RFC preparation is available, when it is added to use, it is necessary to determine the conditions such as appropriate amount ratios to various factors other than RFC in the DNA replication system. The present invention has an advantage that these need not be considered. Particularly in the use for PCR, the PCNA of the present invention even without RFC exerts better promoting activity than the wild type PCNA in combination with RFC.

[0059] As to the specific conditions for PCR, many instruction manuals have been already published, and the conditions in the method of the present invention may be controlled appropriately with reference to those references. As the conditions for PCR, for example, the amount of DNA

polymerase to be added, the reaction time of PCR, the temperature of the reaction solution, components of the reaction solution, pH in the reaction solution and the amount of the template polynucleotide to be added are controlled.

[0060] 3. Reagent kit

The reagent kit of the present invention is a reagent kit for DNA replication comprising the aforementioned PCNA of the present invention and if necessary another reagents. The PCNA of the present invention can be suitably used in an embodiment comprising the monomer and/or the multimeric complex composed of the monomers, as one ingredient of the reagents for DNA replication. The reagent kit of the present invention is particularly suitable as the reagent kit for PCR because the PCNA of the present invention can be used particularly suitably in PCR.

[0061] The PCNA in the reagent kit of the present invention may be any form such as a purified protein, a recombinant polynucleotide in which the polynucleotide encoding the protein has been incorporated, and a transformant in which this recombinant polynucleotide has been introduced. The preferable forms of the recombinant polynucleotide and the transformant are as described above. The PCNA of the present invention may be combined with another PCNA. When the PCNA of the present invention is provided in the form of the recombinant DNA or the transformant, the kit may comprise reagents used for expressing the PCNA of the present invention. If necessary, the reagent kit containing the PCNA of the present invention may also contain other components and media which are generally used as biotechnology reagents.

EXAMPLES

[0062] The present invention will be described in more detail with reference to the following Examples, but the present invention is not limited to these Example at all.

[0063] 1. Preparation of Pfu-PCNA and RFC proteins

A Pfu-PCNA mutant protein preparation and an RFC protein preparation were prepared by expressing these genes in large amounts in *Escherichia coli* and purifying the proteins from those protein-expressing microbial cells.

[0064] 1.1: Acquisition of microbial cells and preparation of genomic DNA

1.1.1: Acquisition of Pfu microbial cells and preparation of genomic DNA

Pyrococcus furiosus DSM3638 strain was obtained from Deutsche Sammlung von Mikrooganismen und Zelkuluren GmbH (English name: German Collection of Microorganisms and Cell Cultures, address: Mascheroder Weg 1b, 38124 Braunschweig, Germany). The DSM3638 strain was cultured according to the method described in the reference (Uemori et al., Nucl. Acids Res. 21: 259-265 (1993)). About 1.2 g of microbial cells were obtained from 500 ml of cultured medium. These were suspended in 10 mL of buffer (10 mM Tris-Cl pH8.0, 1mM EDTA, 100 mM NaCl), and 1 mL of 10% SDS was added. After stirring, 50 μL of proteinase K (20 mg/mL) was added and the mixture was left stand at 55°C for 60 minutes. Subsequently, the reaction solution was extracted sequentially with phenol, phenol/chloroform and chloroform, and then ethanol was added to insolubilize DNA. The collected DNA was dissolved in 1 mL of TE solution (10 mL of Tris-Cl, pH 8.0, 1 mM EDTA), and 0.75 mg of RNase A was added to react at 37°C for 60 minutes. Subsequently, the reaction solution was extracted again sequentially with phenol, phenol/chloroform and chloroform, and subsequently

ethanol was added to collect the DNA.

[0065] 1.2: Cloning of PCNA gene

A Pfu-PCNA gene was acquired by cloning (FIG. 3) utilizing PCR with reference to the base sequence information AB 017486 (SEQ ID NOS: 1 and 2) registered in NCBI database. The detail thereof will be described below.

[0066] 1.2.1: PCR primer

Pfu-PCNA-F and Pfu-PCNA-R were used for the amplification of the pfu-PCNA gene. These sequences are designed so that a region corresponding to the region extending from an initiation codon methionine to a termination codon is amplified and further the sites recognized by the restriction enzymes NdeI and XhoI are added at a 5' side. The sequence of each primer was shown in Table 1 (SEQ ID NOS:3 and 4).

[0067]

Table 1 Primers for amplification of PCNA gene

Primer name	Primer sequence 5'==>3'	SEQ ID NO
Pfu-PCNA-F	cat atg cca ttt gaa atc gta tt	3
Pfu-PCNA-R	ctc gag tca ctc ttc aac tct	4

[0068] 1.2.2: Template DNA

As the template for the PCR, the Pfu genomic DNA prepared in the aforementioned 1.1.1 was used.

[0069] 1.2.3: Composition of PCR reaction solution

The PCR reaction solution has the following composition (amounts to be added to 50 μL of the reaction solution).

Template DNA: 100 ng,

Primers: each 10 pmol,

dNTP: each 10 nmol,

Ex Taq*: 1.25 U and

10 x Ex Taq buffer: 5 μL

were mixed, and sterilized water was added thereto up to the total volume of 50 μL.

(*supplied from TAKARA BIO INC.)

[0070] 1.2.4.: Reaction conditions for PCR

The PCR reaction was performed by using the reaction solution prepared in the aforementioned procedure and a PCR apparatus and using a program repeating 30 cycles of the reaction at 95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for one minute.

[0071] 1.2.5: Purification of PCR product

The PCR product obtained in the aforementioned procedure was subjected to 1% agarose gel electrophoresis, and stained with ethidium bromide. Subsequently a gel fragment containing a band around 800 bp was cut out under ultraviolet light irradiation, and the PCR product in the gel fragment was purified using GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience) according to its manipulation manual.

[0072] 1.2.6: Subcloning of PCR product

The purified PCR product was ligated to pUC118-HincII/BAP using TaKaRa BKL kit (TAKARA BIO INC.) according to the manipulation manual. *Escherichia coli* DH5α (TAKARA BIO INC.) was transformed with this ligated PCR product, which was then seeded on an LB agar plate (containing 100 μg/mL of ampicillin, 40 μg/mL of IPTG, and 40 μg/mL of X-GAL),and cultured by leaving stand at 37°C overnight to yield an *Escherichia coli* clone having the PCR product.

[0073] An *Escherichia coli* colony exhibiting white color on the agar plate was cultured in 3 mL of an LB liquid

medium (containing 100 μg/mL of ampicillin) at 37°C overnight with shaking, and then a plasmid DNA was prepared according to the standard method.

[0074] 1.2.7: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA sequence inserted into the recognition site of the restriction enzyme HincII in the plasmid vector pUC118 was examined using a DNA sequencer. As a result, it was confirmed that the open reading frame of the Pfu-PCNA gene was retained, the restriction enzyme NdeI recognition sequence was added to the 5' end and the restriction enzyme XhoI recognition sequence was added the 3' end in the inserted portion. This plasmid vector having the open reading frame of the pfu-PCNA gene was designated as pUC/PPC.

[0075] 1.3: Preparation of PCNA expression plasmid

The plasmid pUC/PPC was doubly cleaved with restriction enzymes NdeI and XhoI to prepare a PCNA gene fragment. The gene fragment was inserted into an expression vector to produce the expression vector of Pfu-PCNA.

[0076] 1.3.1: Preparation of PCNA DNA fragment

The plasmid pUC/PPC was doubly cleaved with restriction enzymes NdeI and XhoI in the following reaction system.

[0077]

Plasmid DNA: 5 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme XhoI: 5 units

[0078] Sterile water was added to the aforementioned mixture up to the total volume of 50 μL, and the plasmid

DNA was cleaved with the restriction enzymes at 37°C for 30 minutes. After completing the reaction, the reaction product was run on 2% agarose gel electrophoresis. A band (around about 800 bp) corresponding to the Pfu-PCNA gene was cut out and the PCNA DNA fragment was purified from the gel using GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience) according to its manipulation manual.

[0079] 1.3.2: pET-21a Expression vector

A vector DNA pET-21a (Novagen, US) was doubly cleaved with the restriction enzymes NdeI and XhoI by the following reaction.

[0080]

Plasmid DNA: 2 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme XhoI: 5 units

[0081] Sterile water was added to the aforementioned mixture up to the total volume of 50 μL, which was then left stand at 37°C for 2 hours. After completing the reaction, the reaction product was run on 1% agarose gel electrophoresis. A band (around about 5.4 kb) corresponding to the straight form of the vector DNA pET-21a was cut out and the pET-21a DNA fragment was purified from the gel using GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience) according to its manipulation manual.

[0082] 1.3.3: Ligation reaction and transformation

The Pfu-PCNA DNA fragment (100 ng) and the pET-21a DNA fragment (50 ng) obtained in the aforementioned procedure were reacted using DNA Ligation Kit V2 (TAKARA BIO INC.) as follows.

[0083] PCNA DNA fragment: 100 ng

pET-21a DNA fragment: 50 ng

DNA Ligation Kit V2 enzyme solution: 5 μL

Sterile water was added to the aforementioned mixture up to the total volume of 10 μL, which was then reacted at 16°C for 30 minutes.

[0084] 100 μL of *Escherichia coli* (*E. coli*) BL21 (DE3) (Novagen) was transformed with this ligation product (3μL). The solution of transformed *E. coli* was seeded on the LB agar plate (100 μg/mL ampicillin) and left stand to culture at 37°C overnight. Three colonies in *E. coli* colonies formed on the agar plate were cultured with shaking in 3 mL of the LB liquid medium (containing 100 μg/mL ampicillin) at 37°C overnight, and a plasmid DNA was prepared according to standard methods.

[0085] 1.3.4: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA sequence inserted in the plasmid vector pET-21a and the sequence in the vicinity of the inserted site were examined using the DNA sequencer. As a result, the ORF (open reading frame) of the Pfu-PCNA gene was completely inserted between NdeI and XhoI sites which were multicloning sites. This plasmid retaining the Pfu-PCNA gene was designated as pPPCNA (FIG. 4).

[0086] As shown in FIG. 4, it was confirmed that a T7 promoter and rbs (ribosome binding site) which pET-21a had, the PCNA gene ORF (open reading frame) and a T7 terminator were aligned in this order in pPPCNA. It was expected that this plasmid would express the PCNA gene in a large amount.

[0087] 1.4: Introduction of mutation into PCNA gene

For the purpose of producing the PCNA having a higher function, the amino acid in Pfu-PCNA was substituted. The amino acid was substituted by substituting the base in the

codon encoding that amino acid. An amino acid mutation was introduced into a mutation site shown in Table 2.

[0088]

Table 2 Mutation site in PCNA

Mutant ID	Mutation site
Pfu-PCNA01	M73L
Pfu-PCNA10	M73L+D143A
Pfu-PCNA12	M73L+R82C
Pfu-PCNA13	M73L+D143R
Pfu-PCNA16	M73L+R82C+D143R
Pfu-PCNA70	M73L+D143K
Pfu-PCNA71	M73L+D143H
Pfu-PCNA72	M73L+R109E
Pfu-PCNA77	M73L+D147R
Pfu-PCNA78	M73L+E139A
Pfu-PCNA79	M73L+E139R

[0089] 1.4.1: Introduction of substitution mutation

The mutation was introduced into the PCNA gene by utilizing the plasmid to be mutated, an oligo pair for mutation introduction (SEQ ID NOS:5 to 12 in Table 3) and Quick Change II Site-Directed Mutagenesis Kit (Stratagene) according to its manipulation manual.

[0090]

Table 3 Oligo DNA sequence for introducing mutation into PCNA

Primer name	Sequence 5'=>3'	SEQ ID NO
Pfu_M73L-F	CA ATT GGA GTT AAC CTG GAC CAC CTA AAG	5
Pfu_M73L-R	CTT TAG GTG GTC CAG GTT AAC TCC AAT TG	6
Pfu_D143A-F	TT CTT GGA GAA GTC CTA AAA GCT GCT GTT AAA GAT GCC TCT CTA GTG AGT GAC AG	7
Pfu_D143A-R	CT GTC ACT CAC TAG AGA GGC ATC TTT AAC AGC AGC TTT TAG GAC TTC TCC AAG AA	8
Pfu_R82C-F	CCT AAA GAA GAT CCT AAA GTG CGG TAA AGC AAA GG	9
Pfu_R82C-R	CCT TTG CTT TAC CGC ACT TTA GGA TCT TCT TTA GG	10
Pfu_D143R-F	GGA GAA GTC CTA AAA CGT GCT GTT AAA GAT GCC	11
Pfu_D143R-R	GGC ATC TTT AAC AGC ACG TTT TAG GAC TTC TCC	12
Pfu_D143K-F	GGA GAA GTC CTA AAA AAA GCT GTT AAA GAT	62
Pfu_D143K-R	ATC TTT AAC AGC TTT TTT TAG GAC TTC TCC	63
Pfu_D143H-F	GGA GAA GTC CTA AAA CAT GCT GTT AAA GAT	64
Pfu_D143H-R	ATC TTT AAC AGC ATG TTT TAG GAC TTC TCC	65
Pfu_R109E-F	CAA GGA ACT GCA ACA GAA ACA TTT AGA GTT CCC C	66
Pfu_R109E-R	GGG GAA CTC TAA ATG TTT CTG TTG CAG TTC CTT G	67
Pfu_D147R-F	CCT AAA AGA TGC TGT TAA AAG AGC CTC TCT AGT GAG TGA C	68
Pfu_D147R-R	GTC ACT CAC TAG AGA GGC TCT TTT AAC AGC ATC TTT TAG G	69
Pfu_E139A-F	GGT TGT AGT TCT TGG AGC AGT CCT AAA AGA TGC TG	70
Pfu_E139A-R	CAG CAT CTT TTA GGA CTG CTC CAA GAA CTA CAA CC	71
Pfu_E139R-F	GGT TGT AGT TCT TGG AAG AGT CCT AAA AGA TGC TG	72
Pfu_E139R-R	CAG CAT CTT TTA GGA CTC TTC CAA GAA CTA CAA CC	73

[0091] After introducing the mutation, the DNA sequence was checked by sequencing to confirm that the mutation had

been introduced into an objective site and no mutation other than the objective one was present. The design for each PCNA mutant will be described below.

[0092] 1.4.1.1: Pfu-PCNA01

According to the report for the Pfu-PCNA (Non-patent Document 6), the following has been reported: when the natural Pfu-PCNA is prepared as the recombinant protein using *Escherichia coli* as the host, in addition to the original protein as a result of translation from Met, another protein of about 20 kDa with the N terminus beginning from the 73rd residue is produced as a byproduct. It is also reported that when Met at position 73 is substituted with Leu using the gene engineering technique, the production of this protein of about 20 kDa is inhibited. It has been further reported that the Pfu-PCNA produced in this way has the nature indistinguishable from the nature of the wild type PCNA protein. Based on these facts, the mutant Pfu_M73L is addressed as a quasi-wild type in the present specification.

[0093] This mutant Pfu_M73L was designated as Pfu-PCNA01, and this mutant was produced. Pfu-PCNA01 was produced using pPPCNA as a template plasmid and the oligo pair for introducing the mutation (Pfu_M73L-F and Pfu_M73L-R).

[0094] 1.4.1.2: Pfu-PCNA10

Pfu-PCNA10 is a mutant obtained by a single residue substitution by substituting the amino acid D at position 143 with A in Pfu-PCNA01, and its structure and nature have been reported by Matsumiya (Non-patent Document 9). According to the report, aspartic acid at position 143 is located on the interface when the Pfu-PCNA forms the homotrimer and is a part of the amino acid residues involved in trimer formation. It is further reported that, when this aspartic acid at position 143 is substituted with

alanine, the trimer formation is inhibited but stimulation itself to the DNA polymerase activity is kept.

[0095] This PCNA mutant having double mutations of D143A and M73L was designated as Pfu-PCNA10, and produced using the Pfu-PCNA01-producing plasmid as the template and oligos Pfu_D143A-F and Pfu_D143A-R for introducing the mutation.

[0096] 1.4.1.3: Pfu-PCNA12

Pfu-PCNA12 is the mutant obtained by the single residue substitution by substituting the amino acid R at position 82 with C. According to Matsumiya (Non-patent Document 9), it has been reported that arginine at position 82 is located on the interface when the pfu-PCNA forms the homotrimer.

[0097] This PCNA mutant having the double mutations of R82C and M73L was designated as Pfu-PCNA12, and produced using the Pfu-PCNA01-producing plasmid as the template and oligos Pfu_R82C-F and Pfu_R82C-R for introducing the mutation.

[0098] 1.4.1.4: Pfu-PCNA13

Pfu-PCNA 13, aiming at examining how intentional inhibition of PCNA trimer formability would affect on DNA replication, was prepared by substituting the residue at position 143 with arginine (basic amino acid), whereby the electric nature thereof became completely different from aspartic acid (acidic amino acid) in the quasi-wild type (Pfu-PCNA01) and alanine (neutral amino acid) in Pfu-PCNA10. Specifically, the mutant Pfu-PCNA13 was produced using the Pfu-PCNA01-producing plasmid as the template and oligos Pfu_D143R-F and Pfu_D143R-R for introducing the mutation.

[0099] 1.4.1.5: Pfu-PCNA16

This mutant was produced aiming at examining the effect of substituting two amino acid residues that

probably have close relationship with trimer formation, i.e., arginine at position 82 and aspartic acid at position 143. Specifically, the mutant Pfu-PCNA16 was produced using the Pfu-PCNA12-producing plasmid as the template and oligos Pfu_D143R-F and Pfu_D143R-R for introducing the mutation.

[0100] 1.4.1.6: Pfu-PCNA70

For the purpose of comparing activities depending on the amino acid introduced into the position 143, this mutant was produced by substituting the amino acid at position 143 with lysine (basic amino acid). Even though both belong to the category of basic amino acid, guanidinium group in the side chain of arginine has a pK_R value of 12.48 whereas butyl ammonium group in the side chain of lysine has a pK_R value of 10.54. Thus lysine is less basic. The expression plasmid was produced using the Pfu-PCNA01-producing plasmid as the template and oligos Pfu_D143K-F and Pfu_D143K-R for introducing the mutation.

[0101] 1.4.1.7: Pfu-PCNA71

For the purpose of comparing activities depending on the amino acid introduced into the position 143, this mutant was produced by substituting the amino acid at position 143 with histidine (basic amino acid). The side chain of histidine has the pK_R value of 6.0 and is dissociated at physiological pH. At pH 6.0, 50% of imidazole group in histidine is charged and the remaining 50% is not charged. Thus, histidine becomes neutral at higher pH in the range of physiological pH. Therefore, it was anticipated that the amino acid to be introduced would exert a different nature from that of arginine and lysine. The expression plasmid was produced using the Pfu-PCNA01-producing plasmid as the template and oligos Pfu_D143H-F and Pfu_D143H-R for introducing the mutation.

[0102] 1.4.1.8: Pfu-PCNA72

The mutant was produced by substitution with glutamic acid (acidic amino acid) of arginine (basic amino acid) at position 109, which is one of amino acids located on the interface when the Pfu-PCNA forms the homotrimer and involved in the trimer formation. The expression plasmid was produced using the Pfu-PCNA01-producing plasmid as the template and oligos Pfu_R109E-F and Pfu_R109E-R for introducing the mutation.

[0103] Pfu-PCNA77

The mutant was produced by substitution with arginine (basic amino acid) of aspartic acid (acidic amino acid) at position 147, which is one of the amino acids located on the interface when the Pfu-PCNA forms the homotrimer and involved in the trimer formation. The expression plasmid was produced using the Pfu-PCNA01-producing plasmid as the template and oligos Pfu_D147R-F and Pfu_D147R-R for introducing the mutation.

[0104] 1.4.1.10: Pfu-PCNA78

The mutant was produced by substitution with alanine (neutral amino acid) of glutamic acid (acidic amino acid) at position 139, which is one of the amino acids located on the interface when the Pfu-PCNA forms the homotrimer and involved in the trimer formation. The expression plasmid was produced using the Pfu-PCNA01-producing plasmid as the template and oligos Pfu_E139A-F and Pfu_E139A-R for introducing the mutation.

[0105] 1.4.1.11: Pfu-PCNA79

The mutant was produced by substitution with arginine (basic amino acid) of glutamic acid (acidic amino acid) at position 139, which is one of the amino acids located on the interface when the Pfu-PCNA forms the homotrimer and involved in the trimer formation. The expression plasmid

was produced using the Pfu-PCNA01-producing plasmid as the template and oligos Pfu_E139R-F and Pfu_E139R-R for introducing the mutation.

[0106] 1.4.2: Production of expression strain

Escherichia coli BL21 CodonPlus (DE3) RIL (Stratagene) was transformed with the plasmid vectors for expressing the mutant PCNA obtained in the aforementioned procedure, to yield *E. coli* strains each expressing mutant gene.

[0107] 1.4.3: Culturing of microbial cells and induction of expression

Each mutant PCNA expression strain was cultured in 1.5 Liters of the LB medium (containing 50 μg/mL of ampicillin) at 37°C with shaking. The expression was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.1 mM when OD_{600} at a logarithmic growth phase was 0.3 to 0.5, and after inducing the expression, the culturing after the induction was continued for about 3 hours. Microbial cells after the culturing were collected by centrifugation (4°C, 6,000 xg, 6 minutes).

[0108] 1.4.4: Purification of PCNA protein (FIG. 5)

As shown in FIG. 5, a preparation was obtained by collecting the microbial cells by centrifugation (S51), disrupting the microbial cells (ultrasonic disruption, S52), boiling for 5 minutes (S53), performing polyethyleneimine precipitation (S54), performing ammonium sulfate precipitation (S55), performing ion exchange chromatography (S56, using HiTrap Q as a column) and performing gel filtration chromatography (S57, using Superdex 200). It was confirmed by SDS-PAGE that the protein was purified well.

[0109] 1.4.1.1: Disruption of microbial cells

The microbial cells precipitated by centrifugation

were suspended in 25 mL of buffer A (50 mM Tris-HCl pH8.5, 0.1 M NaCl, 2 mM 2-mercaptoethanol, 10% glycerol) or buffer B (50 mM Tris-HCl pH8.0, 0.1 M NaCl, 0.1 mM EDTA, 10% glycerol, 0.5 mM DTT). The microbial cells were disrupted by ultrasonic treatment.

[0110] 1.4.4.2: Treatment with heat

A disrupted microbial cell suspension was boiled for 5 minutes and then centrifuged (18,500 xg, 4°C, 25 minutes), to collect a supernatant.

[0111] 1.4.4.3: Polyethyleneimine precipitation

Polyethyleneimine (Sigma P-3143) and NaCl at final concentrations of 0.2% (w/v) and 0.58 M, respectively, were added to the supernatant, which was then stirred on ice for 30 minutes. This solution was centrifuged (18,500 xg, 4°C, 25 minutes) to yield the supernatant.

[0112] 1.4.4.4: Ammonium sulfate precipitation

5.61 g of ammonium sulfate (final concentration of 80%) was added to 10 mL of the supernatant and stirred on ice for 30 minutes to precipitate a protein. 80 mL of 50 mM Tris-HCl (pH 8.5) buffer in which ammonium sulfate had been 80% saturated was added to this solution and a precipitation was collected by centrifugation (30,000 xg, 4°C, 25 minutes). Subsequently, this precipitation was dissolved in buffer C (50 mM Tris-HCl pH 8.0, 0.1 M NaCl) and the resulting solution was dialyzed against the same buffer C.

[0113] 1.4.4.5: Ion exchange chromatography

A dialyzed sample was applied on an ion exchange chromatography (HiTrap Q: Amersham Bioscience) using FPLC protein purification system (Amersham Bioscience) for Pfu-PCNA01, 10, 12, 13 and 16, and AKTA explorer 10S (Amersham Bioscience) for other Pfu-PCNA70, 71, 72, 77, 78 and 79.

Elution was performed with a linear gradient of 0.1 to 0.8 M NaCl/17.5 mL, and a flow rate was 1 mL/minute.

[0114] 1.4.4.6: Gel filtration chromatography

The peak fraction in the HiTrap Q ion exchange chromatography was further purified by gel filtration chromatography using Superdex 200 (Amersham Bioscience) to obtain a preparation for subsequent assays.

[0115] The obtained preparation was applied on polyacrylamide gel electrophoresis to confirm its molecular size and good purification (FIGs. 6 and 40).

[0116] 1.5: Cloning of Pfu-RFC gene

RFC is composed of two subunits RFCL and RFCS, which are located in tandem on the genome of *Pyrococcus furiosus*. Upon producing an RFC protein preparation, an RFCL gene and an RFCS gene were individually introduced in distinct expression vectors, the respective expression vectors were introduced into the same host and the genes were expressed simultaneously. The expression plasmids were produced with reference to Non-patent Document 7. The detail thereof will be described below.

[0117] 1.5.1: Cloning of RFCL gene and preparation of expression plasmid

A Pfu-RFCL gene (NCBI Gene ID 1467921) was obtained by PCR using the Pfu genome as the template (base sequence: SEQ ID NO:13, amino acid sequence: SEQ ID NO:14). RFCL-F and RFCL-R primers were used as the primers for PCR (SEQ ID NOS 15 and 16 in Table 4). For convenience of cloning, a restriction enzyme NdeI recognition site was added to the RFCL-F primer and a XhoI recognition site was added to the RFCL-R primer.

[0118]

Table 4 Primers for amplification of RFCL gene

Primer name	Primer sequence 5'==>3'	SEQ ID NO
RFCL-F Primer	AGC CAT ATG CCA GAG CTT CCC TGG GTA GAA	15
RFCL-R Primer	AGC TCG AGT CAC TTT TTA AGA AAG TCA AAG AGA G	16

[0119] The Pfu genomic DNA prepared in the aforementioned 1.1 was used as the template for PCR.

[0120] The PCR reaction solution has the following composition (amounts to be added to 50 μL of the reaction system).

Template DNA: 100 ng,
Primers: each 10 pmol,
dNTP: each 10 nmol,
Ex Taq*: 1.25 U and
10 x Ex Taq buffer: 5 μL

were mixed, and sterilized water was added thereto up to the total volume of 50 μL.

(*supplied from TAKARA BIO INC.)

[0121] 1.5.1.1: Reaction conditions for PCR

The PCR reaction was performed by using the reaction solution prepared in the aforementioned procedure, using the PCR apparatus and using the program repeating 30 cycles of the reaction at 95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for one minute.

[0122] 1.5.1.2: Purification of PCR product

The PCR product obtained in the aforementioned

procedure was subjected to 1% agarose gel electrophoresis, and stained with ethidium bromide. Subsequently a gel fragment containing a band around 1.4 kb was cut out under the ultraviolet light irradiation, and the PCR product in the gel fragment was purified using GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience) according to its manipulation manual.

[0123] 1.5.1.3: Subcloning of PCR product

The purified PCR product was ligated to pUC118-HincII/BAP (TAKARA BIO INC.) using TaKaRa BKL kit (TAKARA BIO INC.) according to the manipulation manual. *Escherichia coli* DH5α (TAKARA BIO INC.) was transformed with this ligated PCR product, which was then seeded on the LB agar plate (containing 100 μg/mL of ampicillin, 40 μg/mL of IPTG, and 40 μg/mL of X-GAL),and cultured by leaving stand at 37°C overnight to yield an *Escherichia coli* clone having the PCR product.

[0124] An *Escherichia coli* colony exhibiting the white color on the agar plate was cultured in 3 mL of the LB liquid medium (containing 100 μg/mL of ampicillin) at 37°C overnight with shaking, and then a plasmid DNA was prepared according to the standard method.

[0125] 1.5.1.4: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA sequence inserted into the recognition site of the restriction enzyme HincII in the plasmid vector pUC118 was examined using the DNA sequencer. As a result, it was confirmed that the open reading frame of the Pfu-RFCL gene is retained, the restriction enzyme NdeI recognition sequence was added to the 5' end and the restriction enzyme XhoI recognition sequence was added to the 3' end in the inserted portion. This plasmid was designated as

pUC118/RFCL.

[0126] 1.5.1.5: Preparation of RFCL expression plasmid

The plasmid pUC118/RFCL was doubly cleaved with the restriction enzymes NdeI and XhoI to prepare an RFCL gene fragment. The gene fragment was inserted into the expression vector to produce the expression vector for Pfu-RFCL.

[0127] 1.5.1.6: Preparation of RFCL DNA fragment

The plasmid pUC118/RFCL was doubly cleaved with the restriction enzymes NdeI and XhoI in the following reaction system.

[0128] Plasmid DNA: 5 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme XhoI: 5 units

[0129] Sterile water was added to the aforementioned mixture up to the total volume of 50 μL, and the plasmid DNA was cleaved with the restriction enzymes at 37°C for 2 hours. After completing the reaction, the reaction product was run on 1% agarose gel electrophoresis. A band (around about 1.4 kb) corresponding to the Pfu-RFCL gene was cut out and the RFCL DNA fragment was purified from the gel using GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience) according to its manipulation manual.

[0130] 1.5.1.7: pET-29a expression vector

The vector DNA pET-29a (Novagen, US) was doubly cleaved with the restriction enzymes NdeI and XhoI by the following reaction.

[0131] Plasmid DNA: 2 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme XhoI: 5 units

[0132] Sterile water was added to the aforementioned mixture up to the total volume of 50 μL, which was then left stand at 37°C for 2 hours. After completing the reaction, the reaction product was run on 1% agarose gel electrophoresis. A band (around about 5.4 kb) corresponding to the straight form of pET-29a vector was cut out and the pET-29a DNA fragment was purified from the gel using GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience) according to its manipulation manual.

[0133] 1.5.1.8: Ligation reaction and transformation

The Pfu-RFCL DNA fragment (100 ng) and the pET-29a DNA fragment (50 ng) obtained in the aforementioned procedure were reacted using DNA Ligation Kit V2 (TAKARA BIO INC.) as follows.

[0134] RFCL DNA fragment: 100 ng

pET-29a DNA fragment: 50 ng

DNA Ligation Kit V2 enzyme solution: 5 μL

Sterile water was added to the aforementioned mixture up to the total volume of 10 μL, which was then reacted at 16°C for 30 minutes.

[0135] 100 μL of *Escherichia coli* (*E. coli*) BL21 (DE3) (Novagen) was transformed with this ligation product (3μL). The solution of transformed *E. coli* was seeded on the LB agar plate (containing 30 μg/mL of kanamycin) and left stand to culture at 37°C overnight. Three colonies in *E. coli* colonies formed on the agar plate were cultured with shaking in 3 mL of the LB liquid medium (containing 30 μg/mL of kanamycin) at 37°C overnight, and a plasmid DNA was prepared according to standard methods.

[0136] 1.5.1.9: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA

sequence inserted in the plasmid vector pET-29a and the sequence in the vicinity of the inserted site were examined using DNA sequencer. As a result, the ORF (open reading frame) of the Pfu-RFCL gene was completely inserted between NdeI and XhoI sites which were in the multicloning site. The plasmid retaining this Pfu-RFCL gene was designated as pRFCL (FIG. 7).

[0137] As shown in FIG. 7, it was confirmed that the T7 promoter and rbs (ribosome binding site) which pET-29a had, the RFCL gene ORF (open reading frame) and the T7 terminator were aligned in this order in pRFCL. It was expected that this plasmid would express the RFCL gene in the large amount.

[0138] 1.5.2: Cloning of RFCS gene and preparation of expression plasmid

According to Non-patent Document 7, it has been reported that a pfu-RFCS gene (Gene ID: 1467922) has one intein (encoded by 1,575 bases) and an N terminal extein is encoded by 177 bases and a C terminal extein is encoded by 804 bases (initiation and termination codons are not included). The base sequence and the amino acid sequence of the Pfu-RFCS are shown in SEQ ID NOS:17 and 18, respectively (both include the intein portion).

[0139] When the RFCS expression vector was produced, first, full length of the Pfu-RFCS gene including the intein was amplified by PCR, and subsequently a mature RFCS (also referred to as RFCSm) was prepared by removing the intein. The intein was removed by amplifying the N terminal extein and the C terminal extein individually by PCR and fusing two extein fragments by PCR reaction.

[0140] 1.5.2.1: PCR reaction of RFCS gene including intein

The PCR reaction was performed with the combination of

the Pfu genomic DNA (prepared in the aforementioned 1.1), RFCS-F and RFCS-R primers (SEQ ID NOS:19 and 20 in Table 5).

[0141]

Table 5 Primers for RFCS gene

Primer name	Primer sequence 5'==>3'	SEQ ID NO
RFCS-F primer	ATG AGC GAA GAG ATT AGA GAA GTT	19
RFCS-R primer	ATC ACT TCT TCC CAA TTA GGG TGA AC	20
RFCSF1 primer	TCA TAT GAG CGA AGA GAT TAG AGA AGT TAA G	21
RFCSF2 primer	GCA GGC CCC CCT GGT GTC GGA AAG ACT ACA GCG GCT TTG GCC CTT G	22
RFCSR2 primer	CAA GGG CCA AAG CCG CTG TAG TCT TTC CGA CAC CAG GGG GGC CTG	23
RFCSR1 primer	AGG TCG ACC ATC ACT TCT TCC CAA TTA GGG TGA AC	24

[0142] The PCR reaction solution has the following composition (amounts to be added to 50 μL of the reaction solution).

Template DNA: 100 ng,

Primers: each 10 pmol,

dNTP: each 10 nmol,

Ex Taq*: 1.25 U and

10 x Ex Taq buffer: 5 μL

were mixed, and sterilized water was added thereto up to the total volume of 50μL.

(*supplied from TAKARA BIO INC.)

[0143] 1.5.2.2: Reaction conditions for PCR

The PCR reaction was performed by using the reaction

solution prepared in the aforementioned procedure, using the PCR apparatus and using the program repeating 30 cycles of the reaction at 95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for one minute.

[0144] 1.5.2.3: Purification of PCR product

The PCR product obtained in the aforementioned procedure was subjected to 1% agarose gel electrophoresis, and stained with ethidium bromide. Subsequently a gel fragment containing a band around 2.6 kb was cut out under the ultraviolet light irradiation, and the PCR product in the gel fragment was purified using GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience) according to its manipulation manual.

[0145] 1.5.2.4: Subcloning of PCR product

The purified PCR product was ligated to pUC118-HincII/BAP using TaKaRa BKL kit (TAKARA BIO INC.) according to the manipulation manual. *Escherichia coli* DH5α (TAKARA BIO INC.) was transformed with this ligated PCR product, which was then seeded on the LB agar plate (containing 100 μg/mL of ampicillin, 40 μg/mL of IPTG, and 40 μg/mL of X-GAL),and cultured by leaving stand at 37°C overnight to yield an *Escherichia coli* clone having the PCR product.

[0146] An *Escherichia coli* colony exhibiting the white color on the agar plate was cultured in 3 mL of the LB liquid medium (containing 100 μg/mL of ampicillin) with shaking at 37°C overnight, and then a plasmid DNA was prepared according to the standard method.

[0147] 1.5.2.5: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA sequence inserted into the recognition site of the restriction enzyme HincII in the plasmid vector pUC118 was examined using the DNA sequencer. As a result, the

inserted fragment conformed to the Pfu-RFCS gene including the intein. This plasmid was designated as pUC/RFCS.

[0148] 1.5.2.6: Primers for amplifying exteins

RFCSF1 primer and RFCSR2 primer (SEQ ID NOS:21 and 23 in Table 5) were prepared as the PCR primers for the N terminal extein. RFCSF2 primer and RFCSR1 primer (SEQ ID NOS:22 and 24 in Table 5) were prepared as the PCR primers for the C terminal extein. For the purpose of performing the cloning easily, the restriction enzyme NdeI sequence was added to the 5' end of the RFCSF1 primer and the restriction enzyme SalI sequence was added to the 5' end of the RFCSR1 primer. Complementary sequences utilized when two extein fragments were fused by the PCR reaction were provided in the RFCSF2 primer and the RFCSR2 primer.

[0149] The PCR reaction for amplifying two exteins was performed using the following composition (amounts to be added to 50 μL of the reaction solution).

pUC/RFCS DNA: 100 ng,
Primers: each 10 pmol,
dNTP: each 10 nmol,
Ex Taq*: 1.25 U and
10 x Ex Taq buffer: 5 μL

were mixed, and sterilized water was added thereto up to the total volume of 50 μL.

(*supplied from TAKARA BIO INC.)

[0150] 1.5.2.7: Reaction conditions for PCR

The PCR reaction was performed by using the reaction solution prepared in the aforementioned procedure, using the PCR apparatus and using the program repeating 30 cycles of the reaction at 95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for one minute.

[0151] 1.5.2.8: Purification of PCR product

The PCR product obtained in the aforementioned procedure was subjected to 2% agarose gel electrophoresis and stained with ethidium bromide. A band of about 180 bases and a band of about 800 bases were observed in the PCR products from the primer set of RFCSF1 and RFCSR2 and the primer set of RFCSF2 and RFCSR1, respectively. The gel fragment including each band was cut out under the ultraviolet light irradiation, and the PCR product in the gel fragment was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience) according to its manipulation manual.

[0152] 1.5.2.9: PCR fusion reaction

Two extein PCR products were fused by subjecting the two fragments to the PCR reaction with one set of primers in one tube to yield a gene fragment encoding the mature RFCS. The detail thereof will be described below.

[0153] An annealing reaction of two extein PCR products was performed with the following composition.

N terminal extein fragment: 2 μL (corresponding to 50 ng),

C terminal extein fragment: 2 μL (corresponding to 50 ng) and

10 x Ex Taq buffer: 5 μL.

Sterile water was added to the aforementioned composition up to the total volume of 44.5 μL.

The aforementioned mixture was heated at 95°C for 3 minutes, and slowly cooled down to 37°C over 30 minutes.

[0154] The followings were added to the aforementioned reaction solution.

dNTP: 5 μL (each 10 nmol) and

Ex Taq*: 0.5 μL (2.5 U).

The mixture was reacted at 72°C for 10 minutes.

(*supplied from TAKARA BIO INC.)

[0155] Each 10 pmol of the RFCSF1 primer and the RFCSR1 primer were added to the aforementioned mixture, and the PCR reaction was performed using the program repeating 30 cycles of the reaction at 95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for one minute.

[0156] 1.5.2.10: Cleavage with restriction enzymes and purification of PCR product

The aforementioned PCR product was purified by ethanol precipitation according to the standard method. The purified DNA fragment was doubly cleaved with the restriction enzymes NdeI and SalI.

PCR product: corresponding to 1 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme SalI: 5 units

Sterile water was added to the aforementioned composition up to the total volume of 50 μL, and the PCR product was cleaved with the restriction enzymes at 37°C for 2 hours. After completing the reaction, the reaction product was run on 2% agarose gel electrophoresis. A band (around about 1 kb) supposed to contain the fused fragment of two inteins (encoding the mature RFCS) was cut out, and the fragment was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience).

[0157] 1.5.2.11: Cleavage of expression vector pET-21a with restriction enzymes

The vector DNA pET-21a (Novagen) was doubly cleaved with the restriction enzymes NdeI and SalI.

[0158] Plasmid DNA: 2 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme SalI: 5 units

[0159] Sterile water was added to the aforementioned mixture up to the total volume of 50 μL, which was then left stand at 37°C for 2 hours. After completing the reaction, the reaction product was run on 1% agarose gel electrophoresis. A band (around about 5.4 kb) corresponding to the straight form of the vector pET-21a was cut out and the pET-21a DNA fragment was purified from the gel using GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience) according to its manipulation manual.

[0160] 1.5.2.12: Ligation reaction and transformation

The DNA fragment of about 1 kb (100 ng) predicted to be the fused fragment of two inteins (encoding the mature RFCS) and the pET-21a DNA fragment (50 ng) obtained in the aforementioned procedure were reacted using DNA Ligation Kit V2 (TAKARA BIO INC.) as follows.

[0161] 1 kb DNA fragment: 100 ng

pET-21a DNA fragment: 50 ng

DNA Ligation Kit V2 enzyme solution: 5 μL

Sterile water was added to the aforementioned mixture up to the total volume of 10 μL, which was then reacted at 16°C for 30 minutes.

[0162] 100 μL of *Escherichia coli* (*E. coli*) BL21 (DE3) (Novagen) was transformed with this ligation product (3 μL). The solution of *E. coli* was seeded on the LB agar plate (containing 100 μg/mL of ampicillin) and left stand to culture at 37°C overnight. Three colonies in *E. coli* colonies formed on the agar plate were cultured with shaking in 3 mL of the LB liquid medium (containing 100 μg/mL of ampicillin) at 37°C overnight, and a plasmid DNA was prepared according to standard methods.

[0163] 1.5.2.13: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA sequence inserted in the plasmid vector pET-21a and the sequence in the vicinity of the inserted site were examined using the DNA sequencer. As a result, the sequence of mature RFCSm (984 bases) without intein was identified between NdeI site and SalI site in the multicloning site (designated as pRFCSm).

[0164] As shown in FIG. 8, it was confirmed that the T7 promoter and rbs (ribosome binding site) which pET-21a had, the mature RFCS gene ORF (open reading frame) and the T7 terminator were aligned in this order in pRFCSm. It was expected that this plasmid would express the mature RFCS gene in the large amount.

[0165] 1.6: Production of Pfu-RFC gene expression strain

Escherichia coli BL21-CodonPlus (DE3)-RIL was simultaneously transformed with pRFCL and pRFCSm, and a Pfu-RFC expression strain was obtained by selecting the transformant having both plasmids doubly with ampicillin and kanamycin.

[0166] 1.7: Expression of Pfu-RFC gene

The aforementioned expression strain was cultured with shaking in 1.5 liters of the LB medium (containing 50 μg/mL of ampicillin and 30 μg/mL of kanamycin) at 37°C. The expression was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.1 mM when OD_{600} at the logarithmic growth phase was 0.3 to 0.5, and after inducing the expression, the culturing was continued for about 3 hours. Microbial cells after the culturing were collected by centrifugation (4°C, 6,000 xg, 6 minutes).

[0167] 1.8: Purification of Pfu-RFC protein

As shown in FIG. 9, a preparation was obtained by collecting the microbial cells by centrifugation (S91), disrupting the microbial cells by ultrasonic disruption, (S92), boiling for 5 minutes (S93), performing the polyethyleneimine precipitation (S94), performing the ammonium sulfate precipitation (S95), performing the affinity chromatography (S96, using HiTrap Heparin) and performing the gel filtration chromatography (S97, using Superdex 200). It was confirmed by SDS-PAGE to assure the purity of 90% or more.

[0168] 1.8.1: Disruption of microbial cells

The microbial cells precipitated by centrifugation were suspended in 25mL of buffer B (aforementioned). The microbial cells were disrupted by ultrasonic treatment.

[0169] 1.8.2: Treatment with heat

A disrupted microbial cell suspension was boiled for 5 minutes and then centrifuged (18,500 xg, 4°C, 25 minutes), to collect a supernatant.

[0170] 1.8.3: Polyethyleneimine precipitation

Polyethyleneimine (Sigma P-3143) was added at a final concentration of 0.18% (w/v), which was then stirred on ice for 30 minutes. This solution was centrifuged (18,500 xg, 4°C, 25 minutes) to yield a supernatant.

[0171] 1.8.4: Ammonium sulfate precipitation

5.61 g of ammonium sulfate (final concentration of 80%) was added to 10 mL of the supernatant and stirred on ice for 30 minutes to precipitate a protein. The precipitation was collected by centrifugation (18,500 xg, 4°C, 25 minutes). Subsequently, this precipitation was dissolved in the buffer C (aforementioned) and the resulting solution was dialyzed against the same buffer C.

[0172] 1.8.5: Affinity chromatography

The dialyzed sample was purified using HiTrap Heparin HP column (Amersham Bioscience).

[0173] The sample was eluted with the linear gradient of 0.1 to 0.8 M of NaCl/17.5 mL, and the flow rate was 1 mL/minute.

[0174] 1.8.6: Gel filtration chromatography

The peak fraction in the HiTrap Heparin affinity chromatography was further purified by gel filtration chromatography using Superdex 200 (aforementioned) to obtain a preparation for assays. The resulting preparation was applied onto the polyacrylamide gel electrophoresis to confirm the molecular size and good purification of the preparation (FIG. 10).

[0175] 2. Evaluation of PCNA mutants

The effects of various PCNA mutants prepared in the aforementioned procedure on the DNA replication system, particularly on the PCR reaction system were examined. Specifically, the PCR reaction was performed with the addition of the PCNA mutant alone, RFC alone or the combination thereof, or with the addition of none of them to the PCR reaction system, and its reaction product was subjected to the electrophoresis for comparing the state of the amplified target region.

[0176] 2.1: Evaluation of various PCNA mutants

Effect of addition of various PCNA mutants on Pyrobest DNA Polymerase (TAKARA BIO INC.),a commercially distributed DNA synthesis enzyme derived from genus *Pyrococcus*, was examined using the PCR reaction system. Pyrobest DNA Polymerase is the heat resistant α type DNA polymerase derived from *Pyrococcus* sp. and having 3' --> 5' exonuclease activity (proof reading activity). This enzyme is characterized by performing a highly correct amplification equivalent to that by Pfu DNA polymerase

derived from *Pyrococcus furiosus*, and Vent DNA polymerase.

[0177] 2.1.1: Composition of reaction solution

The reaction solution has the composition of the standard PCR reaction solution except for adding an accessory protein (sometimes abbreviated as "AP"). The attached reaction buffer (10x Pyrobest Buffer II; unpublished composition, TAKARA BIO INC.) was used as the buffer. The composition of the PCR reaction solution is shown below. As the template DNA, lambda DNA (GenBank accession 02459) was used.

[0178]

Table 6

Composition	Amount to be added	Final concentration
Lambda DNA (20ng/μL)	1.25μL	0.5ng/μL
Forward Primer (20pmol/μL)	0.5μL	0.2μM
Reverse Primer (20pmol/μL)	0.5μL	0.2μM
10X Pyrobest buffer II	5μL	1x
dNTP Mix (2.5mM each)	4μL	0.2mM
Pyrobest DNA Polymerase (5U/μL)	0.25μL	0.025U/μL
AP solution (see Table 7)	1.1μL	
Sterile water	37.4μL	
Total	50μL	

The combination of the forward primer and the reverse primer is changed depending on the length to be amplified. For the combination of the primers, see Tables 8 and 9.

[0179]

Table 7 AP solution (Accessory protein solution)

	noAP	RFC0	RFC200	RFC400
PCNAxx	0μL	0.3μL	0.3μL	0.3μL
RFC	0μL	0μL	0.4μL	0.8μL
Buffer*	1.1μL	0.8μL	0.4μL	0μL
Total	1.1μL	1.1μL	1.1μL	1.1μL

Neat concentration of PCNAxx; 100ng/μL, neat concentration of RFC; 500ng/μL

Buffer*: 25mM TrisCl pH8.0, 50mM NaCl, 50% Glycerol

[0180] 2.1.2: PCR reaction program

In the case of 2 kb amplification

94°C for one minute --> (98°C for 5 seconds --> 68°C for one minute) 30 cycles --> being kept at 4°C

[0181] In the case of 8.4 kb amplification

94°C for one minute --> (98°C for 5 seconds --> 68°C for 3.5 minutes) 30 cycles --> being kept at 4°C

[0182] In the case of 15.8 kb amplification

94°C for one minute --> (98°C for 5 seconds --> 68°C for 7 minutes) 30 cycles --> being kept at 4°C

[0183] 2.1.3: Electrophoresis

After completing the PCR reaction, 5 μL of 10 x loading buffer (glycerol 50%, bromophenol blue 0.4%, Xylene cyanol 0.4%) was added to and mixed with 50 μL of the PCR reaction solution, which was then subjected to 1% agarose gel electrophoresis. As an electrophoresis marker, lambda/styI marker (Toyobo Co., Ltd.) was used. The results are shown in FIGs. 11 to 13 (PCNA01, 10, 12, 13, 16), FIGs. 41 to 43 (PCNA01, 10, 13, 70, 71) and FIGs. 44 to 46 (PCNA13, 72, 77, 78, 79) which are grouped according to the size of the amplified fragments.

[0184]

Table 8 Primer sequence for evaluating PCNA

Primer name	Positions for primer (in lambda DNA)	Primer sequence (5' to 3')	SEQ ID NO
F02	23,119-23,138	GTC GTT TCT GCA AGC TTG GC	25
F11	24,634-24,654	GCT GCT GAA ACG TTG CGG TTG	26
F24	18,483-18,504	CGT CGG GGA CAT TGT AAA GGC G	27
R03	25,142-25,122	CCG AGA TAA AAA CAA ACC CGC	28
R14	26,914-26,894	GGC ATT CCT ACG AGC AGA TGG	29
R16	40,479-40,460	TCC CGT TCT TCC CTG GTA GC	30

[0185]

Table 9 Amplified region in lambda DNA

Size	Forward Primer	Reverse Primer	lambda-DNA Position		
2kb	F02	R03	23,119	-	25,142
8.4kb	F24	R14	18,483	-	26,914
15.8kb	F11	R16	24,634	-	40,479

[0186] 2.1.4: Results 1

The results in FIGs. 11 to 13 will be described.

PCNA01

When the amplified size was 2 kb or 8.4 kb, the amplification was observed only when 400 ng of RFC was added. In other cases, the addition of PCNA01 showed the inhibitory effect compared with the case of adding no accessory protein (no AP). This is the result that, when the wild type PCNA is used, the DNA can not be amplified unless the appropriate amount of RFC is used in combination.

The result indicates that the addition of the wild type PCNA alone obviously inhibits the reaction in the PCR, differently from the result of the primer extension analysis (Non-patent Documents 6 and 9) in which the addition of the PCNA alone exhibits the DNA synthesis promoting activity.

[0187] PCNA10

When the amplified size was 2 kb or 8.4 kb and when 200 ng or 400 ng of RFC was added, the amplification was observed. However, when the amplified size was 15.8 kb, only the addition of 400 ng RFC resulted in the amplification. The amplified amount was larger than that in PCNA01, but the presence of RFC was required as was the case of PCNA01. It is suggested thereby that the optimal amount of RFC to be added may be different depending on the amplified size.

[0188] PCNA12

In any case of the amplified sizes 2 kb, 8.4 kb and 15.8 kb, it was observed that the addition of RFC promoted the amplified amount. Even when no RFC was added, the amplification was also observed. However, only the slight promotion of the amplification was observed compared with the case of the PCR enzyme alone.

[0189] PCNA13

In any case of the amplified sizes 2 kb, 8.4 kb and 15.8 kb, the amplification in the large amount was observed, and the amplified amount did not depend on the amount of added RFC. Addition of PCNA13 exerted effect without requiring the RFC, and it is thus conceivable that PCNA 13 remarkably promotes the amplification by PCR.

[0190] PCNA16

In any case of the amplified sizes 2 kb, 8.4 kb and 15.8 kb, the amplification was observed. The amplification

was observed even when no RFC was added, although its level was equivalent to the amplification level in the case of the PCR enzyme alone. The effect of the RFC addition was scarcely observed.

[0191] For five species of PCNA, the effect of their addition to the PCR reaction system using Pyrobest DNA polymerase was examined. It was recognized that addition of PCNA13 exhibited much higher promotion effect on the amplification than in the case of no addition, and that PCNA 13 did not require the addition of RFC.

[0192] 2.1.5: Results 2

The results in FIGs. 41 to 43 will be described.

PCNA01 (quasi-wild type)

Similarly to the results in 2.1.4, in any amplified sizes other than the case of adding 400 ng of RFC, no amplification was observed, and PCNA01 worked in an inhibitory manner compared with the case of adding no PCNA (no AP).

[0193] PCNA10 (D143A)

When the amplified size was 2 kb, clear amplification was observed in the cases of adding 200 ng and 400 ng of RFC. When the amplified size was 8.4 kb, the clear amplification was observed in the cases of adding 400 ng of RFC. When the amplified size was 15.8 kb, no amplification was observed even when 400 ng of RFC was added. Similarly to the result in 2.1.4, PCNA10 was better in terms of amplified amount than PCNA01. However, the presence of RFC was indispensable for the amplification. It was suggested that the optimal amount of RFC to be added might be different depending on the amplified size.

[0194] PCNA71 (D143H)

No amplification was observed when no RFC was added, although when the amplified size was 2 kb or 8.4 kb, the

amplification was observed when 200 ng or 400 ng of RFC was added, and when the amplified size was 15.4 kb, the amplification was observed when 400 ng of RFC was added. The amplified amount was larger than in the case of PCNA10, but the addition of RFC was required for the amplification.

[0195] PCNA70 (D143K)

Not depending on the presence or absence of RFC and the amount of added RFC, the amplification in the large amount was observed in any sizes examined.

[0196] PCNA13 (D143R)

Similarly to the result in 2.1.4, the amplification in the large amount was observed in any sizes examined not depending on the addition of RFC, and the amplification level was better than in the case of PCNA70.

[0197] For four species of mutants (PCNA10, 13, 70 and 71) obtained by introducing the mutation into the position 143 in the PCNA, the effect of their addition to the PCR reaction system was examined. Only PCNA70 (D143K) and PCNA13 (D143R) exhibited the promotion effect not depending on the addition of RFC. PCNA13 obtained by substitution with the arginine residue having higher basicity exhibited the strongest promotion effect. Therefore, it is conceivable that, for leading to the PCR promotion effect of PCNA alone by introducing the mutation into the D143 residue, it is necessary to substitute with the basic amino acid residue, and that it is important that the positive charge which the substituted residue has under the PCR reaction conditions (pH 8.0 or higher) causes the charge repulsion against the ion pair network in the interface between the monomers.

[0198] 2.1.6: Results 3

The results in FIGs. 44 to 46 will be described.

PCNA13 (D143R)

Similarly to the results in 2.1.4 and 2.1.5, the amplification in the large amount was observed in any sizes examined not depending on the RFC.

[0199] PCNA77 (D147R)

Not depending on the presence or absence of RFC and the amount of added RFC, the amplification in the large amount was observed in any sizes examined. PCNA77 was inferior in terms of amplified amount to PCNA13.

[0200] PCNA72 (R109E)

The difference in comparison with the case of adding no PCNA (no AP) was scarcely observed in any cases regardless of amplified sizes, the presence or absence of RFC and the amount of added RFC.

[0201] PCNA79 (E139R)

In all cases with various amplified sizes, the promotion effect was observed when 200 ng to 400 ng of RFC was simultaneously added. However, the presence of RFC was essential for the amplification, as the addition of PCNA79 alone inhibited the reaction.

[0202] PCNA78 (E139A)

In all cases with various amplified sizes, the promotion effect was observed when 200 ng to 400 ng of RFC was simultaneously added. However, the presence of RFC was essential for the amplification, as the addition of PCNA78 alone inhibited the reaction. Comparing with PCNA79, PCNA78 was superior or inferior thereto depending on the amplified size when 200 ng of RFC was simultaneously added. However, when 400 ng of RFC was simultaneously added, PCNA78 was inferior to PCNA79 in terms of amplified amount. It is conceivable that the substitution with the basic amino acid residue (arginine) exerts a higher effect than the substitution with the neutral amino acid residue (alanine) when the substitution is introduced into the E139

residue as well.

[0203] The effect on the PCR reaction system of addition thereto the mutants of Pfu-PCNA obtained as a result of introduction of the mutation by substituting any of four amino acid residues among amino acid residues located on the interface when the Pfu-PCNA forms the homotrimer and involved in the formation of the trimer. The results were classified into three types. The first group inhibits reaction when added alone whereas, when added simultaneously with RFC, the reaction is offset depending on the amount of added RFC; depending on the added amount, the amplification may become larger than in the case of adding no PCNA (no AP). The second group brings about almost no difference from the no addition (no AP) group, either when added alone or when added simultaneously with RFC; this group is thus considered as not being involved in the reaction. The third group promotes the reaction regardless the presence or absence of RFC. It is conceivable that the point would be an ability to form the trimer, and that these phenomena are due to an extent to weaken the ion pair network. That is, if the trimer structure is too firm, the reaction would not progress without aid of RFC. If the ability to form the trimer is lost, PCNA would lose ability to work as the clump. It is thus speculated that the state where the trimer structure is appropriately loosened to an intermediate level would be important for promoting the enzyme activity in the PCR reaction.

[0204] It is conceivable that the ability to form the trimer in an effective manner for promoting the enzyme activity in the PCR reaction is realized by causing the charge repulsion to the ion pair network in the interface between the monomers. It is also conceivable that the

trimer is easily dissociated under the high temperature by loosening the ion pair network which is supposed to contribute to the thermal stability. A reaction mechanism is speculated that the replication reaction is repeated by dissociating the trimer upon temperature elevation for the subsequent cycle in PCR or at high temperature in the initial denaturation step of the cycle.

[0205] The effect of adding them to the PCR reaction system was examined as to the eleven mutants. Those which exhibited the promotion effect not depending on the addition of RFC were three mutants, PCNA13 (D143R), PCNA70 (D143K) and PCNA77 (D147R), and among them, PCNA13 exhibited the strongest overwhelming amplification effect. It was also observed that simultaneous addition of RFC appears to have tendency to rather reduce the amplified amount.

[0206] 2.2: Effect of PCNA13 on various DNA polymerase for PCR

Based on the aforementioned results, PCNA13 having the particularly excellent nature among various PCNA mutants were further examined as to the effect of adding the same to the PCR reaction system using commercially available seven DNA polymerases for PCR. Specifically, the PCR reactions with a variety of extension time periods were performed for two or three target sequences having the different amplified length, and the effect of addiing PCNA 13 thereto was examined.

[0207] 2.2.1: Effect of adding PCNA13 to Pyrobest DNA polymerase

Pyrobest DNA polymerase is a heat resistant α type DNA polymerase derived from *Pyrococcus* sp. and having the 3' --> 5' exonuclease activity.

[0208] The PCR reaction solution having the composition

recommended in the instructions by the manufacturer was used except that PCNA13 was added to the solution. The reaction buffer (10x Pyrobest Buffer II; unpublished composition, TAKARA BIO INC.) attached to the product was used as the buffer. The composition of the PCR reaction solution is shown below.

[0209]

Table 10

Composition	Amount to be added	Final concentration
Lambda DNA (20ng/μL)	1.25μL	0.5ng/μL
Forward Primer (20pmol/μL) [1)]	0.5μL	0.2μM
Reverse Primer (20pmol/μL) [1)]	0.5μL	0.2μM
10X Pyrobest buffer II	5μL	1x
dNTP Mix (2.5mM each)	4μL	0.2mM
Pyrobest DNA Polymerase (5U/μL)	0.25μL	0.025U/μL
PCNA13 solution or buffer [2)]	0.3μL	0 or 0.6ng/μL
Sterile water	38.2μL	
Total	50μL	

1) The combination of the forward primer and the reverse primer is changed depending on the length to be amplified. For the combination of the primers, see Tables 8 and 9.

2) Neat concentration of PCNA 13; 100ng/μL, Buffer; 25mM TrisCl pH8.0, 50mM NaCl, 50% Glycerol

[0210] PCR reaction program

In the case of 2 kb amplification

94°C for one minute --> (98°C for 5 seconds --> 68°C for 0.5, 1, 2 minute) 30 cycles --> being kept at 4°C

In the case of 8.4 kb amplification

94°C for one minute --> (98°C for 5 seconds --> 68°C for 2, 3.5, 5 minutes) 30 cycles --> being kept at 4°C

In the case of 15.8 kb amplification

94°C for one minute --> (98°C for 5 seconds --> 68°C for 2, 5, 6, 7 minutes) 30 cycles --> being kept at 4°C

[0211] Electrophoresis

After completing the PCR, 5 μL of 10 x loading buffer

(glycerol 50%, bromophenol blue 0.4%, Xylene cyanol 0.4%) was added to and mixed with 50 μL of the PCR reaction solution, and a specific amount of the mixture corresponding to 10 μL of the PCR reaction solution was then subjected to 1% agarose gel electrophoresis. As the electrophoresis marker, lambda/styI marker (Toyobo Co., Ltd.) was used. The results are shown in FIGs. 14 to 16 which are grouped according to the size of the amplified fragment.

[0212] Summary

In the case of 2 kb amplification

As shown in FIG. 14, the promotion of the reaction by the addition of PCNA13 was obviously recognized when the extension time is 0.5 or one minute. With the extension time of 2 minutes, the reaction was almost saturated, but it was observed that the amplified amount was slightly larger when PCNA13 is added.

[0213] In the case of 8.4 kb amplification

As shown in FIG. 15, no effect of adding PCNA13 was observed with the extension time of 2 minutes, but the promotion effect by the addition was observed with the extension time of 3.5 or 5 minutes.

[0214] In the case of 15.8 amplification

As shown in FIG. 16, no effect of adding PCNA13 was observed with the extension time of 2 minutes, but the promotion effect by the addition was observed with the extension time of 5, 6 or 7 minutes.

[0215] 2.2.2: Effect of adding PCNA13 to TaKaRa Ex Taq

TaKaRa Ex Taq (TAKARA BIO INC.) is a heat resistant DNA polymerase having the 3' --> 5' exonuclease activity (proof reading activity). Under the ordinary PCR conditions, TaKaRa Ex Taq can realize the more highly sensitive PCR with higher amplification efficiency and

lower error rate than conventional Taq DNA polymerase.

[0216] The PCR reaction solution having the composition recommended in the instructions by the manufacturer was used except that PCNA 13 was added to the solution. The reaction buffer 10 x Ex Taq buffer (20 mM Mg^{2+} plus, unpublished composition, TAKARA BIO INC.) attached to the product was used as the buffer. The composition of the PCR reaction solution is shown below.

[0217]

Table 11

Composition	Amount to be added	Final concentration
Lambda DNA (20ng/μL)	1.25μL	0.5ng/μL
Forward Primer (20pmol/μL) [1)]	0.5μL	0.2μM
Reverse Primer (20pmol/μL) [1)]	0.5μL	0.2μM
10X Ex Taq buffer (20mM Mg^{2+})	5μL	1x
dNTP Mix (2.5mM each)	4μL	0.2mM
TaKaRa EX Taq (5U/μL)	0.25μL	0.025U/μL
PCNA13 solution or buffer [2)]	0.5μL	0 or 1ng/μL
Sterile water	38μL	
Total	50μL	

1) The combination of the forward primer and the reverse primer is changed depending on the length to be amplified. For the combination of the primers, see Tables 8 and 9.

2) Neat concentration of PCNA 13; 100ng/μL, Buffer; 25mM TrisCl pH8.0, 50mM NaCl, 50% Glycerol

[0218] PCR reaction program

In the case of 2 kb amplification

94°C for one minute --> (98°C for 5 seconds --> 68°C for 30 seconds, 40 seconds, one minute) 30 cycles --> 72°C for 10 minutes --> being kept at 4°C

[0219]

In the case of 8.4 kb amplification

94°C for one minute --> (98°C for 5 seconds --> 68°C for 1.5, 2, 3 minutes) 30 cycles --> 72°C for 10 minutes --> being kept at 4°C

[0220]

In the case of 15.8 kb amplification

94°C for one minute --> (98°C for 5 seconds --> 68°C for 3.5, 4, 5 minutes) 30 cycles --> 72°C for 10 minutes --> being kept at 4°C

[0221] Electrophoresis

After completing the PCR reaction, 5 μL of 10 x loading buffer (glycerol 50%, bromophenol blue 0.4%, Xylene cyanol 0.4%) was added to and mixed with 50 μL of the PCR reaction solution, and a specific amount of the mixture corresponding to 10 μL of the PCR reaction solution was then subjected to 1% agarose gel electrophoresis. As the electrophoresis marker, lambda/styI marker (Toyobo Co., Ltd.) was used. The results are shown in FIGs. 17 to 19 which are grouped according to the size of the amplified fragment.

[0222] Summary

In the case of 2 kb amplification

As shown in FIG. 17, the increase in amplified amount and reaction rate by the addition of PCNA13 was obviously recognized with the extension time of 30 seconds and 40 seconds. With the extension time of one minute, the reaction attained to saturation regardless of the addition or no addition, and it is found that the amplified amount when PCNA13 was added with the extension time of 40 seconds is close to the saturation of the reaction.

[0223] In the case of 8.4 kb amplification

As shown in FIG. 18, with extension time of 1.5 and 2 minutes, the increase in amplified amount and reaction rate by the addition of PCNA13 was obviously recognized. With the extension time of 3 minutes, the reaction attained to saturation regardless of the addition or no addition, and it is found that the amplified amount when PCNA13 was added with the extension time of 2 minutes is close to the

saturation of the reaction.

[0224] In the case of 15.8 kb amplification

As shown in FIG. 19, with extension time of 3.5 and 4 minutes, the increase in amplified amount and reaction rate by the addition of PCNA13 was obviously recognized. With the extension time of 5 minutes, the reaction attained to saturation regardless of the addition or no addition, and it is found that the amplified amount when PCNA13 was added with the extension time of 4 minutes is close to the saturation of the reaction.

[0225] 2.2.3: Effect of adding PCNA13 to Vent DNA polymerase

Vent DNA polymerase is an enzyme for PCR derived from thermophile, *Thermococcus litoralis* and distributed by NEW ENGLAND Bio Labs.

[0226] Upon evaluation, the PCR reaction solution having the standard composition was used except that PCNA13 was added to the solution. The reaction buffer (10x ThermoPol Reaction Buffer; 200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 1% Triton X-100,pH 8.8, at 25°C) attached to the product was used as the buffer. The composition of the PCR reaction solution is shown below.

[0227]

Table 12

Composition	Amount to be added	Final concentration
Lambda DNA (20ng/μL)	1.25μL	0.5ng/μL
Forward Primer (20pmol/μL) [1)]	0.5μL	0.2μM
Reverse Primer (20pmol/μL) [1)]	0.5μL	0.2μM
10X ThermoPol Reaction Buffer	5μL	1x
dNTP Mix (2.5mM each)	4μL	0.2mM
Vent DNA Polymerase (2U/μL)	0.25μL	0.01U/μL
PCNA13 solution or buffer [2)]	0.3μL	0 or 0.6ng/μL
Sterile water	38.2μL	
Total	50μL	

1) The combination of the forward primer and the reverse primer is changed depending on the length to be amplified. For the combination of the primers, see Tables 8 and 9.

2) Neat concentration of PCNA 13; 100ng/μL, Buffer; 25mM TrisCl pH8.0, 50mM NaCl, 50% Glycerol

[0228] PCR reaction program

In the case of 2 kb amplification

95°C for 2 minutes --> (95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for 5, 15, 30 seconds) 30 cycles --> 72°C for 10 minutes --> being kept at 4°C

[0229] In the case of 8.4 kb amplification

95°C for 2 minutes --> (95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for 1, 3, 5 minutes) 30 cycles --> 72°C for 10 minutes --> being kept at 4°C

[0230] Electrophoresis

After completing the PCR reaction, 5 μL of 10 x loading buffer (glycerol 50%, bromophenol blue 0.4%, Xylene

cyanol 0.4%) was added to and mixed with 50 μL of the PCR reaction solution, and a specific amount of the mixture corresponding to 10 μL of the PCR reaction solution was then subjected to 1% agarose gel electrophoresis. As the electrophoresis marker, lambda/styI marker (Toyobo Co., Ltd.) was used. The results are shown in FIGs. 20 and 21 which are grouped according to the size of the amplified fragment.

[0231] Summary

In the case of 2 kb amplification

As shown in FIG. 20, the promotion of the reaction by the addition of PCNA13 was obviously recognized with the extension time of 5 seconds and 15 seconds. With the extension time of 30 seconds, the reaction was almost saturated, but it was observed that the amplified amount was nevertheless slightly larger in the case of adding PCNA13.

[0232] In the case of 8.4 kb amplification

As shown in FIG. 21, the promotion effect (amplified amount, reaction rate) by the addition was observed the extension time was 1, 3 or 5 minutes.

[0233] 2.2.4: Effect of adding PCNA13 to Deep vent DNA polymerase

Deep vent DNA polymerase (New England Biolabs) is a heat resistant DNA polymerase derived from *Pyrococcus* species GB-D1 and has the 3' --> 5' exonuclease activity.

[0234] Upon evaluation, the PCR reaction solution having the standard composition was used except that PCNA13 was added to the solution. The reaction buffer (10x ThermoPol Reaction Buffer; 200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 1% Triton X-100, pH 8.8, at 25°C) attached to the product was used as the buffer. The composition of the PCR reaction solution is shown below.

[0235]

Table 13

Composition	Amount to be added	Final concentration
Lambda DNA (20ng/μL)	1.25μL	0.5ng/μL
Forward Primer (20pmol/μL) [1)]	1μL	0.4μM
Reverse Primer (20pmol/μL) [1)]	1μL	0.4μM
10x ThermoPol Reaction Buffer	5μL	1x
dNTP Mix (2.5mM each)	4μL	0.2mM
Deep Vent DNA Polymerase (2U/μL)	0.5μL	0.02U/μL
PCNA13 solution or buffer [2)]	0.3μL	0 or 0.6ng/μL
Sterile water	36.95μL	
Total	50μL	

1) The combination of the forward primer and the reverse primer is changed depending on the length to be amplified. For the combination of the primers, see Tables 8 and 9.

2) Neat concentration of PCNA 13; 100ng/μL, Buffer; 25mM TrisCl pH8.0, 50mM NaCl, 50% Glycerol

[0236] PCR reaction program

In the case of 2 kb amplification

95°C for 2 minutes --> (95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for 0.5, 1, 2, 3 minutes) 30 cycles --> 72°C for 10 minutes --> being kept at 4°C

[0237] In the case of 8.4 kb amplification

95°C for 2 minutes --> (95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for 5, 7, 9 minutes) 30 cycles --> 72°C for 10 minutes --> being kept at 4°C

[0238] Electrophoresis

After completing the PCR reaction, 5 μL of 10 x loading buffer (glycerol 50%, bromophenol blue 0.4%, Xylene

cyanol 0.4%) was added to and mixed with 50 μL of the PCR reaction solution, and a specific amount of the mixture corresponding to 10 μL of the PCR reaction solution was then subjected to 1% agarose gel electrophoresis. As the electrophoresis marker, lambda/styI marker (Toyobo Co., Ltd.) was used. The results are shown in FIGs. 22 and 23 which are grouped according to the size of the amplified fragment.

[0239] Summary

In the case of 2 kb amplification

As shown in FIG. 22, the promotion of the reaction by the addition of PCNA13 was obviously recognized when the extension time is 0.5, 1 or 2 minutes. With the extension time of 3 minutes, the reaction was saturated regardless of the addition or no addition, but it was found that the amplified amount when PCNA13 was added with the extension time of 2 minutes indicated that the reaction was almost saturated.

[0240] In the case of 8.4 kb amplification

As shown in FIG. 23, the promotion effect (amplified amount, reaction rate) by the addition was observed with the extension time of 5, 7 and 9 minutes.

[0241] 2.2.5: Effect of adding PCNA13 to Pfu Turbo DNA polymerase

Pfu Turbo DNA polymerase (Stratagene) is a product containing a heat resistant DNA polymerase derived from *Pyrococcus furiosus* with an additional PCR reaction accelerator referred to as ArchaeMaxx (registered trade name). It is known that dUTP accessorily produced during the PCR reaction inhibits the PCR reaction. ArchaeMaxx Factor is a factor to degrade dUTP, and the addition of this prevents the PCR reaction from being inhibited to result in enhancing the PCR reaction efficiency.

[0242] Upon evaluation, the PCR reaction solution having the standard composition was used except that PCNA13 was added to the solution. The reaction buffer (10x Cloned Pfu DNA polymerase reaction buffer; 200 mM Tris-HCl (pH 8.8), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 1% Triton X-100, 1mg/mL BSA) attached to the product was used as the buffer. The composition of the PCR reaction solution is shown below.

[0243]

Table 14

Composition	Amount to be added	Final concentration
Lambda DNA (20ng/μL)	1.25μL	0.5ng/μL
Forward Primer (20pmol/μL) [1)]	0.5μL	0.2μM
Reverse Primer (20pmol/μL) [1)]	0.5μL	0.2μM
10x Cloned Pfu DNA polymerase reaction Buffer	5μL	1x
dNTP Mix (2.5mM each)	4μL	0.2mM
Pfu Turbo DNA Polymerase (2.5U/μL)	1μL	0.05U/μL
PCNA13 solution or buffer [2)]	0.3μL	0 or 0.6ng/μL
Sterile water	37.45μL	
Total	50μL	

1) The combination of the forward primer and the reverse primer is changed depending on the length to be amplified. For the combination of the primers, see Tables 8 and 9.

2) Neat concentration of PCNA 13; 100ng/μL, Buffer; 25mM TrisCl pH8.0, 50mM NaCl, 50% Glycerol

[0244] PCR reaction program

In the case of 2 kb amplification

95°C for 2 minutes --> (95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for one minute) 30 cycles --> 72°C

for 10 minutes --> being kept at 4°C

[0245] In the case of 8.4 kb amplification

92°C for 2 minutes --> (92°C for 10 seconds --> 55°C for 30 seconds --> 68°C for 8 minutes) 10 cycles --> (92°C for 10 seconds --> 55°C for 30 seconds --> 68°C for 8 minutes + 10 seconds/cycle) 20 cycles --> being kept at 4°C

[0246] In the case of 15.8 kb amplification

92°C for 2 minutes --> (92°C for 10 seconds --> 55°C for 30 seconds --> 68°C for 15 minutes) 10 cycles --> (92°C for 10 seconds --> 55°C for 30 seconds --> 68°C for 15 minutes + 10 seconds/cycle) 20 cycles --> being kept at 4°C

[0247] Electrophoresis

After completing the PCR reaction, 5 μL of 10 x loading buffer (glycerol 50%, bromophenol blue 0.4%, Xylene cyanol 0.4%) was added to and mixed with 50 μL of the PCR reaction solution, and a specific amount of the mixture corresponding to 10 μL of the PCR reaction solution was then subjected to 1% agarose gel electrophoresis. As the electrophoresis marker, lambda/styI marker (Toyobo Co., Ltd.) was used.

[0248] Summary

As shown in FIG. 24, the promotion of the reaction rate and the increase of the amplified amount by the addition of PCNA13 were observed in any size examined in the PCR reaction.

[0249] 2.2.6: Effect of adding PCNA13 to KOD DNA polymerase

KOD DNA polymerase (Toyobo Co., Ltd.) is a DNA polymerase derived from an ultrathermophilic archaebacterium, *Thermococcus·kodakaraensis* KOD1 strain, and has PCR fidelity about 50 times higher than Taq DNA

polymerase because of having the strong 3' --> 5' exonuclease activity (proof reading activity) in addition to the polymerase activity. Enzymes for highly accurate PCR derived from genus *Pyrococcus* distributed commercially tend to have slow extension rate, but this enzyme has the very fast extension rate which is about twice faster than that of Taq DNA polymerase. Thus, highly accurate PCR can be performed in a short period of time using this enzyme.

[0250] Upon evaluation, the PCR reaction solution having the standard composition was used except that PCNA13 was added to the solution. The reaction buffer (10x PCR buffer #1;1.2M Tris-HCl pH 8.0, 60 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% TritonX-100, 0.01% BSA or 10x PCR buffer #2; 1.2M Tris-HCl pH 8.8, 60 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% TritonX-100, 0.01% BSA) attached to the product was used as the buffer. The composition of the PCR reaction solution is shown below.

[0251]

Table 15

Composition	Amount to be added	Final concentration
Lambda DNA (20ng/μL)	1.25μL	0.5ng/μL
Forward Primer (20pmol/μL) 1)	1.25μL	0.5μM
Reverse Primer (20pmol/μL) 1)	1.25μL	0.5μM
10x enclosed Buffer #1 or #2 2)	5μL	1x
dNTP Mix (2.5mM each)	4μL	0.2mM
25mM $MgCl_2$	2μL	1mM
KOD DNA Polymerase (2.5U/μL)	0.28μL	0.014U/μL
PCNA13 solution or buffer 3)	0.3μL	0 or 0.6ng/μL
Sterile water	36.67μL	
Total	50μL	

1) The combination of the forward primer and the reverse primer is changed depending on the length to be amplified. For the combination of the primers, see Tables 8 and 9.

2) 10x enclosed Buffer #1; 2kb, #2; 8.4kb, 15.8kb

3) Neat concentration of PCNA 13; 100ng/μL, Buffer; 25mM TrisCl pH8.0, 50mM NaCl, 50% Glycerol

[0252] PCR reaction program

In the case of 2 kb amplification

94°C for one minute --> (98°C for 10 seconds --> 68°C for 12 seconds) 30 cycles --> 72°C for 3 minutes --> being kept at 4°C

[0253] In the case of 8.4 kb and 15.8 kb amplification

94°C for one minute --> (98°C for 10 seconds --> 68°C for 2 minutes) 30 cycles --> 72°C for 3 minutes --> being kept at 4°C

[0254] Electrophoresis

After completing the PCR reaction, 5 μL of 10 x loading buffer (glycerol 50%, bromophenol blue 0.4%, Xylene cyanol 0.4%) was added to and mixed with 50 μL of the PCR reaction solution, and a specific amount of the mixture corresponding to 10 μL of the PCR reaction solution was then subjected to 1% agarose gel electrophoresis. As the electrophoresis marker, lambda/styI marker (Toyobo Co., Ltd.) was used.

[0255] Summary

As shown in FIG. 25, the promotion of the reaction rate and the increase of the amplified amount by the addition of PCNA13 were observed in any size examined in the PCR reaction.

[0256] 2.2.7: Effect of adding PCNA13 to Pwo DNA polymerase

Pwo DNA polymerase (Roche Diagnostics) is a DNA

polymerase derived from a thermophilic archaebacterium *Pyrococcus woesei* strain, and has the PCR fidelity higher than Taq DNA polymerase because of having the strong 3' --> 5' exonuclease activity (proof reading activity) in addition to the polymerase activity. Thus, PCR with high accuracy can be performed in a short time using this enzyme.

[0257] Upon evaluation, the PCR reaction solution having the composition recommended in the instructions by the manufacturer was used except that PCNA13 was added to the solution. The reaction buffer (10x PCR buffer; 100 mM Tris-HCl pH 8.85, 50 mM $(NH_4)_2SO_4$, 250 mM KCl, 20 mM $MgSO_4$) attached to the product was used as the buffer. The composition of the PCR reaction solution is shown below.

[0258]

Table 16

Composition	Amount to be added	Final concentration
Lambda DNA (20ng/μL)	1.25μL	0.5ng/μL
Forward Primer (20pmol/μL) [1)]	1.5μL	0.6μM
Reverse Primer (20pmol/μL) [1)]	1.5μL	0.6μM
10x PCR buffer with $MgSO_4$	5μL	1x
dNTP Mix (2.5mM each)	4μL	0.2mM
Pwo DNA Polymerase (5U/μL)	0.14μL	0.014U/μL
PCNA13 solution or buffer [2)]	0.3μL	0 or 0.6ng/μL
Sterile water	36.31μL	
Total	50μL	

1) The combination of the forward primer and the reverse primer is changed depending on the length to be amplified. For the combination of the primers, see Tables 8 and 9.

2) Neat concentration of PCNA 13; 100ng/μL, Buffer; 25mM TrisCl pH8.0, 50mM NaCl, 50% Glycerol

[0259] PCR reaction program

In the case of 2 kb amplification

94°C for one minute --> (98°C for 10 seconds --> 68°C for 30 seconds or one minute) 30 cycles --> 72°C for 3 minutes --> being kept at 4°C

[0260] In the case of 8.4 kb amplification

95°C for 2 minutes --> (95°C for 30 seconds --> 60°C for 30 seconds --> 72°C for 2 or 4 minutes) 30 cycles --> 72°C for 3 minutes --> being kept at 4°C

[0261] Electrophoresis

After completing the PCR reaction, 5 μL of 10 x loading buffer (glycerol 50%, bromophenol blue 0.4%, Xylene cyanol 0.4%) was added to and mixed with 50 μL of the PCR reaction solution, and a specific amount of the mixture corresponding to 10 μL of the PCR reaction solution was then subjected to 1% agarose gel electrophoresis. As the electrophoresis marker, lambda/styI marker (Toyobo Co., Ltd.) was used.

[0262] Summary

As shown in FIG. 26, the promotion of the reaction rate and the increase of the amplified amount by the addition of PCNA13 were observed in any size examined of PCR products with any extension time examined.

[0263] As shown in the aforementioned Examples, it has been proved that PCNA13 excellently promotes extension activity of the representative commercially available seven DNA polymerases for PCR. It has been also shown that, although PCNA 13 used is the mutant of PCNA derived from *Pyrococcus furiosus*, PCNA 13 is effective for not only DNA polymerases derived from *Pyrococcus furiosus* but also DNA polymerases derived from different bacterial species.

[0264] 3: Preparation of KOD-PCNA and KOD-RFC proteins

A PCNA mutant protein preparation and an RFC protein preparation derived from *Thermococcus kodakaraensis* KOD1 strain were prepared by expressing the genes thereof in large amounts in *Escherichia coli* and purifying the proteins from expressing microbial cells.

[0265] 3.1: Acquisition of microbial cell and preparation of genomic DNA

Thermococcus kodakaraensis KOD1 strain was obtained from JCM (JAPAN COLLECTION OF MICROORGANISMS) as 10 mL of a cultured liquid (JCM NO.12,380). Microbial cells were collected by centrifuging this liquid at 6,000 xg at 4°C for 15 minutes. The collected microbial cells were suspended in 1 mL of TBS buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl), washed and collected by centrifugation at 15,000 xg at 4°C for 5 minutes.

[0266] The precipitate was dissolved in 100 μL of lytic buffer (50 mM Tris-HCl·pH 8.0, 50 mM EDTA·pH 8.0, 0.5% SDS), and incubated at 50°C for 3 hours. Subsequently, 100 μL of phenol/chloroform was added thereto. This solution was centrifuged at 15,000 xg at room temperature for 5 minutes to collect about 100 μL of the supernatant. The genomic DNA was collected from this supernatant using Mag Extractor Genome Kit (Toyobo Co., Ltd.) according to its manipulation manual.

[0267] 3.2: Preparation of KOD-PCNA

3.2.1: Cloning of KOD-PCNA gene

A KOD-PCNA gene was acquired by cloning using PCR (FIG. 27) with reference to GenBank ID BD182828 (SEQ ID NOS:31 and 32). The detail thereof will be described below.

[0268] 3.2.1.1: PCR primers

KOD-PCNA-F and KOD-PCNA-R were used for the amplification of the KOD-PCNA gene. This primer set was

designed so as to amplify the region corresponding to the initiation codon methionine to the termination codon of the PCNA gene and further add the restriction enzymes NdeI and XhoI recognition sites to the 5' side. The sequence of each primer was shown in Table 17 (SEQ ID NOS:33 and 34).

[0269]

Table 17 PCR primers for cloning KOD-PCNA

Primer name	Primer sequence 5'==>3'	SEQ ID NO
KOD-PCNA-F	cca tat gcc gtt cga agt tgt ttt tga	33
KOD-PCNA-R	ctc gag tca ctc ctc aac gcg cgg	34

[0270] 3.2.1.2: Composition of PCR reaction solution

The PCR reaction solution has the following composition (amounts to be added to 50 μL of the reaction solution).

Template DNA*: 100 ng,

Primers: each 10 pmol,

dNTP: each 10 nmol,

Ex Taq**: 1.25 U and

10 x Ex Taq buffer: 5 μL

were mixed, and sterilized water was added thereto up to the total volume of 50 μL.

(*template DNA: genomic DNA prepared in 3.1, **EX Taq: supplied from TAKARA BIO INC.)

[0271] 3.2.1.3: Conditions for PCR

The PCR reaction was performed by using the reaction solution prepared in the aforementioned procedure and the PCR apparatus and using the program repeating 30 cycles of the reaction at 95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for one minute.

[0272] 3.2.1.4: Purification of PCR product

The PCR product obtained in the aforementioned procedure was subjected to 1% agarose gel electrophoresis, and stained with ethidium bromide. Subsequently a gel fragment containing a band around 750 bp was cut out under the ultraviolet light irradiation, and the PCR product in the gel fragment was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience) according to its manipulation manual.

[0273] 3.2.1.5: Subcloning of PCR product

The purified PCR product was ligated to pUC118-HincII/BAP (TAKARA BIO INC.) using TaKaRa BKL Kit (TAKARA BIO INC.) according to the manipulation manual. *Escherichia. coli* DH5α (TAKARA BIO INC.) was transformed with this ligated product, which was then seeded on the LB agar plate (containing 100 μg/mL of ampicillin, 40 μg/mL of IPTG, and 40 μg/mL of X-GAL),and cultured by leaving stand at 37°C overnight to yield an *E. coli* clone having the PCR product.

An *E. coli* colony exhibiting the white color on the agar plate was cultured in 3 mL of the LB liquid medium (containing 100 μg/mL of ampicillin) with shaking at 37°C overnight with shaking, and then a plasmid DNA was prepared according to the standard method.

[0274] 3.2.1.6: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA sequence inserted into the recognition site of the restriction enzyme HincII in the plasmid vector pUC118 was examined using the DNA sequencer. As a result, it was confirmed that the open reading frame of the KOD-PCNA gene is retained, the restriction enzyme, NdeI recognition sequence was added to the 5' end and the restriction enzyme,

XhoI recognition sequence was added the 3' end in the inserted portion. This plasmid vector having the open reading frame of the KOD-PCNA gene was designated as pUC/KPC.

[0275] 3.2.2: Construction of expression plasmid of KOD-PCNA gene

The plasmid pUC/KPC was doubly cleaved with the restriction enzymes NdeI and XhoI to prepare a PCNA gene fragment. The gene fragment was inserted into the expression vector to produce the expression vector of KOD-PCNA.

[0276] 3.2.2.1: Preparation of PCNA DNA fragment

The plasmid pUC/KPC was doubly cleaved with restriction enzymes NdeI and XhoI in the following reaction system.

[0277]

Plasmid DNA: 5 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme XhoI: 5 units

[0278] Sterile water was added to the aforementioned mixture up to the total volume of 50 μL, and the plasmid DNA was cleaved with the restriction enzymes at 37°C for 2 hours. After completing the reaction, the reaction product was run on 2% agarose gel electrophoresis. A band (around about 750 bp) corresponding to the KOD-PCNA gene was cut out and the PCNA DNA fragment was purified from the gel using GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience) according to its manipulation manual.

[0279] 3.2.2.2: pET-21a Expression vector

The vector DNA pET-21a (Novagen, US) was doubly cleaved with the restriction enzymes NdeI and XhoI by the

following reaction.

[0280]

Plasmid DNA: 2 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme XhoI: 5 units

[0281] Sterile water was added to the aforementioned mixture up to the total volume of 50 μL, which was then left stand at 37°C for 2 hours. After completing the reaction, the reaction product was run on 1% agarose gel electrophoresis. A band (around about 5.4 kb) corresponding to the straight form of the vector DNA pET-21a was cut out and the pET-21a DNA fragment was purified from the gel using GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience) according to its manipulation manual.

[0282] 3.2.2.3: Ligation reaction and transformation

The KOD-PCNA DNA fragment (100 ng) and the pET-21a DNA fragment (50 ng) obtained in the aforementioned procedure were reacted using DNA Ligation Kit V2 (TAKARA BIO INC.) as follows.

[0283] PCNA DNA fragment: 100 ng

pET-21a DNA fragment: 50 ng

DNA Ligation Kit V2 enzyme solution: 5 μL

Sterile water was added to the aforementioned mixture up to the total volume of 10 μL, which was then reacted at 16°C for 30 minutes.

[0284] 100 μL of *E. coli* BL21 (DE3) (Novagen) was transformed with this ligation product (3 μL). The solution of transformed *E. coli* was seeded on the LB agar plate (100 μg/mL ampicillin) and left stand to culture at

37°C overnight. Three colonies in *E. coli* colonies formed on the agar plate were cultured with shaking in 3 mL of the LB liquid medium (containing 100 μg/mL ampicillin) at 37°C overnight, and a plasmid DNA was prepared according to standard methods.

[0285] 3.2.2.4: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA sequence inserted in the plasmid vector pET-21a and the sequence in the vicinity of the inserted site were examined using the DNA sequencer. As a result, the open reading frame of the KOD-PCNA gene was completely inserted between NdeI and XhoI sites which were multicloning sites. This plasmid retaining the KOD-PCNA gene was designated as pKPCNA (FIG. 28).

[0286] As shown in FIG. 28, it was confirmed that the T7 promoter and rbs (ribosome binding site) which pET-21a had, the PCNA gene ORF (open reading frame) and the T7 terminator were aligned in this order in pKPCNA. It was expected that this plasmid would express the PCNA gene in the large amount.

[0287] 3.2.3: Introduction of mutation into KOD-PCNA gene

For the purpose of producing the PCNA having a higher function, the amino acid in KOD-PCNA was substituted. The amino acid was substituted by substituting the base in the codon encoding that amino acid. An amino acid mutation was introduced into the site to be mutated shown in Table 18.

[0288]

Table 18 Mutated site of KOD-PCNA

Mutant	Mutated site
KOD-PCNA01	M73L
KOD-PCNA13	M73L+E143R

[0289] 3.2.3.1: Introduction of substitution mutation

The mutation was introduced into the PCNA gene by utilizing the plasmid to be mutated, the oligo pair for introducing the mutation (SEQ ID NOS:35 to 38 in Table 19) and Quick Change II Site-Directed Mutagenesis Kit (Stratagene) according to its manipulation manual.

[0290]

Table 19 Primers for introducing mutation into KOD-PCNA

Primer name	Primer sequence 5'==>3'	SEQ ID NO
KOD_M73L-F	C GAG ACA ATC GGC ATC AAC CTG GAC CAG TTC AAG	35
KOD_M73L-R	CTT GAA CTG GTC CAG GTT GAT GCC GAT TGT CTC G	36
KOD_E143R-F	CTC GGT GAG GTT CTC AAG CGT GGC ATA AAG GAC GCT TC	37
KOD_E143R-R	GA AGC GTC CTT TAT GCC ACG CTT GAG AAC CTC ACC GAG	38

[0291] After introducing the mutation, the DNA sequence was identified by sequencing to confirm that the DNA was introduced into the objective site and no mutation other than the objective one was present. The design for each PCNA mutant will be described below.

[0292] KOD-PCNA01

According to the report for the PCNA from *Pyrococcus furiosus* (Non-patent Document 6), the following has been reported: when the wild type Pfu-PCNA is prepared as the recombinant protein using *Escherichia coli* as the host, in addition to the original protein as a result of translation from Met, another protein of about 20 kDa with the N terminus beginning from the 73rd residue is produced as a byproduct, and thus a production efficiency of the objective protein is reduced. It has been also reported

that when Met at position 73 is substituted with Leu using the gene engineering technique, the production of this protein of about 20 kDa is inhibited, and that the Pfu-PCNA produced in this way has the nature indistinguishable from the nature of the wild type PCNA protein. Both KOD-PCNA and Pfu-PCNA are composed of 249 amino acid residues in full length, 84.3% of the amino acid residues is completely identical. Further considering the existence of amino acids having similar nature, they have very high homology. Based on such a situation, the mutant M73L of KOD-PCNA obtained by substituting Met at position 73 in KOD-PCNA with Leu is addressed as the quasi-wild type in the present specification.

[0293] This mutant KOD_M73L was designated as KOD-PCNA01 and was prepared. KOD-PCNA01 was produced by using pKPCNA as a template and the oligo pair (KOD_M73L-F and KOD_M73L-R: SEQ ID NOS:35 and 36 in Table 19) for introducing the mutation.

[0294] KOD-PCNA13

In the case of PCNA from *Pyrococcus furiosus*, it has been known that the amino acid residue at position 143 is involved in the formation of the ion pair network when the monomers form the trimer (Non-patent Document 9). As demonstrated in the aforementioned Examples 1 and 2, our group has found that the reaction of DNA polymerase in the DNA synthesis is remarkably promoted by the mutant PCNA obtained by reversely changing the electric nature of the amino acid residue at this position. In the case of wild type PCNA from *Thermococcus kodakaraensis*, the amino acid residue corresponding to this position is glutamic acid (acidic amino acid), and the electric nature of the amino acid residue at this position can be completely changed by substituting this amino acid residue with arginine (basic

amino acid residue. This mutant was produced for the purpose of examining the effect when this mutant is added to the DNA synthesis. Specifically, the mutant was produced by using the KOD-PCNA01-producing plasmid as a template and the oligonucleotides KOD_E143R-F and KOD_E143R-R for introducing the mutation (SEQ ID NOS:37 and 38 in Table 19).

[0295] 3.2.3.2: Production of KOD-PCNA expression strains

E. coli BL21 CodonPlus (DE3)RIL (Stratagene) was transformed with the expression vectors of mutant PCNA obtained in the aforementioned procedure to yield an *E. coli* strain expressing mutant gene.

[0296] 3.2.4: Purification of KOD-PCNA protein

As shown in FIG. 29, preparations were obtained by collecting the microbial cells by centrifugation (S291), disrupting the microbial cells (ultrasonic disruption, S292), boiling for 5 minutes (S293), performing the polyethyleneimine precipitation (S529), performing the ammonium sulfate precipitation (S295), performing the ion exchange chromatography (S296, using HiTrap Q as a column) and performing the gel filtration chromatography (S297, using Superdex 200). It was confirmed by SDS-PAGE that both preparations assured the purity of 90% or more.

[0297] 3.2.4.1: Culturing of microbial cells and induction of KOD-PCNA expression

Each mutant PCNA-expressing strain was cultured with shaking in 1.5 liters of the LB medium (containing 50 μg/mL of ampicillin) at 37°C. The expression was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.1 mM when OD_{600} at the logarithmic growth phase was 0.3 to 0.5, and after inducing the

expression, the culturing was continued for about 3 hours. Microbial cells after the culturing were collected by centrifugation (4°C, 6,000 xg, 6 minutes).

[0298] 3.2.4.2: Disruption of microbial cells

The microbial cells precipitated by centrifugation were suspended in 25 mL of buffer B (50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 0.1 mM EDTA, 10% glycerol, 0.5 mM DTT), and disrupted by ultrasonic treatment.

[0299] 3.2.4.3: Treatment with heat

A solution of the disrupted microbial cells was boiled for 5 minutes and then centrifuged (18,500 xg, 4°C, 25 minutes), to collect a supernatant.

[0300] 3.2.4.4: Polyethyleneimine precipitation

Polyethyleneimine (Sigma P-3143) and NaCl at final concentrations of 0.2% (w/v) and 0.58 M, respectively, were added to the supernatant, which was then stirred on ice for 30 minutes. This solution was centrifuged (18,500 xg, 4°C, 25 minutes) to yield a supernatant.

[0301] 3.2.4.5: Ammonium sulfate precipitation

5.61 g of ammonium sulfate (final concentration of 80%) was added to 10 mL of the supernatant and stirred on ice for 30 minutes to precipitate a protein. 80 mL of 50 mM Tris-HCl (pH 8.5) buffer in which ammonium sulfate had been 80% saturated was added to this solution and the precipitation was collected by centrifugation (30,000 xg, 4°C, 25 minutes). Subsequently, this precipitation was dissolved in buffer C (50 mM Tris-HCl pH 8.0, 0.1M NaCl) and the resulting solution was dialyzed against the same buffer C.

[0302] 3.2.4.6: Ion exchange chromatography

The dialyzed sample was subjected to the ion exchange chromatography (HiTrap Q; Amersham Bioscience) using AKTA

explorer 10S (Amersham Bioscience). The sample was eluted with the linear gradient of 0.1 to 0.8 M NaCl/17.5 mL at a flow rate of 1 mL/minute to yield peak fractions of KOD-PCNA01 and KOD-PCNA13.

[0303] 3.2.4.7: Gel filtration chromatography

Each peak fraction in HiTrap Q ion exchange chromatography was further purified by gel filtration chromatography using Superdex 200 (Amersham Bioscience) to obtain the preparation for the subsequent assays.

[0304] The obtained preparation was applied onto the polyacrylamide gel electrophoresis to confirm the molecular size and good purification of the preparation (FIG. 30).

[0305] 3.3: Purification of KOD-RFC

RFC from *Thermococcus kodakaraensis* KOD is composed of two subunits RFCL and RFCS, which are located in tandem on the genome. Upon producing the RFC protein preparation, an RFCL gene and an RFCS gene were individually inserted in distinct expression vectors, and the respective expression vectors were introduced into the same host to express simultaneously. The expression plasmid was produced with reference to Non-patent Document 10. The detail thereof will be described below.

[0306] 3.3.1: Cloning of KOD-RFCL gene and construction of expression plasmid (FIG. 31)

The KOD-RFCL (GenBank ID;182,830) gene was obtained by PCR using the KOD genome as the template (SEQ ID NO:39).

[0307] 3.3.1.1: PCR primers

KOD-RFCL-F primer and KOD-RFCL-R primer (SEQ ID NOS:40 and 41 in Table 20) were used as the primers for PCR. For convenience of cloning, the restriction enzyme NdeI recognition site was added to the KOD-RFCL-F primer, and the restriction enzyme XhoI recognition site was added to the KOD-RFCL-R primer.

[0308]

Table 20 Primers for cloning KOD-RFCL

Primer name	Primer sequence 5'==>3'	SEQ ID NO
KOD-RFCL-F	cca tat gac gga agt ccc atg ggt tg	40
KOD-RFCL-R	ctc gag tca ctt ctt gag gaa gtc gaa cag	41

[0309] 3.3.1.2: Composition of PCR reaction solution

The KOD genomic DNA prepared in the aforementioned 3.1 was used as the template for PCR. The PCR reaction solution has the following composition (amounts to be added to 50 μL of the reaction solution).

Template DNA: 100 ng,
Primers: each 10 pmol,
dNTP: each 10 nmol,
Ex Taq*: 1.25 U and
10 x Ex Taq buffer: 5 μL

were mixed, and sterilized water was added thereto up to the total volume of 50 μL.

(*supplied from TAKARA BIO INC.)

[0310] 3.3.1.3: Reaction conditions for PCR

The PCR reaction was performed by using the reaction solution prepared in the aforementioned procedure and the PCR apparatus and using the program repeating 30 cycles of the reaction at 95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for one minute

[0311] 3.3.1.4: Purification of PCR product

The PCR product obtained in the aforementioned procedure was subjected to 1% agarose gel electrophoresis, and stained with ethidium bromide. Subsequently a gel fragment containing a band around 1.5 kb was cut out under the ultraviolet light irradiation, and the PCR product in

the gel fragment was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham) according to its manipulation manual.

[0312] 3.3.1.5: Subcloning of PCR product

The purified PCR product was ligated to pUC118-HincII/BAP using TaKaRa BKL kit (TAKARA BIO INC.) according to the manipulation manual. *Escherichia coli* DH5α (TAKARA BIO INC.) was transformed with this ligated PCR product, which was then seeded on the LB agar plate (containing 100 μg/mL of ampicillin, 40 μg/mL of IPTG, and 40 μg/mL of X-GAL),and cultured by leaving stand at 37°C overnight to yield an *E. coli* clone having the PCR product.

[0313] An *E. coli* colony exhibiting the white color on the agar plate was cultured in 3 mL of the LB liquid medium (containing 100 μg/mL of ampicillin) at 37°C overnight with shaking, and then a plasmid DNA was prepared according to the standard method.

[0314] 3.3.1.6: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA sequence inserted into the recognition site of the restriction enzyme HincII in the plasmid vector pUC118 was examined using the DNA sequencer. As a result, it was confirmed that the open reading frame of the KOD-RFCL gene was retained, the restriction enzyme NdeI recognition sequence was added to the 5' end and the restriction enzyme XhoI recognition sequence was added the 3' end in the inserted portion. This plasmid vector was designated as pUC118/KRFCL.

[0315] 3.3.1.7: Preparation of expression plasmid of KOD-RFCL

The plasmid pUC118/KRFCL was doubly cleaved with the restriction enzymes NdeI and XhoI to prepare an RFCL gene

fragment. The gene fragment was inserted into the expression vector to produce the expression vector of KOD-RFCL.

[0316] 3.3.1.8 Preparation of RFCL DNA fragment

The plasmid pUC118/KRFCL was doubly cleaved with the restriction enzymes NdeI and XhoI in the following reaction system.

[0317]

Plasmid DNA: 5 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme XhoI: 5 units

[0318] Sterile water was added to the aforementioned mixture up to the total volume of 50 μL, which was then cleaved with the restriction enzymes at 37°C for 2 hours. After completing the reaction, the reaction product was run on 1% agarose gel electrophoresis. A band (around about 1.5 kb) corresponding to the KOD-RFCL gene was cut out and the RFCL DNA fragment was purified from the gel using GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience) according to its manipulation manual.

[0319] 3.3.1.9: Expression vector pET-29a

The vector DNA pET-29a (Novagen, US) was doubly cleaved with the restriction enzymes NdeI and XhoI by the following reaction.

[0320]

Plasmid DNA: 2 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme XhoI: 5 units

[0321] Sterile water was added to the aforementioned mixture up to the total volume of 50 μL, which was then

left stand at 37°C for 2 hours. After completing the reaction, the reaction product was run on 1% agarose gel electrophoresis. A band (around about 5.4 kb) corresponding to the straight form of the vector pET-29a was cut out and the pET-29a DNA fragment was purified from the gel using GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience) according to its manipulation manual.

[0322] 3.3.1.10: Ligation reaction and transformation

The KOD-RFCL DNA fragment (100 ng) and the pET-29a DNA fragment (50 ng) obtained in the aforementioned procedure were reacted using DNA Ligation Kit V2 (TAKARA BIO INC.) as follows.

[0323] RFCL DNA fragment: 100 ng

pET-29a DNA fragment: 50 ng

DNA Ligation Kit V2 enzyme solution: 5 μL

Sterile water was added to the aforementioned mixture up to the total volume of 10 μL, which was then reacted at 16°C for 30 minutes.

[0324] 100 μL of *E. coli* BL21 (DE3) (Novagen) was transformed with this ligation product (3 μL). The solution of transformed *E. coli* was seeded on the LB agar plate (containing 30 μg/mL of kanamycin) and left stand to culture at 37°C overnight. Three colonies in *E. coli* colonies formed on the agar plate were cultured with shaking in 3 mL of the LB liquid medium (containing 30 μg/mL of kanamycin) at 37°C overnight, and a plasmid DNA was prepared according to standard methods.

[0325] 3.3.1.11: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA sequence inserted in the plasmid vector pET-29a and the sequence in the vicinity of the inserted site were examined using DNA sequencer. As a result, in all plasmid DNA, the

ORF (open reading frame) of the KOD-RFCL gene was completely inserted between NdeI and XhoI sites which were multicloning sites. This plasmid retaining the KOD-RFCL gene was designated as pKRFCL (FIG. 32).

[0326] As shown in FIG. 32, it was confirmed that the T7 promoter and rbs (ribosome binding site) which pET-29a had, the open reading frame of the RFCL gene and the T7 terminator were aligned in this order in pKRFCL. It was expected that this plasmid would express the RFCL gene in the large amount.

[0327] 3.3.2: Cloning of KOD-RFCS gene and construction of expression plasmid

According to Non-patent Document 10, it has been reported that the KOD-RFCS gene (GenBank ID: BD182,829) is encoded by 2,601 bases in full length and contains one intein, and that the N terminal extein is encoded by 177 bases and the C terminal extein is encoded by 804 bases (not including the termination codon). The base sequence of the KOD-RFCS gene (mature type sequence obtained by removing the intein portion) is shown in SEQ ID NO:42 in Sequence Listing.

[0328] Upon producing the RFCS expression vector as shown in FIG. 33, first the KOD-RFCS gene in full length including the intein was cloned (S331 to S334) using PCR, subsequently two extein fragments were individually amplified by PCR reaction (S335 to S336), and then the mature RFCS (also referred to as RFCSm) where the two exteins had been bound and the intein had been removed was prepared. This mature RFCS was incorporated in the expression vector (S337 to S339).

[0329] 3.3.2.1: Cloning of RFCS gene in full length including intein

3.3.2.1.1: PCR primers

KOD-RFCS-F primer and KOD-RFCS-R primer (SEQ ID NOS:43 and 44 in Table 21) were used as the PCR primers.

[0330]

Table 21 Primers for cloning KOD-RFCS

Primer name	Primer sequence 5'==>3'	SEQ ID NO
KOD-RFCS-F	atg tcc gag gaa gtg aag gaa g	43
KOD-RFCS-R	tca ctt acc cat aat cgt gaa ctg	44

[0331] 3.3.2.1.2: Composition for PCR reaction

The KOD genomic DNA prepared in the aforementioned 3.1 was used as the template for PCR. The PCR reaction solution has the following composition (amounts to be added to 50 μL of the reaction solution).

Template DNA: 100 ng,

Primers: each 10 pmol,

dNTP: each 10 nmol,

Ex Taq*: 1.25 U and

10 x Ex Taq buffer: 5 μL

were mixed, and sterilized water was added thereto up to the total volume of 50μL.

(*supplied from TAKARA BIO INC.)

[0332] 3.3.2.1.3: Reaction conditions for PCR

The PCR reaction was performed by using the reaction solution prepared in the aforementioned procedure and the PCR apparatus and using the program repeating 30 cycles of the reaction at 95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for one minute.

[0333] 3.3.2.1.4: Purification of PCR product

The PCR product obtained in the aforementioned procedure was subjected to 1% agarose gel electrophoresis,

and stained with ethidium bromide. Subsequently a gel fragment containing a band around 2.6 kb was cut out under the ultraviolet light irradiation, and the PCR product in the gel fragment was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham) according to its manipulation manual.

[0334] 3.3.2.1.5: Subcloning of PCR product

The purified PCR product was ligated to pUC118-HincII/BAP (TAKARA BIO INC.) using TaKaRa BKL Kit (TAKARA BIO INC.) according to the manipulation manual. *Escherichia coli* DH5α (TAKARA BIO INC.) was transformed with this ligated PCR product, which was then seeded on the LB agar plate (containing 100 μg/mL of ampicillin, 40 μg/mL of IPTG, and 40 μg/mL of X-GAL),and cultured by leaving stand at 37°C overnight to yield an *E. coli* clone having the PCR product.

[0335] An *E. coli* colony exhibiting the white color on the agar plate was cultured in 3 mL of the LB liquid medium (containing 100 μg/mL of ampicillin) at 37°C overnight with shaking, and then a plasmid DNA was prepared according to the standard method.

[0336] 3.3.2.1.6: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA sequence inserted in the restriction enzyme HincII recognition site of the plasmid vector pUC118 was examined using the DNA sequencer. As a result, the inserted portion completely comprises the base sequence of two exteins in the RFCS gene, and this plasmid was designed as pUC/KRFCS.

[0337] 3.3.2.2: Binding of exteins (removal of intein)

3.3.2.2.1: Primers for binding exteins

RFCS-Nde-F primer and RFCS-Ex1-R primer (SEQ ID NOS:45 and 48 in Table 22) were prepared as the PCR primers for

the N terminal extein. RFCS-Ex2-F primer and RFCS-Sal-R primer (SEQ ID NOS:47 and 46 in Table 22) were prepared as the PCR primers for the C terminal extein. For the purpose of performing the cloning easily, the restriction enzyme NdeI sequence was added to the 5' end of the RFCS-Nde-F primer, and the restriction enzyme SalI sequence was added to 5' end of RFCS-Sal-R primer. The complementary sequences utilized when the two extein fragments were fused by PCR were provided in the RFCS-Ex1-R primer and the RFCS-Ex2-F primer.

[0338]

Table 22 Primers for binging KOD-RFCS exteins (for removal of intein)

Primer name	Primer sequence 5'==>3'	SEQ ID NO
RFCS-Nde-F	cca tat gtc cga gga agt gaa gga ag	45
RFCS-Sal-R	gtc gac tca ctt acc cat aat cgt gaa ctg	46
RFCS-Ex2-F	cgt cgg gaa gac aac cgc tgc act ggc ttt ag	47
RFCS-Ex1-R	cag cgg ttg tct tcc cga cgc cgg gtg gc	48

[0339] 3.3.2.2.2: Composition of PCR reaction solution

The PCR reaction for amplifying two exteins was performed using the following composition (amounts to be added to 50 μL of the reaction system).

pUC/KRFCS DNA: 50 ng

Primers: each 10 pmol

dNTP: each 10 nmol

Ex Taq*: 1.25 U and

10 x Ex Taq buffer: 5 μL

were mixed, and sterilized water was added thereto up to the total volume of 50 μL.

(*supplied from TAKARA BIO INC.)

[0340] 3.3.2.2.3.: Reaction conditions for PCR

The PCR reaction was performed by using the reaction solution prepared in the aforementioned procedure and the PCR apparatus and using the program repeating 30 cycles of the reaction at 95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for one minute.

[0341] 3.3.2.2.4: Purification of PCR product

PCR products obtained in the aforementioned procedure were subjected to 2% agarose gel electrophoresis, and stained with ethidium bromide. A band of about 180 bases was observed in the product of PCR with the set of the RFCS-Nde-F primer and the RFCS-Ex1-R primer. A band of about 800 bases was observed in the product of PCR with the set of the RFCS-Ex2-F primer and the RFCS-Sal-R primer. The gel fragment containing each band was cut out under the ultraviolet light irradiation, and the PCR product in the gel fragment was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham) according to its manipulation manual.

[0342] 3.3.2.2.5: PCR fusion reaction

The PCR products of two exteins were fused by subjecting them to PCR in one tube using one set of the primers to yield the gene fragment encoding the mature RFCS. The detail thereof will be described below.

[0343] An annealing reaction of two extein PCR products was performed with the following composition.

N terminal extein fragment: 2 μL (corresponding to 50 ng),

C terminal extein fragment: 2 μL (corresponding to 50 ng) and

10 x Ex Taq buffer: 5 μL.

Sterile water was added to the aforementioned

composition up to the total volume of 44.5 μL.

The aforementioned mixture was heated at 95°C for 3 minutes, and slowly cooled down to 37°C over 30 minutes.

[0344] The followings were added to the aforementioned reaction solution.

dNTP: 5 μL (each 10 nmol) and

Ex Taq*: 0.5 μL (2.5 U).

This mixture was reacted at 72°C for 10 minutes. (*supplied from TAKARA BIO INC.)

[0345] 10 pmol each of RFCS-Nde-F primer and RFCS-Sal-R primer was added to the aforementioned mixture, and the PCR reaction was performed using the program repeating 30 cycles of the reaction at 95°C for 30 seconds --> 55°C for 30 seconds --> 72°C for one minute.

[0346] 3.3.2.2.6: Cleavage with restriction enzymes and purification of PCR product

The PCR product obtained in the aforementioned procedure was purified by ethanol precipitation according to the standard method. The purified DNA fragment was doubly cleaved with the restriction enzymes NdeI and SalI.

PCR product: corresponding to 1 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme SalI: 5 units

Sterile water was added to the aforementioned composition up to the total volume of 50 μL, and the PCR product was cleaved with the restriction enzymes at 37°C for 2 hours. After completing the reaction, the reaction product was run on 2% agarose gel electrophoresis. A band (around about 1 kb) supposed to contain the fused fragment of two inteins (encoding the mature RFCS) was cut out, and

the fragment was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience).

[0347] 3.3.2.2.7: Cleavage of expression vector pET-21a with restriction enzymes

The vector DNA pET-21a (Novagen) was doubly cleaved with the restriction enzymes NdeI and SalI.

[0348] Plasmid DNA: 2 μg

10 x Restriction enzyme buffer: 5 μL

Restriction enzyme NdeI: 5 units

Restriction enzyme SalI: 5 units

[0349] Sterile water was added to the aforementioned mixture up to the total volume of 50 μL, which was then left stand at 37°C for 2 hours. After completing the reaction, the reaction product was run on 1% agarose gel electrophoresis. A band (around about 5.4 kb) corresponding to the straight form of the vector DNA pET-21a was cut out and the pET-21a DNA fragment was purified from the gel using GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience) according to its manipulation manual.

[0350] 3.3.2.2.8: Ligation reaction and transformation

The DNA fragment of about 1 kb (100 ng) predicted to be the fused fragment of two inteins (encoding the mature RFCS) and the pET-21a DNA fragment (50 ng) obtained in the aforementioned procedure were reacted using DNA Ligation Kit V2 (TAKARA BIO INC.) as follows.

[0351] 1 kb DNA fragment: 100 ng

pET-21a DNA fragment: 50 ng

DNA Ligation Kit V2 enzyme solution: 5 μL

Sterile water was added to the aforementioned mixture up to the total volume of 10 μL, which was then reacted at 16°C for 30 minutes.

[0352] 100 μL of *E. coli* BL21 (DE3) (Novagen) was transformed with this ligation product (3 μL). The solution of transformed *E. coli* was seeded on the LB agar plate (containing 100 μg/mL of ampicillin) and left stand to culture at 37°C overnight. Three colonies in *E. coli* colonies formed on the agar plate were cultured with shaking in 3 mL of the LB liquid medium (containing 100 μg/mL of ampicillin) at 37°C overnight, and a plasmid DNA was prepared according to standard methods.

[0353] 3.3.2.2.9: Confirmation of sequence by sequencing

Concerning the aforementioned plasmid DNA, the DNA sequence inserted in the plasmid vector pET-21a and the sequence in the vicinity of the inserted site were examined using the DNA sequencer. As a result, the sequence of mature RFCSm (984 bases) binding two exteins (containing no intein) was identified between NdeI site and SalI site which were multicloning sites (designated as pKRFCSm).

[0354] As shown in FIG. 34, it was confirmed that the T7 promoter and rbs (ribosome binding site) which pET-21a had, the mature RFCS gene open reading frame and the T7 terminator were aligned in this order in pKRFCSm. It was expected that this plasmid would express the mature RFCS gene in the large amount.

[0355] 3.3.3: Preparation of KOD-RFC expression strain

Escherichia coli BL21-CodonPlus (DE3)-RIL was simultaneously transformed with pKRFCL and pKRFCSm, and the expression strain having both plasmids was obtained by double selection of the transformant with ampicillin and kanamycin.

[0356] 3.3.4: Purification of KOD-RFC protein

As shown in FIG. 35, a preparation was obtained by collecting the microbial cells by centrifugation (S351),

disrupting the microbial cells (ultrasonic disruption, S352), boiling for 5 minutes (S353), performing the polyethyleneimine precipitation (S354), performing the ammonium sulfate precipitation (S355), performing the affinity chromatography (S356, using HiTrap Heparin HP as a column) and performing the gel filtration chromatography (S357, using Superdex 200). It was confirmed by SDS-PAGE that the purity of 90% or more was assured in all preparations.

[0357] 3.3.4.1: Culturing of microbial cells and induction of expression

The aforementioned expression strain was cultured with shaking in 1.5 liters of the LB medium (containing 50 μg/mL of ampicillin and 30 μg/mL of kanamycin) at 37°C. The expression was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.1 mM when OD_{600} at the logarithmic growth phase was 0.3 to 0.5, and after inducing the expression, the culturing was continued for about 3 hours. Microbial cells after the culturing were collected by centrifugation (4°C, 6,000 xg, 6 minutes).

[0358] 3.3.4.2: Disruption of microbial cells

The microbial cells precipitated by centrifugation were suspended in 25 mL of the buffer B (aforementioned), and disrupted by ultrasonic treatment.

[0359] 3.3.4.3: Treatment with heat

A disrupted microbial cell solution was boiled for 5 minutes, and subsequently centrifuged (18,500 xg, 4°C, 25 minutes to collect the supernatant.

[0360] 3.3.4.4: Polyethyleneimine precipitation

Polyethyleneimine (Sigma P-3143) was added at a final concentration of 0.18% (w/v), which was then stirred on ice

for 30 minutes. This solution was centrifuged (18,500 xg, 4°C, 25 minutes) to yield a supernatant.

[0361] 3.3.4.5: Ammonium sulfate precipitation

5.61 g of ammonium sulfate (final concentration of 80%) was added to 10 mL of the supernatant and stirred on ice for 30 minutes to precipitate a protein. The precipitation was collected by centrifugation (18,500 xg, 4°C, 25 minutes). Subsequently, this precipitation was dissolved in the buffer C (aforementioned) and the resulting solution was dialyzed against the same buffer C.

[0362] 3.3.4.6: Affinity chromatography

The dialyzed sample was purified using HiTrap Heparin HP column (Amersham Bioscience).

[0363] The sample was eluted with the linear gradient of 0.1 to 0.8 M of NaCl/17.5 mL at the flow rate of 1 mL/minute to yield a peak fraction.

[0364] 3.3.4.7: Gel filtration chromatography

The peak fraction in the HiTrap Heparin affinity chromatography was further purified by gel filtration chromatography using Superdex 200 (aforementioned) to obtain a preparation for assays. The resulting preparation was applied onto the polyacrylamide gel electrophoresis to confirm the molecular size and good purification of the preparation (FIG. 36).

[0365] 4: Evaluation of KOD-PCNA mutant

The effect of the PCNA mutant prepared in the aforementioned procedure on the DNA amplification system, particularly the PCR reaction system was examined. Specifically, the PCR reaction was performed with the addition of the PCNA mutant alone, RFC alone or the combination thereof, or with the addition of none of them to the PCR reaction system. The level of the amplification of the target region were compared by subjecting the PCR

product to the electrophoresis. Two commercially available DNA polymerases for PCR, KOD DNA polymerase (Toyobo Co., Ltd.) and Pyrobest DNA polymerase (TAKARA BIO INC.) were used for the PCR reaction.

[0366] 4.1: Effect of adding KOD-PCNA to KOD DNA polymerase

KOD DNA polymerase is a DNA polymerase derived from the thermophilic archaebacterium, *Thermococcus kodakaraensis* KOD-1 strain. The KOD DNA polymerase is a so-called α-type DNA polymerase having the strong 3' --> 5' exonuclease activity (proof reading activity) in addition to the polymerase activity, and exhibits the PCR fidelity higher than Taq DNA polymerase generally used because of having the 3' --> 5' exonuclease activity. Other α-type highly accurate enzymes for PCR often have the slow extension rate. However, it has been reported that the present enzyme has the very fast extension rate (Non-patent Document 11).

[0367] 4.1.1: Template DNA and reaction primers

Lambda DNA (GenBank accession 02459) was used as the template DNA. The target sequence for PCR amplification was the sequence from 23,119 to 25,142 in the lambda DNA. The sequences of the primers used at that time were shown in Table 8, and were also shown in Table 23 additionally.

[0368]

Table 23 Primers for PCR assay

Primer name	Primer sequence 5'==>3'	SEQ ID NO
F02	GTC GTT TCT GCA AGC TTG GC	25
R03	CCG AGA TAA AAA CAA ACC CGC	28

[0369] 4.1.2: Composition of reaction solution

The composition of the reaction solution was shown in Tables 24 and 25.

[0370]

Table 24

Composition	Amount to be added	Final concentration
Lambda DNA (20ng/μL)	1.25μL	0.5ng/μL
F02 Primer (20pmol/μL)	1.25μL	0.5μM
R03 Primer (20pmol/μL)	1.25μL	0.5μM
10x enclosed Buffer #1	5μL	1x
dNTP Mix (2.5mM each)	4μL	0.2mM
25mM $MgCl_2$	2μL	1mM
KOD DNA Polymerase (2.5U/μL)	0.28μL	0.014U/μL
Accessory protein solution (see Table 25)	1.1μL	-
Sterile water	33.87μL	-
Total	50μL	

[0371]

Table 25

	Composition of accessory protein solution	KOD-PCNA01 (100ng/μl)	KOD-PCNA13 (100ng/μl)	KOD-RFC (500ng/μl)	Buffer*
1	No KOD-PCNA nor KOD-RFC	0μL	0μL	0μL	1.1μL
2	KOD-PCNA01(0.6ng/μL)	0.3μL	0μL	0μL	0.8μL
3	KOD-PCNA01(0.6ng/μL)+ KOD-RFC(4ng/μL)	0.3μL	0μL	0.4μL	0.4μL
4	KOD-PCNA01(0.6ng/μL)+ KOD-RFC(8ng/μL)	0.3μL	0μL	0.8μL	0μL
5	KOD-PCNA13(0.6ng/μL)	0μL	0.3μL	0μL	0.8μL
6	KOD-PCNA13(0.6ng/μL)+ KOD-RFC(4ng/μL)	0μL	0.3μL	0.4μL	0.4μL
7	KOD-PCNA13(0.6ng/μL)+ KOD-RFC(8ng/μL)	0μL	0.3μL	0.8μL	0μL

* Buffer: 25mM Tris-HCl pH8.0, 50mM NaCl, 50% Glycerol

[0372] 4.1.3: PCR program

The aforementioned reaction solution was reacted in the following program.

Reaction at 94°C for one minute --> (98°C for 5 seconds --> 68°C for 15 seconds) 30 cycles --> being kept at 4°C

[0373] 4.1.4: Electrophoresis

After completing the PCR reaction, 5 μL of 10 x loading buffer (glycerol 50%, bromophenol blue 0.4%, Xylene cyanol 0.4%) was added to and mixed with 50 μL of the PCR reaction solution, and 10 μL in 50 μL of the reaction solution was then subjected to 1% agarose gel electrophoresis. As the electrophoresis marker, lambda/styI marker (Toyobo Co., Ltd.) was used. The results are shown in FIG. 37.

[0374] 4.1.5: Results

The results were shown in FIG. 37. Under these experimental conditions, the good extension and amplification can be observed with KOD DNA polymerase alone

(lane 1). When the quasi-wild type PCNA01 was added, the reaction inhibition was observed (lane 2), but when RFC was further added and the amount of added RFC was increased, the inhibition was offset and the extension and amplification at the same level as in the case of no addition were observed (lanes 3 and 4). Meanwhile, when KOD-PCNA13 was added, the good extension and amplification not depending on the addition of KOD-RFC were observed. This effect was better than those when no accessory protein was added and when KOD-PCNA01 was added in combination with RFC.

[0375] 4.2: Effect of adding KOD-PCNA to Pyrobest DNA polymerase

Effect of addition of various PCNA mutants on Pyrobest DNA Polymerase (TAKARA BIO INC.),a commercially distributed DNA synthesis enzyme derived from genus *Pyrococcus*, was examined using the PCR reaction system. Pyrobest DNA Polymerase is a heat resistant α type DNA polymerase derived from *Pyrococcus* sp. and having 3' --> 5' exonuclease activity (proof reading activity). This enzyme is characterized by performing a highly accurate amplification equivalent to that by Pfu DNA polymerase derived from *Pyrococcus furiosus*, and Vent DNA polymerase.

[0376] 4.2.1: Template DNA and reaction primers

The same template DNA and primers as in the aforementioned evaluation of KOD DNA polymerase were used.

[0377] 4.2.2: Composition of reaction solution

The compositions of the reaction solutions were shown in Tables 26 and 27

Table 26

Composition	Amount to be added	Final concentration
Lambda DNA (20ng/μL)	1.25μL	0.5ng/μL
F02 Primer (20pmol/μL)	0.5μL	0.2μM
F03 Primer (20pmol/μL)	0.5μL	0.2μM
10x enclosed Buffer II	5μL	1x
dNTP Mix (2.5mM each)	4μL	0.2mM
Pyrobest DNA Polymerase (5U/μL)	0.25μL	0.025U/μL
Accessory protein solution (see Table 27)	1.3μL	-
Sterile water	37.2μL	-
Total	50μL	

[0378]

Table 27

	Composition of accessory protein solution	KOD-PCNA01 (400ng/μl)	KOD-PCNA13 (400ng/μl)	KOD-RFC (1400ng/μl)	buffer*
1	No KOD-PCNA nor KOD-RFC	0μl	0μl	0μl	1.3μl
2	KOD-PCNA01 (2.4ng/μl)	0.3μl	0μl	0μl	1.0μl
3	KOD-PCNA01 (2.4ng/μl) +KOD-RFC (14ng/μl)	0.3μl	0μl	0.5μl	0.5μl
4	KOD-PCNA01 (2.4ng/μl) +KOD-RFC (28ng/μl)	0.3μl	0μl	1.0μl	0μl
5	KOD-PCNA13 (2.4ng/μl)	0μl	0.3μl	0μl	1.0μl

*Buffer: 25mM Tris-HCl pH8.0, 50mM NaCl, 50% Glycerol

[0379] 4.2.3: PCR program

The aforementioned reaction solution was reacted in the following program.

94°C for one minute --> (98°C for 5 seconds --> 68°C for 1.5 minutes) 30 cycles --> being kept at 4°C

[0380] 4.2.4: Electrophoresis

After completing the PCR reaction, 5 μL of 10 x

loading buffer (glycerol 50%, bromophenol blue 0.4%, Xylene cyanol 0.4%) was added to and mixed with 50 μL of the PCR reaction solution, and 10 μL in 50 μL of the reaction solution was then subjected to 1% agarose gel electrophoresis. As the electrophoresis marker, lambda/styI marker (Toyobo Co., Ltd.) was used. The results are shown in FIG. 38.

[0381] 4.2.5: Results

The results were shown in FIG. 38. Under these experiment conditions, the extension and amplification can be observed with Pyrobest DNA polymerase alone (lane 1). When the quasi-wild type PCNA01 was added, the reaction inhibition was observed and no band was detected (lane 2), but when the amount of added RFC was increased, the inhibition was offset and the extension and amplification at the same level as in the case of no addition were observed (lanes 3 and 4). Meanwhile, when KOD-PCNA13 was added, good extension and amplification were observed even when no RFC was added, and this effect exhibited better extension and amplification than in the other 4 conditions (lane 5).

[0382] 4.3: Summary

It was found by the aforementioned experiments that KOD-PCNA13 promoted the PCR reaction of the representative two PCR enzymes. This reaction not only did not require the addition of RFC but also was better in promoting activity than in the case of adding the wild type in combination with RFC. It was proven by the aforementioned results that the excellent effect could be obtained by introducing the identical mutation into not only PCNA derived from *Pyrococcus furiosus* but also PCNA derived from *Thermococcus kodakaraensis* KOD1 strain. In the present invention, it was demonstrated that the identification of

the amino acid residue in the interface region of the PCNA monomer of the present invention not only was effective for PCNA derived from *Pyrococcus furiosus* but also was applicable to PCNA having the same structural context.

[0383] 5: Measurement of fidelity in addition reaction of Pfu-PCNA

The effect of adding Pfu-PCNA to the PCR reaction on template fidelity was examined as to Pyrobest DNA polymerase (supplied from TAKARA BIO INC., hereinafter sometimes abbreviated simply as "Pyrobest") which was the commercially available PCR enzyme with high fidelity. Pyrobest is a DNA polymerase derived from the archaebacterium belonging to genus *Pyrococcus* and has the 3' --> 5' exonuclease, which works as the proof reading activity.

[0384] The fidelity was obtained by examining the sequence of the PCR product and comparing it with the template sequence. An outline was shown in FIG. 39. The PCR reaction was performed using Pyrobest alone or in combination with Pfu-PCNA13 (S511), the end of the amplified DNA fragment was blunted and phosphorylated (S512), this fragment was cloned into a plasmid vector (S513), *Escherichia coli* was transformed with the plasmid vector and the colony was isolated, and the plasmid was extracted (S514, S515). A part (500 bp) of the inserted sequence in each plasmid was examined by the DNA sequencer (S516), and the result was compared with the template sequence to calculate a frequency of error occurrence (S517).

[0385] 5.1: PCR reaction

5.1.1: Combination of PCR enzyme and Pfu-PCNA13

The case of performing the PCR reaction with Pyrobest alone and the case of performing the PCR reaction with

Pyrobest in combination with Pfu-PCNA13 were examined. As the control for them, the PCR reaction with TaKaRa Taq (TAKARA BIO INC., hereinafter sometimes abbreviated simply as "Taq") alone was also examined. Taq is a PolI type PCR enzyme generally used, does not have the 3' --> 5' exonuclease activity, and is described to have lower fidelity than the α-type PCR enzyme having the same activity.

[0386] 5.1.2: Target region of template

Lambda DNA (GenBank accession 02459) was used as the template of the PCR reaction, and seven regions were targeted to the examination. The location on the lambda DNA and the size of each region, and the combination of the primers used for amplifying each region were shown in Table 28, and the primer sequences were shown in Table 29.

[0387]

Table 28

Fragment ID	Amplified region in lambda DNA	Amplified size(bp)	Forward primer	Reverse primer
1	17,973-19,022	1,050	F23	R25
2	19,001-20,050	1,050	F25	R27
3	23,619-24,654	1,036	F09	R11
4	24,634-25,696	1,063	F11	R15
5	26,894-27,492	599	F14	R04
6	40,460-41,185	1,026	F16	R17
7	42,532-43,613	1,082	F19	R21

[0388]

Table 29

Primer name	Start Position	End Position	Primer sequence5'==>3'	SEQ ID NO
F09	23,619	23,637	AGC CTT TGC CTC GCT ATA C	49
F11	24,634	24,654	GCT GCT GAA ACG TTG CGG TTG	26
F14	26,894	26,914	CCA TCT GCT CGT AGG AAT GCC	50
F16	40,460	40,479	GCT ACC AGG GAA GAA CGG GA	51
F19	42,532	42,553	CCA AGA TAG CAC TCG AAC GAC G	52
F23	17,973	17,994	CGA ATC CCA TCT CGG CAA GGA G	53
F25	19,001	19,022	GCA CTT GCG GTG ACA GTC ACT C	54
R04	27,492	27,472	CCA GTG CAA AGC TTT GTG TGC	55
R11	24,654	24,634	CAA CCG CAA CGT TTC AGC AGC	56
R15	25,696	25,677	CCC AGT AGT ACT GCA AGA GG	57
R17	41,485	41,467	CGT GGT GTA ATT CCC TCG C	58
R21	43,613	43,590	GCT CAC CAG TTC GAT GAT TAA CGG	59
R25	19,022	19,001	GAG TGA CTG TCA CCG CAA GTG C	60
R27	20,050	20,029	GCA TCG CCG GCT GAT TTC TTC G	61

[0389] 5.1.3: Composition of PCR reaction solution

As the PCR reaction solution, the PCR reaction solution having the ordinary composition was used (Table 30). When Pyrobest was used as the PCR enzyme, 10x Pyrobest buffer II (unpublished composition) which was the attached buffer was used. When TaKaRa Taq was used, 10x PCR Buffer(100 mM Tris·Cl (pH 8.3), 500 mM KCl,15 mM $MgCl_2$) which was the attached buffer was used. Pfu-PCNA13 was added at a final concentration of 0.6 ng/μg (0.3 μL), and when not added, the same volume (0.3 μL) of sterile water was added.

[0390]

Table 30

Composition	Amount to be added	Final concentration
Lambda DNA (20ng/μL)	1.25μL	0.5ng/μL
Forward Primer (20pmol/μL)	0.5μL	0.2μM
Reverse Primer (20pmol/μL)	0.5μL	0.2μM
10X buffer [1)]	5μL	1x
dNTP Mix (2.5mM each)	4μL	0.2mM
Enzyme (5U/μL)	0.25μL	0.025U/μL
PCNA13 solution or sterile water [2)]	0.3μL	0 or 0.6ng/μL
Sterile water	38.2μL	
Total	50μL	

1)10x Buffer; 10x Pyrobest buffer II (upon using Pyrobest DNA polymearase), or 10x PCR Buffer(upon using TaKaRa Taq)

2)Neat concentration of PCNA13;100ng/μL

[0391] 5.1.4: PCR program

In the case of Pyrobest alone

(98°C for 10 seconds --> 68°C for 2.5 minutes) 30 cycles --> being kept at 4°C

In the case of Pyrobest + Pfu-PCNA13

(98°C for 10 seconds --> 68°C for 1.5 minutes) 30 cycles --> being kept at 4°C

In the case of Taq alone

(94°C for 30 seconds --> 55°C for 30 seconds --> 72°C for 1.5 minutes) 30 cycles --> being kept at 4°C

[0392] 5.2: End blunting and purification of PCR product

Each PCR product obtained in the aforementioned PCR reaction was subjected to 1% agarose gel electrophoresis and the gel was stained with ethidium bromide. Subsequently, a gel containing a DNA fragment having the

objective size was cut out, and the DNA fragment in the gel was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience) according to its manipulation manual. The end of the purified DNA fragment was blunted and phosphorylated using TaKaRa BKL Kit(Blunting Kination Ligation Kit) (supplied from TAKARA BIO INC.) according to its kit manual.

[0393] 5.3: Insertion of PCR product into cloning vector

The DNA fragment (50 ng) whose ends had been blunted and phosphorylated was ligated to the DNA cleaved with the restriction enzyme HincII and treated with BAP (pUC118 HincII/BAP supplied from TAKARA BIO INC.) as follows.

DNA fragment whose ends was blunted and phosphorylated: 50 ng

pUC118 HincII/BAP: 50 ng

DNA ligation kit V2 enzyme solution: 5 μL

Sterile water was added to the aforementioned mixture up to the total volume of 10 μL, which was then reacted at 16°C for 60 minutes.

[0394] 5.4: Isolation of *Escherichia coli* colony and extraction of plasmid

100μL of *Escherichia coli* JM109 (TAKARA BIO INC.) was transformed with this ligation product (3 μL), which was then seeded on the LB agar plate (containing 100 μg/mL of ampicillin, 40 μg/mL of IPTG, and 40 μg/mL of X-GAL),and cultured by leaving stand at 37°C overnight. An *Escherichia coli* colony exhibiting the white color on the agar plate was cultured in 1.5 mL of the TB liquid medium (containing 100 μg/mL of ampicillin) at 37°C overnight with shaking, and then a plasmid DNA was prepared according to the standard.

[0395] 5.5: Sequencing of inserted sequence

The DNA sequence of the extracted plasmid as a template was analyzed using the DNA sequencer.

[0396] In the inserted DNA fragment (fragment amplified by PCR), the sequence of 500 bp was subjected to the analysis.

[0397] 5.6: Comparison with the original sequence

The results were shown in Table 31. Terms used in the Table denote as follows.

"Fragment ID": Identification number of the target region in the PCR reaction.

"Enzyme AP": Combination of the enzyme with Pfu-PCNA 13 in the PCR reaction.

"Lambda DNA position": Position of the base sequence subjected to the analysis in seven PCR amplified fragments.

"Sample": Number of the plasmids subjected to the sequence analysis (i.e., number of the fragments amplified by PCR).

"Base": Number of the bases subjected to the sequence analysis.

"ND": Number of the bases indecipherable due to noise of sequencing data.

"ALL": Total number of the decipherable bases other than "ND".

"Error": Number of the bases identified as the replication error in the total number of the bases.

"Error rates": Rate of the number of the bases identified as the replication error "Error" relative to the number of the all deciphered bases "All".

[0398]

Table 31

Fragment ID	Enzyme, AP / Lambda DNA Position	Pyrobest DNA Polymerase				Pyrobest DNA Polymerase+Pfu-PCNA13				TaKaRa Taq			
		Sample	Base			Sample	Base			Sample	Base		
			ND	Error	All		ND	Error	All		ND	Error	All
1	18,503-19,002	93	23	1	46,477	89	15	1	44,485	92	7	32	45,993
2	19,531-20,030	63	11	0	31,489	70	36	0	34,964	83	21	22	41,479
3	24,135-24,634	58	17	0	28,983	57	9	0	28,491	58	14	8	28,986
4	24,654-25,153	80	10	3	39,990	85	19	3	42,481	77	17	16	38,483
5	26,914-27,413	100	13	0	49,987	58	40	0	28,960	60	35	10	29,965
6	40,966-41,465	73	8	0	36,492	80	28	1	39,972	82	41	12	40,959
7	430,94-43,593	125	17	0	62,483	50	2	0	24,998	86	9	20	42,991
	total	592	99	4	295,901	489	149	5	244,351	538	144	120	268,856
	Error rates (1/base)				1/73,975				1/48,870				1/2,240

[0399] In the case of Pyrobest alone, when 592 samples were analyzed, the replication errors were 4 bases in total 295,901 bases (the error rate is one base per 74 kb). In the case of Pyrobest in combination with Pfu-PCNA13, when 489 samples were analyzed, the replication errors were 5 bases in total 244,351 bases (the error rate is one base per 49 kb). In the case of Taq alone, when 538 samples were analyzed, the replication errors were 120 bases in total 268,856 bases (the error rate is one base per 2.2 kb).

[0400] Comparing among the aforementioned three test groups, in the case of Pyrobest alone and the case of Pyrobest in combination with Pfu-PCNA13, the error rate was one order lower than in the case of Taq alone. Thus, it was observed that the replication fidelity was obviously different between two groups.

[0401] Meanwhile, comparing the former two reactions (Pyrobest alone and Pyrobest in combination with Pfu-

PCNA13), the replication errors were 4 (Pyrobest alone) and 5 (Pyrobest in combination with Pfu-PCNA13), and no significant difference was detected. It is unknown whether the obvious difference occurs or not in the fidelity by adding Pfu-PCNA, but the difference is small, and it is conceivable that at least the fidelity is not extremely worsened by adding Pfu-PCNA. As shown in Examples in FIGs. 14 to 16, it is obvious that PCNA keeps the fidelity at much higher level than Taq which is the PolI type PCR enzyme while it remarkably enhances the extendibility of Pyrobest which is the PCR enzyme with high fidelity. Thus, superiority of the PCNA of the present invention was proved.

INDUSTRIAL APPLICABILITY

[0402] The present invention is useful in biotechnology related industries, particularly useful in related technology involved in DNA synthesis.

Sequence Listing Free Text

[0403]

SEQ ID NO:1 : Pfu-PCNA

SEQ ID NO:2 : Pfu-PCNA

SEQ ID NO:3 : primer Pfu-PCNA-F

SEQ ID NO:4 : primer Pfu-PCNA-R

SEQ ID NO:5 : primer Pfu_M73L-F

SEQ ID NO:6 : primer Pfu_M73L-R

SEQ ID NO:7 : primer Pfu_D143A-F

SEQ ID NO:8 : primer Pfu_D143A-R

SEQ ID NO:9 : primer Pfu_R82C-F

SEQ ID NO:10: primer Pfu_R82C-R

SEQ ID NO:11: primer Pfu_D143R-F

SEQ ID NO:12: primer Pfu_D143R-R

SEQ ID NO:13: Pfu-RFCL

SEQ ID NO:14: Pfu-RFCL

SEQ ID NO:15: primer RFCL-F primer

SEQ ID NO:16: primer RFCL-R primer

SEQ ID NO:17: Pfu-RFCS

SEQ ID NO:18: Pfu-RFCS

SEQ ID NO:19: primer RFCS-F primer

SEQ ID NO:20: primer RFCS-R primer

SEQ ID NO:21: primer RFCSF1 primer

SEQ ID NO:22: primer RFCSF2 primer

SEQ ID NO:23: primer RFCSR2 primer

SEQ ID NO:24: primer RFCSR1 primer

SEQ ID NO:25: primer F02

SEQ ID NO:26: primer F11

SEQ ID NO:27: primer F24

SEQ ID NO:28: primer R03

SEQ ID NO:29: primer R14

SEQ ID NO:30: primer R16

SEQ ID NO:31: KOD-PCNA

SEQ ID NO:32: KOD-PCNA

SEQ ID NO:33: primer KOD-PCNA-F

SEQ ID NO:34: primer KOD-PCNA-R

SEQ ID NO:35: primer KOD-_M73L-F

SEQ ID NO:36: primer KOD-_M73L-R

SEQ ID NO:37: primer KOD-_E143R-F

SEQ ID NO:38: primer KOD-_E143R-R

SEQ ID NO:39: KOD-RFCL

SEQ ID NO:40: primer KOD-RFCL-F

SEQ ID NO:41: primer KOD-RFCL-R

SEQ ID NO:42: KOD-RFCS

SEQ ID NO:43: primer KOD-RFCS-F

SEQ ID NO:44: primer KOD-RFCS-R

SEQ ID NO:45: primer RFCS-Nde-F

SEQ ID NO:46: primer RFCS-Sal-R

SEQ ID NO:47: primer RFCS-Ex2-F
SEQ ID NO:48: primer RFCS-Ex1-R
SEQ ID NO:49: primer F09
SEQ ID NO:50: primer F14
SEQ ID NO:51: primer F16
SEQ ID NO:52: primer F19
SEQ ID NO:53: primer F23
SEQ ID NO:54: primer F25
SEQ ID NO:55: primer R04
SEQ ID NO:56: primer R11
SEQ ID NO:57: primer R15
SEQ ID NO:58: primer R17
SEQ ID NO:59: primer R21
SEQ ID NO:60: primer R25
SEQ ID NO:61: primer R27
SEQ ID NO:62: primer Pfu_D143K-F
SEQ ID NO:63: primer Pfu_D143K-R
SEQ ID NO:64: primer Pfu_D143H-F
SEQ ID NO:65: primer Pfu_D143H-R
SEQ ID NO:66: primer Pfu_R109E-F
SEQ ID NO:67: primer Pfu_R109E-R
SEQ ID NO:68: primer Pfu_D147R-F
SEQ ID NO:69: primer Pfu_D147R-R
SEQ ID NO:70: primer Pfu_E139A-F
SEQ ID NO:71: primer Pfu_E139A-R
SEQ ID NO:72: primer Pfu_E139R-F
SEQ ID NO:73: primer Pfu_E139R-R

CLAIMS

1. A mutant PCNA monomer comprising a site which causes, when an N terminal region of the PCNA monomer and a C terminal region of another PCNA monomer act as an interface to form a multimeric complex, an intermolecular interaction of the monomers in an interface region of the monomers, said PCNA monomer further comprising amino acid residues in said site causing mutual charge repulsion;

said monomer itself or the multimeric complex thereof having an activity to promote DNA replication.

2. The mutant PCNA monomer according to claim 1 wherein said PCNA monomer has an amino acid sequence obtained by substituting an amino acid residue at least at one position selected from the group consisting of positions 82, 84, 109, 139, 143 and 147 in an amino acid sequence according to SEQ ID NO:2 or 32 with another amino acid residue, and

wherein amino acid residues are composed so that one or more amino acid residues selected from the group (i) and one or more amino acid residues selected from the group (ii) cause the mutual charge repulsion:

(i) a group of amino acid residues at positions 82, 84 and 109, and

(ii) a group of amino acid residues at positions 139, 143 and 147.

3. The mutant PCNA monomer according to claim 2 comprising mutations of one or several amino acid residues selected from the group consisting of an addition, an insertion, a substitution and a deletion at position(s) other than the positions 82, 84, 109, 139, 143 and 147.

4. The mutant PCNA monomer according to claim 3 having a sequence obtained by substituting the amino acid residue at position 73 with leucine in an amino acid sequence according to SEQ ID NO:2 or 32.

5. The mutant PCNA monomer according to any one of claims 2 to 4 wherein one or more amino acids selected from said group (i) and one or more amino acids selected from said group (ii) are both acidic amino acids or both basic amino acids.

6. The mutant PCNA monomer according to any one of claims 2 to 5 having the sequence obtained by substituting the amino acid residue at position 143 with arginine in the amino acid sequence according to SEQ ID NO:2 or 32.

7. A polynucleotide encoding an amino acid sequence of the PCNA monomer according to any one of claims 1 to 6.

8. A transformant in which the polynucleotide according to claim 7 has been introduced.

9. A method for producing a mutant PCNA wherein the transformant according to claim 8 is cultured in a medium and a PCNA monomer and/or a multimeric complex composed of said monomer is accumulated in said transformant and/or the medium.

10. A reagent for DNA replication comprising the PCNA monomer according to any one of claims 1 to 6 and/or a multimeric complex composed of said monomer.

11. A kit for DNA replication comprising the reagent

according to claim 10.

12. The kit for DNA replication according to claim 11 further comprising a reagent for PCR.

13. A method for replicating DNA wherein DNA synthesis is performed in the presence of the PCNA monomer according to any one of claims 1 to 6 and/or a multimeric complex composed of said monomer, and DNA polymerase.

14. The method for replicating the DNA according to claim 13 wherein said DNA synthesis is PCR.