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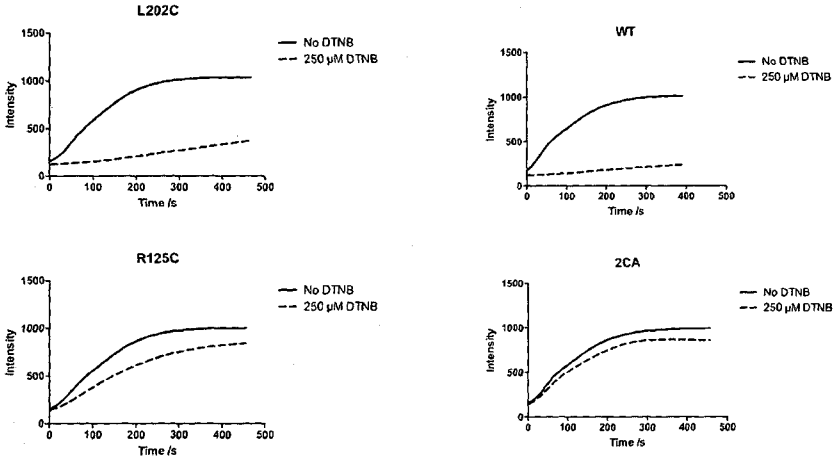
(54) Title: MODIFIED EXONUCLEASE

Figure 8



(57) Abstract: The disclosure relates to modified exonuclease enzymes and their use in DNA manipulation.

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Modified Exonuclease

Introduction

This disclosure relates to modified exonucleases which have advantageous properties and the use of said modified exonucleases in DNA metabolism and DNA manipulation.

Background to the Invention

DNA metabolism involves a number of distinct enzyme activities involved in a variety of processes related to the synthesis, degradation and function of DNA, for example, DNA replication, DNA recombination and repair, regulation of gene expression, stabilisation of chromosomes; and the segregation of chromosomes during mitosis and/or meiosis. Many DNA polymerases, apart from their DNA synthesising properties, have exonuclease activity. These activities digest DNA either in a 3'->5' direction or in a 5'->3' direction. For example the 5'->3' exonuclease domain of *E. coli* DNA polymerase I (ECPolI) has major roles in replication, DNA repair and recombination, including processing the Okazaki fragments formed on the lagging strand during DNA synthesis.

In addition, a number of 5'->3' exonuclease enzymes have been identified which are not DNA polymerases but separate enzymes. Genes encoding many prokaryotic 5'->3' exonucleases have been identified and they show a number of highly similar sequence elements between each other and with respect to DNA polymerases. This implies a conserved biochemical mechanism of action. Recently, a number of eukaryotic 5'->3' exonucleases have been purified and their sequences were shown to be similar to their prokaryotic counterparts (Leiber et al (1997) Bioessays 19, 233-40). Moreover, given the conservation in structural features of these enzymes it is not surprising that mutations in genes encoding 5'>3 exonucleases have deleterious effects on cells carrying these mutations.

This large family of enzymes possess 5'->3' exonuclease activity on duplex DNA with a free 5'-terminus, such as blunt-ended duplexes and on oligonucleotides annealed to a complimentary template. In addition, circular duplex DNA molecules containing a nick are also substrates for exonuclease activity and are converted to partially gapped or fully singled-stranded circular products.

In addition to the 5'->3' exonudease activity, many of these enzymes also display structure-specific endonudease activity. Bifurcated structures are cleaved at or close to the site of branching by the structure-specific endonudease component of the 5'->3' exonudease. Examples of 5'->3' exonucleases containing endonudease activity include, amongst others, T7 gene 6 exonudease and the DNA Pol I enzymes from *Escherichia coli* and *Thermus aquaticus* which show structure specific DNA binding and endonucleolytic cleavage of certain substrates.

Moreover, the phage Exonudease T5 exonudease is an example of a single stranded endonudease which can also process circular DNA molecules. T5 exonudease is an efficient exonudease for either single-stranded DNA or double-stranded DNA and has single-strand specific endonudease activity when used in the presence of magnesium ions which is suppressed in the presence of low magnesium allowing nicked double stranded circular DNA to be gapped to single stranded circular DNA. T5 exonudease is used in the enzymatic assembly of large DNA molecules (Gibson et al (2009) *Nature Methods* 6, 343-345), in plasmid mutagenesis, oligonucleotide site-directed mutagenesis, the creation of plasmid sequencing templates, the removal of denatured DNA from alkaline based purification and the enhancement of transfection efficiency of mini-prep plasmid cDNA libraries.

This disclosure relates to the identification of modified exonucleases which have advantageous properties when compared to native unmodified exonucleases. The modified exonudease [called "2CA_Exo"] is active, resists heavy metal [e.g. mercury] inhibition and oxidation and can be recombinantly produced in high yields. 2CA_Exo also forms the scaffold for several further single mutants in which we have introduced one cysteine at strategic positions around the molecule so as to allow immobilization. We have tested these mutants and they are active, readily expressed and can be used in immobilized form which may facilitate more convenient solid phase-based DNA protocols. We also disclose a derivative of 2CA_Exo in which two new cysteines residues have been introduced. We also disclose single mutants in T5 exonudease that may confer useful properties on the exonudease to modify its exonudease and endonudease activities which can be combined with the modifications in 2CA_Exo or alternatively used in isolation

Statements of Invention

According to a further aspect of the invention there is provided an isolated modified exonuclease enzyme comprising a modified amino acid sequence, or an active enzyme fragment thereof, wherein said modification is of the amino acid sequence in SEQ ID NO: 1 wherein said modification includes amino acid residue D155.

In a preferred embodiment of the invention amino acid residue D155 is substituted with an amino acid residue selected from the group: lysine, arginine or histidine.

In a preferred embodiment of the invention amino acid residue D155 is substituted with the amino acid lysine.

In a preferred embodiment of the invention said modified exonuclease comprises or consists of the amino acid sequence in SEQ ID NO: 2.

In a preferred embodiment of the invention said modified exonuclease comprises or consists of the amino acid sequence in SEQ ID NO: 3.

According to an aspect of the invention there is provided an isolated modified exonuclease enzyme comprising a modified amino acid sequence, or an active enzyme fragment thereof, wherein said modification is of the amino acid sequence in SEQ ID NO: 1 and includes the substitution of cysteine 115 [Cys1 15] with an amino acid selected from the group: alanine, glycine, threonine or valine and/or the substitution of cysteine 266 [Cys266] with alanine or glycine, wherein said modified exonuclease is resistant to oxidation.

In a preferred embodiment of the invention said modified exonuclease comprises substitution of Cys1 15 with alanine, glycine, threonine or valine and substitution of Cys266 with alanine or glycine.

In a preferred embodiment of the invention said modified exonuclease comprises substitution of Cys1 15 with alanine and substitution of Cys256 with alanine.

In a preferred embodiment of the invention said modified exonuclease comprises or consists of the amino acid sequence in SEQ ID NO: 4.

In a preferred embodiment of the invention said modified exonuclease comprises or consists of the amino acid sequence in SEQ ID NO: 5.

In a preferred embodiment of the invention said modified exonuclease is further modified by addition or substitution of one or more amino acids with a cysteine amino acid to facilitate immobilization to a sold phase support.

In a preferred embodiment of the invention said modified exonuclease is modified by cysteine substitution of one or more amino acid residues selected from the group consisting of: Leu202, Thr121, Arg125, Thr161, Tyr265, Glu287.

In an alternative preferred embodiment of the invention said modified exonuclease is modified by cysteine substitution of Asn85 and Asn205.

In a preferred embodiment of the invention said modified exonuclease comprises or consists of an amino acid sequence selected from the group consisting of: SEQ ID NO: 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15.

In a preferred embodiment of the invention said modified exonuclease comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 16, 17, 18 or 19.

According to an aspect of the invention there is provided a nucleic acid molecule comprising a nucleotide sequence encoding a modified exonuclease according to the invention.

According to a further aspect of the invention there is provided a modified exonuclease according to the invention coupled to a solid phase support matrix.

Solid phase supports are well known in the art. For example, Sepharose, beaded agarose or acrylamide beads which are commonly derivatized with activators that allow reaction specifically with the cysteine sulphur at around a neutral pH, pH6-8. Common activators include epichlorohydrin, iodoacetylate, epoxides & bis epoxide, vinyl sulphone or tresyl chloride derivatives. Commercially available supports include QF thiophilic resin (Amocol), Thiopropyl Sepharose 6B (GEHealthcare), UltraLink Iodoacetyl Resin (Pierce).

According to a further aspect of the invention there is provided an exonuclease according to the invention wherein said exonuclease is modified by reaction with an agent that is reactive with at least one thiol containing amino acid in said exonuclease.

In a preferred embodiment of the invention the agent comprises one or more thiol groups.

Thiol modifying agents are known in the art and are reactive towards thiols and are typically used for modifying cysteine residues in peptides and proteins. The Ellman's reagent : 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reacts with thiols by cleaving the disulphide bond in DTNB forming 2-nitro-5-thiobenzoate, whilst halogenated acetamides such as, for example, iodoacetamides ($ICH_2CONHCH_3$) are alkylating agents that form covalent bonds with cysteines. Other thiol modifying agents include 6, 6'-dithionicotinic acid, dithiodinitrobenzoic acid, N alkyl maleimides such as, for example, N-ethylmaleimide, halogenated alkane derivatives such as, for example, iodoethane, bromoethanol, iodoethanol, iodo-acetatic acid or bromo-acetic acid, and vinyl sulfones such as, for example, CH_2CHS0_3R where examples of R include methyl or methylbenzyl (e.g.$CH_2CHS0_3CH_2C_{6H5)}$. The cysteine thiol can also form disulfide bonds with many other thiols including the free amino acid cysteine, beta-mercaptoethanol and other alkyl thiols. Alkylmethanethiosulphonates, for example, 2-aminoethyl-methanethiosulfonate or methylmethanethiosulfonate react specifically and rapidly with thiols to form mixed disulfides. Other thiol modifying agents include mercury derivatives such as parahydroxymercuribenzoate and transition and heavy metal ions including manganese, mercury II compounds, cobalt (II) and lead. Details of agents which can modify proteins can be found in: Roger L. Lundblad, Chemical Reagents for Protein Modification, Third Edition and Chalker JM, Bernardes GJ, Lin YA, Davis BG. Chemical modification of proteins at cysteine: opportunities in chemistry and biology. Chem Asian J. 2009 May 4;4(5):B30-40

In a preferred embodiment of the invention said agent is 5, 5'-dithiobis-(2-nitrobenzoic acid).

In a preferred embodiment of the invention said exonuclease comprises amino acid sequences set forth in SEQ ID NO: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19.

In a preferred embodiment of the invention said exonuclease comprises the amino acid sequence set forth in SEQ ID NO: 16.

According to a further aspect of the invention there is provided a method for the modification of an exonuclease to provide a modified exonuclease with altered exonuclease or endonuclease activity comprising :

i) forming a preparation comprising an exonuclease to be modified;

ii) contacting the preparation with an agent that reacts with one or more thiol containing amino acids in said exonuclease to provide a modified exonuclease; and optionally

iii) separating the modified exonuclease from the agent.

In a preferred method of invention said agent comprises one or more thiol containing groups. Preferably, the method uses 5, 5'-dithiobis-(2-nitrobenzoic acid).

In a preferred method of the invention said exonuclease comprises amino acid sequences set forth in SEQ ID NO: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19.

In a preferred method of the invention said exonuclease comprises the amino acid sequence set forth in SEQ ID NO: 16.

According to a further aspect of the invention there is provided a vector comprising a nucleic acid according to the invention.

In a preferred embodiment of the invention said vector is an expression vector adapted for eukaryotic expression .

In an alternative preferred embodiment of the invention said vector is an expression vector adapted for prokaryotic expression.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different genetic environments or for expression in a host cell. Vectors are typically composed of DNA. Vectors include, but are not limited to, plasmids, phagemids and virus genomes. A cloning vector is one which is able to replicate in a host cell, and which typically is

further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium or just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells which have or have not been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes whose activities are detectable by standard assays known in the art (e.g., β-galactosidase, luciferase or alkaline phosphatase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques (e.g., various fluorescent proteins such as green fluorescent protein, GFP). Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

As used herein, a coding sequence and regulatory sequences are said to be "operably" joined when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual,* Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA). That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell.

According to a further aspect of the invention there is provided a eukaryotic cell transfected with a vector according to the invention.

Eukaryotic cells include CHO cells, COS cells, yeast expression systems and recombinant baculovirus expression in insect cells

According to a further aspect of the invention there is provided a prokaryotic cell according to the invention.

In a preferred embodiment of the invention said prokaryotic cell is a bacterial cell.

According to an aspect of the invention there is provided a cell culture vessel comprising a cell according to the invention.

In a preferred embodiment of the invention said cell culture vessel is a bioreactor; preferably a fermentor.

According to a further aspect of the invention there is provided a method for the recombinant manufacture of an exonuclease according to the invention comprising the steps:

i) providing a cell culture vessel comprising a cell according to the invention;

ii) providing cell culture conditions which facilitate the growth of a cell culture contained in said vessel; and optionally

iii) isolating said recombinant polypeptide from said cell or the surrounding growth medium.

In a preferred method of the invention said cell is a bacterial cell.

Bacterial cultures used in the method according to the invention are grown or cultured in

the manner with which the skilled worker is familiar, depending on the host organism. As a rule, bacteria are grown in a liquid medium comprising a carbon source, usually in the form of sugars, a nitrogen source, usually in the form of organic nitrogen sources such as yeast extract or salts such as ammonium sulfate, trace elements such as salts of iron, manganese and magnesium and, if appropriate, vitamins.

The pH of the liquid medium can either be kept constant, that is to say regulated during the culturing period, or not. The cultures can be grown batchwise, semi-batchwise or continuously. Nutrients can be provided at the beginning of the fermentation or fed in semi-continuously or continuously. The products produced can be isolated from the bacteria as described above by processes known to the skilled worker, for example by extraction, distillation, crystallization, if appropriate precipitation with salt, and/or chromatography. In this process, the pH value is advantageously kept between pH 4 and 12, preferably between pH 6 and 9, especially preferably between pH 7 and 8.

An overview of known cultivation methods can be found in the textbook by Chmiel (BioprozeRtechnik 1. Einfuhrung in die Bioverfahrenstechnik [Bioprocess technology 1. Introduction to Bioprocess technology] (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and peripheral equipment] (Vieweg Verlag, Brunswick/Wiesbaden, 1994)).

The culture medium to be used must suitably meet the requirements of the bacterial strains in question. Descriptions of culture media for various bacteria can be found in the textbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

As described above, these media which can be employed in accordance with the invention usually comprise one or more carbon sources, nitrogen sources, inorganic salts, vitamins and/or trace elements.

Preferred carbon sources are sugars, such as mono-, di- or polysaccharides. Examples of carbon sources are glucose, fructose, mannose, galactose, ribose, sorbose, ribulose, lactose, maltose, sucrose, raffinose, starch or cellulose. Sugars can also be added to the media via complex compounds such as molasses or other by-products from sugar refining. The addition of mixtures of a variety of carbon sources may also be advantageous. Other possible carbon sources are oils and fats such as, for example, soya oil, sunflower oil, peanut oil and/or coconut fat, fatty acids such as, for example, palmitic acid, stearic acid and/or linoleic acid, alcohols and/or polyalcohols such as, for

example, glycerol, methanol and/or ethanol, and/or organic acids such as, for example, acetic acid and/or lactic acid.

Nitrogen sources are usually organic or inorganic nitrogen compounds or materials comprising these compounds. Examples of nitrogen sources comprise ammonia in liquid or gaseous form or ammonium salts such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate or ammonium nitrate, nitrates, urea, amino acids or complex nitrogen sources such as cornsteep liquor, soya meal, soya protein, yeast extract, meat extract and others. The nitrogen sources can be used individually or as a mixture.

Inorganic salt compounds which may be present in the media comprise the chloride, phosphorus and sulfate salts of calcium, magnesium, sodium, cobalt, molybdenum, potassium, manganese, zinc, copper and iron.

Inorganic sulfur-containing compounds such as, for example, sulfates, sulfites, dithionites, tetrathionates, thiosulfates, sulfides, or else organic sulfur compounds such as mercaptans and thiols may be used as sources of sulfur for the production of sulfur-containing fine chemicals, in particular of methionine.

Phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium-containing salts may be used as sources of phosphorus.

Chelating agents may be added to the medium in order to keep the metal ions in solution. Particularly suitable chelating agents comprise dihydroxyphenols such as catechol or protocatechuate and organic acids such as citric acid.

The fermentation media used according to the invention for culturing bacteria usually also comprise other growth factors such as vitamins or growth promoters, which include, for example, biotin, riboflavin, thiamine, folic acid, nicotinic acid, pantothenate and pyridoxine. Growth factors and salts are frequently derived from complex media components such as yeast extract, molasses, cornsteep liquor and the like. It is moreover possible to add suitable precursors to the culture medium. The exact composition of the media compounds heavily depends on the particular experiment and is decided upon individually for each specific case. Information on the optimization of media can be found in the textbook "Applied Microbiol. Physiology, A Practical Approach" (Editors P.M. Rhodes, P.F. Stanbury, IRL Press (1997) pp. 53-73, ISBN 0 19 963577 3). Growth media can also be obtained from commercial suppliers, for example Standard 1 (Merck) or BHI (brain heart infusion, DIFCO) and the like.

All media components are sterilized, either by heat (20 min at 1.5 bar and 121°C) or by filter sterilization. The components may be sterilized either together or, if required, separately. All media components may be present at the start of the cultivation or added continuously or batchwise, as desired.

The culture temperature is normally between 15°C and 45°C, preferably at from 25°C to 40°C, and may be kept constant or may be altered during the experiment. The pH of the medium should be in the range from 5 to 8.5, preferably around 7.0. The pH for cultivation can be controlled during cultivation by adding basic compounds such as sodium hydroxide, potassium hydroxide, ammonia and aqueous ammonia or acidic compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled by employing antifoams such as, for example, fatty acid polyglycol esters. To maintain the stability of plasmids it is possible to add to the medium suitable substances having a selective effect, for example antibiotics. Aerobic conditions are maintained by introducing oxygen or oxygen-containing gas mixtures such as, for example, ambient air into the culture. The culture is continued until formation of the desired product is at a maximum. This aim is normally achieved within 10 to 160 hours.

The fermentation broth can then be processed further. The biomass may, according to requirement, be removed completely or partially from the fermentation broth by separation methods such as, for example, centrifugation, filtration, decanting or a combination of these methods or be left completely in said broth. It is advantageous to process the biomass after its separation.

However, the fermentation broth can also be thickened or concentrated without separating the cells, using known methods such as, for example, with the aid of a rotary evaporator, thin-film evaporator, falling-film evaporator, by reverse osmosis or by nanofiltration. Finally, this concentrated fermentation broth can be processed to obtain the fatty acids present therein.

According to an aspect of the invention there is provided a modified exonuclease according to the invention for use in plasmid mutagenesis.

According to an aspect of the invention there is provided a modified exonuclease according to the invention for use in site directed mutagenesis.

According to an aspect of the invention there is provided a modified exonuclease according to the invention for use in the preparation of plasmid sequencing templates.

According to an aspect of the invention there is provided a modified exonuclease according to the invention for use in plasmid preparation .

According to an aspect of the invention there is provided a modified exonuclease according to the invention for use in enhancing DNA transfection of plasmid cDNA libraries.

According to an aspect of the invention there is provided a modified exonuclease according to the invention for use in enzymatic assembly of DNA molecules, for example DNA fragments obtained by polymerase chain reaction synthesis such as genomic fragments

The simultaneously assembly of large DNA fragments without the prerequisite restriction digests creating overhangs, is referred to as the Gibson method. The Gibson method relies on T5 exonucleases which create single stranded 5' ends enabling homologous regions to anneal, which are then subsequently ligated by ligases. DNA polymerases present in the reaction are used to fill in any gaps. The whole reaction can conveniently be performed in one reaction vessel in the thermo-cycler. The Gibson method is disclosed in US patent application US20 12/0053087 and US20 10/035768, and is hereby incorporated by reference.

Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of the words, for example "comprising" and "comprises", means "including but not limited to", and is not intended to (and does not) exclude other moieties, additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be

understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

An embodiment of the invention will now be described by example only and with reference to the following figures:

Figure 1 illustrates a schematic diagram showing PCR mutagenesis protocol. Arrows represent primers as described in materials and methods. S1 and S2 represent unique restriction sites. The black rectangle represents T5 exonuclease coding sequence. The schematic is not drawn to scale. PCR 1: M13F forward primer was combined with IntR Internal Reverse primer and in a separate reaction, IntF internal Forward primer was combined with M13R (M13 Reverse). In both cases, the template DNA was pJONEXA1 9T5Exo. The resultant products A and B contain the mutated region (shown by a grey stripe) at their 3' and 5' ends respectively as shown above. In PCR 2, the purified products A and B were used as a template for PCR amplification using only the M13F and M13R primers. The resulting product C contains the mutated coding region (indicated by grey stripe) flanked by unique restriction enzyme sites S1 and S2 which were used to subclone the fragment into pJONEX4;

Figure 2 illustrates the expression and activity of T5 Exonuclease Asp1 55->Lys mutant. (A) Coomassie-stained SDS PAGE showing expression of T5 exonuclease D155K mutant. Lane M, molecular weight markers; Lane In, induced M72 cell lysate from overproduction of mutant cells. Lane Un, un-induced cell lysate. A strong band running just ahead of the 35 kDa marker can be seen in the induced sample, compared with the uninduced control. (B) Reaction of purified D155K mutant exonuclease with a 5'-overhang DNA substrate (5'-dCTCTGTCGAACACACGCXTGCGTGTGTTC-3') analyzed by denaturing PAGE. Lane C untreated oligonucleotide. The substrate was then treated with 155K mutant (Lane K) or Δ19 T5 exonuclease protein (Lane Δ19) at 2 μM;

Figure 3 illustrates a comparison of expression of Δ19_2CA and A19_2CS mutants and oxidation resistance: (A) U, uninduced cell lysate from overproduction of Δ19_2CS (double Cys->Ser mutant); I, induced cell lysate; M, Bio-Rad precision plus protein standards; Lane 1, Cell Lysate; Lane 2, mixture after sonication; Lane 3, soluble faction after sonication; Lane 4, insoluble faction dissolved in 6M urea. (B) Analysis of Δ19_2CA (double Cys->Ala mutant) expression and cell lysis. Un, uninduced sample; In, protein sample induced at 42°C; M, Fermentas Protein Molecular Weight Marker; Lane 1,

mixture after sonication; Lane 2, soluble faction after sonication. (C) Δ19_2CA purification. Lanes: Δ19, purified Δ19 as control; F1-F7, fractions of NaCl gradient during Δ19-20A purification from ion exchange column. (D) T5 exonuclease mutants at the concentration of 0.2mg/ml with (+)/ without (-) 2mM DTT were left on ice for 4 hours. M, Protein Molecular Weight Marker; 19, untreated Δ19 T5 exonuclease as marker; CA, A19_2CA_Exo; L, A19_2CA_202C; +, DTT (dithiothreitol) present; -, DTT was not present. Carrier BSA (bovine serum albumin) was included in the reactions. The band of oxidised Δ19_2CA_202C dimer can clearly be seen in lane L-, whereas L+ shows that DTT stops the oxidation as expected;

Figure 4 illustrates expression of mutant T5 exonuclease proteins. (A) show SDS-PAGE analysis of Δ19_2CA_264C expression. M shows marker lane. Total cell lysate from induced cells (Lanes In) or un-induced controls (Lanes Un). (B) Expression of Δ19_2CA_85C-205C. Lane 1, total cell lysate after sonication; Lane 2, supernatant after centrifugation showing soluble faction after sonication. (C) Panel M, Fermentas Protein Molecular Weight Marker; Lane Un, uninduced sample; In, protein sample induced at 42°C; T121C, Δ19_2CA_121C) sample; T161C, A19_2CA_161C sample; L202C, Δ19_2CA_202C sample; E287C, Δ19_2CA_287C sample. A strong band in the induced samples is present at approx. 30 kDa as indicated by the asterix;

Figure 5 illustrates T5 exonuclease mutants show activity on substrate gels. (A) Coomassie Blue stained gel showing purified nucleases; Lanes: M, Fermentas PageRuler protein ladder; Lane 1, Δ19_Exo positive control; Lane 2, A19_2CA_Exo; Lane 3, A19_2CA_85-205C, double mutant partly oxidised form; Lane 4 and 5, Δ19_2CA_85-205C double mutant reduced form; 6, A19_2CA_125C reduced form. (B) Nuclease activity gel shows same lanes as for (A) with nuclease activity demonstrated as dark bands where the enzymes have degraded high molecular weight DNA cast in the gel. The intact DNA is detected as a bright background by ethidium bromide fluorescence under UV transillumination. (C) Activity gel as for (B) showing activity of 202C mutant Lanes M, Fermentas PageRuler protein ladder; Lane 1, Δ19_2CA positive control; Lane 2 A19_2CA_202C exonuclease;

Figure 6 illustrates the reaction of exonucleases with plasmid DNA. Plasmids were treated with either Δ19, 2CA or 2CA_85-205C mutant exonucleases and analyzed by electrophoresis. Lane M, NEB 1kb DNA ladders; Lane L, linear plasmid DNA; Lane P, plasmid DNA; Lane NR, Δ19_2CA_85-205C in reduced form; Lane NO, A19_2CA_85-

205C in oxidised form; Lane CA, A19_2CA; Lane 19, Δ19; Lane CT, mock treated plasmid DNA treated without enzyme added; A + sign indicates DTT was added to the reaction while a - sign shows reaction where DTT was not added. Note that all the enzymes can remove the RFII (nicked plasmid) from the samples, however, in the reduced form A19_2CA_85-205C, this reaction is slowed down considerably; and

Figure 7 illustrates the action of immobilized T5 Exonuclease mutants on plasmid DNA. (A) Lane M, Hyper ladders I; Lane +, Δ19_2CA_264C immobilized on Sepharose beads; Lane - solution reaction of A19_2CA_264C ; Lane C, control sample which enzyme/ sepharose beads was omitted from the reaction. **(B)** Lane M, Hyper ladders **I;** Lane +, plasmid reacted with Δ19_2CA_202C immobilized on Sepharose beads; Lane -, plasmid reacted with Δ19_2CA_202C in solution; Lane C, control sample in which enzyme/ Sepharose beads was omitted from the reaction; Lane 0, control sample in which unmodified Sepharose beads only were added to the plasmid DNA. Note that all the enzymes selectively remove RFII (nicked plasmid) and the faint contaminant running below the RFI form from the samples.

Figure 8 Structure specific endonuclease activity of L202C mutant is greatly impaired when modified with DTNB. Enzymes were pre-treated with sodium dithiodinitrobenzoate (DTNB) at the final concentration of 5 mM at room temperature for 15 minutes to allow the cysteine residues to become modified. The structure-specific cleavage (endonuclease) activity of wild type and mutant T5 FENS indicated was examined using the realtime FRET assay the oligonucleotide OHP_2. Experiments were carried out with protein at the concentration of 1nM and substrate oligo OHP_2 at the final concentration at 5 nM. Reactions were carried out in duplicate with 3 replicates in HEPES-NaOH pH 7 with 10 mM Mg^{2+} as co-factor. Error estimates shown as dashed lines.

Materials and Methods

Site Directed Mutagenesis

Oligonucleotide site-directed mutagenesis was carried out on a single-stranded M13 derivative carrying the cloned T5 D15 exonuclease gene (1). The phosphorothioate-based high efficiency mutagenesis procedure (2) was used to alter wildtype codons as desired to construct the following mutants.

155Lysine Mutant

Primer Asp1 55Lys: 5'-GTTAATAAAGTATCCCATTTACCATCTGTAGATATTAG-3' was used to substitute Asp155 with a lysine residue. The mutated gene was subcloned into the expression vector pJONEX4 as described for the wild-type exonuclease gene (1, 4) using restriction enzyme digests (SacI and HindIII) to produce pJONEXT5D1 55K which encodes the T5_1 55K mutant.

The mutation was also introduced into a derivative designed to express a slightly shorter T5 exonuclease which lacks residues 2-19 of the wild-type T5 exonuclease (described in Feng, M, Patel, P, Dervan, JJ, Ceska, T, Suck, D, Haq, I & Sayers, JR Roles of divalent metal ions in flap endonuclease-substrate interactions Nature Structural & Molecular Biology 2004, 11: 450 - 456). This background is designated Δ19T5Exo. The mutated gene was cloned between the XbaI and HindIII sites of pJONEXΔ19T5Exo to give the Δ19_1 55K mutant.

SEQ ID NO: 1

YP_006958.1 **1**flap endonuclease [Enterobacteria phage T5] WILD TYPE

MSKSWGKFIEEEEAEMASRRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKG
KSVFRLEHLPEYKGMRDEKYAQRTEEEKALDEQFFEYLKDAFELCKTTFPTFTIRGVEADDMAAYIVKLI
GHLYDHVWLISTDGDWDTLLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFI SLKAIMGDLGDNIRGVE
GIGAKRGYNIIREFGNVLDIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYCVDAIAAVGQDVLDK
FTKDILEIAEQ

SEQ ID NO: 2

T5J55K

MSKSWGKFIEEEEAEMASRRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKG
KSVFRLEHLPEYKGNRDEKYAQRTEEEKALDEQFFEYLKDAFELCKTTFPTFTIRGVEADDMAAYIVKLI
GHLYDHVWLISTDGKWDTLLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVE
GIGAKRGYNIIREFGNVLDIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYCVDAIAAVGQDVLDK
FTKDILEIAEQ

SEQ ID NO: 3

A19_D155K

MRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKGKSVFRLEHLPEYKGNRDE
KYAQRTEEEKALDEQFFEYLKDAFELCKTTFPTFTIRGVEADDMAAYIVKLIGHLYDHVWLISTKGDWDT
LLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVEGIGAKRGYNI IREFGNVL
DIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYCVDAIAAVGQDVLDKFTKDILEIAEQ

2CA_Exo Mutant

A Cysteine-free construct was by converting the Cys1 15 and Cys266 codons to alanine codons with primers;

Cys1 15Ala 5'-GGGAATGTAGTTTTAGCCAACTCGAAAGCATCC-3' and

Cys266Ala 5'-CAGCAATAGCATCCACAGCGTAGGTAGGTAAATCAACC-3\

Dideoxy sequencing was used to determine that only the desired sequence changes had been introduced. A correct clone was designated as M13_2CA_Exo. The mutated gene was subcloned into the expression vector pJONEX4 as described for the wild-type exonuclease gene (1, 4) using restriction enzyme digests (SacI and HindIII) to produce pJONEXT52CA (encoding 2CA_Exo) or between the XbaI and HindIII sites of pJONEXAl 9T5Exo to give the A19_2CA_EXO mutant.

SEQ ID NO:4

2CA_Exo

MSKSWGKFIEEEEAEMASRRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKG
KSVFRLEHLPEYKGNRDEKYAQRTEEEKALDEQFFEYLKDAFELAKTTEPTFTIRGVEADDMAAYIVKLI
GHLYDHVWLISTDGDWDTLLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVE
GIGAKRGYNIIREFGNVLDIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDK
FTKDILEIAEQ

SEQ ID NO: 5

Al9_2CA_Exo

MRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKGKSVFRLEHLPEYKGNRDE
KYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLIGHLYDHVWLISTDGDWDT
LLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVEGIGAKRGYNI IREFGNVL
DIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDKFTKDILEIAEQ

Single Cysteine Mutants

Asn85Cys Arg 125Cys, Asn205Cys & Tyr264Cys in 2CA_Exo Background

The single-stranded M13_2CA_Exo DNA prepared above was used as a template for introduction of single cysteine residues using the mutagenesis procedure described above (2) in conjunction with appropriate primer as listed below:

Asn85Cys 5'-GCGTACTTTTCATCACGACAACCTTTATACTCTGG-3'

Arg 125Cys 5'-ATCGTCTGCTTCTACACCACAAATGGTAAAAGTTGG-3'

Asn205Cys 5'-CCTTCAACACCACGAATACAATCTCCTAGATCTCC-3',

Tyr264Cys 5'-CAGCAATAGCATCCACAGCGCAGGTAGGTAAATCAACC-3'

A mutant containing two cysteines was also created on the 2CA_Exo background by using Asn85Cys and Asn205Cys simultaneously in the mutagenesis procedure.

The mutated genes were subcloned into expression vectors pJONEX4 or pJONEXΔ19T5Exo as described above.

SEQ ID NO: 6
T5 2CA_85C
MSKSWGKFIEEEEAEMASRRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKG
KSVFRLEHLPEYKGCRDEKYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLI
GHLYDHVWLISTDGDWDTLLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVE
GIGAKRGYNIIREFGNVLDIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDK
FTKDILEIAEQ

SEQ ID NO: 7
A19_2CA_85C
MRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKGKSVFRLEHLPEYKGCRDE
KYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLIGHLYDHVWLISTDGDWDT
LLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVEGIGAKRGYNIIREFGNVL
DIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDKFTKDILEIAEQ

SEQ ID NO: 8
2CA_1 25C
MSKSWGKFIEEEEAEMASRRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKG
KSVFRLEHLPEYKGNRDEKYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTICGVEADDMAAYIVKLI
GHLYDHVWLISTDGDWDTLLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVE
GIGAKRGYNIIREFGNVLDIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDK
FTKDILEIAEQ

SEQ ID NO: 9
Δ19_2CA_125C
MRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKGKSVFRLEHLPEYKGNRDE
KYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTICGVEADDMAAYIVKLIGHLYDHVWLISTDGDWDT
LLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVEGIGAKRGYNI IREFGNVL
DIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDKFTKDILEIAEQ

SEQ ID NO: 10
T5 2CA_205C
MSKSWGKFIEEEEAEMASRRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKG

KSVFRLEHLPEYKGNRDEKYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLI
GHLYDHVWLISTDGDWDTLLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDCI RGVE
GIGAKRGYNIIREFGNVLDIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDK
FTKDILEIAEQ

SEQ ID NO: 11
A19_2CA_205C
MRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKGKSVFRLEHLPEYKGNRDE
KYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLIGHLYDHVWLISTDGDWDT
LLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDCIRGVEGIGAKRGYNIIREFGNVL
DIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDKFTKDILEIAEQ

SEQ ID NO: 12
T5 2CA_264C
MSKSWGKFIEEEEAEMASRRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKG
KSVFRLEHLPEYKGNRDEKYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLI
GHLYDHVWLISTDGDWDTLLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVE
GIGAKRGYNIIREFGNVLDIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTCAVDAIAAVGQDVLDK
FTKDILEIAEQ

SEQ ID NO: 13
A19_2CA_264C
MRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKGKSVFRLEHLPEYKGNRDE
KYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLIGHLYDHVWLISTDGDWDT
LLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVEGIGAKRGYNI IREFGNVL
DIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTCAVDAIAAVGQDVLDKFTKDILEIAEQ

SEQ ID NO: 14
T5 2CA_85-205C
MSKSWGKFIEEEEAEMASRRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKG
KSVFRLEHLPEYKGCRDEKYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLI
GHLYDHVWLISTDGDWDTLLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDCI RGVE
GIGAKRGYNIIREFGNVLDIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDK
FTKDILEIAEQ

SEQ ID NO: 15
A19_2CA_85-205C
MRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKGKSVFRLEHLPEYKGCRDE
KYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLIGHLYDHVWLISTDGDWDT
LLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDCIRGVEGIGAKRGYNIIREFGNVL
DIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDKFTKDILEIAEQ

Single Cysteine Mutants Thr121Cys Thr161Cys, Leu202Cys & Glu287Cys in 2CA_Exo Background

These mutants were constructed using pJONEXAl 9_2CA as template in a standard PCR-based mutagenesis protocol as shown schematically in Figure *1. M13 forward (5'-d(GTTTTCCCAGTCACGAC)-3') and M13 reverse primers (5'-d(CAGGAAACAGCTATGAC)-3') were used as external primers in conjunction with

Internal forward and Internal reverse primers:

L202C

Forward 5" ATGGGAGATTGTGGAGATAATATT3'

Reverse 5' AATATTATCTCCACAATCTCCCAT 3'

T121C

Forward 5' CATTCCCATGTTTTACCATTC3'

Reverse 5' GAATGGTAAAACATGGGAATG 3'

T161C

Forward 5' TACTTTATTATGTGATAAAGTTTCTC 3'

Reverse 5' GAGAAACTTTATCACATAATAAAGTAT 3'

E287C

Forward 5' GATATTTTGTGTATTGCAGAAC 3'

Reverse 5' GTTCTGCAATACACAAAATATC 3'

Initial PCRs were carried out under standard conditions using the appropriate Internal forward primer with M13 reverse primer and Internal reverse primer with M13 forward primer, for example L202C Forward was used with M13 Reverse while L202C Reverse was used with M13 Forward in two separate reactions using pJONEXAl 9_2CA as template. The PCR products from the initial reactions were then separated on agarose gels by electrophoresis and purified using a QIAquick Gel Extraction Kit. Appropriate products pairs were combined and used as templates for a second round of PCR and amplified using only the M13 Forward and Reverse primers. The products were again separated by agarose gel elecrophoresis and purified as above. The mutated genes were then cloned into pJONEX4 using appropriate restriction enzymes.

SEQ ID NO: 16

Δ19_2CA_202C

MRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKGKSVFRLEHLPEYKGNRDE
KYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLIGHLYDHVWLISTDGDWDT
LLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDCGDNIRGVEGIGAKRGYNIIREFGNVL
DIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDKFTKDILEIAEQ

SEQ ID NO: 17
Δ19_2CA_1 21C
MRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKGKSVFRLEHLPEYKGNRDE
KYAQRTEEEKALDEQFFEYLKDAFELAKTTFPCFTIRGVEADDMAAYIVKLIGHLYDHVWLISTDGDWDT
LLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVEGIGAKRGYNI IREFGNVL
DIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDKFTKDILEIAEQ

SEQ ID NO: 18
A19_2CA_1 61C
MRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKGKSVFRLEHLPEYKGNRDE
KYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLIGHLYDHVWLISTDGDWDT
LLCDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVEGIGAKRGYNIIREFGNVL
DIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDKFTKDILEIAEQ

SEQ ID NO: 19
A19_2CA_287C
MRNLMIVDGTNLGFRFKHNNSKKPFASSYVSTIQSLAKSYSARTTIVLGDKGKSVFRLEHLPEYKGNRDE
KYAQRTEEEKALDEQFFEYLKDAFELAKTTFPTFTIRGVEADDMAAYIVKLIGHLYDHVWLISTDGDWDT
LLTDKVSRFSFTTRREYHLRDMYEHHNVDDVEQFISLKAIMGDLGDNIRGVEGIGAKRGYNI IREFGNVL
DIIDQLPLPGKQKYIQNLNASEELLFRNLILVDLPTYAVDAIAAVGQDVLDKFTKDILCIAEQ

Protein expression and purification

The mutated genes were subcloned into the expression vector pJONEX4 as described for the wild-type exonuclease gene and transfected into the *E.coli* M72(lambda) cells as described (1, 4). This system uses a heat shock promoter to control expression of the cloned exonuclease gene. Recombinant proteins were expressed as described (1,4). The proteins were purified essentially as described (1). Protein concentration was determined using the Bradford assay (5).

SDS-PAGE substrate gel

A discontinuous SDS-PAGE gel containing 20μg/ml Type XIV DNA from herring testes in the resolving gel was prepared as described (6). The products of partial proteolysis were separated on this gel, the protein renatured *in situ* and $MgCl_2$ added to a concentration of 10 mM. After staining with ethidium bromide, exonuclease activity was

visualised as a shadow against a fluorescent background when viewed on a UV transilluminator. The gel was then Coomassie stained.

Continuous Nuclease FRET Assays

FRET assays were carried out using OHP_2 substrate (5'CY3-CTCTGTCGAACACACGCXTGCGTGTGTTC-3' where X is a fluorescein-carrying dT residue and the Cy3 dye is attached to the 5'-phosphate group). Reactions were carried out in 25mM HEPES-NaOH pH7, 100 mM KCl, 0.5 mM EDTA, 2 mM DTT, 0.1 mg/ml BSA and 10 mM $MgCl_2$. Substrate was present at 6.25nM to 250nM and cleaved by 8 nM of protein at 37°C. The data were collected at 496nm/ 519nm with HITACH F-2500 FL Spectrophotometer and analysed by standard procedures to give kcat and Km values.

Determination of Dissociation Constant

Fluorescence anisotropy measurements were performed on a DNA flap substrate composed of 5'- dACGAGCGTCTTTA annealed to 5' fluoresceinated- d AAAACGCTGTCTCGCTGAAAGCGAGA CAG CG AAAGACGCTCGT. The reaction was in 25mM HEPES-NaOH pH7, 50 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.1 mg/ml BSA and 5 mM of $CaCl_2$. DNA flap substrates were incubated with 1 nM to 150 nM protein at 37°C for 20 min. Anisotropy data were then collected at 496nm/ 519nm with a HITACH F-2500 FL Spectrophotometer.

Immobilization of single Cys mutant

Thiol activated Sepharose beads were washed with large volume water and then equilibrated with binding buffer (100 mM Tris pH8, 100 mM NaCl, 1 mM EDTA and 5% glycerol). Equal volume of 0.2 mg/ml T5 exonuclease mutants (DTT free) and equilibrated thiol beads were mixed at *4 °C* overnight. The beads were then washed with excess binding buffer to remove unbound protein.

Interaction with Plasmid DNA

Plasmid digestions were performed in 10mM Tris pH8, 10 mM KCl, 0.1 mg/ml BSA, 10 mM $MgCl_2$ and 1 mM DTT. pUC19 DNA was used as substrate in the test. Approximately 100 μg/ml of DNA was incubated with 2ng/ml (66nM) of protein at 37 °C. 18μl of samples were taken at different time points and then quenched by 6ul of 4x

quenching buffer (160 mM EDTA, 0.4% = SDS, 40 mM DTT, 40% glycerol and 0.04% bromophenol blue). All samples were loaded on 0.8% arose gel for electrophoresis.

Plasmid DNA Reaction with Immobilized Single-Cys Mutants

This was carried out using flat bottom 96-well plates on a microtitre-plate shaker. 100 µl of Sepharose-nuclease slurry was mixed with 100 µl of substrate in 10 mM Tris pH8, 10 mM KCl, 0.1 mg/ml BSA, 10 mM $MgCl_2$. pUC19 DNA was used as substrate in the test.. 18 µl of samples were taken at different time points and then quenched by 6ul of 4x quenching buffer (160 mM EDTA, 0.4% SDS, 40 mM DTT, 40% glycerol and 0.04% bromophenol blue). All samples were loaded on 0.8% arose gel for analysis by electrophoresis.

Exonuclease Activity Assay

Exonuclease activity of enzyme was determined using a spectrophotometric method. A mixture of 25 mM potassium glycinate pH9.3, 10 mM MgCl2, 100 mM KCl, 1 mM DTT and 667 µg/ml of high molecular weight herring sperm Type XIV DNA was prepared and pre-warmed at 37°C for 10 minutes before the exonuclease was added. The reaction was performed at 37°C. Aliquots of 100 µl were taken out from reaction at different time points and quenched by mixing with 100 µl of 6% HCl04 (v/v). The samples were kept on ice for 10 minutes and centrifuged at 14000 x g for 5 minutes. A 150 µl portion of supernatant was transferred to 1 ml tube and then diluted with 850 µl of H20. The absorbance of diluted sample at 260 nm was measured using UV spectrophotometer in a 1 cm path length quartz cuvette. Sample that was not incubated with exonuclease was used as blank. Released nucleotides (A260 of 1.2 corresponds to 100 nmoles of nucleotides) was plotted against time and fitted by linear regression. One unit is defined as the amount of enzyme required to release 1 nmole of acid soluble nucleotides in 30 minutes at 37°C.

Exonuclease activity of Wildtype, 2CA and 2CA-202C mutant to thiol modification

Protein at the final concentration of 0.1 mg/ml was incubated with sodium dithiodinitrobenzoate (DTNB) at the final concentration of 5 mM or 1 mM sodium para-hydroxymercuribenzoate (PHMB) at room temperature for 15 minutes to allow the cysteine residues to become modified. Specific activity of DTNB-treated Flap Endonucleases T5FEN was determined using the standard exonuclease activity assay.

Activity of protein that was not treated with PHMB was used for comparison. Data derived from 5 time points each with three or more replicates, errors indicate SEM.

Example 1

Expression and Activity of Δ19_2CA 155K

The purified 155Lys mutant showed reduced but detectable nuclease activity as shown in Figure *2.

Example 2

Expression and Activity of Δ19_2CA Exo

The Δ19_2CA_Exo was found to be in the soluble fraction after lysis of the *E. coli* cell pellet unlike the previously reported analog in which the two cysteines were replaced with serine residues (Δ19_2CS_Exo, see reference 8) Figure *3. Thus, it was purified from soluble fraction of recombinant *E. coli* after lysis in higher yield than the previously described mutant (3.125 mg/g for Δ19_2CA_EXO versus 0.425 mg /g of cell pellet for Δ19_2CS_EXO prepared analogously). In a standard nuclease assay (as described in (1)) the Δ19_2CA T5 exonuclease retained the same specific activity in the presence of 4-hydroxymercuribenzonic acid as in its absence, this is in contrast to the results obtained with the cysteine containing protein where mercury salts inactivate the enzyme completely (8). The Δ19_2CA_EXO mutant was resistant to oxidation as expected (Figure *3D)

Example 3

Single and double-cysteine mutants in 2CA background

Mutant T5 exonucleases lacking the original cysteines present in the wild-type sequence but carrying either one or two newly introduced cysteines were readily expressed (Figure *4) and found to possess nuclease activity as assayed on substrate gels (Figure *5). The DNA binding (dissociation constants, K_d -Table 1) and kinetic parameters (Table 2) of the enzymes were calculated by standard methods.

Example 4

Reaction of Mutants with Plasmid DNA

It has previously been reported that T5 exonuclease can improve plasmid transfection rates and remove contaminating, non-circularly closed DNA such as genomic fragments, linear, nicked and denatured DNA from plasmid preparations (10, 11).

Figure *6 shows that the Δ19_2CA Exo behaves similarly to the_A19 wild type when reacted with plasmid DNA, digesting contaminating bands and leaving the major band of covalently closed circular DNA intact (sssDNA or RFI). The Δ19_2CA_85-205C mutant, showed little activity in its reduced form, but the oxidised form was able to remove the band of nicked (RFII) DNA from the preparation.

Example 5

Reaction of immobilized Mutants with Plasmid DNA

The single-cysteine containing mutants can be immobilized solid supports and retain enzymatic activity. Thus, by way of example, 19_2CA_202C and A19_2CA_264C exonuclease were immobilized on Sepharose beads and their reaction with plasmid DNA was investigated. Although they reacted slowly compared with enzyme in free solution (Figure *7), both were able to degrade RFII DNA from a mixture of RFI and RFII plasmid.

Example 6

Exonuclease activity of wildtype, 2CA and 2CA-202C mutant to thiol modifying enzymes. Specific activity of DTNB-treated T5FEN was determined using the standard exonuclease activity assay. Activity of protein that was not treated with PHMB was used for comparison. Data derived from 5 time points each with three or more replicates, errors indicate SEM.

Table 1

ENZYME	Specific activity in Exonuclease Assay (unit/μg)		
	No modifying agent	DTNB	PHMB

Wild type	1453115	105+57	52+65
2CA	1386169	1671158	1640135
L202C	1320+52	1267+39	1326+71

Example 7

Is a summary of influence of thiol-modifying agent (DTNB) on exonuclease and endonuclease activity of wild type and mutant T5FEN enzymes. Activities are normalized to wild type, unmodified enzyme.

Table 2

	RELATIVE HYDROLYTIC ACTIVITY			
	Exonuclease Activity (%)		Endonuclease Activity (%)	
Enzyme	No DNTB	Modified	No DNTB	Modified
Wildtype	100	1	100	10
2CA	95	110	100	88
R125C	83	95	100	70
L202C	91	87	100	12

Table 3

Dissociation constants of Δ19 and various mutants

Protein	K_d **(nM)**	**Standard Error**
Δ19	13.4	2.7
Δ19 _2CA	13.3	1.9
Δ19 _2CAR125C	27.0	4.0

Δ19 _2CAL202C	21.3	3.2
Δ19 _2CA264C	11.1	1.9
Δ19 _2CA_85205C ^	12.8	1.8
Δ19_2CA_85-205C ¯^	6.3	0.8
Δ19_2CS	20.8	3.5
Δ19_155K	6.4	2.3

^ In the presence of reducing agent DTT
¯^ In the absence of reducing agent

Table 4
Kinetic Parameters of Δ19 and various mutants

	k_{cat} (s^{-1})	Std Error	K_m (nM)	Std Error	k_{cat}/K_m (nM^{-1} min^{-1})
Δ19	0.121	0.003	58.4	4.02	0.12
Δ19_2CA	0.05	0.002	26.6	4.33	0.113
Δ19_2CA_R125C	0.112	0.001	79.3	13.3	0.085
Δ19_2CA_L202C	ND	ND	ND	ND	0.11
Δ19_2CA_264C	ND	ND	ND	ND	0.09
Δ19 _2CA_85205C ^	ND	ND	ND	ND	0.026
Δ19_2CA_85-205C ¯^	ND	ND	ND	ND	0.04

^ In the presence of reducing agent DTT
¯^ In the absence of reducing agent
ND Not determined.

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Claims

1. A modified exonuclease enzyme comprising a modified amino acid sequence, or an active enzyme fragment thereof, wherein said modification is of the amino acid sequence in SEQ ID NO: 1 and includes the substitution of cysteine 115 [Cys1 15] with an amino acid selected from the group: alanine, glycine, threonine or valine and/or the substitution of cysteine 266 [Cys266] with alanine or glycine, wherein said modified is resistant to oxidation.

2. The exonuclease according to claim 1 wherein said modified exonuclease comprises substitution of Cys1 15 with alanine, glycine, threonine or valine and substitution of Cys266 with alanine or glycine.

3. The exonuclease according to claim 2 wherein said modified exonuclease comprises substitution of Cys1 15 with alanine and substitution of Cys266 with alanine.

4. The exonuclease according to any one of claims 1-3 wherein said modified exonuclease comprises or consists of the amino acid sequence in SEQ ID NO: 4.

5. The exonuclease according to any one of claims 1-3 wherein said modified exonuclease comprises or consists of the amino acid sequence in SEQ ID NO: 5.

6. The exonuclease according to any one of claims 1-5 wherein said modified exonuclease is further modified by addition or substitution of one or more amino acids with a cysteine amino acid.

7. The exonuclease according to claim 6 wherein In a preferred said modified exonuclease is modified by cysteine substitution of one or more amino acid residues selected from the group consisting of: Leu202, Thr1 21, Arg 125, Thr1 6 1, Tyr265, Glu287.

8. The exonuclease according to claim 7 wherein In an alternative said modified exonuclease is modified by cysteine substitution of Asn85 and Asn205.

9. The exonuclease according to any one of claims 1-5 wherein said modified exonuclease comprises or consists of an amino acid sequence selected from the group consisting of: SEQ ID NO: 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15.

10. The exonuclease according to any one of claims 1-5 wherein said modified exonuclease comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 16, 17, 18 or 19.

11. An exonuclease according to any one of claims 1-10 wherein said exonuclease is modified by reaction with an agent that is reactive with at least one thiol containing amino acid in said exonuclease.

12. The exonuclease according to claim 11 wherein the agent comprises one or more thiol groups.

13. The exonuclease according to claim 12 wherein said agent is 5,5'-dithiobis-(2-nitrobenzoic acid).

14. The exonuclease according to any one of claims 11-13 wherein said exonuclease comprises amino acid sequences set forth in SEQ ID NO: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15. 16, 17, 18 or 19.

15. The exonuclease according to claim 14 wherein said exonuclease comprises the amino acid sequence set forth in SEQ ID NO: 16.

16. A modified exonuclease enzyme comprising a modified amino acid sequence, or an active enzyme fragment thereof, wherein said modification is of the amino acid sequence in SEQ ID NO: 1 wherein said modification includes amino acid residue D155 characterized in that said modified exonuclease has retained or enhanced endonuclease activity and reduced or substantially absent exonuclease activity.

17. The exonuclease according to claim 16 wherein amino acid residue D155 is substituted with an amino acid residue selected from the group: lysine, arginine or histidine.

18. The exonuclease according to claim 17 wherein amino acid residue D155 is substituted with the amino acid lysine.

19. The exonuclease according to any one of claims 16-18 wherein said modified exonuclease comprises or consists of the amino acid sequence in SEQ ID NO: 2.

21. The exonuclease according to any one of claims 16-18 wherein said modified exonuclease comprises or consists of the amino acid sequence in SEQ ID NO: 3.

22. The exonuclease according to any one of claims 1-21 coupled to a solid phase support matrix.

23. A nucleic acid molecule comprising a nucleotide sequence encoding a modified exonuclease according to any one of claims 1-21.

24. A vector comprising a nucleic acid according to claim 23.

25. The vector according to claim 24 wherein said vector is an expression vector adapted for eukaryotic expression.

26. The vector according to claim 24 wherein said vector is an expression vector adapted for prokaryotic expression.

27. A eukaryotic cell transfected with a vector according to claim 24 or 25.

28. A prokaryotic cell transfected with a vector according to claim 24 or 26.

29. The prokaryotic cell according to claim 28 wherein said prokaryotic cell is a bacterial cell.

30. A cell culture vessel comprising a cell according to any one of claims 27-29.

31 The cell culture vessel according to claim 30 wherein said cell culture vessel is a bioreactor.

32. The cell culture vessel according to claim 31 wherein said bioreactor is a fermentor.

33. A method for the recombinant manufacture of an exonuclease comprising the steps:

i) providing a cell culture vessel comprising a cell according to any one of claims 30-32;

ii) providing cell culture conditions which facilitate the growth of a cell culture contained in said vessel; and optionally

iii) isolating said recombinant polypeptide from said cell or the surrounding growth medium.

34. The method according to claim 33 wherein said cell is a bacterial cell.

35. A modified exonuclease according to any one of claims 1-21 for use in plasmid mutagenesis.

36 A modified exonuclease according to any one of claims 1-21 for use in site directed mutagenesis.

37. A modified exonuclease according any one of claims 1-21 for use in the preparation of plasmid sequencing templates.

38. A modified exonuclease according to any one of claims 1-21 for use in plasmid preparation.

39. A modified exonuclease according to any one of claims 1-21 for use in enhancing DNA transfection of plasmid cDNA libraries.

40. A modified exonuclease according to any one of claims 1-21 for use in enzymatic assembly of DNA molecules.

41. The modified exonuclease according to claim 40 wherein said DNA molecules are genomic DNA fragments.

42. The modified exonuclease according to claim 41 wherein said exonuclease comprises an amino acid sequence set forth in SEQ ID NO: 16.

43. A method for the modification of an exonuclease to provide a modified exonuclease with altered exonuclease and/or endonuclease activity comprising :

i) forming a preparation comprising an exonuclease to be modified;

ii) contacting the preparation with an agent that reacts with one or more thiol containing amino acids in said exonuclease to provide a modified exonuclease; and optionally

iii) separating the modified exonuclease from the agent.

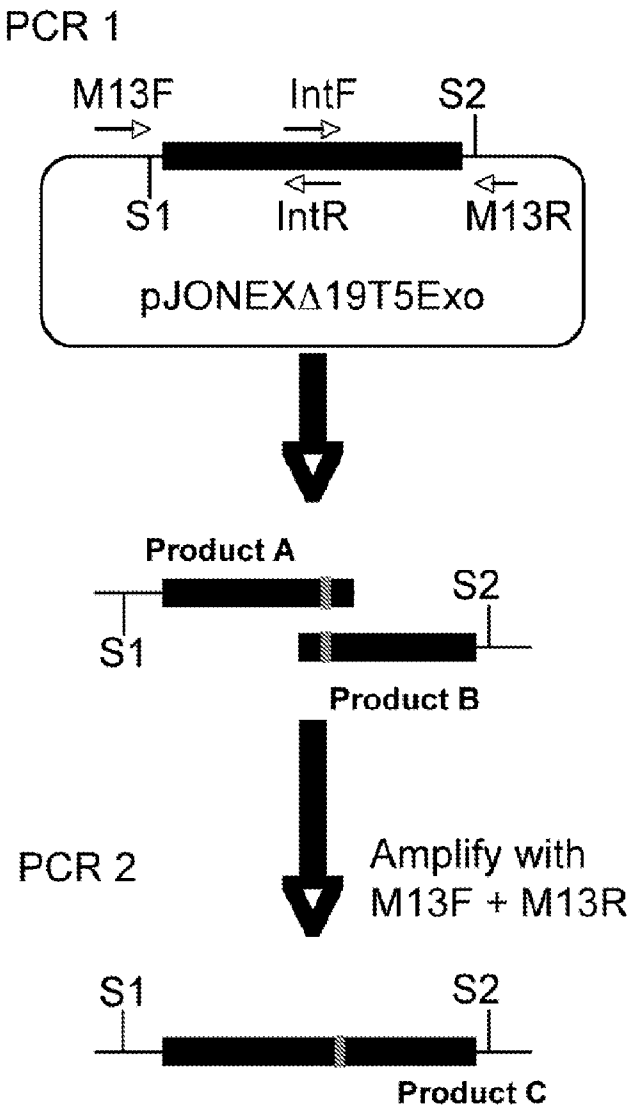
44. The method according to claim 43 wherein the agent comprises one or more thiol containing groups.

45. The method according to claim 44 wherein the method uses5,5'-dithiobis-(2-nitrobenzoic acid)

46. The method according to any one of claims 43-45 wherein said exonuclease comprises amino acid sequences set forth in SEQ ID NO: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19.

47. The method according to claim 46 wherein said exonuclease comprises the amino acid sequence set forth in SEQ ID NO: 16.

Figure 1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S2
S1
IntR
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S2
S1
Product B
PCR 2
Amplify with
M13F + M13R
S1
S2
Product C

Figure 2

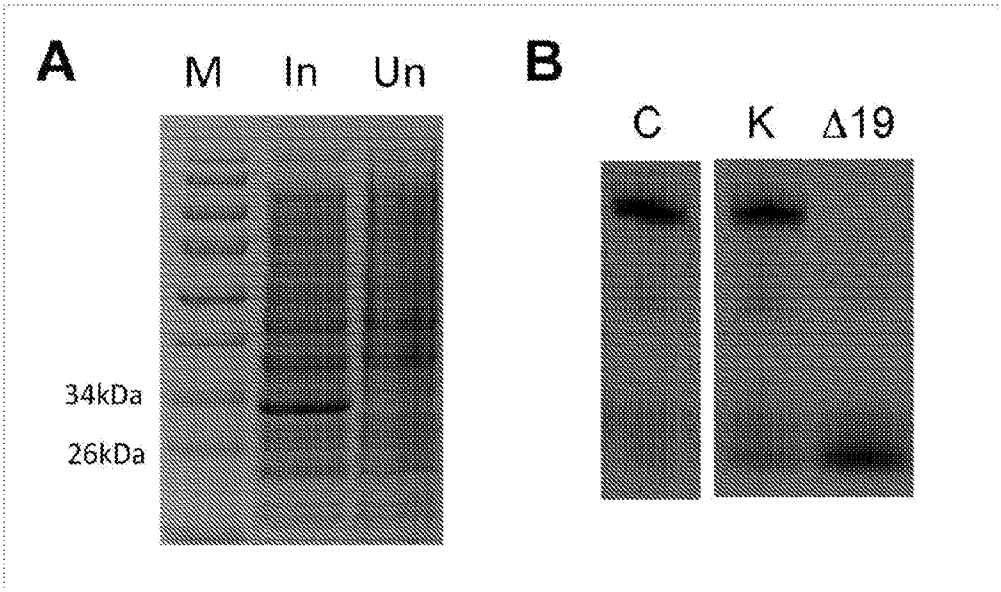


Figure 3

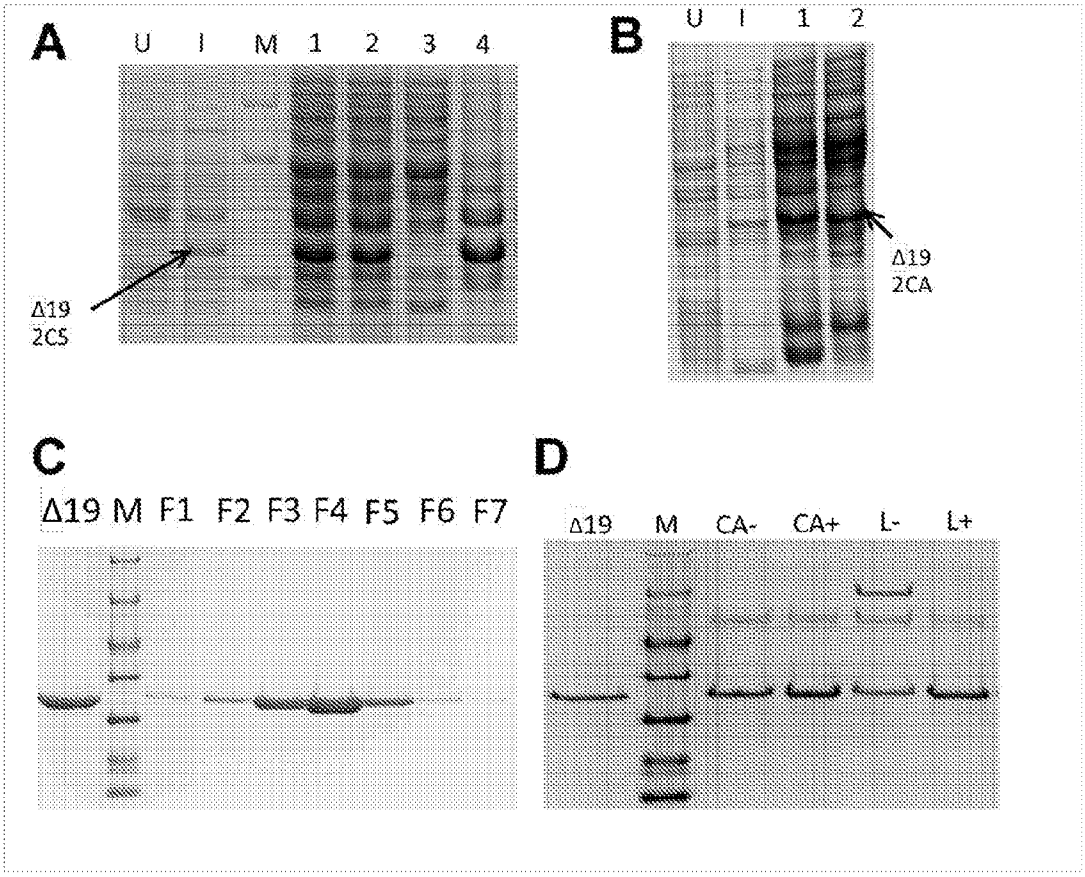
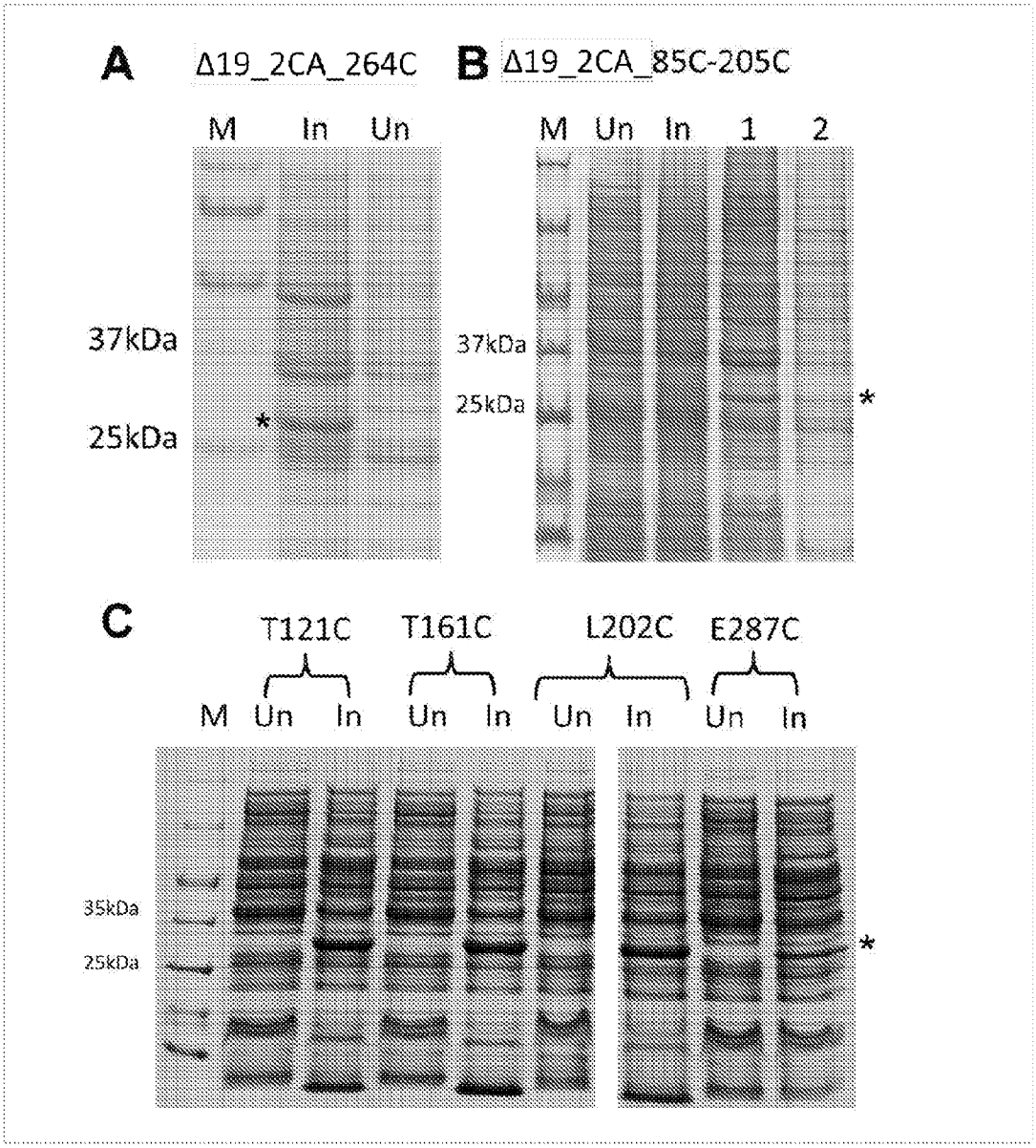


Figure 4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In
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2
37kDa
25kDa
*
C
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25kDa
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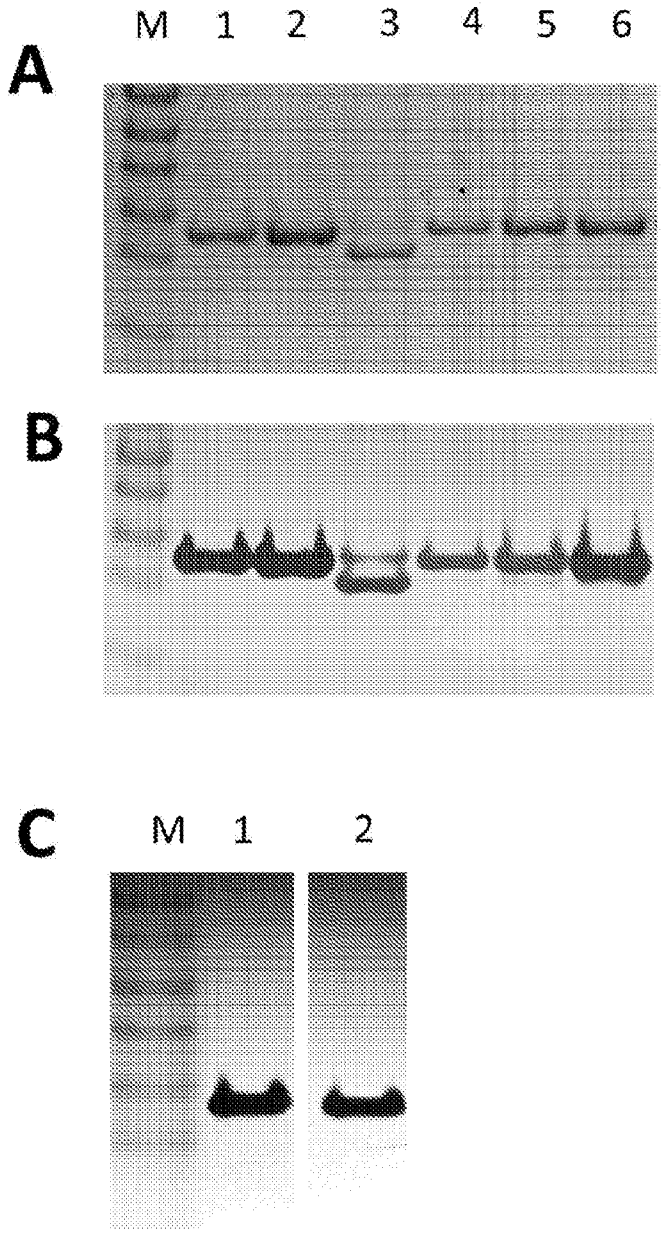
Figure 5



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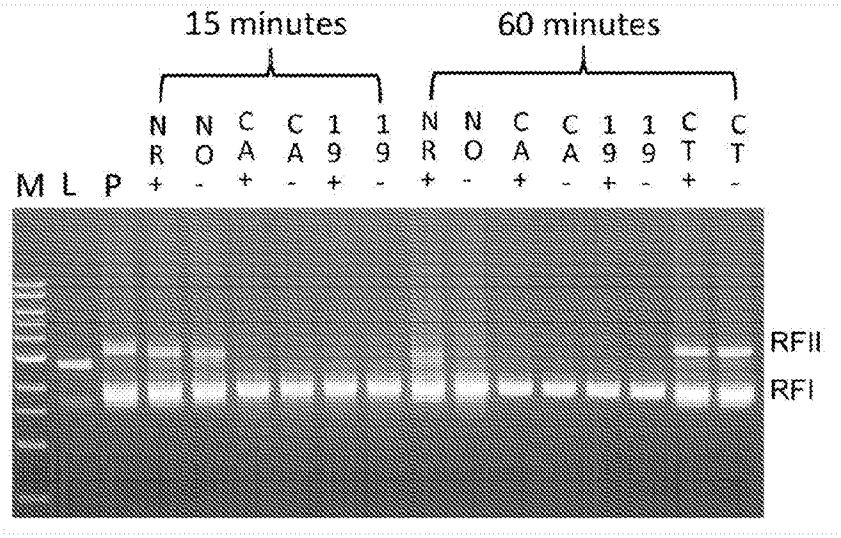
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60 minutes
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CA+
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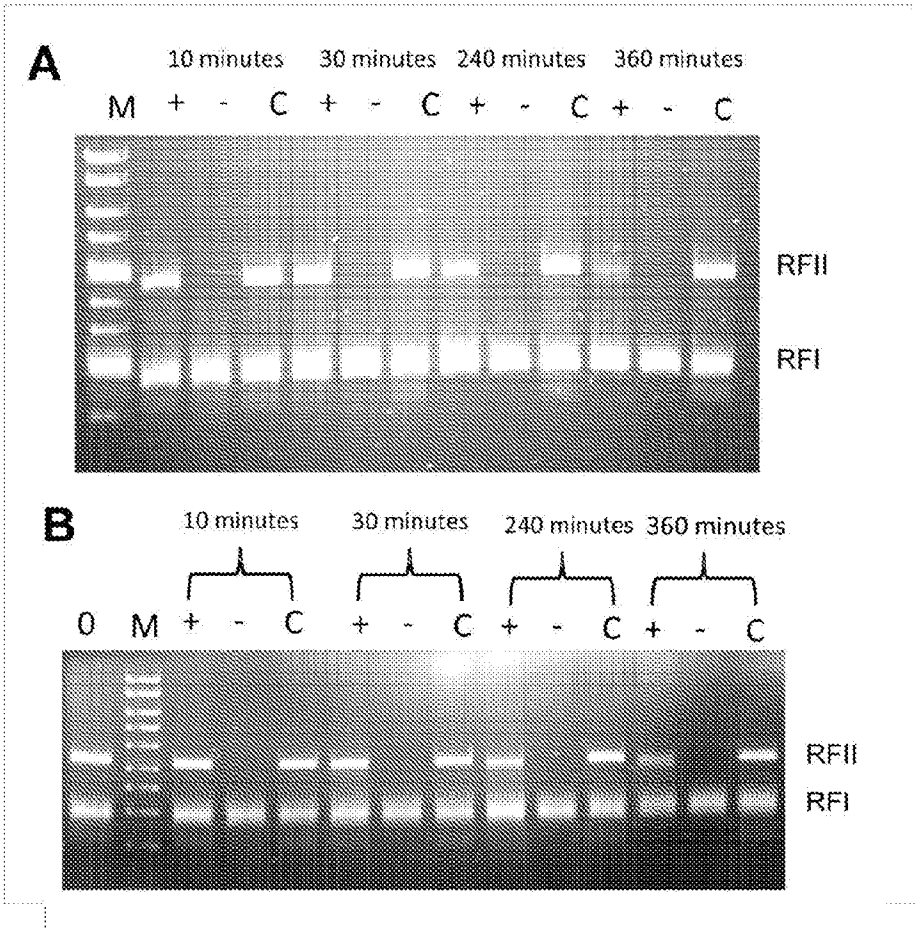
CT-
M L P
RFII
RFI

Figure 7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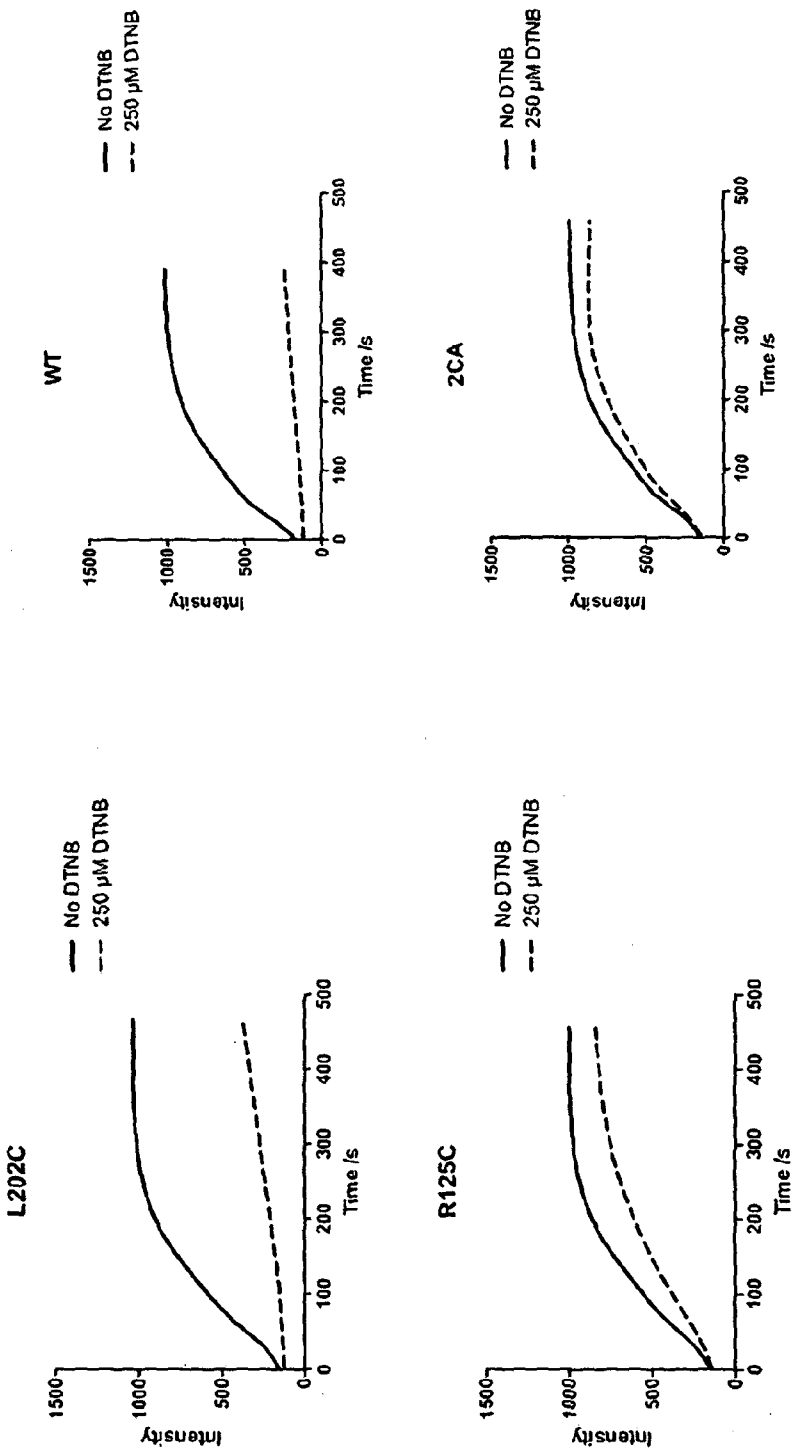
RFI

Figure 8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Intensity
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300
400
500
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1500