Annex A

1. An altered family B type archeal polymerase enzyme which is capable of improved incorporation of nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group, compared to a control family B type archeal polymerase enzyme, the polymerase comprising three amino acid substitution mutations in the motif A region,

wherein

the first amino acid of the motif A region is mutated to an amino acid selected from aromatic amino acids, amino acids with aliphatic side chains, glutamate (Q), cysteine (C) or serine (S), but excluding proline;

the second amino acid of the motif A region is mutated to an amino acid selected from alanine (A), serine (S) or glycine (G); and

the third amino acid of the motif A region is mutated to an amino acid selected from serine (S), alanine (A) or glycine (G), or amino acids having beta-branched side chains; the family B type archeal DNA polymerase is selected from Vent, Deep Vent, 9°N and Pfu polymerase;

the family B type archeal DNA polymerase lacks exonuclease activity;

each nucleotide comprises a modified nucleotide or nucleoside molecule comprising a purine or pyrimidine base and a ribose or deoxyribose sugar moiety having a removable 3' -OH blocking group covalently attached thereto, such that the 3' carbon atom has attached a group of the structure $-OCH_2N_3$;

the control family B type archeal polymerase enzyme has the amino acid sequence shown as SEQ ID NO: 15; and

the improved incorporation is an increased rate of incorporation.

2. ~~The polymerase according to claim 1 which exhibits an increased rate of incorporation of nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group, compared to the control polymerase.~~

3. The polymerase according to claim 1 ~~or 2~~ which is capable of incorporating ~~nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group~~ the nucleotides of claim 1 containing each of the four bases A, T, C and G.

4. The polymerase according to any one of claims 1 ~~to~~ or ~~3~~2 which is capable of incorporating ~~nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group~~ the nucleotides of claim 1 at a reaction temperature of 30°C.

~~54~~. The polymerase according to any one of claims 1 to ~~4~~3 which is capable of incorporating ~~nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group~~ the nucleotides of claim 1 at reaction temperatures across the full range of from 30°C to 80°C.

~~65~~. The polymerase according to any one of claims 1 to ~~5~~4 which is capable of incorporating ~~nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group~~ the nucleotides of claim 1 at a substrate concentration of about 25 micromolar.

~~7. The polymerase according to any previous claim wherein the family B type archael DNA polymerase is selected from Vent, Deep Vent, 9°N and Pfu polymerase.~~

~~86~~. The polymerase according to any previous claim ~~7~~ wherein the polymerase is a Vent™ DNA polymerase.

~~97~~. The polymerase according to any one of claims ~~7~~1 to 5 wherein the polymerase is a 9°N DNA polymerase.

~~108~~. The polymerase according to any one of claims 1 to ~~9~~7 wherein the first amino acid of the motif A region is mutated to tyrosine (Y) or phenylalanine (F).

~~119~~. The polymerase according to any one of claims 1 to ~~9~~7 wherein the first amino acid of the motif A region is mutated to isoleucine (I), alanine (A) or valine (V).

~~1210~~. The polymerase according to any one of claims 1 to ~~9~~7 wherein the first amino acid of the motif A region is mutated to serine (S).

~~1311~~. The polymerase according to claims 1-~~9~~7 wherein the second amino acid of the motif A region, is mutated to alanine (A).

~~1412~~. The polymerase according to any one of claims 1 to ~~9~~7 wherein the third amino acid of the motif A region, is mutated to an amino acid selected from isoleucine (I), threonine (T), valine (V) or leucine (L).

~~1513~~. The polymerase according to claim 1 wherein the motif A region has one of the following amino acid sequences:
YST, FAI, AAA, YAS, YAV, YGI, YSG, SGG, CST, IAL, CGG, SAL, SAA, CAA, YAA, QAS, VSS, VAG, VAV, FAV, AGI, YSS, AAT, FSS or VAL.

~~1614~~. The polymerase according to any one of claims 1 to ~~15~~13 which further comprises at least one amino acid substitution mutation in the motif B region.

~~1715~~. The polymerase according to claim ~~16~~14 wherein the polymerase comprises A485L.

~~18. The polymerase according to any one of claims 1 to 17 which lacks exonuclease activity.~~

~~1916~~. An altered 9°N polymerase comprising the amino acid sequence of any one of SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 18 or SEQ ID NO: 12.

20. An altered 9°N polymerase according to any one of claims 1 to 18, comprising the control amino acid sequence as set out in SEQ ID NO: 14 but having three changes in the amino acid sequence between residues 408 and 410 and optionally one or more changes between residues 484 and 486, wherein the altered 9°N polymerase exhibits improved incorporation of nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group as compared to the control 9°N polymerase having the amino acid sequence shown as SEQ ID NO: 14.

21. The polymerase according to any one of claims 1 to 20 wherein the nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group comprise a modified nucleotide or nucleoside molecule comprising a purine or pyrimidine base and a ribose or deoxyribose sugar moiety having a removable 3' -OH blocking group covalently attached thereto, such that the 3' carbon atom has attached a group of the structure

-O-Z

wherein Z is any of $-C(R')_2-O-R''$, $-C(R')_2-N(R'')_2$, $-C(R')_2-N(H)R''$, $-C(R')_2-S-R''$ and $-C(R')_2-F$,

wherein each R'' is or is part of a removable protecting group;

each R' is independently a hydrogen atom, an alkyl, substituted alkyl, arylalkyl, alkenyl, alkynyl, aryl, heteroaryl, heterocyclic, acyl, cyano, alkoxy, aryloxy, heteroaryloxy or amido group, or a detectable label attached through a linking group; or $(R')_2$ represents an alkylidene group of formula $=C(R''')_2$ wherein each R''' may be the same or different and is selected from the group comprising hydrogen and halogen atoms and alkyl groups; and

wherein said molecule may be reacted to yield an intermediate in which each R'' is exchanged for H or, where Z is $-C(R')_2-F$, the F is exchanged for OH, SH or NH_2, preferably OH, which intermediate dissociates under aqueous conditions to afford a molecule with a free 3' OH;

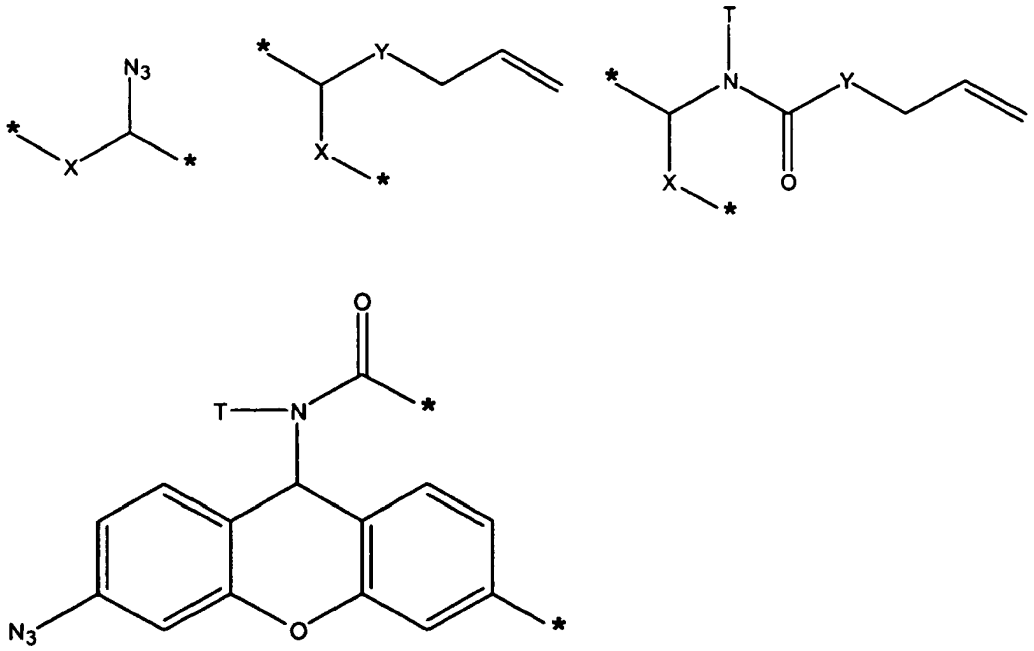
with the proviso that where Z is $-C(R')_2-S-R''$, both R' groups are not H.

22. The polymerase according to claim 21 wherein R' of the modified nucleotide or nucleoside is an alkyl or substituted alkyl.

23. The polymerase according to claim 21 wherein -Z of the modified nucleotide or nucleoside is of formula $-C(R')_2-N_3$.

24. The polymerase according to claim 23 wherein Z is an azidomethyl group.

~~25~~17. The polymerase according to any one of claims 1 to ~~20~~16 wherein the nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group comprises a nucleotide or nucleoside having a base attached to a detectable label via a cleavable linker, characterised in that the cleavable linker contains a moiety selected from the group comprising:



(wherein X is selected from the group comprising 0, S, NH and NQ wherein Q is a C1-10 substituted or unsubstituted alkyl group, Y is selected from the group comprising O, S, NH and N (allyl), T is hydrogen or a C1-10 substituted or unsubstituted alkyl group and * indicates where the moiety is connected to the remainder of the nucleotide or nucleoside).

~~26~~18. A nucleic acid molecule encoding an altered polymerase as defined in any one of claims 1 to ~~20~~16.

~~27~~19. A nucleic acid molecule encoding an altered 9°N polymerase enzyme, the nucleic acid molecule comprising one of nucleotide sequences SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 17 or SEQ ID NO: 11.

~~28~~20. An expression vector comprising the nucleic acid molecule of any of claims ~~26~~18 or ~~27~~19.

~~29~~21. A method for incorporating nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group into DNA comprising allowing the following components to interact:

- A polymerase according to any one of claims 1 to ~~25~~17

- DNA template; and

- Nucleotide solution containing the nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group.

~~30~~22. The method according to claim ~~29~~21 wherein the incorporation of the nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group is detected in order to determine the DNA sequence of the DNA template.

Annex B

1. An altered family B type archeal polymerase enzyme which is capable of improved incorporation of nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group, compared to a control family B type archeal polymerase enzyme, the polymerase comprising three amino acid substitution mutations in the motif A region,
wherein
the first amino acid of the motif A region is mutated to an amino acid selected from aromatic amino acids, amino acids with aliphatic side chains, glutamate (Q), cysteine (C) or serine (S), but excluding proline;
the second amino acid of the motif A region is mutated to an amino acid selected from alanine (A), serine (S) or glycine (G); and
the third amino acid of the motif A region is mutated to an amino acid selected from serine (S), alanine (A) or glycine (G), or amino acids having beta-branched side chains;
the family B type archeal DNA polymerase is selected from Vent, Deep Vent, 9°N and Pfu polymerase;
the family B type archeal DNA polymerase lacks exonuclease activity;
each nucleotide comprises a modified nucleotide or nucleoside molecule comprising a purine or pyrimidine base and a ribose or deoxyribose sugar moiety having a removable 3' -OH blocking group covalently attached thereto, such that the 3' carbon atom has attached a group of the structure $-OCH_2N_3$;
the control family B type archeal polymerase enzyme has the amino acid sequence shown as SEQ ID NO: 15;
the improved incorporation is an increased rate of incorporation; and
the comparison between the family B type archeal DNA polymerase enzyme and the control family B type archeal polymerase enzyme is assessed by measuring the time to 50% product conversion on the first cycle of incorporation or the second cycle of incorporation in accordance with the protocol of Example 2.

~~2. The polymerase according to claim 1 which exhibits an increased rate of incorporation of nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group, compared to the control polymerase.~~

2. The polymerase according to claim 1 wherein the comparison is based on the time to 50% product conversion on first cycle of incorporation.

3. The polymerase according to claim 1 wherein the comparison is based on the time to 50% product conversion on second cycle of incorporation.

~~34~~ The polymerase according to any one of claims 1 ~~or~~to ~~2~~3 which is capable of incorporating ~~nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group~~ the nucleotides of claim 1 containing each of the four bases A, T, C and G.

~~45~~. The polymerase according to any one of claims 1 to ~~34~~ which is capable of incorporating ~~nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group~~ the nucleotides of claim 1 at a reaction temperature of 30°C.

~~56~~. The polymerase according to any one of claims 1 to ~~45~~ which is capable of incorporating ~~nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group~~ the nucleotides of claim 1 at reaction temperatures across the full range of from 30°C to 80°C.

~~67~~. The polymerase according to any one of claims 1 to ~~56~~ which is capable of incorporating ~~nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group~~ the nucleotides of claim 1 at a substrate concentration of about 25 micromolar.

~~7. The polymerase according to any previous claim wherein the family B type archael DNA polymerase is selected from Vent, Deep Vent, 9°N and Pfu polymerase.~~

8. The polymerase according to any previous claim ~~7~~ wherein the polymerase is a Vent™ DNA polymerase.

9. The polymerase according to any one of claims ~~7~~1 to 7 wherein the polymerase is a 9°N DNA polymerase.

10. The polymerase according to any one of claims 1 to 9 wherein the first amino acid of the motif A region is mutated to tyrosine (Y) or phenylalanine (F).

11. The polymerase according to any one of claims 1 to 9 wherein the first amino acid of the motif A region is mutated to isoleucine (I), alanine (A) or valine (V).

12. The polymerase according to any one of claims 1 to 9 wherein the first amino acid of the motif A region is mutated to serine (S).

13. The polymerase according to claims 1-9 wherein the second amino acid of the motif A region, is mutated to alanine (A).

14. The polymerase according to any one of claims 1 to 9 wherein the third amino acid of the motif A region, is mutated to an amino acid selected from isoleucine (I), threonine (T), valine (V) or leucine (L).

15. The polymerase according to any one of claims 1 to 3 wherein the motif A region has one of the following amino acid sequences:

YST, FAI, AAA, YAS, YAV, YGI, YSG, SGG, CST, IAL, CGG, SAL, SAA, CAA, YAA, QAS, VSS, VAG, VAV, FAV, AGI, YSS, AAT, FSS or VAL.

16. The polymerase according to any one of claims 1 to 15 which further comprises at least one amino acid substitution mutation in the motif B region.

17. The polymerase according to claim 16 wherein the polymerase comprises A485L.

~~18. The polymerase according to any one of claims 1 to 17 which lacks exonuclease activity.~~

~~19~~18. An altered 9°N polymerase comprising the amino acid sequence of any one of SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 18 or SEQ ID NO: 12.

~~20. An altered 9°N polymerase according to any one of claims 1 to 18, comprising the control amino acid sequence as set out in SEQ ID NO: 14 but having three changes in the amino acid sequence between residues 408 and 410 and optionally one or more changes between residues 484 and 486, wherein the altered 9°N polymerase exhibits improved incorporation of nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group as compared to the control 9°N polymerase having the amino acid sequence shown as SEQ ID NO: 14.~~

~~21. The polymerase according to any one of claims 1 to 20 wherein the nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group comprise a modified nucleotide or nucleoside molecule comprising a purine or pyrimidine base and a ribose or deoxyribose sugar moiety having a removable 3' -OH blocking group covalently attached thereto, such that the 3' carbon atom has attached a group of the structure~~

~~-O-Z~~

~~wherein Z is any of $-C(R')_2-O-R''$, $-C(R')_2-N(R'')_2$, $-C(R')_2-N(H)R''$, $-C(R')_2-S-R''$ and $-C(R')_2-F$,~~

~~wherein each R'' is or is part of a removable protecting group;~~

~~each R' is independently a hydrogen atom, an alkyl, substituted alkyl, arylalkyl, alkenyl, alkynyl, aryl, heteroaryl, heterocyclic, acyl, cyano, alkoxy, aryloxy, heteroaryloxy or amido group, or a detectable label attached through a linking group; or $(R')_2$ represents an alkylidene group of formula $=C(R''')_2$ wherein each R''' may be the same or different and is selected from the group comprising hydrogen and halogen atoms and alkyl groups; and~~

~~wherein said molecule may be reacted to yield an intermediate in which each R'' is exchanged for H or, where Z is $-C(R')_2-F$, the F is exchanged for OH, SH or NH_2,~~

~~preferably OH, which intermediate dissociates under aqueous conditions to afford a molecule with a free 3' OH;~~

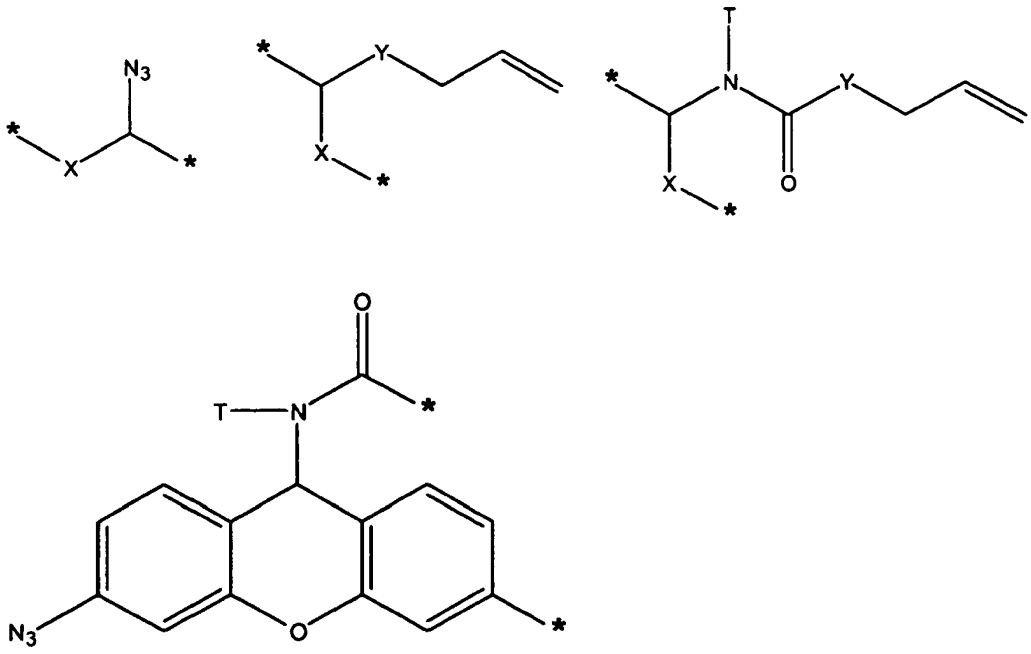
~~with the proviso that where Z is $-C(R')_2-S-R''$, both R' groups are not H.~~

~~22. The polymerase according to claim 21 wherein R' of the modified nucleotide or nucleoside is an alkyl or substituted alkyl.~~

~~23. The polymerase according to claim 21 wherein -Z of the modified nucleotide or nucleoside is of formula $-C(R')_2-N_3$.~~

~~24. The polymerase according to claim 23 wherein Z is an azidomethyl group.~~

~~25~~19. The polymerase according to any one of claims 1 to ~~20~~18 wherein the nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group comprises a nucleotide or nucleoside having a base attached to a detectable label via a cleavable linker, characterised in that the cleavable linker contains a moiety selected from the group comprising:



(wherein X is selected from the group comprising 0, S, NH and NQ wherein Q is a C1-10 substituted or unsubstituted alkyl group, Y is selected from the group comprising O, S, NH and N (allyl), T is hydrogen or a C1-10 substituted or unsubstituted alkyl group and * indicates where the moiety is connected to the remainder of the nucleotide or nucleoside).

~~26~~20. A nucleic acid molecule encoding an altered polymerase as defined in any one of claims 1 to ~~20~~18.

~~27~~21. A nucleic acid molecule encoding an altered 9°N polymerase enzyme, the nucleic acid molecule comprising one of nucleotide sequences SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 17 or SEQ ID NO: 11.

~~28~~22. An expression vector comprising the nucleic acid molecule of any of claims ~~26~~20 or ~~27~~21.

~~29~~23. A method for incorporating nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group into DNA comprising allowing the following components to interact:

- A polymerase according to any one of claims 1 to ~~25~~19
- DNA template; and
- Nucleotide solution containing the nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group.

~~30~~24. The method according to claim ~~29~~23 wherein the incorporation of the nucleotides which have been modified at the 3' sugar hydroxyl such that the substituent is larger in size than the naturally occurring 3' hydroxyl group is detected in order to determine the DNA sequence of the DNA template.