

Single Molecule Real Time (SMRT) DNA sequencing technology and an isothermal amplification method for next generation sequencing published in 2013 by Ma *et al.* (Ma, Z. *et al. Proc Natl Acad Sci U S A* 110(35): 14320-14323).

5 The current toolbox of polymerase enzymes which function well at ambient temperature is, however, very limited. Typically, different isothermal methods require reaction temperatures between 30-65°C which are mainly determined by the working range of the polymerases used in the reactions and are prone to inhibition by salt.

10 A cold-adapted polymerase from a *Psychrobacillus* sp. (PB) belonging to the A-family of DNA polymerases has been characterized. This enzyme possesses high polymerase activity at ambient temperatures but still has good stability at elevated temperatures up to 40 °C. Of particular interest, the marine derived enzyme also possesses good salt tolerance and strong strand-displacement activity (SDA) as well as proficient processivity at 25 °C, and is comparable with the state-
15 of-the-art commercial enzymes (WO 2017/162765).

In many IA methods only a polymerase is required and the effectiveness of the method is heavily dependent on the SDA of that polymerase. Therefore anything which served to increase SDA of the PB or other polymerases used in IA would be highly desirable.

20 The present inventors have surprisingly found that a single point mutation in the finger domain of certain polymerases in the A family, in particular replacement of a single Asp residue, leads to significantly enhanced SDA.

Summary

25 Therefore, in a first example, the present disclosure provides a DNA polymerase, said DNA polymerase including the sequence of SEQ ID NO. 1 or a sequence which is at least 70%, preferably at least 75%, 78%, 80%, 82%, 85%, 88%, 90%, 92% or 95%, identical thereto, but wherein the aspartic acid residue at position 18 of SEQ ID NO. 1, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue.

30 In one aspect, the present disclosure refers to a DNA polymerase including the sequence of SEQ ID NO. 1 or a sequence which is at least 70% identical thereto, but wherein the aspartic acid residue at position 18 of SEQ ID NO. 1, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue.

35 SEQ ID NO. 1 is a fragment of the amino acid sequence of the PB polymerase I, it spans amino acids 405 to 436 in the truncated (lacking the 5'- 3'-exonuclease domain) wild type PB sequence. This region (405-436) within the finger domain is highly conserved amongst some of the DNA polymerase A family (also known as pol I family), see Figures 1 and 5. DNA polymerases of the
40 disclosure would typically be classed as of the DNA polymerase I or A type.

Preferred DNA polymerases of the disclosure comprise the sequence of SEQ ID NO. 6, 7, 8, 9 or 10 but wherein the aspartic acid residue at position 18 of each sequence has been replaced by a non-negatively charged amino acid residue.

5 In some examples, the DNA polymerase of the disclosure comprises an amino acid sequence that has single or multiple amino acid alterations (additions, substitutions, insertions or deletions) compared to SEQ ID NO:1. Such sequences preferably may contain up to 8, 7 or 6, e.g. only 1, 2, 3, 4 or 5, preferably 1, 2 or 3, more preferably 1 or 2, altered amino acids in addition to the replacement of the
10 aforementioned Asp residue. Substitutions can be with conservative or non-conservative amino acids. Preferably said alterations are conservative amino acid substitutions.

A preferred polymerase of the disclosure is a modified PB polymerase, further preferred polymerases are modified polymerases from the species *Geobacillus stearothermophilus* (known as Bst), from *Bacillus subtilis* (known as Bsu), from *Bacillus smithii* (known as Bsm) and *Ureibacillus thermosphaericus*
15 (known as Ubts).

The term "DNA polymerase" refers to an enzyme which catalyses the 5'→3' synthesis of DNA from individual nucleotides, the reaction being based on primer extension and standard Watson – Crick rules of base pairing to a template strand.
20 Likewise, "DNA polymerase activity" refers to the 5'→3' synthesis of DNA from individual nucleotides, the reaction being based on primer extension and standard Watson – Crick rules of base pairing to a template strand. Enzymatically active (catalytically active) fragments of naturally occurring or modified polymerases are included within the term "DNA polymerase". The polymerase may also, but may
25 not, have 3'→5' exonuclease and/or 5'→3' exonuclease activity. Preferably the DNA polymerases of the present disclosure lack 5'→3' exonuclease activity.

The present inventors have found that replacement of the aforementioned aspartic acid residue significantly increases SDA in several different DNA polymerases in the family known as DNA polymerase A, the enzymes which may
30 benefit from modification in accordance with the present disclosure are characterised by a high sequence identity with SEQ ID NO. 1 (a particular region of the finger domain of the PB enzyme) and an aspartic acid residue at position 18 or the equivalent position in other enzymes/sequences. An "equivalent aspartic acid residue in other sequences" than SEQ ID NO. 1 (or other sequences) can be
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readily identified by using standard sequence alignment techniques such as Clustal X2 (Larkin, M.A. et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics, 23:2947-2948).

5 Of course, SEQ ID NO. 1 does not itself define a fully functional DNA polymerase. In preferred examples the DNA polymerase of the present disclosure is based on the amino acid sequence of PB DNA polymerase I, preferably which lacks the 5'-3'- exonuclease domain that is present in the wild-type *Psychrobacillus* species DNA polymerase I sequence. In preferred examples, the 5'-3'-
10 exonuclease domain is absent from the DNA polymerase enzyme as 5'-3'- exonuclease activity is typically unwanted as it may degrade primers and/or products in an amplification mixture. This truncated wild-type PB sequence is referred to herein as SEQ ID NO. 2.

The disclosure provides a DNA polymerase comprising or consisting of the amino acid sequence of SEQ ID NO:2 or an amino acid sequence which is at least
15 60% identical to SEQ ID NO:2 but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue.

In another aspect, the present disclosure refers to a DNA polymerase comprising the amino acid sequence of SEQ ID NO. 2, or an amino acid sequence
20 which is at least 60% identical to SEQ ID NO. 2, but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue.

In preferred examples and examples, the DNA polymerase of the disclosure
25 comprises (or consists of) an amino acid sequence that is at least 70%, or 75%, preferably at least 80%, 85%, 90% or 95%, e.g. at least 98% or 99% or 99.5%, identical to SEQ ID NO:2 but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue. It will be understood that
30 position 18 in SEQ ID NO. 1 and 422 in SEQ ID NO. 2 are equivalent, SEQ ID NO. 1 is a fragment from position 405 to 436 of SEQ ID NO. 2.

Figure 5 shows an alignment of PB, Bst and Upts DNA polymerases. The key Asp residue is at position 422 in each case.

In further preferred examples, the DNA polymerase of the disclosure
35 comprises (or consists of) an amino acid sequence that is at least 60%, 70% or 75%, preferably at least 80%, 85%, 90% or 95%, e.g. at least 98% or 99% or 99.5%, identical to SEQ ID NO. 11 or 12 but wherein the aspartic acid residue at position 422 of SEQ ID NO. 11 or 12, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid
40 residue. Numbering is based on a sequence alignment according to Figure 5. SEQ

ID NOs. 11 and 12 are the (truncated) variants of the wild type Bst and Ubt polymerase sequences respectively.

5 In another aspect, the present disclosure refers to a DNA polymerase comprising the amino acid sequence of SEQ ID NO. 11 or 12, or an amino acid sequence which is at least 60% identical to SEQ ID NO. 11 or 12, but wherein the aspartic acid residue at position 422 of SEQ ID NO. 11, or 12, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue.

10 Preferably, the DNA polymerase of the disclosure comprises or consists of the amino acid sequence of SEQ ID NO:2, 11 or 12 but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, 11 or 12, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue .

15 In one example, the DNA polymerase comprises (or consists of) the amino acid sequence of SEQ ID NO:4 (incorporating also the 5'→3' exonuclease domain) or a variant or fragment thereof but wherein the aspartic acid residue at position 719 of SEQ ID NO. 4, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue. The types of variants and fragments of SEQ ID NO:2 described herein apply, *mutatis mutandis*,
20 to variants and fragments of SEQ ID NO:4, e.g. variants will have at least 70% preferably at least 80% or 90% sequence identity to SEQ ID.NO:4.

DNA polymerases of the disclosure include enzymatically active fragments of native polymerases. Enzymatically active fragments are fragments that have DNA polymerase activity. Enzymatically active fragments may be at least 400, at
25 least 450, at least 475, at least 500, at least 525, at least 550, at least 560, at least 570 or at least 575 amino acids in length. Preferred fragments are at least 525, at least 550, at least 560, at least 570 or at least 575 amino acids in length. The fragments are at least 70%, preferably at least 80%, at least 85% or at least 90%, more preferably at least 95% (e.g. at least 98% or 99% or 99.5%), or 100%
30 identical to the corresponding portion of SEQ ID NO:2, 11 or 12 but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, 11 or 12, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue.

DNA polymerase activity may be assessed using a molecular beacon that
35 bears a loop structure and uses FAM as fluorescence donor and Dabcyl as an acceptor (non-fluorescent quencher) within an 8mer stem. This stem bears a 3'- extension that allows binding of a primer and acts as template for the DNA polymerase. The stem will be opened by the DNA polymerase when the extension proceeds. The following separation of the two labels is recorded by restoration of
40 FAM emission. A suitable assay of this type is described in the Examples.

The DNA polymerases of the present disclosure have good strand displacement activity. This is an important property as in many isothermal amplification methods success relies on the inherent strand displacement activity of the DNA polymerase used in the reaction setup. The term "strand displacement" describes the ability of the polymerase to displace downstream DNA encountered during synthesis.

Suitable assays to assess strand displacement activity of a DNA polymerase are known in the art and a skilled person is readily able to select a suitable assay. In an exemplary strand displacement activity assay, a "cold" primer and a reporter strand that is labelled with a fluorophore (e.g. TAMRA) at its 3' end are annealed to a template strand that has a quencher (e.g. BHQ2) at its 5' end (the fluorophore is thus quenched by the close proximity of the quencher) such that there is a one nucleotide gap between the 3' end of the annealed "cold" primer and the 5' end of the annealed reporter strand; upon strand displacement activity of the DNA polymerase the fluorophore labelled oligonucleotide (reporter strand) is displaced from the template strand and as a consequence the fluorophore and quencher are no longer in close proximity and an increase in fluorescence can be measured.

Strand displacement activity may be assessed in an assay having the steps of (i) providing a template DNA molecule that has a quencher (fluorescence quencher) at its 5' end, (ii) annealing to said template DNA molecule a cold primer (i.e. non-fluorescent oligonucleotide) and a reporter strand (reporter oligonucleotide) that is labelled with a fluorophore at its 3' end wherein there is a one nucleotide gap between the 3' end of the annealed "cold" primer and the 5' end of the annealed reporter strand, whereby the quencher quenches the fluorophore by virtue of their close proximity to each other, (iii) incubating said template-cold primer-reporter strand complex with a DNA polymerase, Me^{2+} and dNTPs and (iv) measuring the increasing fluorescence of the previously quenched fluorophore, wherein said fluorescence is indicative of strand displacement activity.

Preferred primers, reporter strands and template strands are as described in the Examples.

In a preferred example strand displacement activity (SDA) is as assessed in accordance with the strand displacement activity assay described in the Example section. SDA is preferably measured at about the optimum temperature for that polymerase. For PB and other mesophiles that may be around 25°C-37°C.

The present disclosure allows the SDA of a wild type DNA polymerase to be enhanced. In the case of PB the SDA is already high compared to most commercially available polymerases but SDA can still be significantly increased (see Fig. 3) by implementing the amino acid modification at position 18/422 described herein. For thermostable polymerases included in the disclosure, e.g. Ubts and Bst, the native SDA is quite low at ambient temperatures (25-37°C). However the SDA is still significantly enhanced at 37°C when the aspartic acid residue is replaced with a non-negatively charged amino acid residue. Different polymerases may be useful in different scenarios, e.g. Bst and Ubts are thermostable (T_m of 66°C and 62°C respectively), and so the ability to enhance SDA for any of these enzymes is very useful (see Figs. 6 and 7).

Thus, in preferred examples, the DNA polymerases of the disclosure have at least 30%, preferably at least 50%, more preferably at least 100% greater SDA than a DNA polymerase with exactly the same sequence but with aspartic acid at position 18 or 422, relative to SEQ ID NOs. 1 and 2 respectively, or at the equivalent position in other sequences. Preferably the % increase observed will at least be seen under the conditions at which each enzyme exhibits its maximum SDA. In other words, for the best that each enzyme can perform, the polymerase of the disclosure will preferably have at least 30%, more preferably at least 50%, most preferably at least 100% higher SDA than its aspartic acid containing equivalent.

The aspartic acid residue discussed above, the modification of which is key to the benefits provided by the present disclosure, is replaced by a residue without a negative charge. The replacement will typically involve substitution with another amino acid residue but in some examples the aspartic acid residue may have been modified to remove its negative charge. Thus, the residue at position 18/422 of the polymerase of the disclosure will be either neutral or positively charged. Neutral amino acids include polar amino acids and hydrophobic amino acids. Suitable replacement amino acids include Ser, Thr, Asn, Gln, Ala, Ile, Leu, Tyr, Val, Lys and Arg. Non-standard, i.e. non-genetically coded amino acids, may be incorporated which are neutral or positively charged. Ala is particularly preferred.

The inventors have also found that some of the polymerases of the disclosure exhibit improved SDA performance at elevated [NaCl] and [KCl] as compared to enzymes which contain the Asp residue discussed above (see tables 2 and 4). Enhanced salt tolerance is thus a further benefit which may be provided by the present disclosure.

Preferred DNA polymerases of the present disclosure have useful levels of polymerase activity across a range of salt (NaCl and/or KCl) concentrations. Put another way, preferred DNA polymerases of the present disclosure exhibit across a broad range of salt concentrations a substantial proportion of the DNA polymerase activity observed at the salt concentration at which maximum polymerase activity is observed. Suitable assays for determining DNA polymerase activity are described elsewhere herein. A preferred assay for determining DNA polymerase activity is as described in the Example section.

In some examples, across a concentration range from about 20mM to 200mM NaCl or KCl or a mixture thereof, DNA polymerases of the present disclosure exhibit a substantial proportion (e.g. at least 40%, preferably at least 50%, more preferably at least 60%) of their maximum polymerase activity.

In a further example the present disclosure provides molecules (e.g. proteins, such as fusion proteins) comprising DNA polymerases of the present disclosure.

As used throughout the entire application, the terms "a" and "an" are used in the sense that they mean "at least one", "at least a first", "one or more" or "a plurality" of the referenced components or steps, except in instances wherein an upper limit or exclusion is thereafter specifically stated. The operable limits and parameters of combinations, as with the amounts of any single agent, will be known to those of ordinary skill in the art in light of the present disclosure.

Nucleic acid molecules comprising nucleotide sequences that encode DNA polymerases of the present disclosure as defined herein or fragments thereof, or nucleic acid molecules substantially homologous thereto, form yet further examples of the disclosure. A preferred nucleic acid molecule is a nucleic acid encoding a DNA polymerase I of SEQ ID NO:2, or a sequence substantially homologous thereto (e.g. at least 60%, 70%, 75%, 80%, 85%, 90% or 95% identical thereto), but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue.

In another aspect, the present disclosure refers to a nucleic acid molecule comprising a nucleotide sequence that encodes a DNA polymerase as disclosed herein.

A preferred nucleic acid molecule comprises (or consists of) the nucleotide sequence as set forth in SEQ ID NO: 13, 14 or 15, or is a sequence substantially homologous thereto. Optionally, the final three nucleotides of SEQ ID NO: 13, 14 or 15 may be omitted. Nucleic acid sequences of the disclosure include sequences having at least 70% or 75%, preferably at least 80%, and even more preferably at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or 99.5%, sequence identity to SEQ

ID NO: 13, 14 or 15. Nucleic acid sequences of the disclosure thus include single or multiple base alterations (additions, substitutions, insertions or deletions) to the sequence of SEQ ID NO: 13, 14 or 15.

5 A particularly preferred nucleic acid molecule comprises or consists of the nucleotide sequence as set forth in SEQ ID NO: 13, 14 or 15.

The present disclosure also extends to nucleic acid molecules comprising (or consisting of) nucleotide sequences which are degenerate versions of nucleic acid molecules described herein, e.g. degenerate versions of a nucleic acid molecule comprising (or consisting of) SEQ ID NO: 13, 14 or 15.

10 Nucleic acid molecules of the disclosure are preferably "isolated" or "purified".

Homology (e.g. sequence identity) may be assessed by any convenient method. However, for determining the degree of homology (e.g. identity) between sequences, computer programs that make multiple alignments of sequences are
15 useful, for instance Clustal W (Thompson, Higgins, Gibson, *Nucleic Acids Res.*, 22:4673-4680, 1994). If desired, the Clustal W algorithm can be used together with BLOSUM 62 scoring matrix (Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA*, 89:10915-10919, 1992) and a gap opening penalty of 10 and gap extension penalty of 0.1, so that the highest order match is obtained between two sequences
20 wherein at least 50% of the total length of one of the sequences is involved in the alignment. Clustal X is a convenient windows interface for Clustal W (Thompson, J.D. et al (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25:4876-4882).

25 Other methods that may be used to align sequences are the alignment method of Needleman and Wunsch (Needleman and Wunsch, *J. Mol. Biol.*, 48:443, 1970) as revised by Smith and Waterman (Smith and Waterman, *Adv. Appl. Math.*, 2:482, 1981) so that the highest order match is obtained between the two sequences and the number of identical amino acids is determined between the two
30 sequences. Other methods to calculate the percentage identity between two amino acid sequences are generally art recognized and include, for example, those described by Carillo and Lipton (Carillo and Lipton, *SIAM J. Applied Math.*, 48:1073, 1988) and those described in Computational Molecular Biology, Lesk, e.d. Oxford University Press, New York, 1988, Biocomputing: Informatics and Genomics
35 Projects.

Generally, computer programs will be employed for such calculations.

Programs that compare and align pairs of sequences, like ALIGN (Myers and Miller, *CABIOS*, 4:11-17, 1988), FASTA (Pearson and Lipman, *Proc. Natl. Acad. Sci. USA*, 85:2444-2448, 1988; Pearson, *Methods in Enzymology*, 183:63-98, 1990) and
5 gapped BLAST (Altschul *et al.*, *Nucleic Acids Res.*, 25:3389-3402, 1997), BLASTP, BLASTN, or GCG (Devereux, Haeberli, Smithies, *Nucleic Acids Res.*, 12:387, 1984) are also useful for this purpose. Furthermore, the Dali server at the European Bioinformatics institute offers structure-based alignments of protein sequences (Holm, *Trends in Biochemical Sciences*, 20:478-480, 1995; Holm, *J. Mol. Biol.*, 233:123-38, 1993; Holm, *Nucleic Acid Res.*, 26:316-9, 1998).
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By way of providing a reference point, sequences according to the present disclosure having 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99%, sequence identity etc. may be determined using the ALIGN program with default parameters (for instance available on Internet at the GENESTREAM network
15 server, IGH, Montpellier, France).

A "conservative amino acid substitution", as used herein, is one in which the amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art.
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DNA polymerases of the present disclosure comprise genetically encoded amino acids, but may also contain one or more non-genetically encoded amino acids.

When used in connection with a protein or polypeptide molecule such as a DNA polymerase, the term "isolated" or "purified" typically refers to a protein
25 substantially free of cellular material or other proteins from the source from which it is derived. In some examples, such isolated or purified proteins are substantially free of culture medium when produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized.

In one further example the present disclosure provides an expression vector (preferably a recombinant expression vector) containing a nucleic acid molecule of the disclosure, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the protein sequence encoded by the nucleic acid molecule of the disclosure.
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In another aspect, the present disclosure refers to an expression vector containing a nucleic acid molecule as disclosed herein and the necessary regulatory sequences for the transcription and translation of the protein sequence encoded by said nucleic acid molecule.
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Possible expression vectors include but are not limited to cosmids or plasmids, so long as the vector is compatible with the host cell used. The
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expression vectors are "suitable for transformation of a host cell", which means that the expression vectors contain a nucleic acid molecule of the disclosure and regulatory sequences selected on the basis of the host cells to be used for expression, which are operatively linked to the nucleic acid molecule. Operatively
5 linked is intended to mean that the nucleic acid is linked to regulatory sequences in a manner that allows expression of the nucleic acid.

Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes and are well known in the art. Selection of appropriate regulatory sequences is dependent on the host cell
10 chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of
15 replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector.

The recombinant expression vectors of the disclosure may also contain a selectable marker gene that facilitates the selection of host cells transformed or transfected with a recombinant molecule of the disclosure.

20 The recombinant expression vectors may also contain genes that encode a fusion moiety that provides increased expression of the recombinant protein; increased solubility of the recombinant protein and/or aids in the purification of the target recombinant protein by acting as a ligand in affinity purification (for example appropriate "tags" to enable purification and/or identification may be present, e.g.,
25 His tags or myc tags).

Recombinant expression vectors can be introduced into host cells to produce a transformed host cell. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g., a vector) into a cell by one of many possible techniques known in
30 the art. Suitable methods for transforming and transfecting host cells can be found in Sambrook *et al.*, 1989 (Sambrook, Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989) and other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic host cells and eukaryotic cells. Preferably, proteins of the disclosure may be expressed in bacterial host cells, such as *Escherichia coli*.

5 N-terminal or C-terminal fusion proteins comprising DNA polymerases and proteins of the disclosure conjugated to other molecules, such as proteins (e.g. epitope tags), may be prepared by fusing through recombinant techniques.

A yet further example provides a host cell or virus comprising one or more expression constructs or expression vectors of the disclosure. Also provided are host cells or viruses comprising one or more of the nucleic acid molecules of the
10 disclosure. A host cell or virus capable of expressing a DNA polymerase of the disclosure forms a yet further example. Preferred host cells include Rosetta 2 (DE3) cells (Novagen).

In another aspect, the present disclosure refers to a host cell or virus comprising one or more expression vectors as disclosed herein or one or more
15 nucleic acid molecules as disclosed herein.

DNA polymerases of the disclosure may be produced recombinantly in a host cell and isolated and purified therefrom. The DNA polymerases of the disclosure may therefore be considered recombinant enzymes, in particular isolated recombinant enzymes. In certain examples the DNA polymerase is produced by
20 recombinant techniques in a host cell that is not, or not from, an organism which is the same as that from which the DNA polymerase was derived.

DNA polymerases of the present disclosure may be generated using recombinant DNA technology. Alternatively, a cell-free expression system can be used for production of the DNA polymerase. Alternatively, DNA polymerases of the
25 present disclosure may be generated using chemical synthesis so that the DNA polymerase is generated by stepwise elongation, one amino acid at a time. Such chemical synthesis techniques (e.g. solid phase synthesis) are well known in the chemistry of proteins.

A further example of the disclosure provides a method of producing a DNA
30 polymerase of the present disclosure comprising a step of culturing the host cells of the disclosure. Preferred methods comprise the steps of (i) culturing a host cell comprising one or more of the recombinant expression vectors or one or more of the nucleic acid molecules of the disclosure under conditions suitable for the expression of the encoded DNA polymerase or protein; and optionally (ii) isolating
35 or obtaining the DNA polymerase or protein from the host cell or from the growth medium/supernatant. Such methods of production may also comprise a step of purification of the DNA polymerase or protein product and/or formulating the DNA

polymerase or product into a composition including at least one additional component, such as an acceptable buffer or carrier.

In another aspect, the present disclosure refers to a method of producing a DNA polymerase as disclosed herein, wherein the method comprises the steps of:

- 5 (i) culturing a host cell comprising one or more of the recombinant expression vectors as disclosed herein or one or more of the nucleic acid molecules as disclosed herein under conditions suitable for the expression of the encoded DNA polymerase; and optionally
- 10 (ii) isolating or obtaining the DNA polymerase from the host cell or from the growth medium or supernatant.

The DNA polymerase may be separated, or isolated, from the host cells/culture media using any of the purification techniques for protein known in the art and widely described in the literature or any combination thereof. Such techniques may include for example, precipitation, ultrafiltration, dialysis, various chromatographic techniques, e.g. size exclusion chromatography, ion-exchange chromatography, affinity chromatography, electrophoresis, centrifugation etc. As discussed above, the DNA polymerase of the disclosure may be modified to carry amino acid motifs or other protein or non-protein tags, e.g. polyhistidine tags (e.g. His₆-tag), to assist in isolation, solubilisation and/or purification or identification.

20 In another example, the present disclosure provides the use of a DNA polymerase of the disclosure for nucleotide (e.g. dNTP) polymerisation. Accordingly, DNA polymerases of the disclosure may be used to extend a nucleic acid (DNA) strand by one or more nucleotides.

25 In another aspect, the present disclosure refers to use of a DNA polymerase as disclosed herein for nucleotide polymerization.

In another example, the present disclosure provides the use of a DNA polymerase of the disclosure in a nucleic acid (DNA) amplification or sequencing reaction.

30 In another aspect, the present disclosure refers to use of a DNA polymerase as disclosed herein in a nucleic acid amplification or sequencing reaction.

In another example, the present disclosure provides the use of a DNA polymerase of the disclosure in a molecular beacon assay or in a strand displacement assay, e.g. as described herein.

35 Preferably, in uses and methods of the present disclosure, DNA polymerases of the present disclosure are used at a constant temperature, i.e. without thermal cycling. Accordingly, the use of DNA polymerases of the disclosure in isothermal reactions is particularly preferred.

40 The use of DNA polymerases of the disclosure in isothermal amplification reactions is particularly preferred. Isothermal reactions are performed at a constant temperature. Many isothermal amplification techniques are known in the art and include Loop mediated isothermal amplification (LAMP), rolling circle amplification (RCA), strand displacement amplification (SDA), multiple displacement amplification (MDA) and cross priming amplification (CPA).

45 In another example, the present disclosure provides a method of nucleotide polymerisation using a DNA polymerase of the present disclosure. Preferably, said method comprises providing a reaction mixture comprising a DNA polymerase of the present disclosure, a template nucleic acid molecule, an oligonucleotide primer

which is capable of annealing to a portion of the template nucleic acid molecule and one or more species of nucleotide (e.g. deoxynucleoside triphosphates, dNTPs) and incubating said reaction mixture under conditions whereby the oligonucleotide primer anneals to the template nucleic acid molecule and said DNA polymerase extends said oligonucleotide primer by polymerising one or more nucleotides. Suitable conditions are well known in the art. Preferably a constant temperature is used and preferred temperatures are set out elsewhere herein. Optionally, the generation of the polynucleotide product is detected (e.g. via gel electrophoresis).

In another aspect, the present disclosure refers to a method of nucleotide polymerisation using a DNA polymerase as disclosed herein, said method comprising:

(i) providing a reaction mixture comprising a DNA polymerase as disclosed herein, a template nucleic acid molecule, an oligonucleotide primer which is capable of annealing to a portion of the template nucleic acid molecule and one or more species of nucleotide; and

(ii) incubating said reaction mixture under conditions whereby the oligonucleotide primer anneals to the template nucleic acid molecule and said DNA polymerase extends said oligonucleotide primer by polymerising one or more nucleotides.

In another example, the present disclosure provides a method of amplifying a nucleic acid (DNA) using a DNA polymerase of the present disclosure. Typically, said method comprises providing a reaction mixture comprising a DNA polymerase of the present disclosure, a template nucleic acid molecule, an oligonucleotide primer(s) (e.g. 2 or more primers such as 2, 3, 4, 5 or 6 primers) which is capable of annealing to a portion of the template nucleic acid molecule, and nucleotides (e.g. deoxynucleoside triphosphates, dNTPs) and incubating said reaction mixture under conditions whereby the oligonucleotide primer(s) anneals to the template nucleic acid molecule and said DNA polymerase extends said oligonucleotide primer(s) by polymerising one or more nucleotides to generate a polynucleotide. Suitable conditions are well known in the art. Preferred methods of nucleic acid amplification are isothermal amplification methods. Isothermal amplification methods of the disclosure are performed at a constant temperature and preferred temperatures are set out elsewhere herein. Optionally, the generation of the polynucleotide product is detected (e.g. via gel electrophoresis).

In another aspect, the present disclosure refers to a method of amplifying a nucleic acid using a DNA polymerase as disclosed herein, said method comprising:

5 (i) providing a reaction mixture comprising a DNA polymerase as disclosed herein, a template nucleic acid molecule, an oligonucleotide primer(s) which is capable of annealing to a portion of the template nucleic acid molecule, and nucleotides; and

10 (ii) incubating said reaction mixture under conditions whereby the oligonucleotide primer(s) anneals to the template nucleic acid molecule and said DNA polymerase extends said oligonucleotide primer(s) by polymerising one or more nucleotides to generate a polynucleotide.

Exemplary isothermal amplification methods include Loop mediated isothermal amplification (LAMP), rolling circle amplification (RCA), strand displacement amplification (SDA), multiple displacement amplification (MDA) and cross priming amplification (CPA).

15 In some examples, particularly those using DNA polymerases based on the PB sequence, the constant temperature used in the methods and uses of the present disclosure is a low-to-moderate temperature, for example, is chosen from within the range 0°C to about 42°C, preferably is chosen from within the range about 10°C to about 40°C, or about 20°C to about 40°C, or about 25°C to about
20 40°C, or about 30°C to about 40°C or about 35°C to about 40°C, or about 37°C to about 40°C. In some examples, the constant temperature is chosen from within the range about 10°C to about 15°C, or about 10°C to about 20°C. In some

examples, the constant temperature is chosen from within the range about 10°C to about 30°C. In some examples, the constant temperature is chosen from within the range about 20°C to about 30°C. In some examples, the constant temperature is chosen from within the range about 10°C to about 25°C. In some
5 examples, the constant temperature is chosen from within the range about 20°C to about 25°C. A constant temperature of about 25°C is preferred. In some examples, the constant temperature is 25°C.

With other polymerases of the disclosure, for example those based on sequences from organisms which are thermophilic, the constant temperature may
10 be moderate to high, e.g. is chosen from within the range 25°C-65°C, preferably 40°C-65°C.

A temperature may be considered constant when no active steps are taken to modify the temperature during the reaction, e.g. no thermal cycling. A 'constant' temperature may still allow temperature fluctuations during the method e.g. of up to
15 about 5°C, typically no more than 3°C or 2°C.

DNA polymerases of the present disclosure may be used in point-of-care molecular diagnostics platforms.

DNA polymerases of the present disclosure may be used in whole genome amplification.

20 DNA polymerases of the present disclosure may be used in next-generation sequencing methods. So-called "next generation" or "second generation" sequencing approaches (in reference to the Sanger dideoxynucleotide method as the "first generation" approach) have become widespread. These newer techniques are characterised by high throughputs, e.g. as a consequence of the use of parallel,
25 e.g. massively parallel sequencing reactions, or through less time-consuming steps. Various high throughput sequencing methods provide single molecule sequencing and employ techniques such as pyrosequencing, reversible terminator sequencing, cleavable probe sequencing by ligation, non-cleavable probe sequencing by ligation, DNA nanoballs, and real-time single molecule sequencing.

30 References herein to DNA polymerases of the disclosure encompass active fragments unless otherwise clear from the context.

Uses and methods of the present disclosure are typically performed *in vitro*.

The present disclosure also provides compositions comprising a DNA polymerase of the disclosure. Such compositions preferably comprise a buffer.
35 Optionally, compositions of the present disclosure further comprise one or more of

the necessary reagents to carry out a nucleic acid amplification reaction (e.g. an isothermal amplification reaction), e.g. oligonucleotide primers capable of annealing to a region of the template DNA to be amplified and/or nucleotides (e.g. dNTPs). Typically compositions will be aqueous and buffered with a standard buffer such as Tris, HEPES, etc.

In another aspect, the present disclosure refers to a composition comprising a DNA polymerase as disclosed herein and a buffer.

The disclosure further includes kits comprising one or more of the DNA polymerases of the disclosure, or one or more compositions of the disclosure, or one or more of the nucleic acid molecules of the disclosure, or one or more expression vectors of the disclosure, or one or more host cells or viruses of the disclosure. Preferably said kits are for use in the methods and uses as described herein, e.g., in nucleic acid amplification methods, such as isothermal amplification reactions. Preferably said kits comprise instructions for use of the kit components, for example for nucleic acid amplification.