PHI29 DNA POLYMERASE MUTANTS WITH IMPROVED PRIMER RECOGNITION

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of the priority date of U.S. Provisional Application 62/849,252, filed May 17, 2019, the contents of which are incorporated herein in their entirely.

SEQUENCE LISTING

[0002] This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

BACKGROUND

[0003] Phi29 DNA polymerase (Phi29 DNApol) is a monomeric enzyme (66 kDa) in charge of replicating the bacteriophage genome (19285 bp) by catalyzing both protein-primed initiation at both ends of the linear dsDNA molecule, and full elongation of each DNA strand (Blanco and Salas, 1984; 1985). Phi29 DNApol belongs to family B of DNA polymerases (Bernad et al, 1987), showing the common right-hand fold containing the palm, thumb and finger subdomains, but also two additional domains called TPR1 and TPR2 (Rodriguez et al, 2005; Kamtekar et al, 2006; Berman et al, 2007). Phi29 DNApol shows unique properties that enable its application in numerous DNA amplification and DNA sequencing technologies and platforms: highly processive DNA synthesis, enabling the enzyme to incorporate more than 70000 nucleotides per DNA binding event in the absence of processivity factors (Blanco et al, 1989); exceptional strand-displacement, which allows polymerization coupled to the unwinding of double-stranded DNA, in the absence of helicase-type enzymes (Blanco et al, 1989); high fidelity of synthesis, with very low error insertion rates (10^{-4} to 10^{-6}) and efficient proofreading of inserted errors, which collectively enhance fidelity up to one error in 10^6 to 10^8 nucleotides incorporated (Esteban et al, 1993 and 1994).

[0004] These properties make Phi29 DNApol the best choice for isothermal multiple displacement amplification (MDA) (Dean et al, 2002) and rolling circle amplification (RCA) (Lizardi et al, 1998). These DNA amplification technologies are based on the combination of Phi29 DNApol with either random synthetic primers (RPs), mainly hexanucleotides or hexamers, or a DNA primase capable of synthesizing DNA primers *in situ* during the reaction (Picher et al, 2016).

[0005] Amplification of DNA is frequently required for the current sequencing technologies, since the amount of DNA available from certain samples (e.g., single cells) is not enough for the sequencing process. Unfortunately, DNA amplification has the risk of introducing errors, generating asymmetries (bias), and even promoting co-amplification of minute levels of contaminating DNA. Therefore, key parameters determining the quality of the amplification are the absence of contaminations and artefacts in the reaction products, coverage breadth and uniformity, low nucleotide error rates, and the ability to recover single nucleotide variants (SNVs), copy number variants (CNVs) and structural variants.

[0006] A source for potential amplification bias in the current MDA methods based on random hexamers is the priming inequality arising from different sequence-dependent hybridization kinetics of the oligonucleotides. Even more important is the propensity to generate primer-derived input-independent DNA amplification artefacts, caused by the exponential amplification of self-pairing hexamers.

[0007] It has been shown that the use of longer primers instead of hexamers with a reaction temperature of 40°C decreases DNA amplification artefacts significantly (Alsmadi et al, 2009). The most likely reason behind this behavior is that the higher temperature reduces the likelihood of stable self-pairing of primers and therefore their subsequent amplification. However, to carry out the amplification reaction at a temperature as high as 40°C (10°C above the temperature optimum of Phi29 DNApol) thermostable or thermoresistant Phi29 DNApol variants are required. In this regard, some mutated Phi29 DNApols have been described to show improved thermostability (Povilaitis et al, 2016).

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] The accompanying drawings, which are incorporated herein and form a part of the specification, illustrate exemplary embodiments and, together with the description, further serve to enable a person skilled in the pertinent art to make and use these embodiments and others that will be apparent to those skilled in the art. The invention will be more particularly described in conjunction with the following drawings wherein:

[0009] **Figure 1.** 3D-structure of Phi29 DNApol complexed with DNA and dNTP (PDB id: 2PYL). Most of the protein is indicated in white, with the exception of THUMB (dark green), TPR2 (cyan) and TPR1 (yellow) subdomains. The N-terminal 3´-5´ exonuclease domain is not fully depicted (only two segments are indicated (yellow), one containing Arg^{96}, and the other containing Lys^{64}). Primer strand (cyan), shows numbers corresponding to each nucleotide position. Template strand (light green), incoming nucleotide (magenta) and the two activating metal ions (beige) are also indicated.

[00010] **Figure 2. A)** Schematic view of the wild-type (WT) Phi29 DNApol amino acid residues involved in interaction with the first 10 nucleotide of the primer strand, as derived from the crystal structure (PDB id: 2PYL). Nucleotide numbered as 1 is the most 3´-terminal, frequently described as "primer terminus", and the one closest to the enzyme active site. **B)** The scheme shows the gain of novel interactions with the primer strand, originated by the different mutations indicated (magenta). The colored arrows indicate if the interaction involves the phosphodiester (red), the sugar (orange) or the base (green). Mutants T499K and T499R are predicted to interact with the same positions of the complementary/template strand (indicated with dashed-line arrows).

[00011] **Figure 3.** Amplification efficiency of WT Phi29 DNApol or the designed mutants, in combination with TthPrimPol or random primers of different lengths: trimers (3N), tetramers (4N), pentamers (5N), hexamers (6N), heptamers (7N) or octamers (8N), using 1 ng of human genomic DNA as input in the reaction.

[00012] **Figure 4.** Balance of exonuclease and polymerase activities of WT Phi29 DNApol, mutant K64R, mutant M97K and double mutant K64R / M97K, as a function of the deoxynucleotides (dNTP) concentration provided.

[00013] **Figure 5.** Amplification efficiency of WT Phi29 DNApol, mutant K64R, mutant M97K and double mutant K64R / M97K, in combination with random primers of different lengths: trimers (3N), tetramers (4N), pentamers (5N), hexamers (6N), heptamers (7N) or octamers (8N), using 1 ng of human genomic DNA as input in the reaction. For each primer length N the columns are shown in the following order from left to right: WT Phi29 DNApol, mutant K64R, mutant M97K double mutant K64R / M97K.

[00014] **Figure 6.** Amplification yield observed in the absence of input DNA with either WT Phi29 DNApol, mutant K64R, mutant M97K or double mutant K64R / M97K, in combination with random synthetic primers of different sizes: trimers (3N), tetramers (4N), pentamers (5N), hexamers (6N), heptamers (7N) or octamers (8N), under low ionic strength conditions. For each primer length N the columns are shown in the following order from left to right: WT Phi29 DNApol, mutant K64R, mutant M97K double mutant K64R / M97K.

[00015] **Figure 7.** Amplification yield observed in the absence of input DNA with either WT Phi29 DNApol, mutant K64R, mutant M97K or double mutant K64R / M97K, in combination with random synthetic primers of different sizes: trimers (3N), tetramers (4N), pentamers (5N), hexamers (6N), heptamers (7N) or octamers (8N), under high ionic strength conditions. For each primer length N the columns are shown in the following order from left to right: WT Phi29 DNApol, mutant K64R, mutant M97K double mutant K64R / M97K.

[00016] **Figure 8.** Amplification efficiency of WT Phi29 DNApol, mutant K64R, mutant M97K and double mutant K64R / M97K, in combination with random primers of different lengths: trimers (3N), tetramers (4N), pentamers (5N), hexamers (6N), heptamers (7N) or octamers (8N), using 1 ng of human genomic DNA as input in the reaction, under high ionic strength conditions. For each primer length N the columns are shown in the following order from left to right: WT Phi29 DNApol, mutant K64R, mutant M97K double mutant K64R / M97K.

[00017] **Figure 9.** Amplification efficiency of WT Phi29 DNApol, mutant K64R, mutant M97K and double mutant K64R / M97K, in combination with random primers of different lengths (tetramers (4N), pentamers (5N), hexamers (6N)), using different human genomic DNA inputs (1, 10, 100 pg and 1 ng) in the reaction, under low and high ionic strength conditions. For each primer length N the columns are shown in the following order from left to right: WT Phi29 DNApol, mutant K64R, mutant M97K double mutant K64R / M97K.

[00018] **Figure 10.** Amplification of 1, 10, 100 pg and 1 ng of human genomic DNA by multiple displacement amplification (MDA) combining Phi29 DNApol variants and *Tth*PrimPol. For each amount of DNA the columns are shown in the following order from left to right: WT Phi29 DNApol, mutant K64R, mutant M97K double mutant K64R / M97K.

[00019] **Figure 11.** Estimated coverage values obtained from the CovCheck analysis of amplification reactions using hexamers (6N), pentamers (5N) and tetramers (4N), in combination with Phi29 DNApol variants and different human genomic DNA inputs in each case to reach conditions in which coverage differences can be observed.

[00020] **Figure 12.** Estimated coverage values obtained from the CovCheck analysis of amplification reactions carried out by *Tth*PrimPol in combination with Phi29 DNApol variants.

SUMMARY

[00021] Modified DNA polymerases can be useful in multiple applications like DNA sequencing, DNA amplification, library preparation, DNA genotyping, etc. The present invention provides recombinant Phi29 DNA polymerases including mutations that confer improved properties, particularly desirable for these or other applications. These amino acid sequence changes can improve performance in multiple displacement DNA amplification (MDA) by using shorter random synthetic primers, which results in reduced amplification artefacts, better sequence-dependent hybridization kinetics, and therefore, resulting in an improved coverage breadth and uniformity. “Phi29” is sometimes written “ϕ29”.

[00022] The recombinant Phi29 DNA polymerase comprises one or two mutations from the group consisting of K64R and M97K.

DETAILED DESCRIPTION

I. Definitions

[00023] "Isolated" means a molecule is the predominant species present, i.e., on a molar basis, more abundant than any other individual macromolecular species in the composition. Generally, an isolated molecule can comprise more than 80%, more than 90%, more than 95%, more than 98%, or more than 99% of the macromolecular species present in the composition is the purified species of interest. Solvent species, small molecules (<500 Daltons), stabilizers (e.g., BSA), and elemental ion species are not considered macromolecular species for purposes of this definition.

[00024] As used herein, the term "recombinant nucleic acid" refers nucleic acid molecule comprising two or more attached nucleotide sequences not normally attached to each other in nature.

[00025] As used herein, the term "recombinant cell" refers to a cell, e.g., an animal, plant, fungal or microbial (e.g., bacterial) cell, that comprises a recombinant nucleic acid.

[00026] Terms used to describe sequence relationships between two or more nucleotide sequences or amino acid sequences include "reference sequence," "selected from," "comparison window," "identical," "percentage of sequence identity," "substantially identical," "complementary," and "substantially complementary."

[00027] A "reference sequence" is a defined sequence used as a basis for a sequence comparison and may be a subset of a larger sequence, e.g., a complete cDNA, protein, or gene sequence.

[00028] Because two nucleic acids or polypeptides each may comprise (1) a sequence (i.e., only a portion of the complete nucleic acid or polypeptide sequence) that is similar between the two nucleic acids, or (2) a sequence that is divergent between the two nucleic acids, sequence comparisons between two (or more) nucleic acids or polypeptides are typically performed by comparing sequences of the two nucleic acids over a "comparison window" to identify and compare local regions of sequence similarity.

[00029] A "comparison window" refers to a conceptual segment of typically at least 12 consecutive nucleotides or 4 consecutive amino acid residues that is compared to a reference sequence. The comparison window frequently has a length of at least 15 or at least 25 nucleotides or at least 5 or at least 8 amino acids. The comparison window may comprise additions or deletions (i.e., gaps) of about 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window

may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI) or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by any of the various methods is selected.

[00030] A subject nucleotide sequence or amino acid sequence is "identical" to a reference sequence if the two sequences are the same when aligned for maximum correspondence over the length of the nucleotide or amino acid sequence.

[00031] The "percentage of sequence identity" between two sequences is calculated by comparing two optimally aligned sequences over a comparison window, determining the number of positions at which the identical nucleotide or amino acid occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

[00032] Unless otherwise specified, the comparison window used to compare two sequences is the length of the shorter sequence.

[00033] Methods are described further in Natl. Acad. Sci. USA 85:2444; Higgins & Sharp (1988) Gene 73:237-244; Higgins & Sharp, CABIOS 5:151-153 (1989); Corpet et al. (1988) Nucleic Acids Research 16:10881-90; Huang et al. (1992) Computer Applications in the Biosciences 8:155-65; and Pearson et al. (1994) Methods in Molecular Biology 24:307-31. Alignment is also often performed by inspection and manual alignment.

[00034] A subject nucleotide sequence or amino acid sequence is "substantially identical" to a reference sequence if the subject amino acid sequence or nucleotide sequence has at least 80% sequence identity over a comparison window. Thus, sequences that have at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, at least 98% sequence identity or at least 99% sequence identity with the reference sequence are also "substantially identical". Two sequences that are identical to each other are, of course, also "substantially identical".

[00035] As used herein, the term "transcription regulatory sequence" refers to a first nucleotide sequence that regulates transcription of a second nucleotide sequence to which it is operatively linked.

[00036] As used herein, a nucleotide sequence is "operatively linked" with a transcription regulatory sequence when the transcription regulatory sequence functions in a cell to

regulate transcription of the nucleotide sequence. This includes promoting transcription of the nucleotide sequence through an interaction between a polymerase and a promoter.

[00037] A "promoter" is a transcription regulatory sequence at least sufficient to promote the transcription of a nucleotide sequence in DNA into an RNA transcript. A transcript transcribed from a promoter typically includes sequences from the promoter downstream of the transcription start site, as well as downstream sequences that, in the case of mRNA, encode an amino acid sequence. Promoters are the best-characterized transcriptional regulatory sequences because of their predictable location immediately upstream of transcription start sites. Promoters include sequences that modulate the recognition, binding and transcription initiation activity of the RNA polymerase. These sequences can be cis acting or can be responsive to trans acting factors. Promoters, depending upon the nature of the regulation, can be constitutive or regulated. They are often described as having two separate segments: core and extended promoter regions.

[00038] The core promoter includes sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. The core promoter includes the transcriptional start site, an RNA polymerase binding site, and other general transcription binding sites and is where the pre-initiation complex forms and the general transcription machinery assembles. The pre-initiation complex is generally within 50 nucleotides (nt) of the transcription start site (TSS).

[00039] The core promoter also includes a sequence for a ribosome binding site, necessary for translation of an mRNA into a polypeptide.

[00040] The extended promoter region includes the so-called proximal promoter, which extends to about 250 nucleotides upstream of the transcriptional start site (i.e., -250 nt). It includes primary regulatory elements such as specific transcription factor binding sites. It has been found that many genes have transcription regulatory elements located further up-stream. In particular, a fragment that includes most of the transcription regulatory elements of a gene can extend up to 700 nt or more up-stream of the transcription start site. In certain genes, transcription regulatory sequences have been found thousands of nucleotides upstream of the transcriptional start site.

[00041] As used herein, a first nucleotide sequence is "heterologous" to a second nucleotide sequence if the first nucleotide sequence is not attached, e.g., operatively linked, with the second nucleotide sequence in nature. By extension, a polypeptide is "heterologous" to transcription regulatory sequence if it is encoded by a nucleotide sequence heterologous the transcription regulatory sequence.

[00042] As used herein, the term "allelic variant" refers to a naturally occurring variation of a gene.

[00043] As used herein, the term "artificial variant" refers to a gene or protein comprising one or more genetic modifications to a naturally occurring gene or protein.

[00044] As used herein, the term "mutation" as used herein, generally refers to an alteration, variant or polymorphism in a nucleotide sequence compared with wildtype. Such alteration, variant or polymorphism can be with respect to a reference genome, eg., in a genomic database. Mutations include, without limitation, single nucleotide variations (SNVs), substitutions, insertions or deletions (also referred to collectively as "indels"), and repeats.

II. Introduction

[00045] A novel strategy to reduce amplification artefacts and amplification biases derived from sequence-dependent hybridization kinetics could take advantage of using DNA primers shorter than the current gold-standard hexamers. That strategy, which requires obtaining Phi29 DNApol variants able to recognize, stably bind and efficiently use shorter DNA primers, would allow to improve current DNA amplification technologies significantly.

[00046] The availability of the 3D-structure of Phi29 DNApol complexed with DNA and incoming nucleotide (Berman et al, 2007) allowed us to perform a detailed inspection of the amino acid residues directly involved in interactions with the primer strand (Fig. 1). These ligands of the primer strand (see the scheme at Fig. 2A) are:

- **R96** (interacts with the phosphodiester between nucleotide 7 and 8 of the primer).
- **R306** (interacts with the phosphodiester between nucleotide 8 and 9 of the primer).
- **R308** (interacts with the phosphodiester between nucleotide 9 and 10 of the primer).
- **K498** (interacts with the sugar of the first 3' nucleotide of the primer).
- **Y500** (interacts with the phosphodiester between nucleotide 1 and 2 of the primer).
- **K529** (interacts with the phosphodiester between nucleotide 1 and 2 of the primer).

[00047] Based on these few contacts, Phi29 DNApol establishes direct interactions which span the first 10 bases of the primer strand, suggesting that such a size would confer the maximal binding stability to the primer. It is quite surprising that there is a lack of contacts in the interval between nucleotides 3 and 6. Strikingly, current MDA procedures with Phi29 DNApol are based on the provision of random hexamers, which will be poorly stabilized just

by contacts with the phosphodiester bond between the two first nucleotides, and with the base of the 3'-terminal nucleotide. Therefore, hexamers do not have an optimal size to be used as initial primers to be bound and extended by Phi29 DNApol. Very likely, these sub-optimal primers were selected to have complements in any DNA sample at sufficiently short intervals to enable efficient and even amplification, while minimizing self-hybridization artefacts, known as primer-dimers.

[00048] On the other hand, the alternative TruePrime DNA amplification technology (Picher et al, 2016) takes advantage of a DNA primase (*Tth*PrimPol) to synthesize the DNA primers on demand, but it has not been established which is the optimal primer size that is delivered by *Tth*PrimPol to Phi29 DNApol, and what is the fate of those primers that remain shorter than the minimum size required for optimal elongation by Phi29 DNApol.

[00049] Based on this information and caveats, we explored the possibility of generating Phi29 DNApol mutants (inventive variants) with an improved affinity for short primers, ideally in the limit between 4 and 6 nucleotides. For this purpose, we followed two different approaches: 1) reinforcing some existing interactions, 2) creating new (non-existing) enzyme:DNA ligands in the primer region.

[00050] Such an improved variants are expected to be valuable in RPs-based MDA procedures, likely reducing primer-dimer artefacts, and the formation of amplification chimeras. Additionally, in the context of the TruePrime DNA amplification technology, the use of short primers that could be generated by *Tth*PrimPol, could increase the efficiency of amplification, and/or lead to an improved coverage.

[00051] Again, a detailed analysis of the 3D-structure of Phi29 DNApol (Berman et al, 2007) allowed the selection of 5 amino acid residues as candidates for a "gain of function" mutations. These residues are : Lys64 (located at ExoII motif), Met97 (neighbor to Arg96, a primer ligand of WT Phi29 DNApol), Thr499 (neighbor to Lys498 and Tyr500, two primer ligands of WT Phi29 DNApol), Thr534 and Lys538 (close to Lys529, a primer ligand of WT Phi29 DNApol).The mutations selected at these residues (summarized in Fig. 2B) were:

- K64**R**, producing the gain of interaction with the phosphodiester between residues 4 and 5 of the primer strand.
- K64**KG**; K64**KK**; L63**LG**; L63**LH**, as +1 insertion mutations flanking Lys64, designed in agreement with the heterogeneity observed at different ExoII motifs of B family DNA polymerases. These changes are also predicted to gain interaction with residues 4 and 5 of the primer strand.

- R96**K**, predicted to weaken the interaction with the phosphodiester bond between residues 7 and 8 of the primer.
- M97**K**, producing the gain of interaction with the nitrogen base of the nucleotide 5 of the primer strand.
- M97**R**, producing the gain of interaction with the bases of the amino acid residues 4 and 5 of the primer strand.
- T499**K**, producing the gain of interaction with the sugar of the amino acid residue 5 of the template strand.
- T499**R**, producing the gain of interaction with the sugar of the amino acid residues 4 and 5 of the template strand.
- K529**R**, producing the gain of a double interaction with the phosphodiester bonds between residues 1 and 3 of the primer strand.
- T534**K**, producing the gain of interaction with the sugar of the amino acid residue 4 of the primer strand.
- T534**R**, producing the gain of interaction with the phosphodiester between residues 3 and 4 of the primer strand.
- K538**R**, producing the gain of interaction with the phosphodiester between residues 2 and 3 of the primer strand.

[00052] The indicated mutants, designed to increase the affinity of Phi29 DNApol for short primers, were expressed and purified following standard protocols to obtain WT Phi29 DNApol. It cannot be predicted if any particular gain of interaction with the primer strand originated by the mutations introduced will have an adverse effect on Phi29 DNApol features as translocation, processivity, or the appropriate (*Tth*PrimPol) and randomly-primed DNA amplification technologies.

III. Nucleic Acids, Expression Constructs, Recombinant Cells and Mutant Polymerase Polypeptides

A. Nucleic Acids

[00053] Provided herein are nucleic acids having nucleotide sequences that encode mutant Phi29 polymerases with improved primer recognition. Nucleotide sequence for a wild type Phi29 polymerase is provided in SEQ ID NO.: 1. Nucleic acids encoding mutant Phi29

polymerases sequences encoding have one or both mutations K64R and M97K. In some embodiments nucleotide sequences encoding one or both of these mutations are substantially identical to the sequence of SEQ ID NO.: 1.

B. Expression Constructs

[00054] Also provided herein are expression constructs comprising a transcription regulatory sequence operatively linked to a nucleotide sequence encoding a mutant Phi29 polymerase as described herein. The expression construct can take the form of a plasmid or any other form appropriate for expression in a cell of interest.

C. Recombinant Cells

[00055] Also provided herein are recombinant cells comprising an expression construct as described herein. In certain embodiments the cells are bacterial cells. Such recombinant cells are useful for reproducing the nucleic acid molecules of this disclosure and for producing mutant Phi29 polymerases of this disclosure. Mutant Phi29 polymerases can be produced by culturing recombinant cells comprising an expression construct. The transcription regulatory sequence used can comprise a constitutive promoter.

D. Mutant Phi29 Polymerases

[00056] Also provided herein are mutant Phi29 polymerases with improved primer recognition. The mutant Phi29 polymerases of this disclosure have amino acid sequences that are substantially identical to the amino acid sequence of SEQ ID NO.: 1 (also deposited as UniProtKB - P03680) and that comprise one or both amino acid substitutions K64R and M97K.

[00057] Polymerases having substantially identical amino acid sequences can be based on naturally occurring sequences, such as allelic variants, provided they include one or both of the amino acid substitutions K64R and M97K. Such variants can have at most or no more than any of 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid substitutions, additions or deletions compared with the wild type sequence SEQ ID NO.: 1, again provided that one or both of amino acid substitutions K64R and M97K are present.

[00058] Preferably, the amino acid sequence of the DNA polymerases of the invention have an identity of at least 80% with SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 4. More preferably, the amino acid sequence of polymerases of the invention have an identity of at least 90% with SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 4. Still more preferably, the amino acid sequence of polymerases of the invention is SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 4.

IV. Methods of Use

[00059] Provided herein are methods of performing primer extension and/or nucleic acid polymerization using the mutant Phi29 polymerases described herein. Methods of primer extension are useful in nucleic acid replication, amplification and sequencing.

[00060] Primer extension involves hybridization of a primer to a nucleic acid molecule template followed by a polymerization reaction catalyzed by a polymerase that adds nucleotides to the 3' terminus of the primer. Primers can be added to the reaction mixture exogenously, or can be produced by a primase/polymerase. Primases are enzymes that catalyzes the synthesis of an oligonucleotide, called a primer, complementary to a nucleic acid template. One such primase is such as *Tth*PrimPol.

[00061] Synthetic primers are typically used in nucleic acid amplification. Such primers typically are between about six and about 25 nucleotides in length. When specific sequences are to be amplified, primers can have sequences complementary to the target sequence. For purposes of whole genome amplification or other nondirected amplification methodologies, random primers can be used. Random primers typically comprise a collection or set of oligonucleotides in which each of the bases is present at each position in the oligonucleotide in one or more of the primers in the set. In certain situations, one or more of the positions (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) can be filled by a fixed base or a combination of two or three bases.

A. Amplification

[00062] Amplification of nucleic acids, for example by polymerase chain reaction (PCR) as introduced by Mullis (US 5,656,493) is an indispensable technique used in medical and biological research. It has been successfully used in a variety of applications like cloning, manipulating or sequencing of nucleic acids, DNA-based functional and phylogenetic analysis of genes, detection and diagnosis of diseases, as well as in forensic science and paternity testing.

B. Rolling Circle Amplification

[00063] Rolling circle amplification is a method of amplifying a covalently closed DNA molecule such as a single stranded, covalently closed DNA molecule. The template DNA molecule is primed with a primer, for example a primer provided by a primase/polymerase. A DNA polymerase performs primer extension on the primer around the closed DNA molecule. The polymerase displaces the hybridized copy and continues polynucleotide extension around the template to produce a concatenated amplification product.

C. Multiple Displacement Amplification (MDA)

[00064] Multiple displacement amplification (MDA) is an isothermal, non-PCR-based DNA amplification method in which priming and extension from a template produces ssDNA chains which can be continuously re-primed and copied by strand-displacement synthesis, producing a multi-branched DNA structure. After an initial denaturation of the double-stranded DNA sample, multiple strand displacement (MDA) amplification produces a multi-branched structure as DNA synthesis can be continuously primed and extended from many positions in the amplified molecules, without required further rounds of denaturation. Branches are displaced from each other as new primers are extended from one DNA molecule template into the branched area. MDA is further described in, for example, WO2011/047307A1, published April 21, 2011 ("Multiple Displacement Amplification"). MDA could be described in brief as: isothermal polymerization that extends primers at multiple priming sites on self-generated ssDNA templates.

[00065] In certain embodiments MDA employs random trimers, tetramers, pentamers, hexamers, heptamers or octamers as primers to prime amplification at multiple sites on an initial template and amplified copies thereof. In certain embodiments of the disclosed methods, priming is accomplished with a DNA primase/polymerase, such as *Tth*PrimPol.

[00066] In certain embodiments, amplification of double-stranded, linear polynucleotides involves using: 1) random synthetic primers and/or a DNA-directed primase/polymerase, such as *Tth*PrimPol; 2) a modified DNA polymerase having strand-displacement activity, such as Phi29 DNApol; 3) dNTPs. In certain embodiments, the dNTP substrates are unmodified. In other embodiments, dNTPs can be modified by the attachment of a labeled group, for example, a fluorescent molecule. As used herein, the term "label" refers to a chemical moiety attached to a molecule, such as a nucleic acid molecule. Detectable labels include, for example, fluorescent labels, luminescent labels, enzymatic labels, colorimetric labels such as colloidal gold or colored glass or plastic beads and radioactive labels. In combination, these three reagents promote multiple displacement amplification (MDA) of a given DNA, multiply primed either by random synthetic primers or by the primase/polymerase and extended by the DNA polymerase. Furthermore, the combination of random synthetic primers and/or primase/polymerase and DNA polymerase can effect multiple strand displacement amplification through priming of amplified molecules with the primase/polymerase and/or random oligonucleotide primers and primer extension by the DNA polymerase.

1. DNA Polymerase with Strand Displacement Activity

[00067] Amplification methods as MDA can employ a DNA polymerase with strand displacement activity, e.g., a polymerase with strong binding to single-stranded DNA e.g., in preference to double-stranded DNA. Strand displacement activity can be useful in displacing hybridized strands of a DNA molecule while extending a primer position.

[00068] DNA polymerases with strand displacement activity useful in methods disclosed herein include, for example, Phi29 DNApol. Phi29 DNApol can be obtained commercially from, for example, New England Biolabs (Ipswich, MA, USA), ThermoFisher Scientific (Waltham, MA, USA and Expedeon (Cambridge, UK). Phi29 DNApol has both an intrinsic high processivity and strand-displacement ability coupled to DNA polymerization, being able to generate DNA fragments longer than 70 kb from a single enzyme : DNA binding event (Blanco et al., 1989). Such a potential enables Phi29 DNApol to replicate DNA templates containing secondary structures such as hairpin loops. The enzyme also has a 3'→5' exonuclease proofreading activity (Blanco and Salas, 1985; Garmendia et al., 1992) and provides up to 1000-fold higher fidelity compared to Taq DNA polymerase-based methods.

2. Deoxyribonucleoside Triphosphates

[00069] Primer creation and primer extension can be accomplished by the combination of a specialized DNA primase/polymerase as *Tth*PrimPol, capable of synthesizing DNA primers (Picher et al, 2016), and a elongating DNA polymerase, as Phi29 DNApol, just by providing deoxyribonucleotide substrates e.g., dNTPs. Typically, these include the four standard bases, A, T, G and C. However, in certain embodiments non-natural nucleotides, such as inosine can be included. In certain embodiments nucleotides may bear a label for detection or capture of polynucleotides into which they are incorporated.

D. DNA sequencing

[00070] As of today, a number of different sequencing techniques exist, that are commonly categorized under "first generation sequencing", "second generation sequencing" (often called "next generation sequencing" or NGS), and "third generation sequencing", also known as single molecule sequencing (SMS). First generation sequencing refers mainly to the methods of Maxam and Gilbert (Maxam and Gilbert, 1977) or Sanger (Sanger et al, 1977; Sanger and Coulson, 1978), of which only the latter is used today.

[00071] Second, or next generation sequencing refers to techniques that produce many sequences at the same time using advanced technical (optical) detection methods of base positions. An overview over existing methods is given in (Metzker, 2010).

[00072] Third generation or single molecule sequencing (SMS) techniques do not require prior amplification, and templates are not clones or ensembles of DNA, but single molecules whose sequence is often copied/read and online-recorded in "real time", as an outcome of the activity of a polymerase (Sam et al, 2011; Thompson and Milos, 2011).

[00073] As used herein, the term "high throughput sequencing" refers to the simultaneous or near simultaneous sequencing of thousands of nucleic acid molecules. Platforms for high throughput sequencing include, without limitation, massively parallel signature sequencing (MPSS), Polony sequencing, 454 pyrosequencing, Illumina (Solexa) sequencing, SOLiD sequencing, Ion Torrent semiconductor sequencing, DNA nanoball sequencing (Complete Genomics/BGI Shenzhen), Heliscope single molecule sequencing, single molecule real time (SMRT) sequencing (PacBio), and nanopore DNA sequencing (e.g., Oxford Nanopore).

[00074] Methods described herein can be used for, without limitation, whole genome sequencing, exome sequencing and amplicon sequencing. However, amplified molecules themselves, can be subject to amplification of specific amplicons. Sequence capture using baits directed to gene sequences in the genome can be used to isolate amplified molecules representing the exome. By reverse transcribing mRNA into double stranded cDNA an amplified transcriptome can be produced for sequencing.

V. Kits

[00075] Also provided herein are kits for use in performing the methods disclosed herein. As used herein, the term "kit" refers to a collection of items intended for use together.

[00076] Certain kits disclosed herein include 2, 3, 4, 5, 6, 7, elements selected from: (1) a PrimPol enzyme (e.g., *Tth*PrimPol); (2) a DNA polymerase (e.g., Phi29 DNApol); (3) random trimers; (4) random tetramers; (5) random pentamers; (6) random heptamers; (7) random octamers; (8) random primers; (9) dNTPs; (10) reaction buffer; (11) a buffer for use with any of the aforementioned elements. Kits can include containers to hold reagents. Containers, themselves, can be placed into a shipping container. The container can be transmitted by hand delivery or by a common carrier, such as a national postal system or a delivery service such as FedEx. Kits also can contain a container for shipping collected blood to a central facility, such as a box or a bag. Kits can also typically include instructions for use as well as and software for data analysis and interpretation.

EXEMPLARY EMBODIMENTS

[00077] 1. A Phi29 type DNA polymerase that comprises one or both of the mutations K64R or M97K.

[00078] 2. A Phi29 type DNA polymerase that has an amino acid sequence having an identity of at least 80% with SEQ ID NO: 2; SEQ ID NO. 3 or SEQ ID NO 4.

[00079] 3. A method for replicating, amplifying or sequencing a template DNA which comprises contacting said DNA with a reaction mixture comprising at least: a) the DNA polymerase according to any of embodiments 1 to 2, b) a buffer, c) magnesium chloride, d) a primer, and e) nucleoside triphosphates. 4. A kit for carrying out a method according to embodiment 3 comprising: a) the DNA polymerase according to any of embodiments 1 to 2, b) a buffer, and c) magnesium chloride.

[00080] 5. A kit for carrying out a method according to embodiment 3 comprising the DNA polymerase according to any of embodiments 1 to 2, and one or more of: (a) a PrimPol enzyme (e.g., TthPrimPol); (b) random trimers; (c) random tetramers; (d) random pentamers; (e) random heptamers; (f) random octamers; (g) dNTPs; (h) reaction buffer; (i) a buffer for use with any of the aforementioned elements.

[00081] 6. A Phi29 type DNA polymerase, wherein the Phi29 type DNA polymerase has an amino acid sequence having at least 80%, 85%, 90%, 95%, 98% or 99% 99.5% sequence identity with SEQ ID NO:1, and wherein the Phi29 type DNA polymerase comprises one or both amino acid substitutions K64R and M97K. 7. The Phi29 type DNA polymerase of embodiment 6, having a sequence of SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4. 8. The Phi29 type DNA polymerase of embodiment 6, having no more than 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid substitutions, additions or deletions in addition to one or both of amino acid substitutions K64R and M97K.

[00082] 9. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a Phi29 type DNA polymerase, wherein the Phi29 type DNA polymerase has an amino acid sequence having at least 80%, 85%, 90%, 95%, 98% or 99% 99.5% sequence identity with SEQ ID NO:1, and wherein the Phi29 type DNA polymerase comprises one or both amino acid substitutions K64R and M97K. 10. The isolated nucleic acid molecule of embodiment 9, wherein the Phi29 type DNA polymerase has a sequence of SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4. 11. The isolated nucleic acid molecule of embodiment 9, wherein the Phi29 type DNA polymerase has no more than 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid substitutions, additions or deletions in addition to one or both of amino acid substitutions K64R and M97K. 12. A recombinant nucleic acid comprising an transcription regulatory sequence operatively linked with a Phi29 type DNA polymerase of any of embodiments 9-11. 13. The recombinant nucleic acid of embodiment 12, wherein the transcription regulatory

sequence comprises a bacterial or mammalian promoter. 14. The recombinant nucleic acid of embodiment 12 contained in a vector selected from a plasmid vector, a viral vector, a cosmid, and a transposon. 15. The recombinant nucleic acid of embodiment 14, comprising a cloning site positioned relative to the nucleotide sequence encoding the Phi29 type DNA polymerase such that an transcription regulatory sequence inserted into the cloning site becomes operatively linked with the nucleotide sequence encoding the Phi29 type DNA polymerase.

[00083] 16. A recombinant cell comprising a recombinant nucleic acid of any of embodiments 12-15.

[00084] 17. A method comprising: a) contacting a nucleic acid template molecule with a Phi29 type DNA polymerase of any of embodiments 1, 2, 6-8, and reagents sufficient for primer extension; and b) performing primer extension with the polymerase using the nucleic acid template.

[00085] 18. The method of embodiment 17, wherein the reagents sufficient for primer extension comprise oligonucleotide primers.

[00086] 19. The method of embodiment 18, wherein the oligonucleotide primers comprise one or more of trimers, tetramers, pentamers, hexamers, hexamers, octomers, nonamers or 10-mers.

[00087] 20. The method of embodiment 19, wherein the primers are random primers.

[00088] 21. The method of embodiment 18, wherein the oligonucleotide primers have links between five and 25 nucleotides.

[00089] 22. The method of embodiment 17, wherein the reagents sufficient for primer extension comprise a primase/polymerase (e.g., TthPrimPol).

[00090] 23. The method of embodiment 17, wherein primer extension is performed at a temperature about, or above, any of 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, or 42°C.

[00091] 24. The method of embodiment 17, wherein the template nucleic acid molecule is present in an amount no greater than 1 ng, 100 pg, 10 pg, or 1 pg.

[00092] 25. The method of embodiment 17, wherein the primer extension comprises (i) multiple displacement amplification (“MDA”) or (2) rolling circle amplification.

[00093] 26. The method of embodiment 17, wherein the primer extension comprises multiple annealing and looping-based amplification cycles (MALBAC).

[00094] 27. The method of any of embodiments 17-26, wherein the Phi29 type DNA polymerase comprises both of substitutions K64R and M97K.

EXAMPLES

Example 1: Screening to detect which mutants are able to use shorter random synthetic primers than WT Phi29 DNA pol in multiple displacement amplification reactions

[00095] Shown in Fig. 3 is the amplification of 1 ng of human genomic DNA by multiple displacement amplification (MDA) combining Phi29 DNApol variants and *Tth*PrimPol or random synthetic primers of different sizes: trimers (3N), tetramers (4N), pentamers (5N), hexamers (6N), heptamers (7N) or octamers (8N).

[00096] As observed in Fig. 3, WT Phi29 DNApol efficiently used pentamers, hexamers, heptamers and octamers, as well as *Tth*PrimPol, to amplify human genomic DNA. Trimers and tetramers were not suitable to carry out the amplification.

[00097] From the group of Phi29 DNApol variants generated, six of them (K538R, T534K, T534R, L63LH, K64KG and K64KK) were completely inactive in MDA independently of the primer size or the alternative use of *Tth*PrimPol. Another set of mutants (K529R, M97R, R96K, L63LG and T499K) revealed worse amplification performance than WT Phi29 DNApol, showing lower amplification yields and/or limitations to use certain primer sizes. For example, mutant M97R was able to use pentamers and hexamers efficiently, while heptamers and octamers did not trigger the amplification. Similarly, mutant R96K was only able to use hexamers from the set of random synthetic primers. Strikingly, insertion mutant L63LG was able to amplify the DNA with pentamers, hexamers, heptamers and octamers, but the combination with *Tth*PrimPol did not produce any amplified material. On the contrary, mutant T499K was only able to slightly amplify the DNA in the presence of *Tth*PrimPol, while none of the random synthetic primers promoted MDA.

[00098] Mutant T499R showed a behavior approximately similar to WT Phi29 DNApol.

[00099] Finally, mutants K64R and M97K showed significant improvements with respect to WT Phi29 DNApol. Both of them were the only ones able to use tetramers, while WT Phi29 DNApol and the rest of the mutants did not show any amplification yield.

[000100] The two “gain of function” mutations were introduced into the same polypeptide to generate the double mutant K64R / M97K, which was deeply characterized in comparison to WT Phi29 DNApol and single mutants K64R and M97K, as it is shown in the following examples.

Example 2: Polymerase activity is favored versus exonuclease activity in Phi29 DNApol mutant M97K and double mutant K64R / M97K

[000101] Shown in Fig. 4 is the analysis of the dynamic equilibrium between 3′-5′ exonuclease and 5′-3′ polymerization activities of the most relevant inventive mutants with respect to the WT Phi29 DNApol (K64R, M97K and double mutant K64R / M97K). A DNA duplex formed by a 5'-labelled primer (5' GATCACAGTGAGTAC, SEQ ID NO: 5) hybridized to a template (5' AGAAGTGTATCTGGTACTCACTGTGATC, SEQ ID NO: 6) was used to analyze the coupling between DNA synthesis and DNA degradation as a function of dNTP concentration (0, 10, 25, 50 100 and 500 nM). In the absence of dNTPs, the exonucleolytic degradation of the primer-terminus is observed. The degradation pattern reflects the level of exonuclease activity of the inventive variants with respect to the WT Phi29 DNApol. As the concentration of dNTPs increases, the exonuclease activity is progressively overcome by the 5′-3′ polymerization and net dNMP incorporation can be observed as an increase in the size of the labelled primer, defining the concentration of dNTPs needed to obtain an efficient elongation of the primer for each mutant. As observed in Fig. 4, mutant K64R showed a Pol/Exo equilibrium approximately similar to that displayed by the WT enzyme, reaching the 28-mer position at 25 nM dNTPs. On the other hand, mutant M97K and double mutant K64R / M97K reached the same position (28-mer) with the lowest dNTP concentration tested (10 nM), indicating that the polymerase activity of these mutants is favored versus exonuclease.

Example 3: The inventive mutants (K64R, M97K and K64R / M97K) are able to use shorter random synthetic primers than WT Phi29 DNApol in multiple displacement amplification reactions

[000102] Shown in Fig. 5 is the amplification of 1 ng of human genomic DNA by multiple displacement amplification combining Phi29 DNApol selected variants (K64R, M97K and double mutant K64R / M97K) and random synthetic primers of different sizes: trimers (3N), tetramers (4N), pentamers (5N), hexamers (6N), heptamers (7N) or octamers (8N). Amplification yields shown are the mean of two independent experiments that included 3 replicates per condition each. Standard deviation from the two experiments is depicted.

[000103] As observed in Fig. 5, none of the enzymes tested were able to efficiently amplify genomic DNA using random synthetic trimers. Only the double mutant K64R / M97K showed a yield close to 1 µg.

[000104] The three inventive variants were able to use tetramers to trigger the amplification, while no amplification was observed with the WT Phi29 DNApol. Variant K64R showed the lowest amplification yield (2,7 µg), mutant M97K displayed a slightly higher yield (3,8 µg), and double mutant K64R / M97K exhibited a much higher yield (12,9 µg). The

highest yield observed in the double mutant indicates synergic effects of both mutations in the same polypeptide.

[000105] The WT Phi29 DNApol and the three inventive variants were able to use random pentamers efficiently to start off the amplification. Again, the double mutant K64R / M97K produced the highest yield, more than 20 µg of amplified DNA, clearly overcoming the performance of single variants and WT enzyme.

[000106] In the case of random hexamers, a similar comparative pattern is observed, although the amplification yields are higher in all cases.

[000107] Using random heptamers, WT Phi29 DNApol kept the same yield with respect to the results obtained using hexamers, while the three inventive variants tended to decrease the amplification efficiency, producing DNA levels similar to those obtained with random pentamers.

[000108] In the case of octamers, both K64R and M97K single mutants showed amplification yields lower than the WT Phi29 DNApol. On the other hand, double mutant K64R / M97K clearly overcame the WT Phi29 DNApol, as it occurred in all conditions tested, confirming robust and efficient amplification values independently of the length of the random synthetic primer used to initiate the amplification.

Example 4: Effect of ionic strength in the background amplification observed in the absence of input DNA in non-template controls (NTC).

[000109] Shown in Fig. 6 is the amplification yields observed in the absence of input DNA when combining WT Phi29 DNApol or the selected inventive variants (K64R, M97K and K64R / M97K) and random synthetic primers of different sizes: trimers (3N), tetramers (4N), pentamers (5N), hexamers (6N), heptamers (7N) or octamers (8N).

[000110] Under the low ionic strength conditions tested (20 mM KCl; 57 mM NaCl), both M97K single mutant and K64R / M97K double mutant showed significant amplification yields in the absence of input DNA when using pentamers and hexamers, but also tetramers in the case of the double mutant (see Fig. 6). However, the amplification yields are significantly lower than the ones obtained when DNA (1 ng) is used as input in the same conditions (see Fig. 5), pointing to a different amplification mechanism involved. It is well-known in the field the primer-dimer amplification capacity of Phi29 DNApol in the absence of input DNA (Alsmadi et al, 2009), and the stability of primer-dimers may be enhanced in M97K single mutant and K64R / M97K double mutant under the conditions tested.

[000111] Shown in Fig. 7 are the amplification yields observed in the absence of input DNA but increasing the ionic strength conditions by the addition of ammonium sulfate [$(NH_4)_2SO_4$].

In the presence of ammonium sulfate (45 mM), amplification levels observed in the absence of input DNA are completely eliminated in all variants and all primer sizes.

Example 5: High ionic strength conditions reinforce the robustness and efficiency of double mutant K64R / M97K for the amplification of DNA using random primers of different lengths.

[000112] Shown in Fig. 8 is the amplification of 1 ng of human genomic DNA by multiple displacement amplification (MDA) combining WT Phi29 DNApol or the selected inventive variants (K64R, M97K and K64R / M97K) and random synthetic primers of different sizes: trimers (3N), tetramers (4N), pentamers (5N), hexamers (6N), heptamers (7N) or octamers (8N), under high ionic strength conditions (20 mM KCl; 57 mM NaCl; 45 mM $(NH_4)_2SO_4$). Amplification yields shown are the mean of two independent experiments that included 3 replicates per condition each. Standard deviation from the two experiments is depicted.

[000113] As observed in Fig. 8, none of the enzymes tested were able to efficiently amplify genomic DNA using random synthetic trimers. Only the double mutant K64R / M97K showed a yield close to 600 ng.

[000114] As opposed to what was observed in previous conditions (see Fig. 5), tetramers were efficiently used only by M97K single mutant and K64R / M97K double mutant, while single mutant K64R produced only a minor yield close to 1 µg. Remarkably, the amplification yields observed for M97K single mutant and K64R / M97K double mutant increased in comparison to the ones obtained in the absence of ammonium sulfate (from 4 to 12, and from 13 to 16 µg respectively).

[000115] In the case of pentamers and hexamers, M97K single mutant and K64R / M97K double mutant showed similar results, clearly overcoming the amplification yields obtained with the WT enzyme or the K64R variant. As it was shown in the absence of ammonium sulfate, WT Phi29 DNApol showed higher yields than K64R variant.

[000116] In the case of heptamers, only double mutant K64R / M97K maintained the amplification yield obtained with shorter random synthetic primers and/or in the absence of ammonium sulfate. Both WT Phi29 DNApol and K64R variant significantly decreased the yield, showing the same values in these conditions. The yield obtained with the M97K mutant was also reduced in comparison to previous conditions.

[000117] Lastly, octamers were only efficiently deployed by the double mutant K64R / M97K, while the other three enzymes showed very low amplification yields.

[000118] Phi29 DNApol double mutant K64R / M97K keeps intact the amplification performance under both low and high ionic strength conditions, likely as a consequence of

the gain of function acquired by the additional contacts of the enzyme with the nitrogen base from primer nucleotide 5 and the phosphodiester bond between nucleotides 4 and 5 (see Fig. 2). These additional contacts enable the enzyme to proficiently stabilize primers of different sizes under different ionic strength conditions.

Example 6: Double mutation K64R / M97K results in a very sensitive amplification of minute amounts of DNA independently of the primer size tested.

[000119] Shown in Fig. 9 is the amplification of different amounts of human genomic DNA (1, 10, 100 pg and 1 ng) by multiple displacement amplification (MDA) combining WT Phi29 DNApol or the selected inventive variants (K64R, M97K and K64R / M97K) and random synthetic primers of different sizes: tetramers (4N), pentamers (5N) or hexamers (6N), under low (20 mM KCl; 57 mM NaCl) or high (20 mM KCl; 57 mM NaCl; 45 mM $(NH_4)_2SO_4$) ionic strength conditions.

[000120] Under low ionic strength conditions (Fig. 9, higher panels), double mutant K64R / M97K produces the most consistent and the highest amplification yields in all conditions tested.

[000121] In the case of random synthetic tetramers, as it was previously shown (see Fig. 5), WT Phi29 DNApol was not able to amplify any of the DNA inputs tested. Variant K64R produced a detectable yield only with 1 ng of DNA input, lacking sensitivity to amplify lower DNA amounts. On the contrary, both M97K and M97K / K64R mutants efficiently amplified the DNA inputs tested, the double mutant producing higher yields in all cases.

[000122] In the case of random synthetic pentamers, all enzymes were able to use them to start off the amplification, but showing different levels of sensitivity and efficiency. WT Phi29 DNApol showed a significant decrease in the amplification yield when the amount of DNA input descended, while the three inventive variants maintained a reasonable efficiency in all conditions tested. Double mutant M97K / K64R displayed the highest amplification efficiency among the three inventive variants independently of the DNA input amount, therefore showing the best sensitivity.

[000123] In the case of random synthetic hexamers, all enzymes were able to use them efficiently to initiate the amplification of each DNA input tested, showing notable amplification yields in every case. The three inventive variants overcame WT Phi29 DNApol, showing higher amplification yields when low DNA inputs were analyzed. As it occurred with pentamers, double mutant M97K / K64R displayed the highest amplification efficiency among the three inventive variants independently of the DNA input amount.

[000124] As it was previously shown (see Fig. 6), both M97K and M97K / K64R variants showed significant amplification yields in the absence of input DNA (non-template controls, NTC) when using pentamers and hexamers. For this reason, the same sensitivity analysis was carried out under high ionic strength conditions (20 mM KCl; 57 mM NaCl; 45 mM $(NH_4)_2SO_4$) to prevent this artefactual effect derived from primer-dimer amplification.

[000125] Under high ionic strength conditions (see Fig. 9, lower panels), random synthetic tetramers showed a similar pattern of usage among the variants tested in comparison to low ionic strength conditions, although the amplification yields were increased in most cases. The exception to this rule was the variant M97K that showed lower yields with 1 pg and 10 pg DNA inputs.

[000126] In the case of random synthetic pentamers under high ionic strength conditions, M97K and M97K / K64R variants showed the best performance in terms of sensitivity and efficiency, showing higher amplification yields in comparison to low ionic strength conditions with the same DNA input amounts. The increase in the ionic strength of the reaction produced a reduction in the amplification efficiency of variant K64R when limiting amounts of DNA were tested (1 and 10 pg), while the efficiency was similar (100 pg) or higher (1ng) with the other two inputs. Surprisingly, WT Phi29 DNApol overcame K64R variant under these conditions in all cases.

[000127] In the case of random synthetic hexamers under high ionic strength conditions, double mutant K64R / M97K was the only variant that increased the yields observed with all DNA inputs in comparison to the results obtained under low ionic strength conditions. Single mutant M97K showed lower yields with the lowest inputs (1 and 10 pg), while increasing the yields with 100 pg and 1 ng DNA inputs, indicating a decrease in sensitivity. Variant K64R and WT Phi29 DNApol showed a similar behavior. As it occurred with pentamers, WT Phi29 DNApol produced higher amplification yields than K64R variant under these conditions in all cases.

[000128] In summary, double mutant K64R / M97K showed the best performance in terms of amplification efficiency and sensitivity under both low and high ionic strength conditions during the amplification reaction with all DNA primers tested.

Example 7: Amplification efficiency and sensitivity are not modified by the use of the inventive variants when primers are generated by *Tth*PrimPol

[000129] Shown in Fig. 10 is the amplification of 1, 10, 100 pg and 1 ng of human genomic DNA by multiple displacement amplification (MDA) combining WT Phi29 DNApol or the selected inventive variants (K64R, M97K and K64R / M97K) and *Tth*PrimPol, a DNA primase

capable of synthesizing primers in the course of the reaction for Phi29 DNApol (Picher et al, 2016).

[000130] As observed in Fig. 10, there are no significant yield differences between the WT Phi29 DNA pol and the inventive variants tested, which results in similar sensitivity and efficiency levels under this setup.

Example 8: Selected inventive variants (K64R, M97K and K64R / M97K) improve the amplification coverage measured by CovCheck technology

[000131] CovCheck technology allows the coverage analysis of whole genome amplifications using a PCR panel including 24 different primer pairs that amplify small portions from each human chromosome. CovCheck technology has been validated by comparing CovCheck coverage values with real coverage obtained through low-pass whole genome sequencing, obtaining excellent correlation values (https://www.expedeon.com/products/genomics/dna-rna-products/covcheck-pcr-kits/).

[000132] In order to analyze the amplification coverage obtained with each variant, a limited amount of input material was selected: 30 pg of human genomic DNA. This DNA amount is equivalent to 5 human diploid genomes, and it might be the lowest amount ensuring that sufficient copies of each chromosome will be available for the amplification. Below this level, certain regions or complete chromosomes could be absent in the input for the amplification due to the random distribution of molecules in a purified DNA sample, resulting in regions not covered in the amplified material due to the absence of the template, and not due to amplification failures.

[000133] Shown in Fig. 11 is the estimated coverage values obtained from the CovCheck analysis of amplification reactions using hexamers, pentamers and tetramers, in combination with WT Phi29 DNApol or the selected inventive variants and 30 pg of human genomic DNA input. Coverage values are means of 6 independent reactions per condition.

[000134] In the case of random synthetic hexamers, amplification coverage is improved when using the three inventive variants in comparison to the value obtained by the WT Phi29 DNApol.

[000135] In the case of random synthetic pentamers, all enzymes showed coverage values above 90% in these conditions. Therefore, no significant differences could be observed. However, M97K variant stood out with a perfect coverage in the 6 replicates tested.

[000136] In the case of random synthetic tetramers, only M97K and M97K / K64R variants produced amplified DNA, which is consistent with the sensitivity of amplification shown by WT Phi29 DNApol and K64R variant when combined with tetramers (Fig. 9). The CovCheck

analysis revealed an excellent amplification coverage in both cases (99%), pointing to the benefit of using the inventive variants in combination with the shortest primers possible, in order to maximize the amplification coverage and uniformity, preventing amplification biases and loss of sequences.

[000137] In the case of using an enzyme (*Tth*PrimPol) make DNA primers for Phi29 DNApol, Fig. 12 shows the estimated coverage values obtained from the CovCheck analysis of amplification reactions carried out by the combination of *Tth*PrimPol with WT Phi29 DNApol or the inventive variants, using 30 pg (5 genome equivalents) of human genomic DNA as input in the amplification reaction. Coverage values are means of 12 independent reactions per condition. The CovCheck analysis also revealed an improvement in amplification coverage when using the inventive variants, supporting the advantage of those to enhance the uniformity of the amplified material with respect to the original DNA input.

References (incorporated herein by reference)

[000138] Blanco L, Salas M. Characterization and purification of a phage phi 29-encoded DNA polymerase required for the initiation of replication. *Proc Natl Acad Sci U S A*. 1984 Sep;81(17):5325-9.

[000139] Blanco L, Salas M. Characterization of a 3'-5' exonuclease activity in the phage phi 29-encoded DNA polymerase. *Nucleic Acids Res.* 1985 Feb 25;13(4):1239-49.

[000140] Bernad A, Zaballos A, Salas M, Blanco L. Structural and functional relationships between prokaryotic and eukaryotic DNA polymerases. *EMBO J.* 1987 Dec 20;6(13):4219-25.

[000141] Rodriguez I, Lázaro JM, Blanco L, Kamtekar S, Berman AJ, Wang J, Steitz TA, Salas M, de Vega M. A specific subdomain in ø29 DNA polymerase confers both processivity and strand-displacement capacity *Proc. Natl. Acad. Sci. USA* 2005 May 3;102(18):6407-12.

[000142] Kamtekar, S. Berman, AJ, Wang, J, Lázaro, JM, de Vega, M, Blanco L, Salas M and Steitz TA. The phi29 DNA polymerase:protein-primer structure suggests a model for the initiation to elongation transition *EMBO J.* 2006 Mar 22;25(6):1335-43. Epub 2006 Mar 2.

[000143] Berman AJ, Kamtekar S, Goodman JL, Lázaro JM, de Vega M, Blanco L, Salas M, and Steitz TA. Structures of phi29 DNA polymerase complexed with substrate: the mechanism of translocation in B-family polymerases. *EMBO J.* 2007 Jul 25;26(14):3494-505.

[000144] Blanco L, Bernad A, Lázaro JM, Martín G, Garmendia C, Salas M. Highly efficient DNA synthesis by the phage phi 29 DNA polymerase. Symmetrical mode of DNA replication. *J Biol Chem.* 1989 May 25;264(15):8935-40.

[000145] Esteban JA, Salas M, Blanco L. Fidelity of phi 29 DNA polymerase. Comparison between protein-primed initiation and DNA polymerization. *J Biol Chem.* 1993 Feb 5;268(4):2719-26.

[000146] Esteban JA, Soengas MS, Salas M, Blanco L. 3'-->5' exonuclease active site of phi 29 DNA polymerase. Evidence favoring a metal ion-assisted reaction mechanism. *J Biol Chem.* 1994 Dec 16;269(50):31946-54.

[000147] Dean FB, Hosono S, Fang L, Wu X, Faruqi AF, Bray-Ward P, Sun Z, Zong Q, Du Y, Du J, Driscoll M, Song W, Kingsmore SF, Egholm M, Lasken RS. Comprehensive human genome amplification using multiple displacement amplification. *Proc Natl Acad Sci U S A.* 2002 Apr 16;99(8):5261-6.

[000148] Lizardi PM, Huang X, Zhu Z, Bray-Ward P, Thomas DC, Ward DC. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat Genet.* 1998 Jul;19(3):225-32.

[000149] Picher AJ, Budeus B, Wafzig O, Kruger C, García-Gómez S, Martínez-Jiménez MI, Díaz-Talavera A, Weber D, Blanco L & Schneider A. TruePrime is a novel method for whole-genome amplification from single cells based on *Tth*PrimPol. *Nat Comm* 2016, Nov 29; 7:13296.

[000150] Alsmadi O, Alkayal F, Monies D, Meyer BF. Specific and complete human genome amplification with improved yield achieved by phi29 DNA polymerase and a novel primer at elevated temperature. *BMC Res Notes.* 2009 Mar 24;2:48. doi: 10.1186/1756-0500-2-48.

[000151] Povilaitis T, Alzbutas G, Sukackaite R, Siurkus J, Skirgaila R. In vitro evolution of phi29 DNA polymerase using isothermal compartmentalized self-replication technique. *Protein Eng Des Sel.* 2016 Dec;29(12):617-628. Epub 2016 Sep 26.

[000152] US5656493A, "System for automated performance of the polymerase chain reaction", February 18, 1994.

[000153] Maxam AM, Gilbert W. A new method for sequencing DNA. *Proc Natl Acad Sci U S A.* 1977 Feb;74(2):560-4.

[000154] Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A.* 1977 Dec;74(12):5463-7.

[000155] Sanger F, Coulson AR. The use of thin acrylamide gels for DNA sequencing. *FEBS Lett.* 1978 Mar 1;87(1):107-10.

[000156] Metzker ML. Sequencing technologies - the next generation. *Nat Rev Genet.* 2010 Jan;11(1):31-46.

[000157] Sam LT, Lipson D, Raz T, Cao X, Thompson J, Milos PM, Robinson D, Chinnaiyan AM, Kumar-Sinha C, Maher CA. A comparison of single molecule and amplification based sequencing of cancer transcriptomes. *PLoS One.* 2011 Mar 1;6(3):e17305.

[000158] Thompson JF, Milos PM. The properties and applications of single-molecule DNA sequencing. *Genome Biol.* 2011;12(2):217.

[000159] WO 2011/047307A1, "Multiple Displacement Amplification", April 21, 2011.

[000160] Garmendia C, Bernad A, Esteban JA, Blanco L, Salas M. The bacteriophage phi 29 DNA polymerase, a proofreading enzyme. *J Biol Chem.* 1992 Feb 5;267(4):2594-9.

[000161] As used herein, the following meanings apply unless otherwise specified. The word "may" is used in a permissive sense (i.e., meaning having the potential to), rather than the mandatory sense (i.e., meaning must). The words "include", "including", and "includes" and the like mean including, but not limited to. The singular forms "a," "an," and "the" include plural referents. Thus, for example, reference to "an element" includes a combination of two or more elements, notwithstanding use of other terms and phrases for one or more elements, such as "one or more." The term "or" is, unless indicated otherwise, non-exclusive, i.e., encompassing both "and" and "or." The term "any of" between a modifier and a sequence means that the modifier modifies each member of the sequence. So, for example, the phrase "at least any of 1, 2 or 3" means "at least 1, at least 2 or at least 3". . In certain embodiments, inventions that "comprise" varies elements also may "consisting essentially of" these elements. The term "consisting essentially of" refers to the inclusion of recited elements and other elements that do not materially affect the basic and novel characteristics of a claimed combination.

[000162] It should be understood that the description and the drawings are not intended to limit the invention to the particular form disclosed, but to the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the present invention as defined by the appended claims. Further modifications and alternative embodiments of various aspects of the invention will be apparent to those skilled in the art in view of this description. Accordingly, this description and the drawings are to be construed

as illustrative only and are for the purpose of teaching those skilled in the art the general manner of carrying out the invention. It is to be understood that the forms of the invention shown and described herein are to be taken as examples of embodiments. Elements and materials may be substituted for those illustrated and described herein, parts and processes may be reversed or omitted, and certain features of the invention may be utilized independently, all as would be apparent to one skilled in the art after having the benefit of this description of the invention. Changes may be made in the elements described herein without departing from the spirit and scope of the invention as described in the following claims. Headings used herein are for organizational purposes only and are not meant to be used to limit the scope of the description.

[000163] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

SEQUENCE LISTING

Amino acids in italics are not expressed in some embodiments

SEQ ID NO 1: Wild-type Phi29 DNA polymerase (UniProtKB - P03680)

Met Lys His Met Pro Arg Lys Met Tyr Ser Cys Asp Phe Glu Thr
1 5 10 15

Thr Thr Lys Val Glu Asp Cys Arg Val Trp Ala Tyr Gly Tyr Met
20 25 30

Asn Ile Glu Asp His Ser Glu Tyr Lys Ile Gly Asn Ser Leu Asp
35 40 45

Glu Phe Met Ala Trp Val Leu Lys Val Gln Ala Asp Leu Tyr Phe
50 55 60

His Asn Leu Lys Phe Asp Gly Ala Phe Ile Ile Asn Trp Leu Glu
65 70 75

Arg Asn Gly Phe Lys Trp Ser Ala Asp Gly Leu Pro Asn Thr Tyr
80 85 90

Asn Thr Ile Ile Ser Arg Met Gly Gln Trp Tyr Met Ile Asp Ile
95 100 105

Cys Leu Gly Tyr Lys Gly Lys Arg Lys Ile His Thr Val Ile Tyr
110 115 120

Asp Ser Leu Lys Lys Leu Pro Phe Pro Val Lys Lys Ile Ala Lys
125 130 135

Asp Phe Lys Leu Thr Val Leu Lys Gly Asp Ile Asp Tyr His Lys
140 145 150

Glu Arg Pro Val Gly Tyr Lys Ile Thr Pro Glu Glu Tyr Ala Tyr
155 160 165

Ile Lys Asn Asp Ile Gln Ile Ile Ala Glu Ala Leu Leu Ile Gln
170 175 180

Phe Lys Gln Gly Leu Asp Arg Met Thr Ala Gly Ser Asp Ser Leu
185 190 195

Lys Gly Phe Lys Asp Ile Ile Thr Thr Lys Lys Phe Lys Lys Val
200 205 210

Phe Pro Thr Leu Ser Leu Gly Leu Asp Lys Glu Val Arg Tyr Ala
215 220 225

Tyr Arg Gly Gly Phe Thr Trp Leu Asn Asp Arg Phe Lys Glu Lys
230 235 240

Glu Ile Gly Glu Gly Met Val Phe Asp Val Asn Ser Leu Tyr Pro

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                245                 250                 255

Ala Gln Met Tyr Ser Arg Leu Leu Pro Tyr Gly Glu Pro Ile Val
                260                 265                 270

Phe Glu Gly Lys Tyr Val Trp Asp Glu Asp Tyr Pro Leu His Ile
                275                 280                 285

Gln His Ile Arg Cys Glu Phe Glu Leu Lys Glu Gly Tyr Ile Pro
                290                 295                 300

Thr Ile Gln Ile Lys Arg Ser Arg Phe Tyr Lys Gly Asn Glu Tyr
                305                 310                 315

Leu Lys Ser Ser Gly Gly Glu Ile Ala Asp Leu Trp Leu Ser Asn
                320                 325                 330

Val Asp Leu Glu Leu Met Lys Glu His Tyr Asp Leu Tyr Asn Val
                335                 340                 345

Glu Tyr Ile Ser Gly Leu Lys Phe Lys Ala Thr Thr Gly Leu Phe
                350                 355                 360

Lys Asp Phe Ile Asp Lys Trp Thr Tyr Ile Lys Thr Thr Ser Glu
                365                 370                 375

Gly Ala Ile Lys Gln Leu Ala Lys Leu Met Leu Asn Ser Leu Tyr
                380                 385                 390

Gly Lys Phe Ala Ser Asn Pro Asp Val Thr Gly Lys Val Pro Tyr
                395                 400                 405

Leu Lys Glu Asn Gly Ala Leu Gly Phe Arg Leu Gly Glu Glu Glu
                410                 415                 420

Thr Lys Asp Pro Val Tyr Thr Pro Met Gly Val Phe Ile Thr Ala
                425                 430                 435

Trp Ala Arg Tyr Thr Thr Ile Thr Ala Ala Gln Ala Cys Tyr Asp
                440                 445                 450

Arg Ile Ile Tyr Cys Asp Thr Asp Ser Ile His Leu Thr Gly Thr
                455                 460                 465

Glu Ile Pro Asp Val Ile Lys Asp Ile Val Asp Pro Lys Lys Leu
                470                 475                 480

Gly Tyr Trp  Ala His Glu Ser Thr Phe Lys Arg Ala Lys Tyr Leu
                485                 490                 495

Arg Gln Lys Thr Tyr Ile Gln Asp Ile Tyr Met Lys Glu Val Asp
                500                 505                 510

Gly Lys Leu Val Glu Gly Ser Pro Asp Asp Tyr Thr Asp Ile Lys
                515                 520                 525
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Phe Ser Val Lys Cys Ala Gly Met Thr Asp Lys Ile Lys Lys Glu
                530                 535                 540

Val Thr Phe Glu Asn Phe Lys Val Gly Phe Ser Arg Lys Met Lys
                545                 550                 555

Pro Lys Pro Val Gln Val Pro Gly Gly Val Val Leu Val Asp Asp
                560                 565                 570

Thr Phe Thr Ile Lys
                575
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SEQ ID NO 2: K64R Phi29DNApol mutant

```
Met Lys His Met Pro Arg Lys Met Tyr Ser Cys Asp Phe Glu Thr
1               5                   10                  15

Thr Thr Lys Val Glu Asp Cys Arg Val Trp Ala Tyr Gly Tyr Met
                20                  25                  30

Asn Ile Glu Asp His Ser Glu Tyr Lys Ile Gly Asn Ser Leu Asp
                35                  40                  45

Glu Phe Met Ala Trp Val Leu Lys Val Gln Ala Asp Leu Tyr Phe
                50                  55                  60

His Asn Leu Arg Phe Asp Gly Ala Phe Ile Ile Asn Trp Leu Glu
                65                  70                  75

Arg Asn Gly Phe Lys Trp Ser Ala Asp Gly Leu Pro Asn Thr Tyr
                80                  85                  90

Asn Thr Ile Ile Ser Arg Met Gly Gln Trp Tyr Met Ile Asp Ile
                95                  100                 105

Cys Leu Gly Tyr Lys Gly Lys Arg Lys Ile His Thr Val Ile Tyr
                110                 115                 120

Asp Ser Leu Lys Lys Leu Pro Phe Pro Val Lys Lys Ile Ala Lys
                125                 130                 135

Asp Phe Lys Leu Thr Val Leu Lys Gly Asp Ile Asp Tyr His Lys
                140                 145                 150

Glu Arg Pro Val Gly Tyr Lys Ile Thr Pro Glu Glu Tyr Ala Tyr
                155                 160                 165

Ile Lys Asn Asp Ile Gln Ile Ile Ala Glu Ala Leu Leu Ile Gln
                170                 175                 180

Phe Lys Gln Gly Leu Asp Arg Met Thr Ala Gly Ser Asp Ser Leu
                185                 190                 195

Lys Gly Phe Lys Asp Ile Ile Thr Thr Lys Lys Phe Lys Lys Val
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                200                 205                 210

Phe Pro Thr Leu Ser Leu Gly Leu Asp Lys Glu Val Arg Tyr Ala
                215                 220                 225

Tyr Arg Gly Gly Phe Thr Trp Leu Asn Asp Arg Phe Lys Glu Lys
                230                 235                 240

Glu Ile Gly Glu Gly Met Val Phe Asp Val Asn Ser Leu Tyr Pro
                245                 250                 255

Ala Gln Met Tyr Ser Arg Leu Leu Pro Tyr Gly Glu Pro Ile Val
                260                 265                 270

Phe Glu Gly Lys Tyr Val Trp Asp Glu Asp Tyr Pro Leu His Ile
                275                 280                 285

Gln His Ile Arg Cys Glu Phe Glu Leu Lys Glu Gly Tyr Ile Pro
                290                 295                 300

Thr Ile Gln Ile Lys Arg Ser Arg Phe Tyr Lys Gly Asn Glu Tyr
                305                 310                 315

Leu Lys Ser Ser Gly Gly Glu Ile Ala Asp Leu Trp Leu Ser Asn
                320                 325                 330

Val Asp Leu Glu Leu Met Lys Glu His Tyr Asp Leu Tyr Asn Val
                335                 340                 345

Glu Tyr Ile Ser Gly Leu Lys Phe Lys Ala Thr Thr Gly Leu Phe
                350                 355                 360

Lys Asp Phe Ile Asp Lys Trp Thr Tyr Ile Lys Thr Thr Ser Glu
                365                 370                 375

Gly Ala Ile Lys Gln Leu Ala Lys Leu Met Leu Asn Ser Leu Tyr
                380                 385                 390

Gly Lys Phe Ala Ser Asn Pro Asp Val Thr Gly Lys Val Pro Tyr
                395                 400                 405

Leu Lys Glu Asn Gly Ala Leu Gly Phe Arg Leu Gly Glu Glu Glu
                410                 415                 420

Thr Lys Asp Pro Val Tyr Thr Pro Met Gly Val Phe Ile Thr Ala
                425                 430                 435

Trp Ala Arg Tyr Thr Thr Ile Thr Ala Ala Gln Ala Cys Tyr Asp
                440                 445                 450

Arg Ile Ile Tyr Cys Asp Thr Asp Ser Ile His Leu Thr Gly Thr
                455                 460                 465

Glu Ile Pro Asp Val Ile Lys Asp Ile Val Asp Pro Lys Lys Leu
                470                 475                 480
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```
Gly Tyr Trp  Ala His Glu Ser Thr Phe Lys Arg Ala Lys Tyr Leu
                485                 490                 495

Arg Gln Lys Thr Tyr Ile Gln Asp Ile Tyr Met Lys Glu Val Asp
                500                 505                 510

Gly Lys Leu Val Glu Gly Ser Pro Asp Asp Tyr Thr Asp Ile Lys
                515                 520                 525

Phe Ser Val Lys Cys Ala Gly Met Thr Asp Lys Ile Lys Lys Glu
                530                 535                 540

Val Thr Phe Glu Asn Phe Lys Val Gly Phe Ser Arg Lys Met Lys
                545                 550                 555

Pro Lys Pro Val Gln Val Pro Gly Gly Val Val Leu Val Asp Asp
                560                 565                 570

Thr Phe Thr Ile Lys
                575
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SEQ ID NO 3: M97K Phi29DNApol mutant

```
Met Lys His Met Pro Arg Lys Met Tyr Ser Cys Asp Phe Glu Thr
1               5                   10                  15

Thr Thr Lys Val Glu Asp Cys Arg Val Trp Ala Tyr Gly Tyr Met
                20                  25                  30

Asn Ile Glu Asp His Ser Glu Tyr Lys Ile Gly Asn Ser Leu Asp
                35                  40                  45

Glu Phe Met Ala Trp Val Leu Lys Val Gln Ala Asp Leu Tyr Phe
                50                  55                  60

His Asn Leu Lys Phe Asp Gly Ala Phe Ile Ile Asn Trp Leu Glu
                65                  70                  75

Arg Asn Gly Phe Lys Trp Ser Ala Asp Gly Leu Pro Asn Thr Tyr
                80                  85                  90

Asn Thr Ile Ile Ser Arg Lys Gly Gln Trp Tyr Met Ile Asp Ile
                95                  100                 105

Cys Leu Gly Tyr Lys Gly Lys Arg Lys Ile His Thr Val Ile Tyr
                110                 115                 120

Asp Ser Leu Lys Lys Leu Pro Phe Pro Val Lys Lys Ile Ala Lys
                125                 130                 135

Asp Phe Lys Leu Thr Val Leu Lys Gly Asp Ile Asp Tyr His Lys
                140                 145                 150

Glu Arg Pro Val Gly Tyr Lys Ile Thr Pro Glu Glu Tyr Ala Tyr
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                155                 160                 165

Ile Lys Asn Asp Ile Gln Ile Ile Ala Glu Ala Leu Leu Ile Gln
                170                 175                 180

Phe Lys Gln Gly Leu Asp Arg Met Thr Ala Gly Ser Asp Ser Leu
                185                 190                 195

Lys Gly Phe Lys Asp Ile Ile Thr Thr Lys Lys Phe Lys Lys Val
                200                 205                 210

Phe Pro Thr Leu Ser Leu Gly Leu Asp Lys Glu Val Arg Tyr Ala
                215                 220                 225

Tyr Arg Gly Gly Phe Thr Trp Leu Asn Asp Arg Phe Lys Glu Lys
                230                 235                 240

Glu Ile Gly Glu Gly Met Val Phe Asp Val Asn Ser Leu Tyr Pro
                245                 250                 255

Ala Gln Met Tyr Ser Arg Leu Leu Pro Tyr Gly Glu Pro Ile Val
                260                 265                 270

Phe Glu Gly Lys Tyr Val Trp Asp Glu Asp Tyr Pro Leu His Ile
                275                 280                 285

Gln His Ile Arg Cys Glu Phe Glu Leu Lys Glu Gly Tyr Ile Pro
                290                 295                 300

Thr Ile Gln Ile Lys Arg Ser Arg Phe Tyr Lys Gly Asn Glu Tyr
                305                 310                 315

Leu Lys Ser Ser Gly Gly Glu Ile Ala Asp Leu Trp Leu Ser Asn
                320                 325                 330

Val Asp Leu Glu Leu Met Lys Glu His Tyr Asp Leu Tyr Asn Val
                335                 340                 345

Glu Tyr Ile Ser Gly Leu Lys Phe Lys Ala Thr Thr Gly Leu Phe
                350                 355                 360

Lys Asp Phe Ile Asp Lys Trp Thr Tyr Ile Lys Thr Thr Ser Glu
                365                 370                 375

Gly Ala Ile Lys Gln Leu Ala Lys Leu Met Leu Asn Ser Leu Tyr
                380                 385                 390

Gly Lys Phe Ala Ser Asn Pro Asp Val Thr Gly Lys Val Pro Tyr
                395                 400                 405

Leu Lys Glu Asn Gly Ala Leu Gly Phe Arg Leu Gly Glu Glu Glu
                410                 415                 420

Thr Lys Asp Pro Val Tyr Thr Pro Met Gly Val Phe Ile Thr Ala
                425                 430                 435
```

```
Trp Ala Arg Tyr Thr Thr Ile Thr Ala Ala Gln Ala Cys Tyr Asp
                440                 445                 450

Arg Ile Ile Tyr Cys Asp Thr Asp Ser Ile His Leu Thr Gly Thr
                455                 460                 465

Glu Ile Pro Asp Val Ile Lys Asp Ile Val Asp Pro Lys Lys Leu
                470                 475                 480

Gly Tyr Trp  Ala His Glu Ser Thr Phe Lys Arg Ala Lys Tyr Leu
                485                 490                 495

Arg Gln Lys Thr Tyr Ile Gln Asp Ile Tyr Met Lys Glu Val Asp
                500                 505                 510

Gly Lys Leu Val Glu Gly Ser Pro Asp Asp Tyr Thr Asp Ile Lys
                515                 520                 525

Phe Ser Val Lys Cys Ala Gly Met Thr Asp Lys Ile Lys Lys Glu
                530                 535                 540

Val Thr Phe Glu Asn Phe Lys Val Gly Phe Ser Arg Lys Met Lys
                545                 550                 555

Pro Lys Pro Val Gln Val Pro Gly Gly Val Val Leu Val Asp Asp
                560                 565                 570

Thr Phe Thr Ile Lys
                575
```

SEQ ID NO 4: K64R / M97K Phi29DNApol double mutant

```
Met Lys His Met Pro Arg Lys Met Tyr Ser Cys Asp Phe Glu Thr
1               5                   10                  15

Thr Thr Lys Val Glu Asp Cys Arg Val Trp Ala Tyr Gly Tyr Met
                20                  25                  30

Asn Ile Glu Asp His Ser Glu Tyr Lys Ile Gly Asn Ser Leu Asp
                35                  40                  45

Glu Phe Met Ala Trp Val Leu Lys Val Gln Ala Asp Leu Tyr Phe
                50                  55                  60

His Asn Leu Arg Phe Asp Gly Ala Phe Ile Ile Asn Trp Leu Glu
                65                  70                  75

Arg Asn Gly Phe Lys Trp Ser Ala Asp Gly Leu Pro Asn Thr Tyr
                80                  85                  90

Asn Thr Ile Ile Ser Arg Lys Gly Gln Trp Tyr Met Ile Asp Ile
                95                  100                 105
```

```
Cys Leu Gly Tyr Lys Gly Lys Arg Lys Ile His Thr Val Ile Tyr
                110                 115                 120

Asp Ser Leu Lys Lys Leu Pro Phe Pro Val Lys Lys Ile Ala Lys
                125                 130                 135

Asp Phe Lys Leu Thr Val Leu Lys Gly Asp Ile Asp Tyr His Lys
                140                 145                 150

Glu Arg Pro Val Gly Tyr Lys Ile Thr Pro Glu Glu Tyr Ala Tyr
                155                 160                 165

Ile Lys Asn Asp Ile Gln Ile Ile Ala Glu Ala Leu Leu Ile Gln
                170                 175                 180

Phe Lys Gln Gly Leu Asp Arg Met Thr Ala Gly Ser Asp Ser Leu
                185                 190                 195

Lys Gly Phe Lys Asp Ile Ile Thr Thr Lys Lys Phe Lys Lys Val
                200                 205                 210

Phe Pro Thr Leu Ser Leu Gly Leu Asp Lys Glu Val Arg Tyr Ala
                215                 220                 225

Tyr Arg Gly Gly Phe Thr Trp Leu Asn Asp Arg Phe Lys Glu Lys
                230                 235                 240

Glu Ile Gly Glu Gly Met Val Phe Asp Val Asn Ser Leu Tyr Pro
                245                 250                 255

Ala Gln Met Tyr Ser Arg Leu Leu Pro Tyr Gly Glu Pro Ile Val
                260                 265                 270

Phe Glu Gly Lys Tyr Val Trp Asp Glu Asp Tyr Pro Leu His Ile
                275                 280                 285

Gln His Ile Arg Cys Glu Phe Glu Leu Lys Glu Gly Tyr Ile Pro
                290                 295                 300

Thr Ile Gln Ile Lys Arg Ser Arg Phe Tyr Lys Gly Asn Glu Tyr
                305                 310                 315

Leu Lys Ser Ser Gly Gly Glu Ile Ala Asp Leu Trp Leu Ser Asn
                320                 325                 330

Val Asp Leu Glu Leu Met Lys Glu His Tyr Asp Leu Tyr Asn Val
                335                 340                 345

Glu Tyr Ile Ser Gly Leu Lys Phe Lys Ala Thr Thr Gly Leu Phe
                350                 355                 360

Lys Asp Phe Ile Asp Lys Trp Thr Tyr Ile Lys Thr Thr Ser Glu
                365                 370                 375

Gly Ala Ile Lys Gln Leu Ala Lys Leu Met Leu Asn Ser Leu Tyr
                380                 385                 390
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```
Gly Lys Phe Ala Ser Asn Pro Asp Val Thr Gly Lys Val Pro Tyr
                395                 400                 405

Leu Lys Glu Asn Gly Ala Leu Gly Phe Arg Leu Gly Glu Glu Glu
                410                 415                 420

Thr Lys Asp Pro Val Tyr Thr Pro Met Gly Val Phe Ile Thr Ala
                425                 430                 435

Trp Ala Arg Tyr Thr Thr Ile Thr Ala Ala Gln Ala Cys Tyr Asp
                440                 445                 450

Arg Ile Ile Tyr Cys Asp Thr Asp Ser Ile His Leu Thr Gly Thr
                455                 460                 465

Glu Ile Pro Asp Val Ile Lys Asp Ile Val Asp Pro Lys Lys Leu
                470                 475                 480

Gly Tyr Trp  Ala His Glu Ser Thr Phe Lys Arg Ala Lys Tyr Leu
                485                 490                 495

Arg Gln Lys Thr Tyr Ile Gln Asp Ile Tyr Met Lys Glu Val Asp
                500                 505                 510

Gly Lys Leu Val Glu Gly Ser Pro Asp Asp Tyr Thr Asp Ile Lys
                515                 520                 525

Phe Ser Val Lys Cys Ala Gly Met Thr Asp Lys Ile Lys Lys Glu
                530                 535                 540

Val Thr Phe Glu Asn Phe Lys Val Gly Phe Ser Arg Lys Met Lys
                545                 550                 555

Pro Lys Pro Val Gln Val Pro Gly Gly Val Val Leu Val Asp Asp
                560                 565                 570

Thr Phe Thr Ile Lys
                575
```

WHAT IS CLAIMED IS:

1. A Phi29 type DNA polymerase that comprises one or both of the mutations K64R or M97K.

2. A Phi29 type DNA polymerase that has an amino acid sequence having an identity of at least 80% with SEQ ID NO: 2; SEQ ID NO. 3 or SEQ ID NO 4.

3. A method for replicating, amplifying or sequencing a template DNA which comprises contacting said DNA with a reaction mixture comprising at least:

a) the DNA polymerase according to any of claims 1 to 2,

b) a buffer,

c) magnesium chloride,

d) a primer, and

e) nucleoside triphosphates.

4. A kit for carrying out a method according to claim 3 comprising: a) the DNA polymerase according to any of claims 1 to 2, b) a buffer, and c) magnesium chloride.

5. A kit for carrying out a method according to claim 3 comprising the DNA polymerase according to any of claims 1 to 2, and one or more of:

(a) a PrimPol enzyme (e.g., TthPrimPol);

(b) random trimers;

(c) random tetramers;

(d) random pentamers;

(e) random heptamers;

(f) random octamers;

(g) dNTPs;

(h) reaction buffer;

(i) a buffer for use with any of the aforementioned elements.

6. A Phi29 type DNA polymerase, wherein the Phi29 type DNA polymerase has an amino acid sequence having at least 80%, 85%, 90%, 95%, 98% or 99% 99.5% sequence identity with SEQ ID NO:1, and wherein the Phi29 type DNA polymerase comprises one or both amino acid substitutions K64R and M97K.

7. The Phi29 type DNA polymerase of claim **6**, having a sequence of SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4.

8. The Phi29 type DNA polymerase of claim **6**, having no more than 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid substitutions, additions or deletions in addition to one or both of amino acid substitutions K64R and M97K.

9. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a Phi29 type DNA polymerase, wherein the Phi29 type DNA polymerase has an amino acid sequence having at least 80%, 85%, 90%, 95%, 98% or 99% 99.5% sequence identity with SEQ ID NO:1, and wherein the Phi29 type DNA polymerase comprises one or both amino acid substitutions K64R and M97K.

10. The isolated nucleic acid molecule of claim **9**, wherein the Phi29 type DNA polymerase has a sequence of SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4.

11. The isolated nucleic acid molecule of claim **9**, wherein the Phi29 type DNA polymerase has no more than 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid substitutions, additions or deletions in addition to one or both of amino acid substitutions K64R and M97K.

12. A recombinant nucleic acid comprising an transcription regulatory sequence operatively linked with a Phi29 type DNA polymerase of any of claims **9-11**.

13. The recombinant nucleic acid of claim **12**, wherein the transcription regulatory sequence comprises a bacterial or mammalian promoter.

14. The recombinant nucleic acid of claim **12** contained in a vector selected from a plasmid vector, a viral vector, a cosmid, and a transposon.

15. The recombinant nucleic acid of claim **14**, comprising a cloning site positioned relative to the nucleotide sequence encoding the Phi29 type DNA polymerase such that an transcription regulatory sequence inserted into the cloning site becomes operatively linked with the nucleotide sequence encoding the Phi29 type DNA polymerase.

16. A recombinant cell comprising a recombinant nucleic acid of any of claims **12-15**.

17. A method comprising:

a) contacting a nucleic acid template molecule with a Phi29 type DNA polymerase of any of claims **1, 2, 6-8**, and reagents sufficient for primer extension; and

b) performing primer extension with the polymerase using the nucleic acid template.

18. The method of claim **17,** wherein the reagents sufficient for primer extension comprise oligonucleotide primers.

19. The method of claim **18,** wherein the oligonucleotide primers comprise one or more of trimers, tetramers, pentamers, hexamers, hexamers, octomers, nonamers or 10-mers.

20. The method of claim **19**, wherein the primers are random primers.

21. The method of claim **18,** wherein the oligonucleotide primers have links between five and 25 nucleotides.

22. The method of claim **17,** wherein the reagents sufficient for primer extension comprise a primase/polymerase (e.g., TthPrimPol).

23. The method of claim **17,** wherein primer extension is performed at a temperature about, or above, any of 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, or 42°C.

24. The method of claim **17,** wherein the template nucleic acid molecule is present in an amount no greater than 1 ng, 100 pg, 10 pg, or 1 pg.

25. The method of claim **17,** wherein the primer extension comprises (i) multiple displacement amplification ("MDA") or (2) rolling circle amplification.

26. The method of claim **17,** wherein the primer extension comprises multiple annealing and looping-based amplification cycles (MALBAC).

27. The method of any of claims **17-26**, wherein the Phi29 type DNA polymerase comprises both of substitutions K64R and M97K.