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(54) Title: DNA POLYMERASES FOR EFFICIENT AND EFFECTIVE INCORPORATION OF METHYLATED-DNTPS

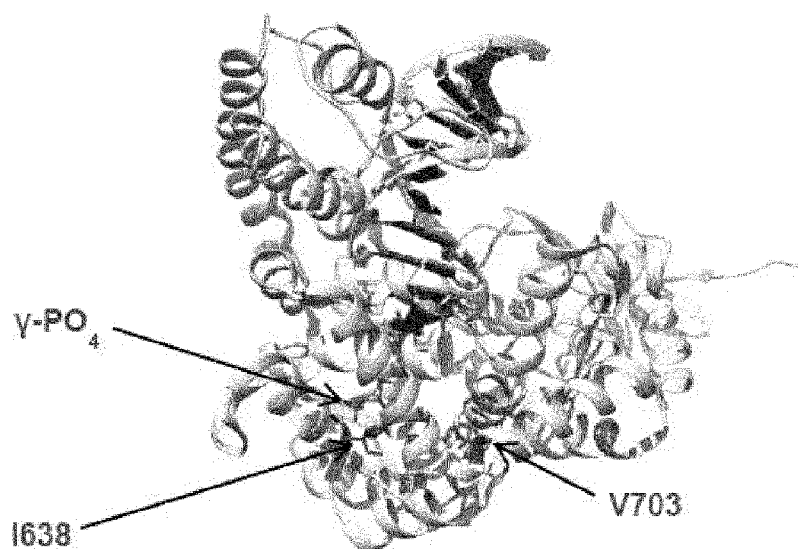


Figure 3

(57) Abstract: Disclosed are DNA polymerases having improved ability to incorporate methylated-dNTPs, relative to a corresponding, unmodified polymerase. The polymerases are useful in a variety of disclosed primer extension methods. Also disclosed are related compositions, including recombinant nucleic acids, vectors, and host cells, which are useful, e.g., for production of the DNA polymerases. Further disclosed are kits and reaction mixtures comprising the improved DNA polymerases as well as methods of primer extension using the improved DNA polymerases.



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DNA POLYMERASES FOR EFFICIENT AND EFFECTIVE INCORPORATION OF METHYLATED-dNTPS

FIELD OF THE INVENTION

The present invention provides DNA polymerases with improved activities, including
5 efficient and effective incorporation of methylated nucleotides (*e.g.*, deoxynucleotide
triphosphates (dNTPs)), as well as methods for use of such polymerases in various
applications, including nucleic acid polynucleotide extension and amplification. Also
provided are kits comprising the DNA polymerases with improved activities, including
efficient and effective incorporation of methylated nucleotides (*e.g.*, dNTPs), and reaction
10 mixtures thereof, as well as nucleic acids that encode the DNA polymerases.

BACKGROUND OF THE INVENTION

DNA polymerases are responsible for the replication and maintenance of the genome, a role
that is central to accurately transmitting genetic information from generation to generation.
DNA polymerases function in cells as the enzymes responsible for the synthesis of DNA.
15 They polymerize deoxyribonucleoside triphosphates in the presence of a metal activator, such
as Mg²⁺, in an order dictated by the DNA template or polynucleotide template that is copied.
In vivo, DNA polymerases participate in a spectrum of DNA synthetic processes including
DNA replication, DNA repair, recombination, and gene amplification. During each DNA
synthetic process, the DNA template is copied once or at most a few times to produce
20 identical replicas. In contrast, *in vitro*, DNA replication can be repeated many times such as,
for example, during polymerase chain reaction (*see, e.g.*, U.S. Patent No. 4,683,202).
In the initial studies with polymerase chain reaction (PCR), the DNA polymerase was added
at the start of each round of DNA replication (*see* U.S. Patent No. 4,683,202, *supra*).
Subsequently, it was determined that thermostable DNA polymerases could be obtained from
25 bacteria that grow at elevated temperatures, and that these enzymes need to be added only
once (*see* U.S. Patent Nos. 4,889,818 and 4,965,188). At the elevated temperatures used
during PCR, these enzymes are not irreversibly inactivated. As a result, one can carry out
repetitive cycles of polymerase chain reactions without adding fresh enzymes at the start of
each synthetic addition process. DNA polymerases, particularly thermostable polymerases,
30 are the key to a large number of techniques in recombinant DNA studies and in medical
diagnosis of disease. For diagnostic applications in particular, a target nucleic acid sequence
may be only a small portion of the DNA or RNA in question, so it may be difficult to detect
the presence of a target nucleic acid sequence without amplification.

The overall folding pattern of DNA polymerases resembles the human right hand and contains three distinct subdomains of palm, fingers, and thumb. (See Beese *et al.*, *Science* 260:352-355, 1993); Patel *et al.*, *Biochemistry* 34:5351-5363, 1995). While the structure of the fingers and thumb subdomains vary greatly between polymerases that differ in size and in cellular functions, the catalytic palm subdomains are all superimposable. For example, motif A, which interacts with the incoming dNTP and stabilizes the transition state during chemical catalysis, is superimposable with a mean deviation of about one Å amongst mammalian pol α and prokaryotic pol I family DNA polymerases (Wang *et al.*, *Cell* 89:1087-1099, 1997). Motif A begins structurally at an antiparallel β-strand containing predominantly hydrophobic residues and continues to an α-helix. The primary amino acid sequence of DNA polymerase active sites is exceptionally conserved. In the case of motif A, for example, the sequence DYSQIELR (SEQ ID NO:6) is retained in polymerases from organisms separated by many millions years of evolution, including, *e.g.*, *Thermus aquaticus*, *Chlamydia trachomatis*, and *Escherichia coli*.

In addition to being well-conserved, the active site of DNA polymerases has also been shown to be relatively mutable, capable of accommodating certain amino acid substitutions without reducing DNA polymerase activity significantly. (See, *e.g.*, U.S. Patent No. 6,602,695). Such mutant DNA polymerases can offer various selective advantages in, *e.g.*, diagnostic and research applications comprising nucleic acid synthesis reactions.

There are at least two steps in the enzymatic process of DNA polymerization: (1) the incorporation of the incoming nucleotide, and (2) the extension of the newly incorporated nucleotide. The overall faithfulness or “fidelity” of the DNA polymerase is generally thought of as a conglomerate of these two enzymatic activities, but the steps are distinct. A DNA polymerase may misincorporate the incoming nucleotide, but if it is not efficiently extended the extension rate will be severely decreased and overall product formation would be minimal. Alternatively, it is possible to have a DNA polymerase misincorporate the incoming nucleotide and readily misextend the newly formed mismatch. In this case, the overall extension rate would be high, but the overall fidelity would be low. An example of this type of enzyme would be ES112 DNA polymerase (E683R Z05 DNA polymerase; see US 7,179,590) when using Mn²⁺ as the divalent metal ion activator. The enzyme has a very high efficiency because unlike typical DNA polymerases that tend to hesitate/stall when a mismatch is encountered, the ES112 DNA polymerase readily extends the mismatch. The phenotype displayed in ES112 is more pronounced during the RT step, presumably because of structural effects of the RNA/DNA heteroduplex vs. the DNA/DNA homoduplex. A second

example would be if the DNA polymerase does not readily misincorporate (may be even less likely to misincorporate), but does have increased capacity to misextend a mismatch. In this case, the fidelity is not significantly altered for the overall product. In general, this type of enzyme is more favorable for extension reactions than the characteristics of ES112 in Mn^{2+} because the fidelity of the product is improved. However, this attribute can be utilized to allow the misextension of a mismatched oligonucleotide primer such as when an oligonucleotide primer of a single sequence is hybridized to a target that has sequence heterogeneity (e.g., viral targets), but the normal or lower misincorporation rate allows for completion of DNA synthesis beyond the original oligonucleotide primer. An example of this type of DNA polymerase is Z05 D580G DNA polymerase (see U.S. Patent Publication No. 2009/0148891). This type of activity is referred to as “mismatch tolerant” because it is more tolerant to mismatches in the oligonucleotide primer. While the examples above have discussed primer extension type reactions, the activity can be more significant in reactions such as RT-PCR and PCR where primer extension is reoccurring frequently. Data suggests that while enzymes such as Z05 D580G are more “tolerant” to mismatches, they also have enhanced ability to extend oligonucleotide primers containing modified bases (e.g., t-butyl benzyl modified bases) or in the presence of DNA binding dyes such as SYBR Green I (see U.S. Patent Publication No. 2009/028053).

Reverse transcription polymerase chain reaction (RT-PCR) is a technique used in many applications to detect/and or quantify RNA targets by amplification. In order to amplify RNA targets by PCR, it is necessary to first reverse transcribe the RNA template into cDNA. Typically, RT-PCR assays rely on a non-thermostable reverse transcriptase (RNA dependent DNA polymerase), derived from a mesophilic organism, for the initial cDNA synthesis step (RT). An additional thermostable DNA polymerase is required for amplification of cDNA to tolerate elevated temperatures required for nucleic acid denaturation in PCR. There are several potential benefits of using thermoactive or thermostable DNA polymerases engineered to perform more efficient reverse transcription for RT-PCR assays. Increased reverse transcriptase activity coupled with the ability to use higher reverse transcription incubation temperatures that allow for relaxing of RNA template secondary structure can result in overall higher cDNA synthesis efficiency and assay sensitivity. Higher temperature incubation could also increase specificity by reducing false priming in the reverse transcription step. Enzymes with improved reverse transcription efficiency can simplify assay design by allowing for reduced RT incubation times and/or enzyme concentration. When using dUTP and UNG, nonspecific extension products containing dUMP that are formed during nonstringent set-up

conditions are degraded by UNG and cannot be utilized either as primers or as templates. When using a non-thermostable reverse transcriptase (RNA dependent DNA polymerase) derived from a mesophilic organism, it is not possible to utilize the dUTP and UNG methodologies. (Myers, T.W. et al., Amplification of RNA: High Temperature Reverse
5 Transcription and DNA Amplification with *Thermus thermophilus* DNA Polymerase, in *PCR Strategies*, Innis, M.A., Gelfand, D.H., and Sninsky, J.J., Eds., Academic Press, San Diego, CA, 58-68, (1995)). However, the use of a thermoactive or thermostable DNA polymerase of the invention for the reverse transcription step enables the reaction to be completely compatible with the utilization of the dUTP/uracil N-glycosylase (UNG) carry-over
10 prevention system (Longo et al., Use of Uracil DNA Glycosylase to Control Carry-over Contamination in Polymerase Chain Reactions. *Gene* 93:125-128, (1990). In addition to providing carry-over contamination control, the use of dUTP and UNG provides a “hot-start” to reduce nonspecific amplification (Innis and Gelfand, 1999).

Nucleic acid amplification methods, such as PCR, are typically performed at high
15 temperatures. It is known that, in PCR, repeated cycles of denaturation, annealing, and extension, at high temperatures, causes significant breakdown of dNTPs, mostly via the hydrolysis of dNTPs to di- and then mono-phosphates. Studies show that dNTPs having a modification of the γ -phosphate (*i.e.*, terminal phosphate) exhibit improved stability at high temperatures (U.S. Patent No. 7,452,698). That is, modifications, such as addition of a methyl
20 group (*i.e.*, methylated-dNTP (me-dNTP)) prevents the hydrolysis reaction from taking place (U.S. Patent No. 7,452,698). Thus, from a standpoint of stability at room temperature and elevated temperature, the use of methylated nucleotides (*i.e.*, methylated-dNTPs) are favorable over conventional nucleotides (*i.e.*, dNTPs). Thus, stable dNTPs would be a tremendous advantage and an improvement in the art. However, in routine PCR reactions,
25 conventional polymerases do not efficiently and effectively incorporate methylated-dNTPs. Therefore, there remains a need in the art for compositions and methods for conduct amplification reactions using more stable methylated-dNTPs.

BRIEF SUMMARY OF THE INVENTION

Provided herein are DNA polymerases having improved activities, including the ability to
30 efficiently and effectively incorporate methylated-dNTPs, relative to a corresponding unmodified control polymerase (*i.e.*, parental polymerase), methods of making such DNA polymerases, and methods of using such DNA polymerases. The improved DNA polymerase has improved ability to efficiently and effectively incorporate methylated-dNTPs as compared with a control DNA polymerase. Herein, the improved DNA polymerase comprises an amino

acid sequence that is substantially identical (*e.g.*, at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identical) to SEQ ID NOs:1-5.

In one aspect a DNA polymerase is provided having increased efficiency in incorporating methylated deoxynucleotide triphosphates (dNTPs) compared with a control DNA polymerase, wherein the amino acid of the DNA polymerase corresponding to position 640 of SEQ ID NO:2 is any amino acid other than I, and/or wherein the amino acid of the DNA polymerase corresponding to position 705 of SEQ ID NO:2 is any amino acid other than V, and wherein the control DNA polymerase has the same amino acid sequence as the DNA polymerase except that the amino acid of the control DNA polymerase corresponding to position 640 of SEQ ID NO:2 is I and/or the amino acid of the control DNA polymerase corresponding to position 705 of SEQ ID NO:2 is V. In one embodiment, the DNA polymerase comprises an amino acid sequence at least 90%, particularly at least 95%, more particularly at least 98% identical to SEQ ID NO:2. In some embodiments, the DNA polymerase and the control DNA polymerase comprises an amino acid sequence at least 90% identical to SEQ ID NO:2. In some embodiments, the DNA polymerase and the control DNA polymerase comprises an amino acid sequence at least 95%, particularly at least 98% identical to SEQ ID NO:2. In another embodiment, the amino acid corresponding to position 640 of SEQ ID NO:2 is M. In another embodiment, the DNA polymerase comprises the amino acid sequence of SEQ ID NO:4. In yet another embodiment, the amino acid corresponding to position 705 of SEQ ID NO:2 is L. In another embodiment, the DNA polymerase comprises the amino acid sequence of SEQ ID NO:5. In another embodiment, the DNA polymerase comprises the amino acid sequence of SEQ ID NO:3. In one embodiment, the DNA polymerase comprises an amino acid sequence at least 90%, particularly at least 95%, more particularly at least 98% identical to SEQ ID NO:2, wherein the amino acid corresponding to position 640 of SEQ ID NO:2 is M. In another embodiment, the DNA polymerase comprises an amino acid sequence at least 90%, particularly at least 95%, more particularly at least 98% identical to SEQ ID NO:2, wherein the amino acid corresponding to position 705 of SEQ ID NO:2 is L. In another embodiment, the DNA polymerase comprises an amino acid sequence at least 90%, particularly at least 95%, more particularly at least 98% identical to SEQ ID NO:2, wherein the amino acid corresponding to position 640 of SEQ ID NO:2 is M and wherein the amino acid corresponding to position 705 of SEQ ID NO:2 is L.

In another aspect a recombinant nucleic acid is provided encoding a DNA polymerase having increased efficiency in incorporating methylated deoxynucleotide triphosphates (dNTPs) compared with a control DNA polymerase, wherein the amino acid of the DNA polymerase

corresponding to position 640 of SEQ ID NO:2 is any amino acid other than I, and/or wherein the amino acid of the DNA polymerase corresponding to position 705 of SEQ ID NO:2 is any amino acid other than V, and wherein the control DNA polymerase has the same amino acid sequence as the DNA polymerase except that the amino acid of the control DNA polymerase
5 corresponding to position 640 of SEQ ID NO:2 is I and/or the amino acid of the control DNA polymerase corresponding to position 705 of SEQ ID NO:2 is V. In certain embodiments, the recombinant nucleic acid encodes the DNA polymerase described above.

In another related aspect an expression vector is provided comprising the recombinant nucleic acid encoding a DNA polymerase having increased efficiency in incorporating methylated
10 deoxynucleotide triphosphates (dNTPs) compared with a control DNA polymerase, wherein the amino acid of the DNA polymerase corresponding to position 640 of SEQ ID NO:2 is any amino acid other than I, and/or wherein the amino acid of the DNA polymerase corresponding to position 705 of SEQ ID NO:2 is any amino acid other than V, and wherein the control DNA polymerase has the same amino acid sequence as the DNA polymerase except that the
15 amino acid of the control DNA polymerase corresponding to position 640 of SEQ ID NO:2 is I and/or the amino acid of the control DNA polymerase corresponding to position 705 of SEQ ID NO:2 is V. In certain embodiments, the expression vector comprises the recombinant nucleic acid encoding the DNA polymerase described above.

In an additional aspect a kit for producing an extended primer is provided comprising at least
20 one container providing a DNA polymerase having increased efficiency in incorporating methylated deoxynucleotide triphosphates (dNTPs) compared with a control DNA polymerase, wherein the amino acid of the DNA polymerase corresponding to position 640 of SEQ ID NO:2 is any amino acid other than I, and/or wherein the amino acid of the DNA polymerase corresponding to position 705 of SEQ ID NO:2 is any amino acid other than V,
25 and wherein the control DNA polymerase has the same amino acid sequence as the DNA polymerase except that the amino acid of the control DNA polymerase corresponding to position 640 of SEQ ID NO:2 is I and/or the amino acid of the control DNA polymerase corresponding to position 705 of SEQ ID NO:2 is V. In another embodiment, the kit further comprises one or more additional containers selected from the group consisting of: (a) a
30 container providing a primer hybridizable, under primer extension conditions, to a predetermined polynucleotide template; (b) a container providing dNTPs; and (c) a container providing a buffer suitable for primer extension. In another embodiment, the dNTPs are methylated dNTP. In certain embodiments, the DNA polymerase is the DNA polymerase described above.

In another aspect a reaction mixture is provided comprising a DNA polymerase having increased efficiency in incorporating methylated deoxynucleotide triphosphates (dNTPs) compared with a control DNA polymerase, wherein the amino acid of the DNA polymerase corresponding to position 640 of SEQ ID NO:2 is any amino acid other than I, and/or wherein the amino acid of the DNA polymerase corresponding to position 705 of SEQ ID NO:2 is any amino acid other than V, and wherein the control DNA polymerase has the same amino acid sequence as the DNA polymerase except that the amino acid of the control DNA polymerase corresponding to position 640 of SEQ ID NO:2 is I and/or the amino acid of the control DNA polymerase corresponding to position 705 of SEQ ID NO:2 is V, at least one primer, a polynucleotide template, and dNTPs. In a related embodiment, the polynucleotide template is RNA. In another embodiment, the polynucleotide template is DNA. In another embodiment, the reaction mixture further comprises Mg^{2+} . In certain embodiments, the DNA polymerase is the DNA polymerase described above. In another embodiment, the reaction mixture further comprises a second thermostable DNA polymerase.

In another aspect a method for conducting primer extension is provided, comprising: contacting a DNA polymerase with a primer, a polynucleotide template, and dNTPs, under conditions suitable for extension of the primer, thereby producing an extended primer, wherein the DNA polymerase incorporates methylated dNTPs with increased efficiency, compared with a control DNA polymerase, wherein the amino acid of the DNA polymerase corresponding to position 640 of SEQ ID NO:2 is any amino acid other than I, and/or wherein the amino acid of the DNA polymerase corresponding to position 705 of SEQ ID NO:2 is any amino acid other than V, and wherein the control DNA polymerase has the same amino acid sequence as the DNA polymerase except that the amino acid of the control DNA polymerase corresponding to position 640 of SEQ ID NO:2 is I and/or the amino acid of the control DNA polymerase corresponding to position 705 of SEQ ID NO:2 is V. In one embodiment, the DNA polymerase comprises an amino acid sequence at least 90%, particularly at least 95%, more particularly at least 98% identical to SEQ ID NO:2. In some embodiments, the DNA polymerase and the control DNA polymerase comprises an amino acid sequence at least 90% identical to SEQ ID NO:2. In some embodiments, the DNA polymerase and the control DNA polymerase comprises an amino acid sequence at least 95%, particularly at least 98% identical to SEQ ID NO:2. In another embodiment, the amino acid corresponding to position 640 of SEQ ID NO:2 is M. In another embodiment, the DNA polymerase comprises the amino acid sequence of SEQ ID NO:4. In yet another embodiment, the amino acid corresponding to position 705 of SEQ ID NO:2 is L. In another embodiment, the DNA polymerase comprises

the amino acid sequence of SEQ ID NO:5. In another embodiment, the DNA polymerase comprises the amino acid sequence of SEQ ID NO:3. In certain embodiments, the DNA polymerase is the DNA polymerase described above.

5 Various DNA polymerases are amenable to mutation as described above. Particularly suitable are thermostable polymerases, including wild-type or naturally occurring thermostable polymerases from various species of thermophilic bacteria, as well as synthetic thermostable polymerases derived from such wild-type or naturally occurring enzymes by amino acid substitution, insertion, or deletion, or other modification. Thus, in some embodiments, the polymerase is a thermostable DNA polymerase. Exemplary unmodified forms of polymerase
10 include, *e.g.*, CS5, CS6, Z05, or Z05D DNA polymerase, or a functional DNA polymerase having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity thereto. Other unmodified polymerases include, *e.g.*, DNA polymerases from any of the following species of thermophilic bacteria (or a functional DNA polymerase having at least 80%, 81%, 82%, 83%,
15 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity to such a polymerase): *Thermotoga maritima*; *Thermus aquaticus*; *Thermus thermophilus*; *Thermus flavus*; *Thermus filiformis*; *Thermus sp. sps17*; *Thermus sp. Z05*; *Thermotoga neopolitana*; *Thermosiphon africanus*; *Thermus caldophilus*, *Deinococcus radiodurans*, *Bacillus stearothermophilus* or *Bacillus caldotenax*. Suitable
20 polymerases also include those having reverse transcriptase (RT) activity and/or the ability to incorporate unconventional nucleotides, such as ribonucleotides or other 2'-modified nucleotides.

In some embodiments, the DNA polymerase is a thermoactive DNA polymerase. While thermostable DNA polymerases possessing efficient reverse transcription activity are
25 particularly suited for performing RT-PCR, especially single enzyme RT-PCR, thermoactive, but not thermostable DNA polymerases possessing efficient reverse transcription activity also are amenable to mutation according to the present invention. For example, the attribute of efficiently and effectively incorporated methylated-dNTPs are useful for the RT step in an RT-PCR and this step does not need to be performed at temperatures that would inactivate a
30 thermoactive but not thermostable DNA polymerase. Following the RT step, a thermostable DNA polymerase could either be added or it could already be included in the reaction mixture to perform the PCR amplification step. For example, the improved DNA polymerase described herein can be combined with a second thermostable DNA polymerase prior to the RT step in a buffer suitable for extension and amplification of RNA and DNA templates, as

described in the Examples. Examples of suitable thermostable DNA polymerases are described in US Patent Nos. 4,889,818, 5,773, 258 and 5,677,152. In some embodiments, the second thermostable DNA polymerase is AmpliTaq® DNA polymerase (Deoxynucleoside triphosphate: DNA Deoxynucleotidyltransferase, E.C.2.7.7.7). In some embodiments, the second thermostable DNA polymerase is a reversibly inactivated thermostable polymerase, as described below. In one embodiment, the reversibly inactivated thermostable polymerase is AmpliTaq Gold® DNA polymerase (Roche Applied Science, Indianapolis, IN, USA). This second methodology would especially benefit by using a chemically modified thermostable DNA polymerase (or other HotStart technology to inactivate the thermostable DNA polymerase) so that it would not be fully active during the RT step. An example of a thermoactive but not thermostable DNA polymerase possessing efficient reverse transcription activity is the DNA polymerase from *Carboxydotherrnus hydrogenoformans* (Chy). See, e.g., US Patent Nos. 6,468,775, 6,399,320, 8,945,882, 9,441,269, and U.S. Patent Publication No. 2017/0029792.

In some embodiments, the DNA polymerase is derived from a *Thermus* species. Thus, in some embodiments, the DNA polymerase has at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% amino acid sequence identity to a polymerase selected from the group consisting of:

- (a) a *Thermus sp.* Z05 DNA polymerase (Z05) (SEQ ID NO:1);
- (b) a *Thermus aquaticus* DNA polymerase (Taq);
- (c) a *Thermus filiformis* DNA polymerase (Tfi);
- (d) a *Thermus flavus* DNA polymerase (Tfl);
- (e) a *Thermus sp. sps17* DNA polymerase (Sps17);
- (f) a *Thermus thermophilus* DNA polymerase (Tth); and
- (g) a *Thermus caldophilus* DNA polymerase (Tca).

The mutant or improved polymerases can include other, non-substitutional modifications. One such modification is a thermally reversible covalent modification that inactivates the enzyme, but which is reversed to activate the enzyme upon incubation at an elevated temperature, such as a temperature typically used for polynucleotide extension. Exemplary reagents for such thermally reversible modifications are described in U.S. Patent Nos. 5,773, 258 and 5,677,152.

In some embodiments, the reverse transcriptase activity is determined by performing real-time RT-PCR amplification and detection of a Hepatitis C Virus (HCV) transcript generated from the first 800 bases of HCV genotype 1b 5'LTR in pSP64 poly(A) (Promega). Two or more

reaction mixtures can have titrated numbers of copies of the Hepatitis C Virus (HCV) transcript (*e.g.*, 1:5 titrations, 1:10 titrations, *e.g.*, 10,000 copies, 1000 copies, 100 copies, 10 copies, 1 copy, 0 copies in several reaction mixtures). The reverse transcriptase ability of a polymerase of the invention can be compared to the reverse transcriptase ability of a reference polymerase (*e.g.*, a naturally occurring, unmodified, or control polymerase), over a preselected unit of time, as described herein. Polymerases with improved reverse transcriptase ability will amplify the transcript with greater efficiency, or will require a lower number of PCR cycles to amplify the transcript (*i.e.*, exhibit a lower C_p value, as calculated herein), in comparison to a naturally occurring or unmodified polymerase. Moreover, in some embodiments, polymerases with improved RT function also have improved replication of long RNA (*e.g.*, at least 500 or 1000 or 2000 or 5000 or more nucleotides long) templates. In some embodiments, the improved reverse transcriptase efficiency includes a shorter reverse transcription time in comparison to a control polymerase. Thus, in some embodiments, polymerases with increased reverse transcriptase efficiency will reverse transcribe an RNA template faster than a control or reference polymerase.

In various other aspects, the present invention provides a recombinant nucleic acid encoding a mutant or improved DNA polymerase as described herein, a vector comprising the recombinant nucleic acid, and a host cell transformed with the vector. In certain embodiments, the vector is an expression vector. Host cells comprising such expression vectors are useful in methods of the invention for producing the mutant or improved polymerase by culturing the host cells under conditions suitable for expression of the recombinant nucleic acid. The polymerases of the invention may be contained in reaction mixtures and/or kits. The embodiments of the recombinant nucleic acids, host cells, vectors, expression vectors, reaction mixtures and kits are as described above and herein.

In yet another aspect, a method for conducting polynucleotide extension is provided. The method generally includes contacting a DNA polymerase having improved ability to efficiently and effectively incorporate methylated-dNTPs with a primer, a polynucleotide template, and nucleoside triphosphates (*i.e.*, methylated-dNTPs) under conditions suitable for extension of the primer, thereby producing an extended primer. The polynucleotide template can be, for example, an RNA or DNA template. The nucleoside triphosphates can include unconventional nucleotides such as, *e.g.*, ribonucleotides and/or labeled nucleotides, and, of course, methylated-dNTPs. Nucleoside triphosphates may include dATP (2'-deoxyadenosine 5'-triphosphate), dCTP (2'-deoxycytidine 5'-triphosphate), dGTP (2'-deoxyguanosine 5'-triphosphate), dTTP (2'-deoxythymidine 5'-triphosphate), dITP (2-deoxyinosine 5'-

triphosphate), and/or dUTP (2'-deoxyuridine 5'-triphosphate). Methylated dNTPs may include methylated-dATP, methylated-dCTP, methylated-dGTP, methylated-DTTP, methylated-ITP, and/or methylated-dUTP. Further, the primer and/or template can include one or more nucleotide analogs. In some variations, the polynucleotide extension method is a
5 method for polynucleotide amplification that includes contacting the mutant or improved DNA polymerase with a primer pair, the polynucleotide template, and the nucleoside triphosphates under conditions suitable for amplification of the polynucleotide. The polynucleotide extension reaction can be, *e.g.*, PCR, isothermal extension, or sequencing (*e.g.*, 454 sequencing reaction). The polynucleotide template can be from any type of
10 biological sample.

The present invention also provides a kit useful in such a polynucleotide extension method. Generally, the kit includes at least one container providing a mutant or improved DNA polymerase as described herein. In certain embodiments, the kit further includes one or more additional containers providing one or more additional reagents. For example, in specific
15 variations, the one or more additional containers provide nucleoside triphosphates; a buffer suitable for polynucleotide extension; and/or one or more primer or probe polynucleotides, hybridizable, under polynucleotide extension conditions, to a predetermined polynucleotide template. The polynucleotide template can be from any type of biological sample.

Further provided are reaction mixtures comprising the polymerases of the invention. The
20 reaction mixtures can also contain a template nucleic acid (DNA and/or RNA), one or more primer or probe polynucleotides, nucleoside triphosphates (including, *e.g.*, deoxy-ribonucleoside triphosphates, ribonucleoside triphosphates, labeled nucleoside triphosphates, unconventional nucleoside triphosphates), buffers, salts, labels (*e.g.*, fluorophores). In some embodiments, the reaction mixtures comprise an iron chelator or a purple dye. In certain
25 embodiments, the reaction mixtures comprise hemoglobin, or a degradation product of hemoglobin. For example, in certain embodiments, the degradation products of hemoglobin include heme breakdown products such as hemin, hematin, hematophoryn, and bilirubin. In other embodiments, the reaction mixtures comprise heparin or a salt thereof. Optionally, the reaction mixture comprises an intercalating dye (including but not limited to those described
30 above or elsewhere herein). In certain embodiments, the reaction mixture contains a template nucleic acid that is isolated from blood. In other embodiments, the template nucleic acid is RNA and the reaction mixture comprises heparin or a salt thereof.

In some embodiments, the reaction mixture comprises two or more polymerases. For example, in some embodiments, the reaction mixture comprises an improved DNA

polymerase having increased reverse transcription efficiency (e.g., increased activity extending an RNA-template) as described herein, and another polymerase having DNA-dependent polymerase activity. In one embodiment, the reaction mixture comprises a blend of an improved DNA polymerase having increased reverse transcription efficiency as described
5 herein, and a second thermostable DNA-dependent polymerase. The second thermostable DNA-dependent polymerase can be a reversibly modified polymerase as described above such that the enzyme is inactive at temperatures suitable for the reverse transcription step, but is activated under suitable conditions, for example, at elevated temperatures of about 90° C to 100° C for a period of time up to about 12 minutes. Suitable conditions for activation of a
10 reversibly inactivated thermostable polymerase are provided, for example, in a Hot Start PCR reaction, as described in the Examples. Examples of suitable second thermostable DNA-dependent polymerases are described in U.S. Patent Nos. 5,773, 258 and 5,677,152.
Further embodiments of the invention are described herein.

DEFINITIONS

15 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although essentially any methods and materials similar to those described herein can be used in the practice or testing of the present invention, only exemplary methods and materials are described. For purposes of the present invention, the following terms are defined
20 below.

The terms “a,” “an,” and “the” include plural referents, unless the context clearly indicates otherwise.

An “amino acid” refers to any monomer unit that can be incorporated into a peptide, polypeptide, or protein. As used herein, the term “amino acid” includes the following twenty
25 natural or genetically encoded alpha-amino acids: alanine (Ala or A), arginine (Arg or R), asparagine (Asn or N), aspartic acid (Asp or D), cysteine (Cys or C), glutamine (Gln or Q), glutamic acid (Glu or E), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), leucine (Leu or L), lysine (Lys or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophan (Trp or W), tyrosine (Tyr or Y), and
30 valine (Val or V). In cases where “X” residues are undefined, these should be defined as “any amino acid.” The structures of these twenty natural amino acids are shown in, e.g., Stryer et al., Biochemistry, 5th ed., Freeman and Company (2002). Additional amino acids, such as selenocysteine and pyrrolysine, can also be genetically coded for (Stadtman (1996) “Selenocysteine,” Annu Rev Biochem 65:83-100 and Ibba et al. (2002) “Genetic code:

introducing pyrrolysine,” Curr Biol. 12(13):R464-R466). The term “amino acid” also includes unnatural amino acids, modified amino acids (e.g., having modified side chains and/or backbones), and amino acid analogs. See, e.g., Zhang et al. (2004) “Selective incorporation of 5-hydroxytryptophan into proteins in mammalian cells,” Proc. Natl. Acad. Sci. U.S.A. 101(24):8882-8887, Anderson et al. (2004) “An expanded genetic code with a functional quadruplet codon” Proc. Natl. Acad. Sci. U.S.A. 101(20):7566-7571, Ikeda et al. (2003) “Synthesis of a novel histidine analogue and its efficient incorporation into a protein in vivo,” Protein Eng. Des. Sel. 16(9):699-706, Chin et al. (2003) “An Expanded Eukaryotic Genetic Code,” Science 301(5635):964-967, James et al. (2001) “Kinetic characterization of ribonuclease S mutants containing photoisomerizable phenylazophenylalanine residues,” Protein Eng. Des. Sel. 14(12):983-991, Kohrer et al. (2001) “Import of amber and ochre suppressor tRNAs into mammalian cells: A general approach to site-specific insertion of amino acid analogues into proteins,” Proc. Natl. Acad. Sci. U.S.A. 98(25):14310-14315, Bacher et al. (2001) “Selection and Characterization of Escherichia coli Variants Capable of Growth on an Otherwise Toxic Tryptophan Analogue,” J. Bacteriol. 183(18):5414-5425, Hamano-Takaku et al. (2000) “A Mutant Escherichia coli Tyrosyl-tRNA Synthetase Utilizes the Unnatural Amino Acid Azatyrosine More Efficiently than Tyrosine,” J. Biol. Chem. 275(51):40324-40328, and Budisa et al. (2001) “Proteins with β -(thienopyrrolyl)alanines as alternative chromophores and pharmaceutically active amino acids,” Protein Sci. 10(7):1281-1292.

To further illustrate, an amino acid is typically an organic acid that includes a substituted or unsubstituted amino group, a substituted or unsubstituted carboxy group, and one or more side chains or groups, or analogs of any of these groups. Exemplary side chains include, e.g., thiol, seleno, sulfonyl, alkyl, aryl, acyl, keto, azido, hydroxyl, hydrazine, cyano, halo, hydrazide, alkenyl, alkynyl, ether, borate, boronate, phospho, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, ester, thioacid, hydroxylamine, or any combination of these groups. Other representative amino acids include, but are not limited to, amino acids comprising photoactivatable cross-linkers, metal binding amino acids, spin-labeled amino acids, fluorescent amino acids, metal-containing amino acids, amino acids with novel functional groups, amino acids that covalently or noncovalently interact with other molecules, photocaged and/or photoisomerizable amino acids, radioactive amino acids, amino acids comprising biotin or a biotin analog, glycosylated amino acids, other carbohydrate modified amino acids, amino acids comprising polyethylene glycol or polyether, heavy atom substituted amino acids, chemically cleavable and/or photocleavable amino acids, carbon-

linked sugar-containing amino acids, redox-active amino acids, amino thioacid containing amino acids, and amino acids comprising one or more toxic moieties.

The term “biological sample” encompasses a variety of sample types obtained from an organism and can be used in a diagnostic or monitoring assay. The term encompasses urine, 5 urine sediment, blood, saliva, and other liquid samples of biological origin, solid tissue samples, such as a biopsy specimen or tissue cultures or cells derived therefrom and the progeny thereof. The term encompasses samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, sedimentation, or enrichment for certain components. The term encompasses a clinical sample, and also 10 includes cells in cell culture, cell supernatants, cell lysates, serum, plasma, biological fluids, and tissue samples.

The term “mutant,” in the context of DNA polymerases of the present invention, means a polypeptide, typically recombinant, that comprises one or more amino acid substitutions relative to a corresponding, functional DNA polymerase.

15 The term “unmodified form,” in the context of a mutant polymerase, is a term used herein for purposes of defining a mutant DNA polymerase of the present invention: the term “unmodified form” or “parental form” refers to a functional DNA polymerase that has the amino acid sequence of the mutant polymerase except at one or more amino acid position(s) specified as characterizing the mutant polymerase. Thus, reference to a mutant DNA 20 polymerase in terms of (a) its unmodified form and (b) one or more specified amino acid substitutions means that, with the exception of the specified amino acid substitution(s), the mutant polymerase otherwise has an amino acid sequence identical to the unmodified form in the specified motif. The “unmodified polymerase” (and therefore also the modified form having improved ability to incorporate methylated-dNTPs) may contain additional mutations 25 to provide desired functionality, e.g., improved transcriptase efficiency, mismatch tolerance, extension rate; improved tolerance of RT and polymerase inhibitors; and/or improved incorporation of dideoxyribonucleotides, ribonucleotides, ribonucleotide analogs, dye-labeled nucleotides, modulating 5'-nuclease activity, modulating 3'-nuclease (or proofreading) activity, or the like. Accordingly, in carrying out the present invention as described herein, the 30 unmodified form of a DNA polymerase is predetermined. The unmodified form of a DNA polymerase can be, for example, a wild-type and/or a naturally occurring DNA polymerase, or a DNA polymerase that has already been intentionally modified. An unmodified form of the polymerase is preferably a thermostable DNA polymerase, such as DNA polymerases from various thermophilic bacteria, as well as functional variants thereof having substantial

sequence identity to a wild-type or naturally occurring thermostable polymerase. Such variants can include, for example, chimeric DNA polymerases such as, for example, the chimeric DNA polymerases described in U.S. Patent Nos. 6,228,628 and 7,148,049. In certain embodiments, the unmodified form of a polymerase has reverse transcriptase (RT) activity.

5 The term “thermostable polymerase,” refers to an enzyme that is stable to heat, is heat resistant, and retains sufficient activity to effect subsequent polynucleotide extension reactions and does not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded nucleic acids. The heating conditions necessary for nucleic acid denaturation are well known in the art
10 and are exemplified in, e.g., U.S. Patent Nos. 4,683,202, 4,683,195, and 4,965,188. As used herein, a thermostable polymerase is suitable for use in a temperature cycling reaction such as the polymerase chain reaction (“PCR”). Irreversible denaturation for purposes herein refers to permanent and complete loss of enzymatic activity. For a thermostable polymerase, enzymatic activity refers to the catalysis of the combination of the nucleotides in the proper manner to
15 form polynucleotide extension products that are complementary to a template nucleic acid strand. Thermostable DNA polymerases from thermophilic bacteria include, e.g., DNA polymerases from *Thermotoga maritima*, *Thermus aquaticus*, *Thermus thermophilus*, *Thermus flavus*, *Thermus filiformis*, *Thermus* species sps17, *Thermus* species Z05 (e.g., Z05D polymerase), *Thermus caldophilus*, *Bacillus caldotenax*, *Thermotoga neopolitana*, and
20 *Thermosipho africanus*.

The term “thermoactive” refers to an enzyme that maintains catalytic properties at temperatures commonly used for reverse transcription or anneal/extension steps in RT-PCR and/or PCR reactions (i.e., 45-80 °C). Thermostable enzymes are those which are not irreversibly inactivated or denatured when subjected to elevated temperatures necessary for
25 nucleic acid denaturation. Thermoactive enzymes may or may not be thermostable. Thermoactive DNA polymerases can be DNA or RNA dependent from thermophilic species or from mesophilic species including, but not limited to, *Escherichia coli*, *Moloney murine leukemia viruses*, and *Avian myoblastosis virus*.

As used herein, a “chimeric” protein refers to a protein whose amino acid sequence represents
30 a fusion product of subsequences of the amino acid sequences from at least two distinct proteins. A chimeric protein typically is not produced by direct manipulation of amino acid sequences, but, rather, is expressed from a “chimeric” gene that encodes the chimeric amino acid sequence. In certain embodiments, for example, an unmodified form of a mutant DNA polymerase of the present invention is a chimeric protein that consists of an amino-terminal

(N-terminal) region derived from a *Thermus* species DNA polymerase and a carboxy-terminal (C-terminal) region derived from Tma DNA polymerase. The N-terminal region refers to a region extending from the N-terminus (amino acid position 1) to an internal amino acid. Similarly, the C-terminal region refers to a region extending from an internal amino acid to the C-terminus.

The term “aptamer” refers to a single-stranded DNA that recognizes and binds to DNA polymerase, and efficiently inhibits the polymerase activity as described in U.S. Patent No. 5,693,502. Use of aptamer and dUTP/UNG in RT-PCR is also discussed, for example, in Smith, E.S. et al, (Amplification of RNA: High-temperature Reverse Transcription and DNA Amplification with a Magnesium-activated Thermostable DNA Polymerase, in PCR Primer: A Laboratory Manual, 2nd Edition, Dieffenbach, C.W. and Dveksler, G.S., Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 211-219, (2003)).

In the context of mutant DNA polymerases, “correspondence” to another sequence (*e.g.*, regions, fragments, nucleotide or amino acid positions, or the like) is based on the convention of numbering according to nucleotide or amino acid position number and then aligning the sequences in a manner that maximizes the percentage of sequence identity. An amino acid “corresponding to position [X] of [specific sequence]” refers to an amino acid in a polypeptide of interest that aligns with the equivalent amino acid of a specified sequence. Generally, as described herein, the amino acid corresponding to a position of a polymerase can be determined using an alignment algorithm such as BLAST as described below. Because not all positions within a given “corresponding region” need be identical, non-matching positions within a corresponding region may be regarded as “corresponding positions.” Accordingly, as used herein, referral to an “amino acid position corresponding to amino acid position [X]” of a specified DNA polymerase refers to equivalent positions, based on alignment, in other DNA polymerases and structural homologues and families. In some embodiments of the present invention, “correspondence” of amino acid positions are determined with respect to a region of the polymerase comprising one or more motifs.

“Recombinant,” as used herein, refers to an amino acid sequence or a nucleotide sequence that has been intentionally modified by recombinant methods. By the term “recombinant nucleic acid” herein is meant a nucleic acid, originally formed *in vitro*, in general, by the manipulation of a nucleic acid by restriction endonucleases, in a form not normally found in nature. Thus an isolated, mutant DNA polymerase nucleic acid, in a linear form, or an expression vector formed *in vitro* by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a

recombinant nucleic acid is made and reintroduced into a host cell, it will replicate non-recombinantly, *i.e.*, using the *in vivo* cellular machinery of the host cell rather than *in vitro* manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes
5 of the invention. A “recombinant protein” is a protein made using recombinant techniques, *i.e.*, through the expression of a recombinant nucleic acid as depicted above.

A nucleic acid is “operably linked” when it is placed into a functional relationship with another nucleic acid sequence. For example, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is
10 operably linked to a coding sequence if it is positioned so as to facilitate translation.

The term “host cell” refers to both single-cellular prokaryote and eukaryote organisms (*e.g.*, bacteria, yeast, and actinomycetes) and single cells from higher order plants or animals when being grown in cell culture.

The term “vector” refers to a piece of DNA, typically double-stranded, which may have
15 inserted into it a piece of foreign DNA. The vector may be, for example, of plasmid origin. Vectors contain “replicon” polynucleotide sequences that facilitate the autonomous replication of the vector in a host cell. Foreign DNA is defined as heterologous DNA, which is DNA not naturally found in the host cell, which, for example, replicates the vector molecule, encodes a selectable or screenable marker, or encodes a transgene. The vector is used to transport the
20 foreign or heterologous DNA into a suitable host cell. Once in the host cell, the vector can replicate independently of or coincidental with the host chromosomal DNA, and several copies of the vector and its inserted DNA can be generated. In addition, the vector can also contain the necessary elements that permit transcription of the inserted DNA into an mRNA molecule or otherwise cause replication of the inserted DNA into multiple copies of RNA.
25 Some expression vectors additionally contain sequence elements adjacent to the inserted DNA that increase the half-life of the expressed mRNA and/or allow translation of the mRNA into a protein molecule. Many molecules of mRNA and polypeptide encoded by the inserted DNA can thus be rapidly synthesized.

The term “nucleotide,” in addition to referring to the naturally occurring ribonucleotide or
30 deoxyribonucleotide monomers, shall herein be understood to refer to related structural variants thereof, including derivatives and analogs, that are functionally equivalent with respect to the particular context in which the nucleotide is being used (*e.g.*, hybridization to a complementary base), unless the context clearly indicates otherwise.

The term “nucleic acid” or “polynucleotide” refers to a polymer that can be corresponded to a ribose nucleic acid (RNA) or deoxyribose nucleic acid (DNA) polymer, or an analog thereof. This includes polymers of nucleotides such as RNA and DNA, as well as synthetic forms, modified (*e.g.*, chemically or biochemically modified) forms thereof, and mixed polymers
5 (*e.g.*, including both RNA and DNA subunits). Exemplary modifications include methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, and the like), pendent moieties (*e.g.*, polypeptides), intercalators (*e.g.*, acridine, psoralen, and the like), chelators, alkylators, and
10 modified linkages (*e.g.*, alpha anomeric nucleic acids and the like). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Typically, the nucleotide monomers are linked via phosphodiester bonds, although synthetic forms of nucleic acids can comprise other linkages (*e.g.*, peptide nucleic acids as described in Nielsen *et al.* (*Science*
15 254:1497-1500, 1991). A nucleic acid can be or can include, *e.g.*, a chromosome or chromosomal segment, a vector (*e.g.*, an expression vector), an expression cassette, a naked DNA or RNA polymer, the product of a polymerase chain reaction (PCR), an oligonucleotide, a probe, and a primer. A nucleic acid can be, *e.g.*, single-stranded, double-stranded, or triple-stranded and is not limited to any particular length. Unless otherwise indicated, a particular
20 nucleic acid sequence optionally comprises or encodes complementary sequences, in addition to any sequence explicitly indicated.

The term “oligonucleotide” refers to a nucleic acid that includes at least two nucleic acid monomer units (*e.g.*, nucleotides). An oligonucleotide typically includes from about six to about 175 nucleic acid monomer units, more typically from about eight to about 100 nucleic acid monomer units, and still more typically from about 10 to about 50 nucleic acid monomer units (*e.g.*, about 15, about 20, about 25, about 30, about 35, or more nucleic acid monomer units). The exact size of an oligonucleotide will depend on many factors, including the ultimate function or use of the oligonucleotide. Oligonucleotides are optionally prepared by any suitable method, including, but not limited to, isolation of an existing or natural sequence,
25 DNA replication or amplification, reverse transcription, cloning and restriction digestion of appropriate sequences, or direct chemical synthesis by a method such as the phosphotriester method of Narang *et al.* (*Meth. Enzymol.* 68:90-99, 1979); the phosphodiester method of Brown *et al.* (*Meth. Enzymol.* 68:109-151, 1979); the diethylphosphoramidite method of Beaucage *et al.* (*Tetrahedron Lett.* 22:1859-1862, 1981); the triester method of Matteucci *et*
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al. (*J. Am. Chem. Soc.* 103:3185-3191, 1981); automated synthesis methods; or the solid support method of U.S. Pat. No. 4,458,066, or other methods known to those skilled in the art. The term “primer” as used herein refers to a polynucleotide capable of acting as a point of initiation of template-directed nucleic acid synthesis when placed under conditions in which
5 polynucleotide extension is initiated (*e.g.*, under conditions comprising the presence of requisite nucleoside triphosphates (as dictated by the template that is copied) and a polymerase in an appropriate buffer and at a suitable temperature or cycle(s) of temperatures (*e.g.*, as in a polymerase chain reaction)). To further illustrate, primers can also be used in a variety of other oligonucleotide-mediated synthesis processes, including as initiators of *de*
10 *novo* RNA synthesis and *in vitro* transcription-related processes (*e.g.*, nucleic acid sequence-based amplification (NASBA), transcription mediated amplification (TMA), etc.). A primer is typically a single-stranded oligonucleotide (*e.g.*, oligodeoxyribonucleotide). The appropriate length of a primer depends on the intended use of the primer but typically ranges from 6 to 40 nucleotides, more typically from 15 to 35 nucleotides. Short primer molecules generally
15 require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template for primer elongation to occur. In certain embodiments, the term “primer pair” means a set of primers including a 5’ sense primer (sometimes called “forward”) that hybridizes with the complement of the 5’ end of the nucleic
20 acid sequence to be amplified and a 3’ antisense primer (sometimes called “reverse”) that hybridizes with the 3’ end of the sequence to be amplified (*e.g.*, if the target sequence is expressed as RNA or is an RNA). A primer can be labeled, if desired, by incorporating a label detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical
25 means. For example, useful labels include ³²P, fluorescent dyes, electron-dense reagents, enzymes (as commonly used in ELISA assays), biotin, or haptens and proteins for which antisera or monoclonal antibodies are available.

The term “conventional” or “natural” when referring to nucleic acid bases, nucleoside triphosphates, or nucleotides refers to those which occur naturally in the polynucleotide being described (*i.e.*, for DNA these are dATP, dGTP, dCTP and dTTP). Additionally, dITP, and 7-
30 deaza-dGTP are frequently utilized in place of dGTP and 7-deaza-dATP can be utilized in place of dATP in *in vitro* DNA synthesis reactions, such as sequencing. Collectively, these may be referred to as dNTPs.

The term “unconventional” or “modified” when referring to a nucleic acid base, nucleoside, or nucleotide includes modification, derivations, or analogues of conventional bases,

nucleosides, or nucleotides that naturally occur in a particular polynucleotide. One example of an unconventional or modified nucleic acid base includes methylated-dNTPs, wherein a methyl group is attached to dNTP. Any one or more of the α -, β -, or γ -phosphate groups can be modified with one or more methyl groups. In a preferred embodiment, the methylated-dNTP has one methyl group added to the γ -phosphate group. Certain unconventional nucleotides are modified at the 2' position of the ribose sugar in comparison to conventional dNTPs. Thus, although for RNA the naturally occurring nucleotides are ribonucleotides (*i.e.*, ATP, GTP, CTP, UTP, collectively rNTPs), because these nucleotides have a hydroxyl group at the 2' position of the sugar, which, by comparison is absent in dNTPs, as used herein, ribonucleotides are unconventional nucleotides as substrates for DNA polymerases. As used herein, unconventional nucleotides include, but are not limited to, compounds used as terminators for nucleic acid sequencing. Exemplary terminator compounds include, but are not limited, to those compounds that have a 2',3' dideoxy structure and are referred to as dideoxynucleoside triphosphates. The dideoxynucleoside triphosphates ddATP, ddTTP, ddCTP and ddGTP are referred to collectively as ddNTPs. Additional examples of terminator compounds include 2'-PO₄ analogs of ribonucleotides (*see, e.g.*, U.S. Patent Application Publication Nos. 2005/0037991 and 2005/0037398). Other unconventional nucleotides include phosphorothioate dNTPs ([α -S]dNTPs), 5'-[α -borano]-dNTPs, [α]-methylphosphonate dNTPs, and ribonucleoside triphosphates (rNTPs). Unconventional bases may be labeled with radioactive isotopes such as ³²P, ³³P, or ³⁵S; fluorescent labels; chemiluminescent labels; bioluminescent labels; hapten labels such as biotin; or enzyme labels such as streptavidin or avidin. Fluorescent labels may include dyes that are negatively charged, such as dyes of the fluorescein family, or dyes that are neutral in charge, such as dyes of the rhodamine family, or dyes that are positively charged, such as dyes of the cyanine family. Dyes of the fluorescein family include, *e.g.*, FAM, HEX, TET, JOE, NAN and ZOE. Dyes of the rhodamine family include Texas Red, ROX, R110, R6G, and TAMRA. Various dyes or nucleotides labeled with FAM, HEX, TET, JOE, NAN, ZOE, ROX, R110, R6G, Texas Red and TAMRA are marketed by Perkin-Elmer (Boston, MA), Applied Biosystems (Foster City, CA), or Invitrogen/Molecular Probes (Eugene, OR). Dyes of the cyanine family include Cy2, Cy3, Cy5, and Cy7 and are marketed by GE Healthcare UK Limited (Amersham Place, Little Chalfont, Buckinghamshire, England).

As used herein, "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the sequence in the comparison window can comprise additions or deletions (*i.e.*, gaps) as compared to the

reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue 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 and multiplying the result by 100 to yield the percentage of sequence identity.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same. Sequences are "substantially identical" to each other if they have a specified percentage of nucleotides or amino acid residues that are the same (*e.g.*, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identity over a specified region)), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. These definitions also refer to the complement of a test sequence. Optionally, the identity exists over a region that is at least about 50 nucleotides in length, or more typically over a region that is 100 to 500 or 1,000 or more nucleotides in length.

The terms "similarity" or "percent similarity," in the context of two or more polypeptide sequences, refer to two or more sequences or subsequences that have a specified percentage of amino acid residues that are either the same or similar as defined by a conservative amino acid substitutions (*e.g.*, 60% similarity, optionally 65%, 70%, 75%, 80%, 85%, 90%, or 95% similar over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Sequences are also "substantially similar" to each other if they are at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, or at least 55% similar to each other. Optionally, this similarity exists over a region that is at least about 50 amino acids in length, or more typically over a region that is at least about 100 to 500 or 1,000 or more amino acids in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters are commonly used, or alternative parameters can be designated. The sequence comparison

algorithm then calculates the percent sequence identities or similarities for the test sequences relative to the reference sequence, based on the program parameters.

A “comparison window,” as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually
5 about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith and Waterman (*Adv. Appl. Math.* 2:482,
10 1970), by the homology alignment algorithm of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), by the search for similarity method of Pearson and Lipman (*Proc. Natl. Acad. Sci. USA* 85:2444, 1988), by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by manual alignment and visual
15 inspection (see, e.g., Ausubel *et al.*, *Current Protocols in Molecular Biology* (1995 supplement)).

Examples of an algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (*Nuc. Acids Res.* 25:3389-402, 1977), and Altschul *et al.* (*J. Mol. Biol.*
20 215:403-10, 1990), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of
25 the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward
30 score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue

alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915, 1989) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-87, 1993). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, typically less than about 0.01, and more typically less than about 0.001.

The term “Cp value” or “crossing point” value refers to a value that allows quantification of input target nucleic acids. The Cp value can be determined according to the second-derivative maximum method (Van Luu-The, et al., “Improved real-time RT-PCR method for high-throughput measurements using second derivative calculation and double correction,” *BioTechniques*, Vol. 38, No. 2, February 2005, pp. 287–293). In the second derivative method, a Cp corresponds to the first peak of a second derivative curve. This peak corresponds to the beginning of a log-linear phase. The second derivative method calculates a second derivative value of the real-time fluorescence intensity curve, and only one value is obtained. The original Cp method is based on a locally defined, differentiable approximation of the intensity values, e.g., by a polynomial function. Then the third derivative is computed. The Cp value is the smallest root of the third derivative. The Cp can also be determined using the fit point method, in which the Cp is determined by the intersection of a parallel to the threshold line in the log-linear region (Van Luu-The, et al., *BioTechniques*, Vol. 38, No. 2, February 2005, pp. 287–293). The Cp value provided by the LightCycler instrument offered by Roche by calculation according to the second-derivative maximum method.

The term “PCR efficiency” refers to an indication of cycle to cycle amplification efficiency. PCR efficiency is calculated for each condition using the equation: % PCR efficiency = $(10^{-(\text{slope})} - 1) \times 100$, wherein the slope was calculated by linear regression with the log copy number

plotted on the y-axis and C_p plotted on the x-axis. PCR efficiency can be measured using a perfectly matched or mismatched primer template.

The term “nucleic acid extension rate” refers to the rate at which a biocatalyst (*e.g.*, an enzyme, such as a polymerase, ligase, or the like) extends a nucleic acid (*e.g.*, a primer or other oligonucleotide) in a template-dependent or template-independent manner by attaching (*e.g.*, covalently) one or more nucleotides to the nucleic acid. To illustrate, certain mutant DNA polymerases described herein have improved nucleic acid extension rates relative to unmodified forms of these DNA polymerases, such that they can extend primers at higher rates than these unmodified forms under a given set of reaction conditions.

The term “tolerance of RT and polymerase inhibitors” refers to the ability of a polymerase to maintain activity (polymerase or reverse transcription activity) in the presence of an amount of an inhibitor that would inhibit the polymerase activity or reverse transcription activity of a control polymerase. In some embodiments, the improved polymerase is capable of polymerase or reverse transcription activity in the presence of an amount of the inhibitor that would essentially eliminate the control polymerase activity.

The term “deoxyribonucleoside triphosphate” or “dNTP” is a generic term referring to the deoxyribonucleotides, dATP, dCTP, dGTP, dTTP, dITP, and/or dUTP. The nucleoside triphosphates containing deoxyribose are called dNTPs, and take the prefix deoxy- in their names and small d- in their abbreviations: deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP), deoxyinosine triphosphate (dITP), and deoxyuridine triphosphate (dUTP). The dNTPs are the building blocks for DNA replication (they lose two of the phosphate groups in the process of incorporation). Each dNTP is made up of a phosphate group, a deoxyribose sugar, and a nitrogenous base. The double helix structure of DNA is made up of the dNTPs, much like monomer units in a polymer.

The term “alkylated primers” refers to oligonucleotides in which one or more of the bases have been modified by the addition of an alkyl group. Use of the modified primers of the invention results in a reduction in non-specific amplification, especially primer dimer formation, and/or a concomitant increase in the yield of the intended target relative to an amplification carried out with unmodified primers (*see*, U.S. Patent No. 6,001,611).

The terms “methylated dNTP,” “methylated-dNTP,” or “me-dNTP” refer to any deoxyribonucleoside triphosphate or dNTP having one or more added methyl groups. Examples include methylated-dATP, methylated-dCTP, methylated-dGTP, and methylated-dTTP. One or more methyl groups may be added to one or more of any of the α -, β -, or γ -

phosphate groups found in dNTPs. In a particular example, the methylated-dNTPs are such that the terminal γ -phosphate group of each dNTP is modified with a methyl group, which yields a more stable dNTP component for use in amplification reactions, such as PCR, as compared with conventional unmethylated dNTPs. In such case using a methylated-dNTP
5 having one methyl group on the γ -phosphate group, because the methyl group is on the γ -phosphate group, the methyl group is not incorporated into the amplicon during the extension phase of PCR, because the γ -phosphate group is cleaved off as pyrophosphate. In this way, a more stable component is employed, while not interfering with PCR reaction.

The terms “fluorinated-dNTP,” “fluoro-dNTP,” “F-dNTP,” or “f-dNTP” refer to any
10 deoxyribonucleoside triphosphate or dNTP that was fluorinated at one of the phosphate groups. Examples include fluoro-dATP, fluoro-dCTP, fluoro-dGTP, fluoro-dTTP, and fluoro-dUTP. The fluorine atom may replace one oxygen at one of any of the α -, β -, or γ -phosphate groups found in dNTPs. In a particular example, the fluoro-dNTPs are such that the terminal γ -phosphate group of each dNTP is fluorinated, which yields a more stable dNTP component
15 for use in amplification reactions, such as PCR, as compared with conventional unmodified dNTPs.

The term “5'-nuclease probe” refers to an oligonucleotide that comprises at least one light emitting labeling moiety and that is used in a 5'-nuclease reaction to effect target nucleic acid detection. In some embodiments, for example, a 5'-nuclease probe includes only a single light
20 emitting moiety (*e.g.*, a fluorescent dye, etc.). In certain embodiments, 5'-nuclease probes include regions of self-complementarity such that the probes are capable of forming hairpin structures under selected conditions. To further illustrate, in some embodiments a 5'-nuclease probe comprises at least two labeling moieties and emits radiation of increased intensity after one of the two labels is cleaved or otherwise separated from the oligonucleotide. In certain
25 embodiments, a 5'-nuclease probe is labeled with two different fluorescent dyes, *e.g.*, a 5' terminus reporter dye and the 3' terminus quencher dye or moiety. In some embodiments, 5'-nuclease probes are labeled at one or more positions other than, or in addition to, terminal positions. When the probe is intact, energy transfer typically occurs between the two fluorophores such that fluorescent emission from the reporter dye is quenched at least in part.
30 During an extension step of a polymerase chain reaction, for example, a 5'-nuclease probe bound to a template nucleic acid is cleaved by the 5' to 3' nuclease activity of, *e.g.*, a *Taq* polymerase or another polymerase having this activity such that the fluorescent emission of the reporter dye is no longer quenched. Exemplary 5'-nuclease probes are also described in, *e.g.*, U.S. Patent Nos. 5,210,015, 5,994,056, and 6,171,785. In other embodiments, a 5'

nuclease probe may be labeled with two or more different reporter dyes and a 3' terminus quencher dye or moiety.

The term "FRET" or "fluorescent resonance energy transfer" or "Foerster resonance energy transfer" refers to a transfer of energy between at least two chromophores, a donor chromophore and an acceptor chromophore (referred to as a quencher). The donor typically transfers the energy to the acceptor when the donor is excited by light radiation with a suitable wavelength. The acceptor typically re-emits the transferred energy in the form of light radiation with a different wavelength. When the acceptor is a "dark" quencher, it dissipates the transferred energy in a form other than light. Whether a particular fluorophore acts as a donor or an acceptor depends on the properties of the other member of the FRET pair. Commonly used donor-acceptor pairs include the FAM-TAMRA pair. Commonly used quenchers are DABCYL and TAMRA. Commonly used dark quenchers include BlackHole Quenchers™ (BHQ), (Biosearch Technologies, Inc., Novato, Cal.), Iowa Black™ (Integrated DNA Tech., Inc., Coralville, Iowa), and BlackBerry™ Quencher 650 (BBQ-650) (Berry & Assoc., Dexter, Mich.).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows real-time PCR data demonstrating improved performance of Z05D-F2 with methylated-dNTPs or fluorinated-dNTPS.

Figure 2 shows real-time PCR data comparing the incorporation of methylated-dNTPs at different concentrations. Incorporation of 1 mM regular dNTPs by parental Z05D is shown as the benchmark, and basis for comparison.

Figure 3 shows the crystal structure of the Klenow fragment of Taq DNA polymerase in the ternary complex with template and ddCTP (PDB Accession Code 4N5S). Taq DNA polymerase is used as a model for Z05D. The corresponding residues I638 and V703 (I640 and V705) are labeled.

Figure 4 shows real-time PCR data comparing the incorporation of regular unmodified dNTPs or methylated-dNTPs by the parental Z05D (SEQ ID NO:2), Z05D-F2 (SEQ ID NO:3), and the single mutants Z05D-I640M (SEQ ID NO:4) and Z05D-V705L (SEQ ID NO:5).

Figure 5 shows RT-PCR data comparing the incorporation of methylated-dNTPs by the parental Z05D (SEQ ID NO:2), Z05D-F2 (SEQ ID NO:3), and the single mutants Z05D-I640M (SEQ ID NO:4) and Z05D-V705L (SEQ ID NO:5). A β -catenin transcript was used as an RNA template with alkylated primer and methylated-dNTPs (at 1 mM or 2 mM). Data for Z05D with regular (unmodified) dNTPs were shown as the benchmark.

DETAILED DESCRIPTION

The present invention provides improved DNA polymerases in which one or more amino acids in the polymerase domain have been mutated relative to an unmodified DNA polymerase. The DNA polymerases of the invention are active enzymes having improved
5 ability to efficiently and effectively incorporate methylated-dNTPs, relative to the unmodified form of the polymerase.

DNA polymerases that more efficiently and effectively incorporate methylated-dNTPs are helpful, for example, in a variety of applications involving assays that employ RT-PCR to detect and/or quantify RNA targets. The DNA polymerases are therefore useful in a variety of
10 applications involving polynucleotide extension as well as reverse transcription or amplification of polynucleotide templates, including, for example, applications in recombinant DNA studies and medical diagnosis of disease.

In some embodiments, a polymerase of the invention is a chimeric polymerase, *i.e.*, comprising polypeptide regions from two or more enzymes. Examples of such chimeric DNA
15 polymerases are described in, *e.g.*, U.S. Patent No. 6,228,628. Particularly suitable are chimeric CS-family DNA polymerases, which include the CS5 and CS6 polymerases and variants thereof having substantial amino acid sequence identity or similarity (typically at least 80% amino acid sequence identity and more typically at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity). The CS5 and CS6 DNA
20 polymerases are chimeric enzymes derived from *Thermus* sp. Z05 and *Thermotoga maritima* (*Tma*) DNA polymerases. They comprise the N-terminal 5'-nuclease domain of the *Thermus* enzyme and the C-terminal 3'-5' exonuclease and the polymerase domains of the *Tma* enzyme. These enzymes have efficient reverse transcriptase activity, can extend nucleotide analog-containing primers, and can incorporate alpha-phosphorothioate dNTPs, dUTP, dITP,
25 and also fluorescein- and cyanine-dye family labeled dNTPs. The CS5 and CS6 polymerases are also efficient Mg²⁺-activated PCR enzymes. The CS5 and CS6 chimeric polymerases are further described in, *e.g.*, U.S. Pat. No. 7,148,049.

In some embodiments, the amino acid substitutions are single amino acid substitutions. The DNA polymerases provided herein can comprise one or more amino acid substitutions in the
30 active site relative to the unmodified polymerase. Amino acid substitutions at these positions confer improved ability to incorporate methylated-dNTPs, yielding a mutant DNA polymerase with an improved ability to incorporate methylated-dNTPs, relative to the unmodified polymerase. In some embodiments, the amino acid at position 640 and/or 705 of Z05D is substituted with an amino acid that does not correspond to the native sequence of

Z05D. In certain embodiments, the amino acid at position 640 of Z05D is substituted with Methionine (Met or M) and/or the amino acid at position 705 of Z05D is substituted with Leucine (Leu or L). Other suitable amino acid substitution(s) at one or more of the identified sites can be determined using, *e.g.*, known methods of site-directed mutagenesis and determination of polynucleotide extension performance in assays described further herein or otherwise known to persons of skill in the art.

In some embodiments, the polymerase of the invention comprises SEQ ID NO:3, and further comprises one or more additional amino acid changes (*e.g.*, by amino acid substitution, addition, or deletion) compared to a native polymerase.

In some embodiments, such functional variant polymerases typically will have substantial sequence identity or similarity to the wild-type or naturally occurring polymerase, typically at least 80% amino acid sequence identity and more typically at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity.

Other suitable amino acid substitution(s) at one or more of the identified sites can be determined using, *e.g.*, known methods of site-directed mutagenesis and determination of polynucleotide extension performance in assays described further herein or otherwise known to persons of skill in the art, *e.g.*, amino acid substitutions described in U.S. Patent Publication Nos. 2009/0148891 and 2009/0280539.

In some embodiments, the DNA polymerase of the present invention is derived from *Thermus sp.* Z05 DNA polymerase (SEQ ID NO:1) or a variant thereof (*e.g.*, carrying the D580G mutation, like Z05D (SEQ ID NO:2) or the like). Thus, in certain variations of the invention, the mutant polymerase comprises at least one amino acid substitution, relative to a *Thermus sp.* Z05D DNA polymerase (or a DNA polymerase that is substantially identical, *e.g.*, at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identical to SEQ ID NO:2), at position 640 and/or position 705. In certain embodiments, the amino acid at position 640 of SEQ ID NO:2 is Methionine (Met or M) (*i.e.*, SEQ ID NOs:3 or 4). In certain embodiments, the amino acid residue at position 705 of SEQ ID NO:2 is Leucine (Leu or L) (*i.e.*, SEQ ID NOs:3 or 5). Exemplary *Thermus sp.* Z05 DNA polymerase mutants include those comprising the amino acid substitution(s) D580G (*i.e.*, Z05D (SEQ ID NO:2)). In some embodiments, the mutant *Thermus sp.* Z05 DNA polymerase comprises, *e.g.*, amino acid residue substitutions D580G and I640M (SEQ ID NO:4). In some embodiments, the mutant *Thermus sp.* Z05 DNA polymerase comprises, *e.g.*, amino acid residue substitutions D580G and V705L (SEQ ID NO:5). In some embodiments, the mutant *Thermus sp.* Z05 DNA polymerase comprises, *e.g.*, amino acid residue substitutions D580G, I640M, and V705L (SEQ ID NO:3). In certain

embodiments, the mutant *Thermus* sp. Z05 DNA polymerase comprises, *e.g.*, one or more amino acid residue substitutions independently selected from D580G, I640M, and/or V705L (SEQ ID NOs:3-5).

It was previously shown that substitutions at the amino acid corresponding to position 580 of SEQ ID NO:1 (Z05), which is known as Z05D (SEQ ID NO:2), can result in DNA polymerases having improved polymerase activity, including, for example, improved extension rates, reverse transcription efficiency, and/or amplification ability (*see*, U.S. Patent Nos. 8,962,293 and 9,102,924; and U.S. Patent Publication No. US 2016-0024548). Thus, it is expected that the improved polymerases that comprise substitutions at the amino acid corresponding to position 580 of SEQ ID NO:1 (Z05) described herein will also have the improved properties described above.

In addition to the mutations and substitutions described herein, the DNA polymerases of the present invention can also include other, non-substitutional modification(s). Such modifications can include, for example, covalent modifications known in the art to confer an additional advantage in applications comprising polynucleotide extension. For example, one such modification is a thermally reversible covalent modification that inactivates the enzyme, but which is reversed to activate the enzyme upon incubation at an elevated temperature, such as a temperature typically used for polynucleotide extension. Exemplary reagents for such thermally reversible modifications are described in U.S. Patent Nos. 5,773, 258 and 5,677,152.

The DNA polymerases of the present invention can be constructed by mutating the DNA sequences that encode the corresponding unmodified polymerase (*e.g.*, a wild-type polymerase or a corresponding variant from which the polymerase of the invention is derived), such as by using techniques commonly referred to as site-directed mutagenesis. Nucleic acid molecules encoding the unmodified form of the polymerase can be mutated by a variety of polymerase chain reaction (PCR) techniques well-known to one of ordinary skill in the art. (*See, e.g.*, *PCR Strategies* (M. A. Innis, D. H. Gelfand, and J. J. Sninsky eds., 1995, Academic Press, San Diego, CA) at Chapter 14; *PCR Protocols: A Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White eds., Academic Press, NY, 1990).

By way of non-limiting example, the two primer system, utilized in the Transformer Site-Directed Mutagenesis kit from Clontech, may be employed for introducing site-directed mutants into a polynucleotide encoding an unmodified form of the polymerase. Following denaturation of the target plasmid in this system, two primers are simultaneously annealed to

the plasmid; one of these primers contains the desired site-directed mutation, the other contains a mutation at another point in the plasmid resulting in elimination of a restriction site. Second strand synthesis is then carried out, tightly linking these two mutations, and the resulting plasmids are transformed into a mutS strain of *E. coli*. Plasmid DNA is isolated from the transformed bacteria, restricted with the relevant restriction enzyme (thereby linearizing the unmutated plasmids), and then retransformed into *E. coli*. This system allows for generation of mutations directly in an expression plasmid, without the necessity of subcloning or generation of single-stranded phagemids. The tight linkage of the two mutations and the subsequent linearization of unmutated plasmids result in high mutation efficiency and allow minimal screening. Following synthesis of the initial restriction site primer, this method requires the use of only one new primer type per mutation site. Rather than prepare each positional mutant separately, a set of "designed degenerate" oligonucleotide primers can be synthesized in order to introduce all of the desired mutations at a given site simultaneously. Transformants can be screened by sequencing the plasmid DNA through the mutagenized region to identify and sort mutant clones. Each mutant DNA can then be restricted and analyzed by electrophoresis, such as for example, on a Mutation Detection Enhancement gel (Mallinckrodt Baker, Inc., Phillipsburg, NJ) to confirm that no other alterations in the sequence have occurred (by band shift comparison to the unmutagenized control). Alternatively, the entire DNA region can be sequenced to confirm that no additional mutational events have occurred outside of the targeted region.

DNA polymerases with more than one amino acid substituted can be generated in various ways. In the case of amino acids located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than ten amino acids, for example) it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed. In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions. An alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: DNA encoding the unmodified polymerase is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The

second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on. Alternatively, the multi-site mutagenesis method of Seyfang & Jin (*Anal. Biochem.* 324:285-291. 2004) may be utilized.

Accordingly, also provided are recombinant nucleic acids encoding any of the DNA polymerases of the present invention. Using a nucleic acid of the present invention, encoding a DNA polymerase, a variety of vectors can be made. Any vector containing replicon and control sequences that are derived from a species compatible with the host cell can be used in the practice of the invention. Generally, expression vectors include transcriptional and translational regulatory nucleic acid regions operably linked to the nucleic acid encoding the DNA polymerase. The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. In addition, the vector may contain a Positive Retroregulatory Element (PRE) to enhance the half-life of the transcribed mRNA (*see Gelfand et al.* U.S. Patent No. 4,666,848). The transcriptional and translational regulatory nucleic acid regions will generally be appropriate to the host cell used to express the polymerase. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of host cells. In general, the transcriptional and translational regulatory sequences may include, *e.g.*, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. In typical embodiments, the regulatory sequences include a promoter and transcriptional start and stop sequences. Vectors also typically include a polylinker region containing several restriction sites for insertion of foreign DNA. In certain embodiments, "fusion flags" are used to facilitate purification and, if desired, subsequent removal of tag/flag sequence, *e.g.*, "His-Tag". However, these are generally unnecessary when purifying a thermoactive and/or thermostable protein from a mesophilic host (*e.g.*, *E. coli*) where a "heat-step" may be employed. The construction of suitable vectors containing DNA encoding replication sequences, regulatory sequences, phenotypic selection genes, and the polymerase of interest are prepared using standard recombinant DNA procedures. Isolated plasmids, viral vectors, and DNA fragments are cleaved, tailored, and ligated together in a

specific order to generate the desired vectors, as is well-known in the art (*see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York, NY, 2nd ed. 1989)).

In certain embodiments, the expression vector contains a selectable marker gene to allow the
5 selection of transformed host cells. Selection genes are well known in the art and will vary with the host cell used. Suitable selection genes can include, for example, genes coding for ampicillin and/or tetracycline resistance, which enables cells transformed with these vectors to grow in the presence of these antibiotics.

In one aspect of the present invention, a nucleic acid encoding a DNA polymerase is
10 introduced into a cell, either alone or in combination with a vector. By “introduced into” or grammatical equivalents herein is meant that the nucleic acids enter the cells in a manner suitable for subsequent integration, amplification, and/or expression of the nucleic acid. The method of introduction is largely dictated by the targeted cell type. Exemplary methods include CaPO₄ precipitation, liposome fusion, LIPOFECTIN®, electroporation, viral
15 infection, and the like.

In some embodiments, prokaryotes are typically used as host cells for the initial cloning steps of the present invention. They are particularly useful for rapid production of large amounts of DNA, for production of single-stranded DNA templates used for site-directed mutagenesis, for screening many mutants simultaneously, and for DNA sequencing of the mutants
20 generated. Suitable prokaryotic host cells include *E. coli* K12 strain 94 (ATCC No. 31,446), *E. coli* strain W3110 (ATCC No. 27,325), *E. coli* K12 strain DG116 (ATCC No. 53,606), *E. coli* X1776 (ATCC No. 31,537), and *E. coli* B; however many other strains of *E. coli*, such as HB101, JM101, NM522, NM538, NM539, and many other species and genera of prokaryotes including bacilli such as *Bacillus subtilis*, other enterobacteriaceae such as *Salmonella*
25 *typhimurium* or *Serratia marcesans*, and various *Pseudomonas* species can all be used as hosts. Prokaryotic host cells or other host cells with rigid cell walls are typically transformed using the calcium chloride method as described in section 1.82 of Sambrook *et al., supra*. Alternatively, electroporation can be used for transformation of these cells. Prokaryote transformation techniques are set forth in, for example Dower, in *Genetic Engineering, Principles and Methods* 12:275-296 (Plenum Publishing Corp., 1990); Hanahan *et al., Meth. Enzymol.*, 204:63, 1991. Plasmids typically used for transformation of *E. coli* include pBR322, pUCI8, pUCI9, pUCII8, pUC119, and Bluescript M13, all of which are described in
30 sections 1.12-1.20 of Sambrook *et al., supra*. However, many other suitable vectors are available as well.

The DNA polymerases of the present invention are typically produced by culturing a host cell transformed with an expression vector containing a nucleic acid encoding the DNA polymerase, under the appropriate conditions to induce or cause expression of the DNA polymerase. Methods of culturing transformed host cells under conditions suitable for protein expression are well-known in the art (*see, e.g., Sambrook et al., supra*). Suitable host cells for production of the polymerases from lambda pL promoter-containing plasmid vectors include *E. coli* strain DG116 (ATCC No. 53606) (*see* US Patent No. 5,079,352 and Lawyer, F.C. *et al., PCR Methods and Applications* 2:275-87, 1993). Following expression, the polymerase can be harvested and isolated. Methods for purifying the thermostable DNA polymerase are described in, for example, Lawyer *et al., supra*. Once purified, the ability of the DNA polymerases to have improved RT efficiency, increased mismatch tolerance, extension rate and/or tolerance of RT and polymerase inhibitors can be tested (*e.g., as described in the examples*).

The improved DNA polymerases of the present invention may be used for any purpose in which such enzyme activity is necessary or desired. Accordingly, in another aspect of the invention, methods of polynucleotide extension (*e.g., PCR*) using the polymerases are provided. Conditions suitable for polynucleotide extension are known in the art. (*See, e.g., Sambrook et al., supra. See also Ausubel et al., Short Protocols in Molecular Biology* (4th ed., John Wiley & Sons 1999). Generally, a primer is annealed, *i.e., hybridized*, to a target nucleic acid to form a primer-template complex. The primer-template complex is contacted with the DNA polymerase and nucleoside triphosphates in a suitable environment to permit the addition of one or more nucleotides to the 3' end of the primer, thereby producing an extended primer complementary to the target nucleic acid. The primer can include, *e.g., one or more nucleotide analog(s)*. In addition, the nucleoside triphosphates can be conventional nucleotides, unconventional nucleotides (*e.g., ribonucleotides or labeled nucleotides*), or a mixture thereof. In some variations, the polynucleotide extension reaction comprises amplification of a target nucleic acid. Conditions suitable for nucleic acid amplification using a DNA polymerase and a primer pair are also known in the art (*e.g., PCR amplification methods*). (*See, e.g., Sambrook et al., supra; Ausubel et al., supra; PCR Applications: Protocols for Functional Genomics* (Innis *et al.* eds., Academic Press 1999). In other, non-mutually exclusive embodiments, the polynucleotide extension reaction comprises reverse transcription of an RNA template (*e.g., RT-PCR*). In some embodiments, the improved polymerases find use in 454 sequencing (Margulies, M *et al.* 2005, *Nature*, 437, 376-380).

Optionally, the primer extension reaction comprises an actual or potential inhibitor of a reference or unmodified polymerase. The inhibitor can inhibit, for example, the nucleic acid extension rate and/or the reverse transcription efficiency of a reference or unmodified (control) polymerase. In some embodiments, the inhibitor is hemoglobin, or a degradation product thereof. For example, in some embodiments, the hemoglobin degradation product is a heme breakdown product, such as hemin, hematoporphyrin, or bilirubin. In some
5 embodiments, the inhibitor is an iron-chelator or a purple pigment. In other embodiments, the inhibitor is heparin. In certain embodiments, the inhibitor is an intercalating dye. In certain embodiments, the inhibitor is melanin, which has been described as a polymerase inhibitor.
10 See, e.g., Ekhardt, *et al.*, *Biochem Biophys Res Commun.* 271(3):726-30 (2000).

The DNA polymerases of the present invention can be used to extend templates in the presence of polynucleotide templates isolated from samples comprising polymerase inhibitors, e.g., such as blood. For example, the DNA polymerases of the present invention can be used to extend templates in the presence of hemoglobin, a major component of blood,
15 or in the presence of a hemoglobin degradation product. Hemoglobin can be degraded to various heme breakdown products, such as hemin, hematin, hematoporphyrin, and bilirubin. Thus, in certain embodiments, the DNA polymerases of the present invention can be used to extend templates in the presence of hemoglobin degradation products, including but not limited to, hemin, hematin, hematoporphyrin, and bilirubin. In certain embodiments, the
20 hemoglobin degradation product is hemin. In some embodiments, the DNA polymerases of the present invention can be used to extend templates in the presence of about 0.5 to 20.0 μM , about 0.5 to 10.0 μM , about 0.5 to 5.0 μM , about 1.0 to 10.0 μM , about 1.0 to 5.0 μM , about 2.0 to 5.0 μM , or about 2.0 to 3.0 μM hemin. In other embodiments, the DNA polymerases of the present invention can be used to extend templates in the presence of at least about 0.5, 1.0,
25 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 10.0, 20.0, or greater than 20 μM hemin. The breakdown products of hemoglobin include iron-chelators and purple pigments. Thus, in some embodiments, the DNA polymerases of the present invention can be used to extend templates in the presence of iron-chelators and/or purple pigments. In other embodiments, the DNA polymerases of the present invention can be used to extend templates in the presence of amounts of hemoglobin
30 degradation products that would inhibit extension of the same template by a reference or control DNA polymerase.

The DNA polymerases of the present invention can be used to extend templates in the presence of heparin. Heparin is commonly present as an anticoagulant in samples isolated from blood. In some embodiments, the DNA polymerases of the present invention can be

used to extend templates in the presence of about 1.0 to 400 ng/ μ l, 1.0 to 300 ng/ μ l, 1.0 to 200 ng/ μ l, 5.0 to 400 ng/ μ l, 5.0 to 300 ng/ μ l, 5.0 to 200 ng/ μ l, 10.0 to 400 ng/ μ l, 10.0 to 300 ng/ μ l, or 10.0 to 200 ng/ μ l heparin. In some embodiments, the DNA polymerases of the present invention can be used to extend templates in the presence of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400 ng/ μ l, or greater than 400 ng/ μ l of heparin. In other embodiments, the DNA polymerases of the present invention can be used to extend templates in the presence of amounts of heparin that would inhibit extension of the same template by a reference or control DNA polymerase.

In some embodiments, an improved polymerase of the invention is used in a reverse transcription reaction. In some embodiments, the reverse transcription reaction is carried out in a mixture containing the RNA template, one or more primer(s), and a thermostable DNA polymerase of the invention. The reaction mixture typically contains all four standard deoxyribonucleoside triphosphates (dNTPs) (or methylated versions of dNTPs) and a buffer containing a divalent cation and a monovalent cation. Exemplary cations include, *e.g.*, Mg²⁺, although other cations, such as Mn²⁺ or Co²⁺ can activate DNA polymerases. In other embodiments, the reverse transcription reaction is carried out with a thermoactive DNA polymerase of the invention. In particular embodiments, the improved polymerase of the invention allows for more efficient amplification of RNA templates without compromising the efficient amplification of a DNA template in the presence of Mn²⁺ or Mg²⁺, as described in the examples.

In some embodiments, the improved polymerase has improved ability to incorporated more stable methylated-dNTPs, as compared to control polymerase. It was not previously appreciated that substitutions at the amino acid corresponding to positions 640 and/or 705 of Z05D (SEQ ID NO:2) could result in the ability to incorporate methylated-dNTPs. Thus, in some embodiments, DNA polymerases having a Ile (I) to Met (M) substitution at the amino acid corresponding to position 640 of SEQ ID NO:2 and/or a Val (V) to Leucine (L) substitution at the amino acid corresponding to position 705 of SEQ ID NO:2 could result in improved ability to incorporate methylated-dNTPs. In some embodiments, the DNA polymerase having improved ability to incorporated more stable methylated-dNTPs, as compared to control polymerase, comprises an Ile (I) to Met (M) substitution, at the amino acid corresponding to position 640 of SEQ ID NO:2 and/or a Val (V) to Leu (L) substitution, at the amino acid corresponding to position 705 of SEQ ID NO:2, and has at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% amino acid sequence identity to SEQ ID NOs:1-5.

Target nucleic acids can come from a biological or synthetic source. The target can be, for example, DNA or RNA. Generally, where amplicons are generated, the amplicons will be composed of DNA, though ribonucleotides or synthetic nucleotides can also be incorporated into the amplicon. Where one wishes to detect an RNA, the amplification process will typically involve the use of reverse transcription, including for example, reverse transcription PCR (RT-PCR).

Specific target sequences can include, *e.g.*, viral nucleic acids (*e.g.*, human immunodeficiency virus (HIV), hepatitis virus B (HBV), cytomegalovirus (CMV), parvo B19 virus, Epstein-Barr virus, hepatitis virus C (HCV), human papilloma virus (HPV), Japanese encephalitis virus (JEV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV), Murray Valley encephalitis virus, and Kunjin virus), bacterial nucleic acids (*e.g.*, *S. aureus*, *Neisseria meningitidis*, *Plasmodium falciparum*, *Chlamydia muridarum*, *Chlamydia trachomatis*), mycobacteria, fungal nucleic acids, or nucleic acids from animals or plants. In some embodiments, the target nucleic acids are animal (*e.g.*, human) nucleic acids or are derived from an animal (*e.g.*, human) sample (*i.e.*, viral or other pathogenic organism nucleic acids may be present in a sample from an animal biopsy, blood sample, urine sample, fecal sample, saliva, etc.). In some embodiments, the target nucleic acids are, for example, human genetic regions that may include variants associated with disease (*e.g.*, cancer, diabetes, etc.). Because in some embodiments the polymerases of the invention have mismatch tolerance, such enzymes are particularly useful, for example, where a diversity of related sequences could be in a target sequence. As an example, the invention can be used to detect viral pathogens, where the viral pathogens have sufficient variation in their genomes to make it difficult or impossible to design a single or small set of primers that will amplify most or all possible viral genomes or in cancer or other disease genetic markers where variation in sequence is known or likely to occur.

Other methods for detecting extension products or amplification products using the improved polymerases described herein include the use of fluorescent double-stranded nucleotide binding dyes or fluorescent double-stranded nucleotide intercalating dyes. Examples of fluorescent double-stranded DNA binding dyes include SYBR-green (Molecular Probes). The double stranded DNA binding dyes can be used in conjunction with melting curve analysis to measure primer extension products and/or amplification products. The melting curve analysis can be performed on a real-time PCR instrument, such as the ABI 5700/7000 (96 well format) or ABI 7900 (384 well format) instrument with onboard software (SDS 2.1). Alternatively,

the melting curve analysis can be performed as an end point analysis. Exemplary methods of melting point analysis are described in U.S. Patent Publication No. 2006/0172324.

In another aspect of the present invention, kits are provided for use in primer extension methods described herein. In some embodiments, the kit is compartmentalized for ease of use and contains at least one container providing an improved DNA polymerase in accordance with the present invention. One or more additional containers providing additional reagent(s) can also be included. In some embodiments, the kit can also include a blood collection tube, container, or unit that comprises heparin or a salt thereof, or releases heparin into solution. The blood collection unit can be a heparinized tube. Such additional containers can include any reagents or other elements recognized by the skilled artisan for use in primer extension procedures in accordance with the methods described above, including reagents for use in, *e.g.*, nucleic acid amplification procedures (*e.g.*, PCR, RT-PCR), DNA sequencing procedures, or DNA labeling procedures. For example, in certain embodiments, the kit further includes a container providing a 5' sense primer hybridizable, under primer extension conditions, to a predetermined polynucleotide template, or a primer pair comprising the 5' sense primer and a corresponding 3' antisense primer. In other, non-mutually exclusive variations, the kit includes one or more containers providing nucleoside triphosphates (conventional and/or unconventional). In specific embodiments, the kit includes alpha-phosphorothioate dNTPs, dUTP, dITP, and/or labeled dNTPs such as, *e.g.*, fluorescein- or cyanin-dye family dNTPs, or methylated-dNTPs. In still other, non-mutually exclusive embodiments, the kit includes one or more containers providing a buffer suitable for a primer extension reaction.

In another aspect of the present invention, reaction mixtures are provided comprising the polymerases with improved ability to incorporate methylated-dNTPs, as described herein. The reaction mixtures can further comprise reagents for use in, *e.g.*, nucleic acid amplification procedures (*e.g.*, PCR, RT-PCR), DNA sequencing procedures, or DNA labeling procedures. For example, in certain embodiments, the reaction mixtures comprise a buffer suitable for a primer extension reaction. The reaction mixtures can also contain a template nucleic acid (DNA and/or RNA), one or more primer or probe polynucleotides, nucleoside triphosphates (including, *e.g.*, deoxyribonucleotides, ribonucleotides, labeled nucleotides, unconventional nucleotides, methylated-dNTPs), salts (*e.g.*, Mn^{2+} , Mg^{2+}), labels (*e.g.*, fluorophores). In some embodiments, the reaction mixtures contain a 5'-sense primer hybridizable, under primer extension conditions, to a predetermined polynucleotide template, or a primer pair comprising the 5'-sense primer and a corresponding 3' antisense primer. In some embodiments, the

reaction mixtures contain alpha-phosphorothioate dNTPs, dUTP, dITP, and/or labeled dNTPs such as, *e.g.*, fluorescein- or cyanin-dye family dNTPs, and/or methylated-dNTPs. In some embodiments, the reaction mixtures comprise an iron chelator or a purple dye. In certain embodiments, the reaction mixtures comprise hemoglobin, or a degradation product of hemoglobin. For example, in certain embodiments, the degradation products of hemoglobin include heme breakdown products such as hemin, hematin, hematophoryn, and bilirubin. In other embodiments, the reaction mixtures comprise heparin or a salt thereof. In certain embodiments, the reaction mixture contains a template nucleic acid that is isolated from blood. In other embodiments, the template nucleic acid is RNA and the reaction mixture comprises heparin or a salt thereof.

In some embodiments, the reaction mixture comprises two or more polymerases. For example, in some embodiments, the reaction mixture comprises a first DNA polymerase having increased reverse transcriptase efficiency compared to a control polymerase, and a second DNA polymerase having DNA-dependent polymerase activity. The second DNA polymerase can be a wild-type or unmodified polymerase, or can be an improved polymerase having increased DNA- dependent polymerase activity. Such reaction mixtures are useful for amplification of RNA templates (*e.g.*, RT-PCR) by providing both a polymerase having increased reverse transcriptase activity and a polymerase having DNA-dependent polymerase activity.

20 **EXAMPLES**

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1: Identification of Modified Polymerase

The modified polymerase was based on a known mutant of wild type Z05 polymerase (SEQ ID NO:1), known as Z05D (SEQ ID NO:2, described in U.S. Patent No. 8,962,293). The Z05D mutant has the amino acid aspartic acid (D) at position 580 replaced with amino acid glycine (G) (*i.e.*, Z05D is a Z05D polymerase with a D580G mutation) (SEQ ID NO:2). The modified polymerase of the invention is known as “Z05 mutant F2” or (“Z05D-F2”) (SEQ ID NO:3). The Z05D mutant F2 was found in a library generated by error-prone PCR, using a PCR-based screen using methylated-dNTPs and alkylated primers. Z05D-F2 was expressed and purified in larger scale (500 ml), and the incorporation of methylated-dNTPs by Z05D-F2 in a PCR-assay was confirmed, as shown in Figure 1. The sequences of the various polymerases employed are depicted in Table 1, below.

Name	SEQ ID NO	Sequence
Z05 Polymerase	1	MKAMLPLFEPKGRVLLVDGHHLAYRTFFALKGLTTSRGEVPQAVYGFASLL KALKEDGYKAVFVVFDAKAPSRHEAYEAYKAGRAPTPEDFPRQLALIKELV DLLGFTRLEVPGFEADDVLATLAKKAEREGYEVRLTADRDLVQLVSDRVAVL HPEGHLITPEWLWEKYGLKPEQWVDFRALVGDPSDNLPGVKGIGEKTKALKLL KEWGSLENILKNLDRVKPEVSRERIKAHLEDLKLSLELSRVRSDLPLEVDFARR REPDREGLRAFLERLEFGSLLHEFGLLEAPAPLEEAPWPPPEGAFVGFVLSRPEP MWAEKALAAACKEGRVHRAKDPLAGLKDLKEVRGLLAKDLAVLALREGLDL APSDDPMLLAYLLDPSNTTPEGVARRYGGEWTEAAHRALLAERLQQNLLER LKGEEKLLWLYQEVEKPLSRVLAHMEATGVRLDVAYLKALSLELAEEIRRLEE EVFRLAGHPFNLSRDQLERVLFDELRLPALGKTQKTGKRSTSAAVLEALREA HPIVEKILQHRELTKLKNTYVDPLPGLVHPRTGRLHTRFNQTATATGRLSSSDP NLQNIPIRTPLGQRIRRAFVAEAGWALVALDYSQIELRVLAHLSGDNLRVVFQ EGKDIHTQTASWMFGVSPEAVDPLMRRAAKTVNFGVLYGMSAHRLSQELAIP YEEAVAFIERYFQSFPKYRAWIEKTLEGRKRGYVETLFGRRRYVPDLNARVK SVREAAERMAFNMPVQGTAAADLMKLAMVKLPHLREMGMARMLLQVHDELL LEAPQARAEEVAALAKEAMEKAYPLAVPLEVEVGIGEDWLSAKG
Z05D polymerase	2	MKAMLPLFEPKGRVLLVDGHHLAYRTFFALKGLTTSRGEVPQAVYGFASLL KALKEDGYKAVFVVFDAKAPSRHEAYEAYKAGRAPTPEDFPRQLALIKELV DLLGFTRLEVPGFEADDVLATLAKKAEREGYEVRLTADRDLVQLVSDRVAVL HPEGHLITPEWLWEKYGLKPEQWVDFRALVGDPSDNLPGVKGIGEKTKALKLL KEWGSLENILKNLDRVKPEVSRERIKAHLEDLKLSLELSRVRSDLPLEVDFARR REPDREGLRAFLERLEFGSLLHEFGLLEAPAPLEEAPWPPPEGAFVGFVLSRPEP MWAEKALAAACKEGRVHRAKDPLAGLKDLKEVRGLLAKDLAVLALREGLDL APSDDPMLLAYLLDPSNTTPEGVARRYGGEWTEAAHRALLAERLQQNLLER LKGEEKLLWLYQEVEKPLSRVLAHMEATGVRLDVAYLKALSLELAEEIRRLEE EVFRLAGHPFNLSRDQLERVLFDELRLPALGKTQKTGKRSTSAAVLEALREA HPIVEKILQHRELTKLKNTYVDPLPGLVHPRTGRLHTRFNQTATATGRLSSSGP NLQNIPIRTPLGQRIRRAFVAEAGWALVALDYSQIELRVLAHLSGDNLRVVFQ EGKDIHTQTASWMFGVSPEAVDPLMRRAAKTVNFGVLYGMSAHRLSQELAIP YEEAVAFIERYFQSFPKYRAWIEKTLEGRKRGYVETLFGRRRYVPDLNARVK SVREAAERMAFNMPVQGTAAADLMKLAMVKLPHLREMGMARMLLQVHDELL LEAPQARAEEVAALAKEAMEKAYPLAVPLEVEVGIGEDWLSAKG
Double Mutant Z05D I650M V705L (Z05D-F2)	3	MKAMLPLFEPKGRVLLVDGHHLAYRTFFALKGLTTSRGEVPQAVYGFASLL KALKEDGYKAVFVVFDAKAPSRHEAYEAYKAGRAPTPEDFPRQLALIKELV DLLGFTRLEVPGFEADDVLATLAKKAEREGYEVRLTADRDLVQLVSDRVAVL HPEGHLITPEWLWEKYGLKPEQWVDFRALVGDPSDNLPGVKGIGEKTKALKLL KEWGSLENILKNLDRVKPEVSRERIKAHLEDLKLSLELSRVRSDLPLEVDFARR REPDREGLRAFLERLEFGSLLHEFGLLEAPAPLEEAPWPPPEGAFVGFVLSRPEP MWAEKALAAACKEGRVHRAKDPLAGLKDLKEVRGLLAKDLAVLALREGLDL APSDDPMLLAYLLDPSNTTPEGVARRYGGEWTEAAHRALLAERLQQNLLER LKGEEKLLWLYQEVEKPLSRVLAHMEATGVRLDVAYLKALSLELAEEIRRLEE

		EVFRLAGHPFNLNSRDQLERVLFDELRLPALGKTQKTGKRSTSAAVLEALREA HPIVEKILQHRELTKLKNTYVDPLPGLVHPRTGRLHTRFNQTATATGRLSSSGP NLQNIPIRTPLGQRIRRAFVAEAGWALVALDYSQIELRVLAHLSGDENLIRVFQ EGKDMHTQTASWMFGVSPEAVDPLMRRAAKTVNFGVLYGMSAHRLSQELAI PYEEAVAFIERYFQSFPKLRRAWIEKTLEEGRKRGYVETLFGRRRYVPDLNARV KSVREAAERMAFNMPVQGTAAADMKLAMVKLPHLREMGMARMLLQVHDEL LLEAPQARAEVAALAKEAMEKAYPLAVPLEVEVGIGEDWLSAKG
Single Mutant Z05D I640M	4	MKAMLPLFEPKGRVLLVDGHHLAYRTFFALKGLTTSRGEVQAVYGFSAKSL KALKEDGYKAVFVVFDAKAPSRHEAYEAYKAGRAPTPEDFPRQLALIKELV DLLGFTRLEVPGFEADDVLATLAKKAEREGYEVRLTADRDLVQLVSDRVAVL HPEGHLITPEWLWEKYGLKPEQWVDFRALVGDPSDNLPGVKGIGEKTKALKLL KEWGSLENILKNLDRVKPESVRERIKAHLEDLKLSELSRVRSDLPLEVDFARR REPDREGLRAFLERLEFGSLLHEFGLLEAPAPLEEAPWPPPEGAFVGFVLSRPEP MWAELKALAACKEGRVHRAKDPLAGLKDLKEVRGLLAKDLAVLALREGLDL APSDDPMLLAYLLDPSNTTPEGVARRYGGEWTEAAHRALLAERLQQNLLER LKGEKLLWLYQEVEKPLSRVLAHMEATGVRLDVAYLKALSLELAEEIRRL EVFRLAGHPFNLNSRDQLERVLFDELRLPALGKTQKTGKRSTSAAVLEALREA HPIVEKILQHRELTKLKNTYVDPLPGLVHPRTGRLHTRFNQTATATGRLSSSGP NLQNIPIRTPLGQRIRRAFVAEAGWALVALDYSQIELRVLAHLSGDENLIRVFQ EGKDMHTQTASWMFGVSPEAVDPLMRRAAKTVNFGVLYGMSAHRLSQELAI PYEEAVAFIERYFQSFPKYRAWIEKTLEEGRKRGYVETLFGRRRYVPDLNARV KSVREAAERMAFNMPVQGTAAADMKLAMVKLPHLREMGMARMLLQVHDEL LLEAPQARAEVAALAKEAMEKAYPLAVPLEVEVGIGEDWLSAKG
Single Mutant Z05D V705L	5	MKAMLPLFEPKGRVLLVDGHHLAYRTFFALKGLTTSRGEVQAVYGFSAKSL KALKEDGYKAVFVVFDAKAPSRHEAYEAYKAGRAPTPEDFPRQLALIKELV DLLGFTRLEVPGFEADDVLATLAKKAEREGYEVRLTADRDLVQLVSDRVAVL HPEGHLITPEWLWEKYGLKPEQWVDFRALVGDPSDNLPGVKGIGEKTKALKLL KEWGSLENILKNLDRVKPESVRERIKAHLEDLKLSELSRVRSDLPLEVDFARR REPDREGLRAFLERLEFGSLLHEFGLLEAPAPLEEAPWPPPEGAFVGFVLSRPEP MWAELKALAACKEGRVHRAKDPLAGLKDLKEVRGLLAKDLAVLALREGLDL APSDDPMLLAYLLDPSNTTPEGVARRYGGEWTEAAHRALLAERLQQNLLER LKGEKLLWLYQEVEKPLSRVLAHMEATGVRLDVAYLKALSLELAEEIRRL EVFRLAGHPFNLNSRDQLERVLFDELRLPALGKTQKTGKRSTSAAVLEALREA HPIVEKILQHRELTKLKNTYVDPLPGLVHPRTGRLHTRFNQTATATGRLSSSGP NLQNIPIRTPLGQRIRRAFVAEAGWALVALDYSQIELRVLAHLSGDENLIRVFQ EGKDIHTQTASWMFGVSPEAVDPLMRRAAKTVNFGVLYGMSAHRLSQELAI YEEAVAFIERYFQSFPKLRRAWIEKTLEEGRKRGYVETLFGRRRYVPDLNARV KSVREAAERMAFNMPVQGTAAADMKLAMVKLPHLREMGMARMLLQVHDELL LLEAPQARAEVAALAKEAMEKAYPLAVPLEVEVGIGEDWLSAKG

Table 1

For all the PCR experiments described in these Examples, the primers used had the nucleic acid sequence of SEQ ID NOs:7 and 8, the probe used had the nucleic acid sequence of SEQ ID NO:9, and the template used had the nucleic acid sequence of SEQ ID NO:10, as shown below in Table 2, below.

Type	Name	SEQ ID NO	Sequence
Forward Primer	EIC3PR1TBB	7	ACAACCGCGCCATACATGTCAAGA<t-Butyl benzyl-dA>
Reverse Primer	EFIC3PR1RTBB	8	GTCGGGCCGCTTATACAGTACCA<t-Butyl benzyl-dA>
Probe	GIC10RCY55BQ 11	9	<CY5.5>TGC GCGTCCCG<BHQ- 2>TTTTGATACTTCGTAACGGTGC<Phosphate>
Template	gBlock RS3011	10	GATCTAGCTTTGCTGCTTGATAGCAATCGGCTATCGA CTAATGACTGTCCTGGCGGTCTCTCGCCATCTCCTACC GCATTGGCTCATAGGTAAGCTCGCTGTCACCCAGTACG GAGGTGCCAGTAGATTATTAGAGACAGTCGCCAATCG ATCGTTATACCGAGATGACTGAGTATCGAAGCTACATT GTAGCCGCACATAGGACCACCCATCTTCATGTTGAAAC ATGAGGATTACCCATGTGGATCTAACTGGGTAGTAACT GCGGGGGCGAATGATGCAGGCTTCAGAAATTAAGCTC AATAGTATCCGGTGTCTCAATCTTTTTTCGGGCCAGGCG GCGGTGGACGACAGACAATTTTACGATTTTGGTTCCGG TCACAACCGCGCCATACATGTCAAGAATGAAGTGGGC GAACGCTAGAAAAGTACGCCAGCAATTAAGTGAGTC GGGGCGAGGTGACTCCCACGTAAAAAGCCCCTACCCC GCACCGTTACGAAGTATCAAACGGGACGCGCACGAA CCGACGATTGGTACTGTATAAGCGGCCCGACGAACTC AAAATCCCAAGTGAATCTATGAAATCTACATCGCGTTT ATAATCTACGGGTGTAAACGGATGAGAATTGGCCAA ACGGAGGCACACACGCGTGCAATGCGCCGACCCTGAG AAAAGTATCATGTGCGTCGGCCACAAAACATGAGGAT TACCCATGTA

5 **Table 2**

While parental Z05D (SEQ ID NO:2) does not incorporate methylated-dNTPs, Z05D-F2 (SEQ ID NO:3) successfully performed PCR using 1 mM dNTPs under standard cobas 6800/8800 conditions. The conditions are shown in Table 3, below.

Component	Concentration/Amount
Tricine pH 8.2	60 mM
KOAc (added)	120 mM
Na Azide	0.027%
EDTA	0.044 mM
DMSO	5.4%
Glycerol (total)	3% w/v
Pierce Tween 20 (total)	0.0168%
UNG	0.2 U/ μ l
NTQ21-46A	220 nM
MnOAc	3.3mM
Alkylated primers (t-butyl benzyl)	0.2 μ M
GIC probe Cy5.5_BHQ (TaqMan Probe)	0.1 μ M

Table 3

However, a roughly 6 cycle delay was observed when using Z05D-F2 and methylated-dNTPs compared to the reference with parental Z05D and regular dNTPs (*i.e.*, unmethylated dNTPs). In addition to methylated-dNTPs, fluorinated-dNTPs (F-dNTPs) were also tested as another candidate stable alternative that would also possibly avoid hydrolysis of nucleotides. F-dNTPs, however, were incorporated by Z05D-F2 with a very low efficiency ($C_p > 40$). Even increasing the concentration of F-dNTPs did not result in a significant improvement of PCR performance. By contrast, increasing the concentration of methylated-dNTPs in the mastermix to 2 mM improved PCR efficiency, and the determined C_p -value was identical to the C_p -value for parental Z05D and non-modified nucleotides under standard conditions, as shown in Figure 2.

Example 2: Modified Polymerases Incorporating Modified dNTPs

The Z05D-F2 variant harbors the two amino acid substitutions: I640M and V705L (SEQ ID NO:3), as show in Figure 3. A deconvolution of the two amino acid substitutions was performed and the single amino acid variants were evaluated regarding their incorporation of methylated-dNTPs. Therefore, the single amino acid variants Z05D-I640M (SEQ ID NO:4) and Z05D-V705L (SEQ ID NO:5) were generated by site-directed mutagenesis, expressed and purified. The single amino acid variants, the double mutant F2 (SEQ ID NO:3) and the parental Z05D protein (SEQ ID NO:2) were assessed for their PCR performance using regular non-modified dNTPs, methylated-dNTPs and F-dNTPs, as show in Figures 4A and 4B. Equal concentration of Z05D variants were tested using a DNA-template with alkylated primers in

the master mix (shown in Table 3, above). With regular dNTPs, the single mutant Z05D-V705L (SEQ ID NO:5) shows the same performance as the parental Z05D (SEQ ID NO:2), whereas both the double mutant Z05D-F2 (SEQ ID NO:3) and the single mutant Z05D-I640M (SEQ ID NO:4) show a delayed Cp-value and a lower fluorescence signal. Z05D-F2 (SEQ ID NO:3) and Z05D-I640M (SEQ ID NO:4), on the other hand, are the only variants that support PCR with methylated dNTPs. Taken together, these data suggest that I640M is the crucial amino acid substitution for methylated-dNTP incorporation. Interestingly, Z05D-I640M (SEQ ID NO:4) shows a 5 cycle delay compared to the double mutant Z05D (Z05D-F2, SEQ ID NO:3). This suggests that while the amino acid substitution V705L is not promoting PCR with methylated-dNTPs, by itself, the amino acid substitution V705L does result in an increase in PCR efficiency with modified nucleotides in the Z05D-F2 double mutant (SEQ ID NO:3). Fluorinated-dNTPs (F-dNTPs) are not incorporated at all or only with a very low efficiency by the different proteins (data not shown).

Example 3: Incorporation of Methylated d-NTPs by Modified Polymerase in HIV Assay

In order to employ methylated-dNTPs as a stable nucleotide alternative in an assay, such as an HIV-assay for the cobas® Liat® System, methylated-dNTPs and Z05D-F2 (SEQ ID NO:3) need to promote RT-PCR. Therefore, to test this, RT-PCR was performed using a β -catenin transcript as an RNA template with alkylated primer and 1mM or 2 mM methylated-dNTPs. By comparison, a reaction using parental Z05D (SEQ ID NO:2) and unmodified nucleotides was performed in parallel. The results are presented in Figure 5. As expected the single mutant Z05D-V705L (SEQ ID NO:5) did not show any growth curve, and the single mutant Z05D-I640M (SEQ ID NO:4) only supported RT-PCR with increased methylated-dNTPs concentration. The double mutant, Z05D-F2 (SEQ ID NO:3), successfully performed RT-PCR, at a concentration of methylated-dNTPs of 1 mM and 2 mM, but Cp-values were delayed by ~9 and ~4 cycles, respectively under these master mix conditions. In order to optimize RT-PCR performance, other components of the master mix (*e.g.*, metal-ion, salt concentration) may require adjustment.

CLAIMS

1. A DNA polymerase having increased efficiency in incorporating methylated deoxynucleotide triphosphates (dNTPs) compared with a control DNA polymerase, wherein the amino acid of the DNA polymerase corresponding to position 640 of SEQ ID NO:2 is any amino acid other than I, and/or wherein the amino acid of the DNA polymerase corresponding to position 705 of SEQ ID NO:2 is any amino acid other than V, and wherein the control DNA polymerase has the same amino acid sequence as the DNA polymerase except that the amino acid of the control DNA polymerase corresponding to position 640 of SEQ ID NO:2 is I and/or the amino acid of the control DNA polymerase corresponding to position 705 of SEQ ID NO:2 is V.
2. The DNA polymerase of claim 1, wherein the DNA polymerase and the control DNA polymerase comprise an amino acid sequence at least 90% identical to SEQ ID NO:2.
3. The DNA polymerase of any one claims 1 to 2, wherein the amino acid corresponding to position 640 of SEQ ID NO:2 is M.
4. The DNA polymerase of any one of claims 1 to 3, wherein the amino acid corresponding to position 705 of SEQ ID NO:2 is L.
5. The DNA polymerase of claim 3, wherein the DNA polymerase comprises the amino acid sequence of SEQ ID NO:4.
6. The DNA polymerase of claim 4, wherein the DNA polymerase comprises the amino acid sequence of SEQ ID NO:5.
7. The DNA polymerase of any one of claims 1 to 4, wherein the DNA polymerase comprises the amino acid sequence of SEQ ID NO:3.
8. A recombinant nucleic acid encoding the DNA polymerase according to any one of claims 1 to 7.
9. An expression vector comprising the recombinant nucleic acid of claim 8.
10. A kit for producing an extended primer comprising at least one container providing a DNA polymerase according to any one of claims 1 to 7.
11. The kit of claim 10, further comprising one or more additional containers selected from the group consisting of:
 - (a) a container providing a primer hybridizable, under primer extension conditions, to a predetermined polynucleotide template;

- (b) a container providing dNTPs; and
- (c) a container providing a buffer suitable for primer extension.

12. The kit of claim 11, wherein the dNTPs are methylated dNTP.
13. A reaction mixture comprising a DNA polymerase according to any one of claims 1 to
5 7, at least one primer, a polynucleotide template, and dNTPs.
14. The reaction mixture of claim 13, wherein the polynucleotide template is RNA.
15. The reaction mixture of claim 13, wherein the polynucleotide template is DNA.
16. The reaction mixture of any one of claims 13 to 15 further comprising Mg^{2+} .
17. The reaction mixture of any one of claims 13 to 16 further comprising a second
10 thermostable DNA polymerase.
18. A method for conducting primer extension, comprising:
contacting a DNA polymerase of any one of claims 1 to 7 with a primer, a
polynucleotide template, and nucleoside triphosphates under conditions suitable for
extension of the primer, thereby producing an extended primer.

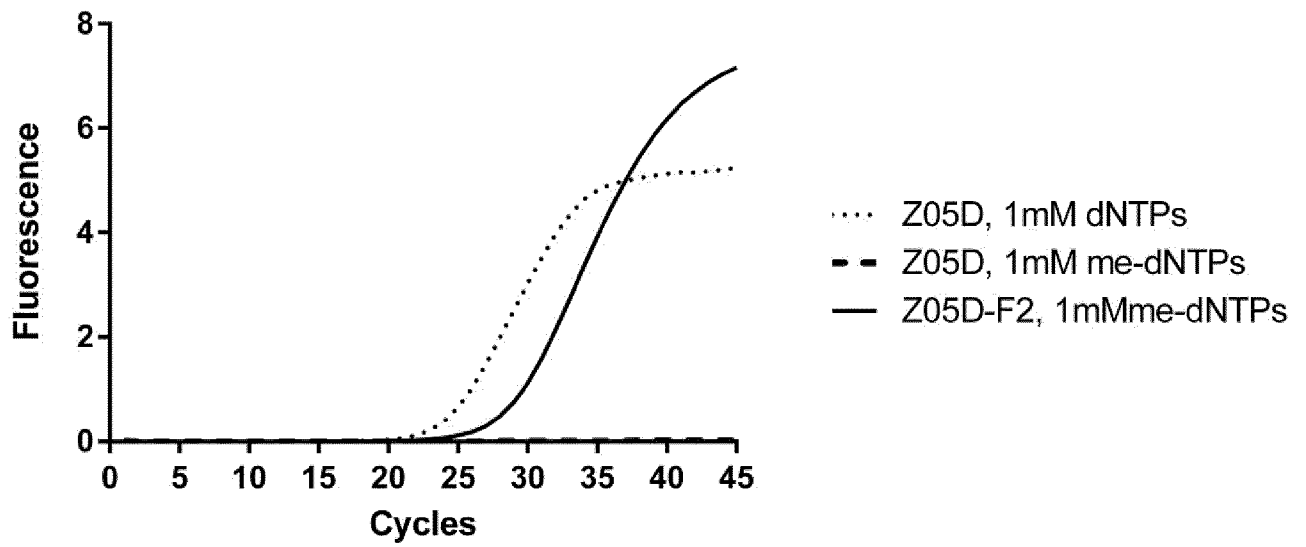


Figure 1

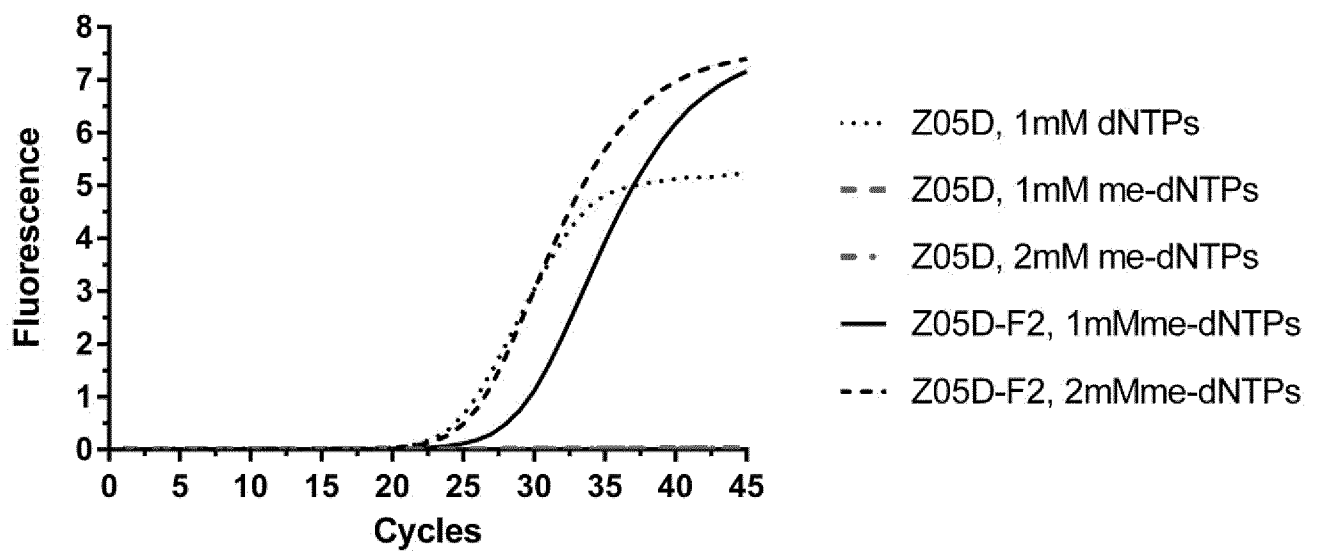


Figure 2

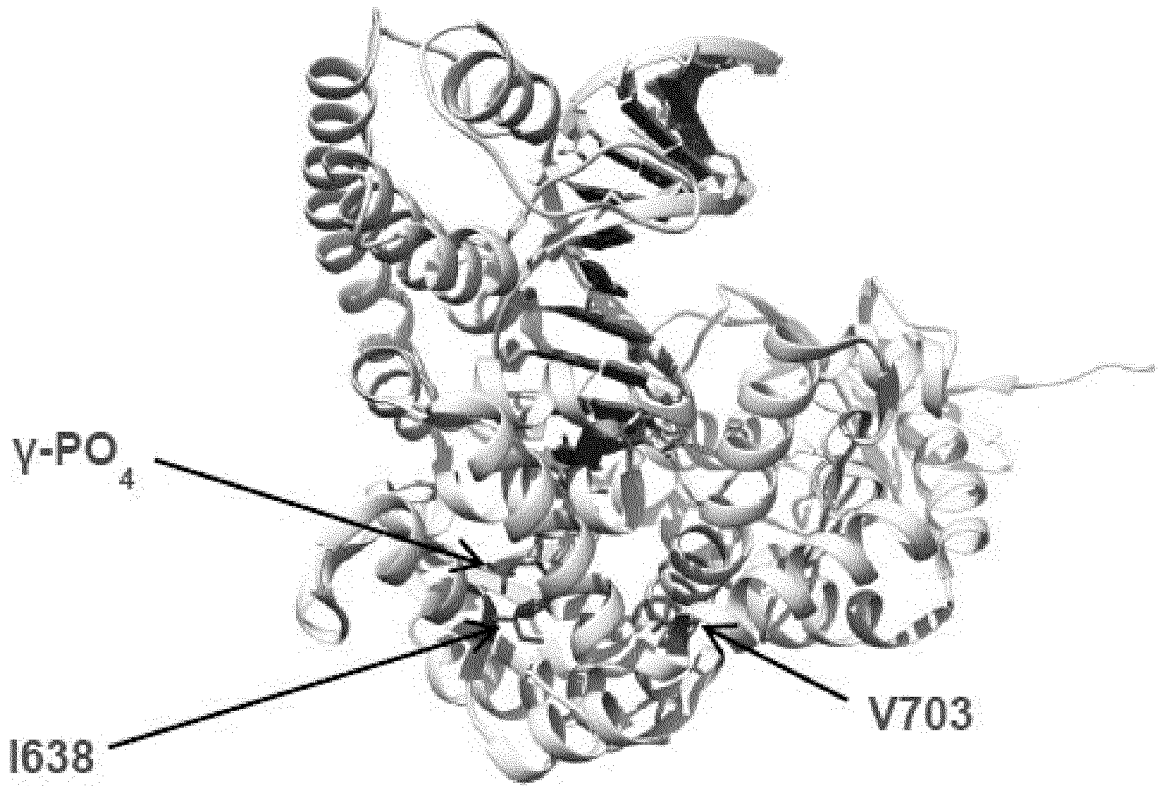


Figure 3

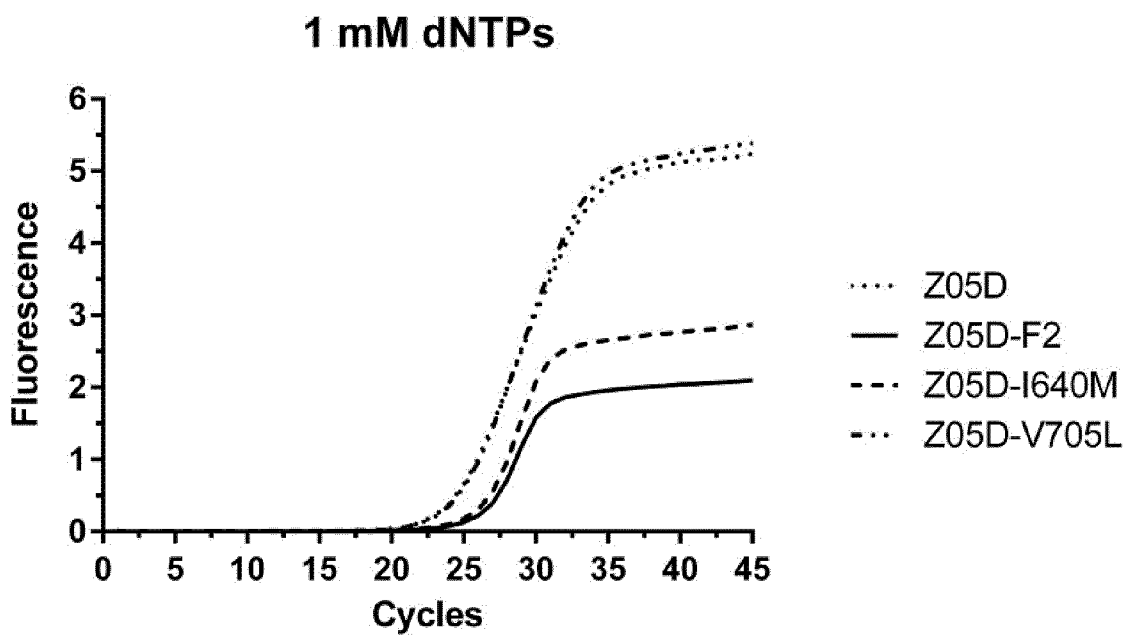


Figure 4A

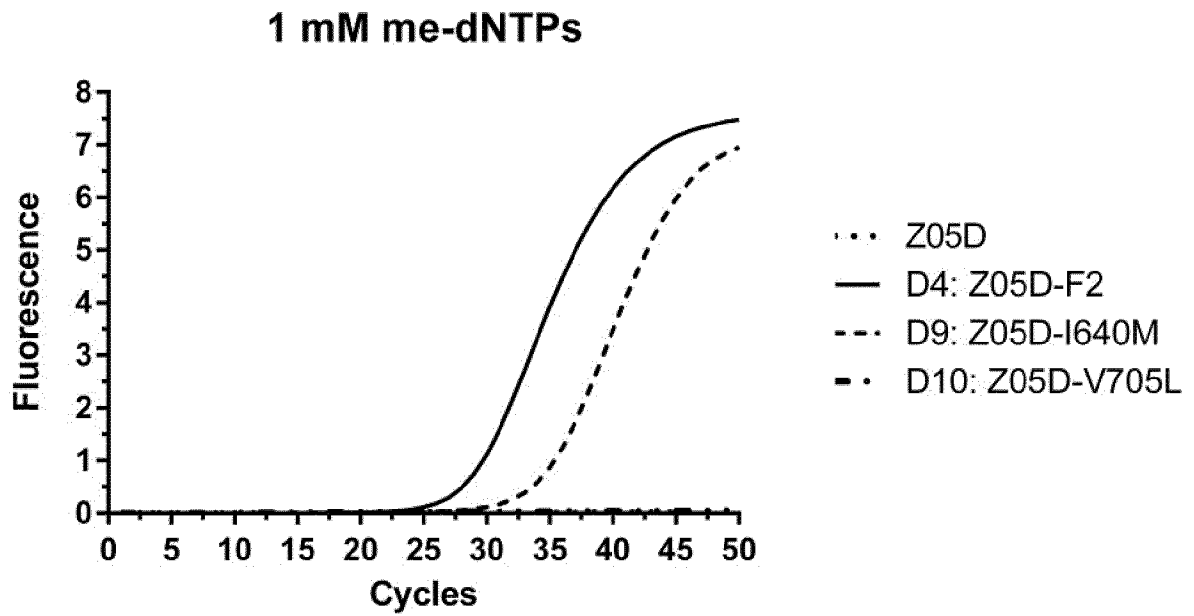


Figure 4B

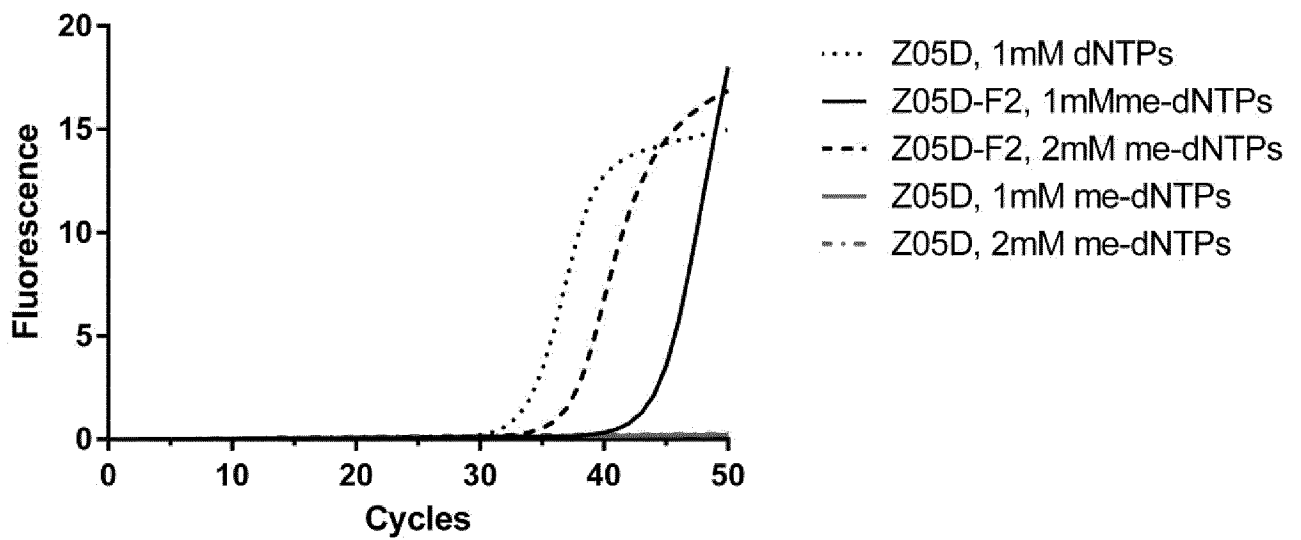


Figure 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/057080

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/12
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/083263 A1 (ROCHE DIAGNOSTICS GMBH [DE]; HOFFMANN LA ROCHE [CH]) 13 June 2013 (2013-06-13) claims 1-3	1-18
X	Zymo Research Corp ET AL: "5-Methylcytosine dNTP Mix [10mM] Product Information", 1 January 2010 (2010-01-01), pages 1-2, XP055583360, Retrieved from the Internet: URL:https://files.zymoresearch.com/protocols/_d1030_5-methylcytosine_dntp_mix_10mm.pdf [retrieved on 2019-04-25] the whole document	1-18

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search 25 April 2019	Date of mailing of the international search report 14/05/2019
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