

**METHOD AND KIT FOR TEMPLATE-INDEPENDENT NUCLEIC ACID
SYNTHESIS**

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority of U.S. Patent
5 Application No. 16/725,420, filed on December 23, 2019,
the entire content of which is incorporated herein by
reference.

FIELD

The disclosure relates to a method and a kit for
10 nucleic acid synthesis, more particularly to a method
and a kit for template-independent nucleic acid
synthesis.

BACKGROUND

De novo DNA synthesis dispensing with a DNA template
15 has been developed during past decades. Among the
currently available template-independent DNA
synthesis methods, the phosphoramidite-based chemical
DNA synthesis has been well-known since early 1980's,
but basically has remained unchanged since then. The
20 phosphoramidite-based chemical DNA Synthesis requires
four consecutive reaction steps, including de-blocking,
coupling, capping, and oxidation steps, to add one
nucleoside to another nucleoside tethered to a solid
support. However, one of the major drawbacks of the
25 phosphoramidite-based chemical DNA synthesis is
inevitable use of hazardous chemicals in the aforesaid

reaction steps.

Due to growing demand for environmental protection, green technology applicable to DNA synthesis has drawn attention of researchers. Therefore, enzymatic DNA synthesis, which can greatly reduce use of hazardous chemicals, seems promising since such synthesis has merits such as longer strand generation, a lower error rate, a faster cycle time, a lower production cost, etc.

Speaking of template-independent enzymatic DNA synthesis, terminal deoxynucleotidyl transferase (TdT) has been found to be a template-independent DNA polymerase that adds all four deoxynucleoside triphosphates (dNTPs) to the 3' termini of DNA strands. TdT belongs to the X Family of low-fidelity DNA polymerases. The TdT-based DNA synthesis requires only two reaction steps, namely, a single-nucleotide addition by TdT and subsequent removal of the 3'-protective group from the extended 3'-end of the single-stranded DNA strand being synthesized. Even though TdT and its homologs have been applied to numerous DNA synthesis platforms, template-independent enzymatic DNA synthesis based on TdT can be hardly commercialized due to unsatisfactory product length, reagent reusability, cycle time, and so forth.

SUMMARY

Therefore, an object of the disclosure is to provide

a method and a kit for synthesizing a nucleic acid, which can alleviate at least one of the drawbacks of the prior art.

5 The method includes providing an initiator having an unprotected nucleoside base and a 3' hydroxyl group at a 3' terminus thereof, providing a nucleic acid polymerase having at least a conservative catalytic polymerase domain of a family-B DNA polymerase, providing a nucleotide monomer, and exposing the
10 initiator to the nucleotide monomer in the presence of the nucleic acid polymerase and at least one type of metal cofactors, which are divalent cations, and in the absence of a template, such that the nucleotide monomer is incorporated to the initiator.

15 The kit includes an initiator as described above, a nucleic acid polymerase as described above, at least one type of metal cofactors as described above, and a nucleotide monomer as described above. The kit is used according to a method as described above.

20 **BRIEF DESCRIPTION OF THE DRAWINGS**

Other features and advantages of the disclosure will become apparent in the following detailed description of the embodiments with reference to the accompanying drawings, of which:

25 FIG. 1 is a *de novo* nucleic acid synthesis scheme using family-B DNA polymerases;

FIG. 2 is an image of denaturing urea-polyacrylamide

gel showing products of template-independent nucleic acid synthesis obtained at different reaction temperatures using KOD1^{exo-} DNA polymerase, in which the symbol "S" indicates the position of initiator DNA;

5 FIG. 3 is an image of denaturing urea-polyacrylamide gel showing products of template-independent nucleic acid synthesis obtained at different reaction temperatures using Vent^{exo-} DNA polymerase, in which the symbol "S" indicates the position of initiator DNA;

10 FIG. 4 is an image of denaturing urea-polyacrylamide gel showing products of template-independent nucleic acid synthesis obtained at different reaction temperatures using Pfu^{exo-} DNA polymerase, in which the symbol "S" indicates the position of initiator DNA; and

15 FIG. 5 is an image of denaturing urea-polyacrylamide gel showing products of template-independent nucleic acid synthesis obtained, in the presence of Mg²⁺ only or in combination with Mn²⁺, using Vent^{exo-} DNA polymerase, KOD1^{exo-} DNA polymerase, or Pfu^{exo-} DNA polymerase, in which the symbol "S" indicates the position of initiator DNA, and the symbols "V", "K" and "P" stand for Vent^{exo-} DNA polymerase, KOD1^{exo-} DNA polymerase, and Pfu^{exo-} DNA polymerase, respectively.

DETAILED DESCRIPTION

25 It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms

a part of the common general knowledge in the art, in Taiwan or any other country.

For the purpose of this specification, it will be clearly understood that the word "comprising" means
5 "including but not limited to", and that the word "comprises" has a corresponding meaning.

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which the
10 present disclosure belongs. One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present disclosure. Indeed, the present disclosure is in no way limited to the methods and materials
15 described.

The applicant surprisingly found that family-B DNA polymerases, which are well-known as template-dependent DNA polymerases, can be used to conduct template-independent nucleic acid synthesis
20 (i.e. *de novo* nucleic acid synthesis). Referring to FIG. 1, a *de novo* nucleic acid synthesis scheme using family-B DNA polymerases is illustrated.

Family-B DNA polymerases (also known as type-B DNA polymerases) are replicative and repair polymerases
25 that intrinsically have a catalytic polymerase domain and a 3' to 5' exonuclease domain, and can be found in bacteria, archaea, eukaryotes, and viruses. The term

"catalytic polymerase domain" refers to a structural portion or region of the amino acid sequence of a protein which possesses the catalytic DNA/RNA polymerase activity of the protein, and which does not contain other catalytic activity, such as editing activity (e.g. proofreading activity of a 3' to 5' exonuclease domain), activity for excision of Okazaki primers during replication, and activity for interaction with other proteins. The catalytic polymerase domains of family-B DNA polymerases have a common overall architecture, which resembles a right hand and consists of thumb, palm, and fingers domains. The most conserved region is the palm domain, which contains the catalytic site.

Examples of family-B DNA polymerases include, but are not limited to, bacterial family-B DNA polymerases (e.g. Pol II), eukaryotic family-B DNA polymerases (e.g. Pol α , Pol δ , and Pol ϵ , and Pol ζ), archaeal family-B DNA polymerases (e.g. Pol B, Pol BI, Pol BII, Pol BIII, 9°N, Kod1, Pfu, Tgo, and Vent), and viral family-B DNA polymerases (e.g. HSV-1, RB69, T4, B103 and Φ 29).

Therefore, the present disclosure provides a method for synthesizing a nucleic acid, which includes:

providing an initiator having an unprotected nucleoside base and a 3' hydroxyl group at a 3' terminus thereof;

providing a nucleic acid polymerase having at least a conservative catalytic polymerase domain of a family-B DNA polymerase;

providing a nucleotide monomer; and

5 exposing the initiator to the nucleotide monomer in the presence of the nucleic acid polymerase and at least one type of metal cofactors, which are divalent cations, and in the absence of a template, such that the nucleotide monomer is incorporated to
10 the initiator.

The terms "nucleic acid", "nucleic acid sequence", and "nucleic acid fragment" as used herein refer to a deoxyribonucleotide or ribonucleotide sequence in single-stranded or double-stranded form, and comprise
15 naturally occurring nucleotides or artificial chemical mimics. The term "nucleic acid" as used herein is interchangeable with the terms "oligonucleotide", "polynucleotide", "DNA", "RNA", "gene", "cDNA", and "mRNA" in use.

20 Generally, a "template" is a polynucleotide that contains the target nucleotide sequence. In some instances, the terms "target sequence", "template polynucleotide", "target nucleic acid", "target polynucleotide", "nucleic acid template", "template
25 sequence", and variations thereof, are used interchangeably. Specifically, the term "template" refers to a strand of nucleic acid on which a

complementary copy is synthesized from nucleotides or nucleotide analogs through the activity of a template-dependent nucleic acid polymerase. Within a duplex, the template strand is, by convention, depicted and described as the "bottom" strand. Similarly, the non-template strand is often depicted and described as the "top" strand. The "template" strand may also be referred to as the "sense" strand, and the non-template strand as the "antisense" strand.

10 The term "incorporated" or "incorporation" refers to becoming a part of a nucleic acid. There is a known flexibility in the terminology regarding incorporation of nucleic acid precursors. For example, the nucleotide dGTP is a deoxyribonucleoside triphosphate. Upon incorporation into DNA, dGTP becomes dGMP, that is, a deoxyguanosine monophosphate moiety. Although DNA does not include dGTP molecules, one may say that one incorporates dGTP into DNA.

20 The term "initiator" refers to a mononucleoside, a mononucleotide, an oligonucleotide, a polynucleotide, or modified analogs thereof, from which a nucleic acid is to be synthesized *de novo*. The term "initiator" may also refer to a Xeno nucleic acids (XNA) or a peptide nucleic acid (PNA) having a 3'-hydroxyl group.

25 According to the present disclosure, the initiator may have a sequence selected from a non-self complementary sequence and a non-self complementarity

forming sequence. The term "self complementary" means that a sequence (e.g. a nucleotide sequence or a PNA sequence) folds back on itself (i.e. a region of the sequence binds or hybridizes to another region of the sequence), creating a duplex, double-strand like structure which can serve as a template for nucleic acid synthesis. Depending on how close together the complementary regions of the sequence are, the strand may form, for instance, hairpin loops, junctions, bulges or internal loops. The term "self complementarity forming" is used to describe a sequence (e.g. a nucleotide sequence, XNA, or a PNA sequence) from which a complementary extended portion is formed when such sequence serves as a template (namely, a self-complementary sequence is formed based on such sequence serving as a template). For instance, the self complementarity forming sequence may be "ATCC". When the "ATCC" sequence serves as a template, an extended portion "GGAT" complementary to such sequence is formed from such sequence (i.e. a self-complementary sequence "ATCCGGAT" is formed).

The term "conservative" or "conserved" is used to describe domains containing amino acid residues that are the same among a plurality of proteins having the same structure and/or function. A region of conserved amino acid residues may be important for protein structure or function. Thus, contiguous conserved

amino acid residues as identified in a three-dimensional protein may be important for protein structure or function.

For instance, as reported in Albà (2001), *Genome*
5 *Biology*, 2(1):reviews3002.1 to reviews3002.4, family-B DNA polymerases have Regions I and II that form part of the active sites of the catalytic polymerase domain, and that may respectively contain conserved amino acid residues "DT" and "SLYPS". Region I may span
10 amino acid residues 512 to 582, amino acid residues 513 to 582 or 583, or amino acid residues 535 to 604. Region II may span amino acid residues 375 to 441 or 442, or amino acid residues 397 to 464.

According to the present disclosure, the nucleic
15 acid polymerase may further have a 3' to 5' exonuclease domain and may be a family-B DNA polymerase selected from the group consisting of a bacterial family-B DNA polymerase, a eukaryotic family-B DNA polymerase, an archaeal family-B DNA polymerase, and a viral family-B
20 DNA polymerase. In some embodiments, the family-B DNA polymerase is selected from the group consisting of a family-B DNA polymerase of *Thermococcus kodakaraensis* KOD1, a family-B DNA polymerase of *Pyrococcus furiosus* (Pfu), and a family-B DNA polymerase of *Thermococcus*
25 *litoralis* (Vent).

According to the present disclosure, the 3' to 5' exonuclease domain of the family-B DNA polymerase may

be inactivated. Alternatively, the 3' to 5' exonuclease activity of the family-B DNA polymerase may be reduced. Still alternatively, the 3' to 5' exonuclease domain of the family-B DNA polymerase may remain unchanged,
5 and an inhibitor may be used to inhibit the 3' to 5' exonuclease domain of the family-B DNA polymerase during the method of the present disclosure.

According to the present disclosure, alternatively, the nucleic acid polymerase may only
10 have the aforesaid conservative catalytic polymerase domain. In some embodiments, the nucleic acid polymerase is designed to only have the aforesaid conservative catalytic polymerase domain originally. In other embodiments, the nucleic acid polymerase was
15 originally a family-B DNA polymerase having a 3' to 5' exonuclease domain, and such domain has been removed from the nucleic acid polymerase.

In some embodiments, the initiator is in single-stranded form.

20 In some embodiments, the initiator has at least five nucleotides. In an exemplary embodiment, the initiator has forty-five nucleotides.

In some embodiments, the initiator is exposed to the nucleotide monomer at a temperature ranging from
25 10°C to 90°C, and/or the initiator is exposed to the nucleotide monomer at a pH of not less than 8.0 (for instance, 8.8).

According to the present disclosure, the nucleotide monomer may be a natural nucleic acid nucleotide whose constituent elements are a sugar, a phosphate group and a nitrogen base. The sugar may be
5 ribose in RNA or 2'-deoxyribose in DNA. Depending on whether the nucleic acid to be synthesized is DNA or RNA, the nitrogen base is selected from adenine, guanine, uracil, cytosine and thymine. Alternatively, the nucleotide monomer may be a nucleotide which is modified
10 in at least one of the three constituent elements. By way of example, the modification can take place at the level of the base, generating a modified product (such as inosine, methyl-5-deoxycytidine, deoxyuridine, dimethylamino-5-deoxyuridine, diamino-2,6-purine or
15 bromo-5-deoxyuridine, and any other modified base which permits hybridization), at the level of the sugar (for example, replacement of a deoxyribose by an analog), or at the level of the phosphate group (for example, boronate, alkylphosphonate, or phosphorothioate
20 derivatives).

According to the present disclosure, the nucleotide monomer may have a phosphate group selected from a monophosphate, a diphosphate, a triphosphate, a tetraphosphate, a pentaphosphate, and a
25 hexaphosphate.

According to the present disclosure, the metal cofactor may be selected from the group consisting of

Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} , Mn^{2+} , Co^{2+} , Fe^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , and their combinations thereof. In an exemplary embodiment, the cofactor is Mg^{2+} . In another embodiment, the cofactor is a combination of Mg^{2+} and Mn^{2+} .

5 According to the present disclosure, the nucleotide monomer may have a removable blocking moiety. Examples of the removable blocking moiety include, but are not limited to, a 3'-O-blocking moiety, a base blocking moiety, and a combination thereof.

10 The nucleotide monomer having a removable blocking moiety is also referred to as a reversible terminator. Therefore, the nucleotide monomer having the 3'-O-blocking moiety is also referred to as 3'-blocked reversible terminator or a 3'-O-modified reversible
15 terminator, and the nucleotide monomer having the base blocking moiety is also referred to as a 3'-unblocked reversible terminator or a 3'-OH unblocked reversible terminator.

 As used herein, the term "reversible terminator"
20 refers to a chemically modified nucleotide monomer. When such a reversible terminator is incorporated into a growing nucleic acid by a polymerase, it blocks the further incorporation of a nucleotide monomer by the polymerase. Such "reversible terminator" base and a
25 nucleic acid can be deprotected by chemical or physical treatment, and following such deprotection, the nucleic acid can be further extended by a polymerase.

Examples of the 3'-O-blocking moiety include, but are not limited to, O-azidomethyl, O-amino, O-allyl, O-phenoxyacetyl, O-methoxyacetyl, O-acetyl, O-(p-toluene)sulfonate, O-phosphate, O-nitrate, O-[4-methoxy]-tetrahydrothiopyranyl, O-tetrahydrothiopyranyl, O-[5-methyl]-tetrahydrofuranlyl, O-[2-methyl,4-methoxy]-tetrahydropyranyl, O-[5-methyl]-tetrahydropyranyl, and O-tetrahydrothiofuranlyl, O-2-nitrobenzyl, O-methyl, and O-acyl.

Examples of the 3'-unblocked reversible terminators include, but are not limited to, 7-[(S)-1-(5-methoxy-2-nitrophenyl)-2,2-dimethyl-propyloxy]methyl-7-deaza-dATP, 5-[(S)-1-(5-methoxy-2-nitrophenyl)-2,2-dimethyl-propyloxy]methyl-dCTP, 1-(5-methoxy-2-nitrophenyl)-2,2-dimethyl-propyloxy]methyl-7-deaza-dGTP, 5-[(S)-1-(5-methoxy-2-nitrophen-yl)-2,2-dimethyl-propyloxy]methyl-dUTP, and 5-[(S)-1-(2-nitrophenyl)-2,2-dimethyl-propyloxy]methyl-dUTP.

According to the present disclosure, the base blocking moiety may be a reversible dye-terminator. Examples of the reversible dye-terminator include, but are not limited to, a reversible dye-terminator of Illumina NovaSeq, a reversible dye-terminator of Illumina NextSeq, a reversible dye-terminator of Illumina MiSeq, a reversible dye-terminator of

Illumina HiSeq, a reversible dye-terminator of Illumina Genome Analyzer IIX, a lightning terminator of LaserGen, and a reversible dye-terminator of Helicos Biosciences Heliscope.

5 Since the reversible terminators are well-known to and commonly used by those skilled in the art, further details of the same are omitted herein for the sake of brevity. Nevertheless, applicable 3'-blocked reversible terminators, applicable 3'-unblocked
10 reversible terminators, and applicable conditions for protection and deprotection (i.e. conditions for adding and eliminating the removable blocking moiety) can be found in, for example, Gardner *et al.* (2012), *Nucleic Acids Research*, 40(15):7404-7415, Litosh *et al.*
15 (2011), *Nucleic Acids Research*, 39(6):e39, and Chen *et al.* (2013), *Genomics Proteomics Bioinformatics*, 11:34-40.

 According to the present disclosure, the initiator may be linked to a solid support and have a 5' end linked
20 to the solid support. The initiator may be directly attached to the support, or may be attached to the support via a linker.

 According to the present disclosure, examples of the solid support include, but are not limited to,
25 microarrays, beads (coated or non-coated), columns, optical fibers, wipes, nitrocellulose, nylon, glass, quartz, diazotized membranes (paper or nylon),

silicones, polyformaldehyde, cellulose, cellulose acetate, paper, ceramics, metals, metalloids, semiconductive materials, magnetic particles, plastics (such as polyethylene, polypropylene, and polystyrene, gel-forming materials [such as proteins (e.g., gelatins), lipopolysaccharides, silicates, agarose, polyacrylamides, methylmethacrylate polymers], sol gels, porous polymer hydrogels, nanostructured surfaces, nanotubes (such as carbon nanotubes), and nanoparticles (such as gold nanoparticles or quantum dots).

In addition, the present disclosure provides a kit for synthesizing a nucleic acid, which includes the aforesaid initiator, the aforesaid nucleic acid polymerase, the aforesaid nucleotide monomer, and the aforesaid at least one type of divalent cations. The kit is used according to the method of the present disclosure.

The disclosure will be further described by way of the following examples. However, it should be understood that the following examples are solely intended for the purpose of illustration and should not be construed as limiting the disclosure in practice.

EXAMPLES

Example 1. Template-independent nucleic acid synthesis using family-B DNA polymerase of *Thermococcus kodakaraensis* KOD1

A synthesis reaction mixture was prepared using suitable amounts of the following ingredients: a single-stranded initiator that has a nucleotide sequence of SEQ ID NO: 1 and a 3' end possessing an unprotected hydroxyl group and a 5' end labeled with fluorescein amidite (FAM); deoxynucleoside triphosphates (dNTPs) serving as nucleotide monomers, including dATP, dGTP, dCTP, and dTTP; a family-B DNA polymerase of *Thermococcus kodakaraensis* KOD1 that has an inactivated 3' to 5' exonuclease domain and that is referred to as KOD1^{exo-} DNA polymerase; and a Tris-HCl buffer (pH 8.8). Specifically, the synthesis reaction mixture contained 100 nM of the initiator, 100 μ M of the dNTPs, and 200 nM of KOD1^{exo-} DNA polymerase.

KOD1^{exo-} DNA polymerase was prepared as follows. A gene construct encoding a family-B DNA polymerase of *Thermococcus kodakaraensis* KOD1 (intein-free and having a normal 3' to 5' exonuclease domain) was synthesized by Genomics BioSci & Tech Co. (New Taipei City, Taiwan). To obtain KOD1^{exo-} DNA polymerase, the inactivation of the conservative 3' to 5' exonuclease domain was achieved by changing Asp¹⁴¹ to Ala (D141A) and Glu¹⁴³ to Ala (E143A), i.e. modifying the conserved amino residues "DIE" of the conservative 3' to 5' exonuclease domain. Specifically, to accomplish the amino acid modifications "D141A" and "E143A", the corresponding nucleotide residues on the aforesaid

gene construct were subjected to site-directed mutagenesis using Q5 Site-directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA). The resulting mutagenized gene construct was expressed in BL21(DE3) cells, and the expressed protein was purified using Akta Pure FPLC system (GE Healthcare Life Sciences, Marlborough, MA, USA) through HisTrap Q and Heparin columns sequentially. KOD1^{exo-} DNA polymerase thus obtained has an amino acid sequence of SEQ ID NO: 2.

10 10 μ L of the nucleic acid synthesis reaction mixture was preincubated for 2 minutes at one of the following temperatures: 10°C, 20°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 70°C, 80°C, and 90°C. Subsequently, a suitable amount of Mg²⁺ serving as cofactors were added into the respective reaction mixture to initiate the template-independent nucleic acid synthesis, which was allowed to proceed for 5 minutes. The synthesis was terminated by adding 10 μ L of 2X quench solution (containing 95% de-ionized formamide and 25 mM ethylenediaminetetraacetic acid (EDTA)).

20

The resulting synthesis reaction products were subjected to denaturation at 98°C for 10 minutes. Subsequently, the synthesis reaction products were analyzed by a 15% denaturing urea-polyacrylamide gel.

25 The synthesis reaction products on the gel were

visualized by Amersham Typhoon Imager (GE Healthcare Life Sciences, Marlborough, MA, USA).

Results:

As shown in FIG. 2, KOD1^{exo-} DNA polymerase is able
5 to perform the template-independent nucleic acid
synthesis at each of the temperatures tested, thereby
indicating that a family-B DNA polymerase can be used
to synthesize a nucleic acid in the absence of a
template.

10 **Example 2. Template-independent nucleic acid synthesis
using family-B DNA polymerase of
Thermococcus litoralis (Vent)**

Template-independent nucleic acid synthesis and
analysis of the reaction products were conducted
15 generally according to the procedures set forth in
Example 1, except for use of a family-B DNA polymerase
of *Thermococcus litoralis* (Vent) which has an
inactivated 3' to 5' exonuclease domain and is referred
to as Vent^{exo-} DNA polymerase accordingly. Vent^{exo-} DNA
20 polymerase was prepared following the same procedure
as that for preparing KOD1^{exo-} DNA polymerase (see
Example 1), except that a gene construct encoding a
family-B DNA polymerase of *Thermococcus litoralis*
(intein-free and having a normal 3' to 5' exonuclease
25 domain) was used. Vent^{exo-} DNA polymerase has an amino
acid sequence of SEQ ID NO: 3.

Results:

As shown in FIG. 3, Vent^{exo-} DNA polymerase is able to perform the template-independent nucleic acid synthesis at each of the temperatures tested, thereby indicating that a family-B DNA polymerase can be used to synthesize a nucleic acid in the absence of a template.

Example 3. Template-independent nucleic acid synthesis using family-B DNA polymerase of *Pyrococcus furiosus* (Pfu)

Template-independent nucleic acid synthesis and analysis of the reaction products were conducted generally according to the procedures set forth in Example 1, except for use of a family-B DNA polymerase of *Pfu* which has an inactivated 3' to 5' exonuclease domain and is referred to as Pfu^{exo-} DNA polymerase accordingly. Pfu^{exo-} DNA polymerase was prepared following the same procedure as that for preparing KOD1^{exo-} DNA polymerase (see Example 1), except that a gene construct encoding a family-B DNA polymerase of *Pfu* (intein-free and having a normal 3' to 5' exonuclease domain) was used. Pfu^{exo-} DNA polymerase has an amino acid sequence of SEQ ID NO: 4.

Results:

As shown in FIG. 4, Pfu^{exo-} DNA polymerase is able to perform template-independent nucleic acid synthesis at each of the temperatures tested, thereby indicating

that a family-B DNA polymerase can be used to synthesize a nucleic acid in the absence of a template.

Example 4. Template-independent nucleic acid synthesis by family-B DNA polymerase with a single type of divalent cation or a combination of different divalent cations

In order to evaluate whether different types of divalent cations may affect the efficiency of template-independent nucleic acid synthesis by a family-B polymerase, the following experiment was performed.

Template-independent nucleic acid synthesis and analysis of the reaction products were conducted generally according to the procedures set forth in Example 1, except that: a respective one of KOD1^{exo-} DNA polymerase (described in Example 1), Vent^{exo-} DNA polymerase (described in Example 2), and Pfu^{exo-} DNA polymerase (described in Example 3) was used; a respective synthesis reaction mixture was preincubated at 70°C; and Mg²⁺ only or Mg²⁺ in combination with Mn²⁺ were added into the respective reaction mixture.

Results:

As shown in FIG. 5, template-independent nucleic acid synthesis with any of the three family-B DNA polymerases was more efficient (more newly synthesized nucleic acids were found) in the presence of two different types of divalent cations, thus manifesting

that the efficiency of template-independent nucleic acid synthesis with a family-B polymerase can be enhanced using a combination of different types of divalent cations.

5 All patents and references cited in this specification are incorporated herein in their entirety as reference. Where there is conflict, the descriptions in this case, including the definitions, shall prevail.

10 While the disclosure has been described in connection with what are considered the exemplary embodiments, it is understood that this disclosure is not limited to the disclosed embodiments but is intended to cover various arrangements included within the
15 spirit and scope of the broadest interpretation so as to encompass all such modifications and equivalent arrangements.

SEQUENCE LISTING

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			35					40					45			
	Glu	Glu	Val	Lys	Lys	Ile	Thr	Ala	Glu	Arg	His	Gly	Thr	Val	Val	Thr
			50				55					60				
5	Val	Lys	Arg	Val	Glu	Lys	Val	Gln	Lys	Lys	Phe	Leu	Gly	Arg	Pro	Val
	65				70				75				80			
	Glu	Val	Trp	Lys	Leu	Tyr	Phe	Thr	His	Pro	Gln	Asp	Val	Pro	Ala	Ile
					85				90				95			
	Arg	Asp	Lys	Ile	Arg	Glu	His	Pro	Ala	Val	Ile	Asp	Ile	Tyr	Glu	Tyr
10				100				105					110			
	Asp	Ile	Pro	Phe	Ala	Lys	Arg	Tyr	Leu	Ile	Asp	Lys	Gly	Leu	Val	Pro
				115				120					125			
	Met	Glu	Gly	Asp	Glu	Glu	Leu	Lys	Met	Leu	Ala	Phe	Ala	Ile	Ala	Thr
				130				135					140			
15	Leu	Tyr	His	Glu	Gly	Glu	Glu	Phe	Ala	Glu	Gly	Pro	Ile	Leu	Met	Ile
	145				150					155				160		
	Ser	Tyr	Ala	Asp	Glu	Gly	Ala	Arg	Val	Ile	Thr	Trp	Lys	Asn	Val	
					165				170				175			
	Asp	Leu	Pro	Tyr	Val	Asp	Val	Val	Ser	Thr	Glu	Arg	Glu	Met	Ile	Lys
20				180					185				190			
	Arg	Phe	Leu	Arg	Val	Val	Lys	Glu	Lys	Asp	Pro	Asp	Val	Leu	Ile	Thr
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	Tyr	Asn	Gly	Asp	Asn	Phe	Asp	Phe	Ala	Tyr	Leu	Lys	Lys	Arg	Cys	Glu
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25	Lys	Leu	Gly	Ile	Asn	Phe	Ala	Leu	Gly	Arg	Asp	Gly	Ser	Glu	Pro	Lys
	225				230					235				240		
	Ile	Gln	Arg	Met	Gly	Asp	Arg	Phe	Ala	Val	Glu	Val	Lys	Gly	Arg	Ile
					245				250				255			
	His	Phe	Asp	Leu	Tyr	Pro	Val	Ile	Arg	Arg	Thr	Ile	Asn	Leu	Pro	Thr
30				260					265				270			

25

	Tyr Thr Leu Glu Ala Val Tyr Glu Ala Val Phe Gly Gln Pro Lys Glu		
	275	280	285
	Lys Val Tyr Ala Glu Glu Ile Thr Thr Ala Trp Glu Thr Gly Glu Asn		
	290	295	300
5	Leu Glu Arg Val Ala Arg Tyr Ser Met Glu Asp Ala Lys Val Thr Tyr		
	305	310	315 320
	Glu Leu Gly Lys Glu Phe Leu Pro Met Glu Ala Gln Leu Ser Arg Leu		
	325	330	335
	Ile Gly Gln Ser Leu Trp Asp Val Ser Arg Ser Ser Thr Gly Asn Leu		
10	340	345	350
	Val Glu Trp Phe Leu Leu Arg Lys Ala Tyr Glu Arg Asn Glu Leu Ala		
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	Pro Asn Lys Pro Asp Glu Lys Glu Leu Ala Arg Arg Arg Gln Ser Tyr		
	370	375	380
15	Glu Gly Gly Tyr Val Lys Glu Pro Glu Arg Gly Leu Trp Glu Asn Ile		
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	Val Tyr Leu Asp Phe Arg Ser Leu Tyr Pro Ser Ile Ile Ile Thr His		
	405	410	415
	Asn Val Ser Pro Asp Thr Leu Asn Arg Glu Gly Cys Lys Glu Tyr Asp		
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	Val Ala Pro Gln Val Gly His Arg Phe Cys Lys Asp Phe Pro Gly Phe		
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	Ile Pro Ser Leu Leu Gly Asp Leu Leu Glu Glu Arg Gln Lys Ile Lys		
	450	455	460
25	Lys Lys Met Lys Ala Thr Ile Asp Pro Ile Glu Arg Lys Leu Leu Asp		
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	Tyr Arg Gln Arg Ala Ile Lys Ile Leu Ala Asn Ser Tyr Tyr Gly Tyr		
	485	490	495
	Tyr Gly Tyr Ala Arg Ala Arg Trp Tyr Cys Lys Glu Cys Ala Glu Ser		
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	Val Thr Ala Trp Gly Arg Glu Tyr Ile Thr Met Thr Ile Lys Glu Ile	
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	Glu Glu Lys Tyr Gly Phe Lys Val Ile Tyr Ser Asp Thr Asp Gly Phe	
	530	535 540
5	Phe Ala Thr Ile Pro Gly Ala Asp Ala Glu Thr Val Lys Lys Lys Ala	
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	Met Glu Phe Leu Lys Tyr Ile Asn Ala Lys Leu Pro Gly Ala Leu Glu	
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	Leu Glu Tyr Glu Gly Phe Tyr Lys Arg Gly Phe Phe Val Thr Lys Lys	
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	Lys Tyr Ala Val Ile Asp Glu Glu Gly Lys Ile Thr Thr Arg Gly Leu	
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	Glu Ile Val Arg Arg Asp Trp Ser Glu Ile Ala Lys Glu Thr Gln Ala	
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15	Arg Val Leu Glu Ala Leu Leu Lys Asp Gly Asp Val Glu Lys Ala Val	
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	Arg Ile Val Lys Glu Val Thr Glu Lys Leu Ser Lys Tyr Glu Val Pro	
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	Pro Glu Lys Leu Val Ile His Glu Gln Ile Thr Arg Asp Leu Lys Asp	
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	Tyr Lys Ala Thr Gly Pro His Val Ala Val Ala Lys Arg Leu Ala Ala	
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	Arg Gly Val Lys Ile Arg Pro Gly Thr Val Ile Ser Tyr Ile Val Leu	
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25	Lys Gly Ser Gly Arg Ile Gly Asp Arg Ala Ile Pro Phe Asp Glu Phe	
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	Asp Pro Thr Lys His Lys Tyr Asp Ala Glu Tyr Tyr Ile Glu Asn Gln	
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	Val Leu Pro Ala Val Glu Arg Ile Leu Arg Ala Phe Gly Tyr Arg Lys	
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 Leu Lys Pro Lys Gly Thr
 770

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 35 40 45
 Glu Glu Ile Lys Ala Ile Lys Gly Glu Arg His Gly Lys Thr Val Arg
 20 50 55 60
 Val Leu Asp Ala Val Lys Val Arg Lys Lys Phe Leu Gly Arg Glu Val
 65 70 75 80
 Glu Val Trp Lys Leu Ile Phe Glu His Pro Gln Asp Val Pro Ala Met
 85 90 95
 25 Arg Gly Lys Ile Arg Glu His Pro Ala Val Val Asp Ile Tyr Glu Tyr
 100 105 110
 Asp Ile Pro Phe Ala Lys Arg Tyr Leu Ile Asp Lys Gly Leu Ile Pro
 115 120 125
 Met Glu Gly Asp Glu Glu Leu Lys Leu Leu Ala Phe Ala Ile Ala Thr
 30 130 135 140

Phe Tyr His Glu Gly Asp Glu Phe Gly Lys Gly Glu Ile Ile Met Ile
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 Ser Tyr Ala Asp Glu Glu Glu Ala Arg Val Ile Thr Trp Lys Asn Ile
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 5 Asp Leu Pro Tyr Val Asp Val Val Ser Asn Glu Arg Glu Met Ile Lys
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 Arg Phe Val Gln Val Val Lys Glu Lys Asp Pro Asp Val Ile Ile Thr
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 Tyr Asn Gly Asp Asn Phe Asp Leu Pro Tyr Leu Ile Lys Arg Ala Glu
 10 210 215 220
 Lys Leu Gly Val Arg Leu Val Leu Gly Arg Asp Lys Glu His Pro Glu
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 Pro Lys Ile Gln Arg Met Gly Asp Ser Phe Ala Val Glu Ile Lys Gly
 245 250 255
 15 Arg Ile His Phe Asp Leu Phe Pro Val Val Arg Arg Thr Ile Asn Leu
 260 265 270
 Pro Thr Tyr Thr Leu Glu Ala Val Tyr Glu Ala Val Leu Gly Lys Thr
 275 280 285
 Lys Ser Lys Leu Gly Ala Glu Glu Ile Ala Ala Ile Trp Glu Thr Glu
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 Glu Ser Met Lys Lys Leu Ala Gln Tyr Ser Met Glu Asp Ala Arg Ala
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 Thr Tyr Glu Leu Gly Lys Glu Phe Phe Pro Met Glu Ala Glu Leu Ala
 325 330 335
 25 Lys Leu Ile Gly Gln Ser Val Trp Asp Val Ser Arg Ser Ser Thr Gly
 340 345 350
 Asn Leu Val Glu Trp Tyr Leu Leu Arg Val Ala Tyr Ala Arg Asn Glu
 355 360 365
 Leu Ala Pro Asn Lys Pro Asp Glu Glu Glu Tyr Lys Arg Arg Leu Arg
 30 370 375 380

Thr Thr Tyr Leu Gly Gly Tyr Val Lys Glu Pro Glu Lys Gly Leu Trp
 385 390 395 400
 Glu Asn Ile Ile Tyr Leu Asp Phe Arg Ser Leu Tyr Pro Ser Ile Ile
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 5 Val Thr His Asn Val Ser Pro Asp Thr Leu Glu Lys Glu Gly Cys Lys
 420 425 430
 Asn Tyr Asp Val Ala Pro Ile Val Gly Tyr Arg Phe Cys Lys Asp Phe
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 Pro Gly Phe Ile Pro Ser Ile Leu Gly Asp Leu Ile Ala Met Arg Gln
 10 450 455 460
 Asp Ile Lys Lys Lys Met Lys Ser Thr Ile Asp Pro Ile Glu Lys Lys
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 Met Leu Asp Tyr Arg Gln Arg Ala Ile Lys Leu Leu Ala Asn Ser Tyr
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 15 Tyr Gly Tyr Met Gly Tyr Pro Lys Ala Arg Trp Tyr Ser Lys Glu Cys
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 Ala Glu Ser Val Thr Ala Trp Gly Arg His Tyr Ile Glu Met Thr Ile
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 545 550 555 560
 Lys Lys Ala Lys Glu Phe Leu Asn Tyr Ile Asn Ser Lys Leu Pro Gly
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 25 Leu Leu Glu Leu Glu Tyr Glu Gly Phe Tyr Leu Arg Gly Phe Phe Val
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				20					25					30		
	Thr	Phe	Arg	Pro	Tyr	Ile	Tyr	Ala	Leu	Leu	Arg	Asp	Asp	Ser	Lys	Ile
			35					40					45			
5	Glu	Glu	Val	Lys	Lys	Ile	Thr	Gly	Glu	Arg	His	Gly	Lys	Ile	Val	Arg
		50				55						60				
	Ile	Val	Asp	Val	Glu	Lys	Val	Glu	Lys	Lys	Phe	Leu	Gly	Lys	Pro	Ile
	65					70					75				80	
	Thr	Val	Trp	Lys	Leu	Tyr	Leu	Glu	His	Pro	Gln	Asp	Val	Pro	Thr	Ile
10					85					90					95	
	Arg	Glu	Lys	Val	Arg	Glu	His	Pro	Ala	Val	Val	Asp	Ile	Phe	Glu	Tyr
				100					105					110		
	Asp	Ile	Pro	Phe	Ala	Lys	Arg	Tyr	Leu	Ile	Asp	Lys	Gly	Leu	Ile	Pro
				115					120					125		
15	Met	Glu	Gly	Glu	Glu	Glu	Leu	Lys	Ile	Leu	Ala	Phe	Ala	Ile	Ala	Thr
		130					135					140				
	Leu	Tyr	His	Glu	Gly	Glu	Glu	Phe	Gly	Lys	Gly	Pro	Ile	Ile	Met	Ile
	145					150					155				160	
	Ser	Tyr	Ala	Asp	Glu	Asn	Glu	Ala	Lys	Val	Ile	Thr	Trp	Lys	Asn	Ile
20				165					170					175		
	Asp	Leu	Pro	Tyr	Val	Glu	Val	Val	Ser	Ser	Glu	Arg	Glu	Met	Ile	Lys
				180					185					190		
	Arg	Phe	Leu	Arg	Ile	Ile	Arg	Glu	Lys	Asp	Pro	Asp	Ile	Ile	Val	Thr
			195						200					205		
25	Tyr	Asn	Gly	Asp	Ser	Phe	Asp	Phe	Pro	Tyr	Leu	Ala	Lys	Arg	Ala	Glu
		210						215						220		
	Lys	Leu	Gly	Ile	Lys	Leu	Thr	Ile	Gly	Arg	Asp	Gly	Ser	Glu	Pro	Lys
	225					230					235				240	
	Met	Gln	Arg	Ile	Gly	Asp	Met	Thr	Ala	Val	Glu	Val	Lys	Gly	Arg	Ile
30					245						250				255	

	His Phe Asp Leu Tyr His Val Ile Thr Arg Thr Ile Asn Leu Pro Thr	
	260	265 270
	Tyr Thr Leu Glu Ala Val Tyr Glu Ala Ile Phe Gly Lys Pro Lys Glu	
	275	280 285
5	Lys Val Tyr Ala Asp Glu Ile Ala Lys Ala Trp Glu Ser Gly Glu Asn	
	290	295 300
	Leu Glu Arg Val Ala Lys Tyr Ser Met Glu Asp Ala Lys Ala Thr Tyr	
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	Glu Leu Gly Lys Glu Phe Leu Pro Met Glu Ile Gln Leu Ser Arg Leu	
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	Val Gly Gln Pro Leu Trp Asp Val Ser Arg Ser Ser Thr Gly Asn Leu	
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	Val Glu Trp Phe Leu Leu Arg Lys Ala Tyr Glu Arg Asn Glu Val Ala	
	355	360 365
15	Pro Asn Lys Pro Ser Glu Glu Glu Tyr Gln Arg Arg Leu Arg Glu Ser	
	370	375 380
	Tyr Thr Gly Gly Phe Val Lys Glu Pro Glu Lys Gly Leu Trp Glu Asn	
	385	390 395 400
	Ile Val Tyr Leu Asp Phe Arg Ala Leu Tyr Pro Ser Ile Ile Ile Thr	
20	405	410 415
	His Asn Val Ser Pro Asp Thr Leu Asn Leu Glu Gly Cys Lys Asn Tyr	
	420	425 430
	Asp Ile Ala Pro Gln Val Gly His Lys Phe Cys Lys Asp Ile Pro Gly	
	435	440 445
25	Phe Ile Pro Ser Leu Leu Gly His Leu Leu Glu Glu Arg Gln Lys Ile	
	450	455 460
	Lys Thr Lys Met Lys Glu Thr Gln Asp Pro Ile Glu Lys Ile Leu Leu	
	465	470 475 480
	Asp Tyr Arg Gln Lys Ala Ile Lys Leu Leu Ala Asn Ser Phe Tyr Gly	
30	485	490 495

Tyr Tyr Gly Tyr Ala Lys Ala Arg Trp Tyr Cys Lys Glu Cys Ala Glu
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 Ser Val Thr Ala Trp Gly Arg Lys Tyr Ile Glu Leu Val Trp Lys Glu
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 5 Leu Glu Glu Lys Phe Gly Phe Lys Val Leu Tyr Ile Asp Thr Asp Gly
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 Leu Tyr Ala Thr Ile Pro Gly Gly Glu Ser Glu Glu Ile Lys Lys Lys
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 Ala Leu Glu Phe Val Lys Tyr Ile Asn Ser Lys Leu Pro Gly Leu Leu
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 15 Leu Glu Ile Val Arg Arg Asp Trp Ser Glu Ile Ala Lys Glu Thr Gln
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 Glu Tyr Lys Ala Ile Gly Pro His Val Ala Val Ala Lys Lys Leu Ala
 675 680 685
 25 Ala Lys Gly Val Lys Ile Lys Pro Gly Met Val Ile Gly Tyr Ile Val
 690 695 700
 Leu Arg Gly Asp Gly Pro Ile Ser Asn Arg Ala Ile Leu Ala Glu Glu
 705 710 715 720
 Tyr Asp Pro Lys Lys His Lys Tyr Asp Ala Glu Tyr Tyr Ile Glu Asn
 30 725 730 735

WHAT IS CLAIMED IS:

1. A method for synthesizing a nucleic acid,
comprising:
 providing an initiator having an unprotected
5 nucleoside base and a 3' hydroxyl group at a 3'
terminus thereof;
 providing a nucleic acid polymerase having at
least a conservative catalytic polymerase domain of
a family-B DNA polymerase;
10 providing a nucleotide monomer; and
 exposing the initiator to the nucleotide monomer
in the presence of the nucleic acid polymerase and
at least one type of metal cofactors which are
divalent cations, and in the absence of a template,
15 such that the nucleotide monomer is incorporated to
the initiator.
2. The method of Claim 1, wherein the initiator has a
sequence selected from a non-self complementary
sequence and a non-self complementarity forming
20 sequence.
3. The method of Claim 1, wherein the initiator is
linked to a solid support and has a 5' end linked
to the solid support.
4. The method of Claim 3, wherein the solid support is
25 selected from a microarray, a bead, a column, an
optical fiber, a wipe, nitrocellulose, nylon, glass,
quartz, a diazotized membrane, a silicone,

- polyformaldehyde, cellulose, cellulose acetate, paper, a ceramic, a metal, a metalloid, a semiconductor material, a magnetic particle, a plastic, a gel-forming material, a gel, a nanostructured surface, a nanotube, and a nanoparticle.
- 5
5. The method of Claim 1, wherein the initiator is exposed to the nucleotide monomer at a temperature ranging from 10°C to 90°C.
- 10
6. The method of Claim 1, wherein the initiator is exposed to the nucleotide monomer at a pH of not less than 8.0.
7. The method of Claim 1, wherein the metal cofactor is selected from the group consisting of Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} , Mn^{2+} , Co^{2+} , Fe^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} and combinations thereof.
- 15
8. The method of Claim 1, wherein the nucleic acid polymerase further has a 3' to 5' exonuclease domain and is a family-B DNA polymerase selected from the group consisting of a bacterial family-B DNA polymerase, a eukaryotic family-B DNA polymerase, an archaeal family-B DNA polymerase, and a viral family-B DNA polymerase.
- 20
9. The method of Claim 8, wherein the family-B polymerase is selected from the group consisting of a family-B DNA polymerase of *Thermococcus kodakaraensis* (KOD1), a family-B DNA polymerase of
- 25

Pyrococcus furious (Pfu), a family-B DNA polymerase of *Thermococcus sp.* (9°N), a family-B DNA polymerase of *Thermococcus gorgonarius* (Tgo), and a family-B DNA polymerase of *Thermococcus litoralis* (Vent).

- 5 10. The method of Claim 8, wherein the 3' to 5' exonuclease domain of the family-B DNA polymerase is modified in a manner selected from the group consisting of inactivation, attenuation, and deletion.
- 10 11. The method of Claim 1, wherein the initiator is in a single-stranded form.
12. The method of Claim 1, wherein the initiator has at least five nucleotide monomers.
13. The method of Claim 1, wherein the nucleotide
15 monomer has a phosphate group selected from a monophosphate, a diphosphate, a triphosphate, a tetraphosphate, a pentaphosphate, and a hexaphosphate.
14. The method of Claim 1, wherein the nucleotide
20 monomer has a removable blocking moiety selected from the group consisting of a 3'-O-blocking moiety, a base blocking moiety, and a combination thereof.
15. A kit for synthesizing a nucleic acid, comprising:
an initiator having an unprotected nucleoside
25 base and a 3' hydroxyl group at a 3' terminus thereof;

a nucleic acid polymerase having at least a conservative catalytic polymerase domain of a family-B DNA polymerase;

a nucleotide monomer; and

5 at least one type of metal cofactors,

wherein the kit is used according to a method as described in Claim 1.

16. The kit of Claim 15, wherein the initiator has a sequence selected from a non-self complementary sequence and a non-self complementarity forming sequence.

17. The kit of Claim 15, wherein the nucleic acid polymerase further has a 3' to 5' exonuclease domain and is a family-B DNA polymerase selected from the group consisting of a bacterial family-B DNA polymerase, a eukaryotic family-B DNA polymerase, an archaeal family-B DNA polymerase, and a viral family-B DNA polymerase.

18. The kit of Claim 17, wherein the family-B polymerase is selected from the group consisting of a family-B DNA polymerase of *Thermococcus kodakaraensis* KOD1, a family-B DNA polymerase of *Pyrococcus furiosus* (Pfu), a family-B DNA polymerase of *Thermococcus sp.* (9°N), a family-B DNA polymerase of *Thermococcus gorgonarius* (Tgo), and a family-B DNA polymerase of *Thermococcus litoralis* (Vent).

19. The kit of Claim 17, wherein the 3' to 5' exonuclease domain of the family-B DNA polymerase is modified in a manner selected from the group consisting of inactivation, attenuation, and deletion.
- 5 20. The kit of Claim 15, wherein the nucleotide monomer has a removable blocking moiety selected from the group consisting of a 3'-O-blocking moiety, a base blocking moiety, and a combination thereof.