

METHOD AND KIT FOR REGENERATING REUSABLE INITIATORS FOR
NUCLEIC ACID SYNTHESIS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority of U.S. Patent
5 Application No. 17/128,677, filed on December 21, 2020,
the entire content of which is incorporated herein by
reference.

FIELD

The disclosure relates to a method and a kit for
10 regenerating reusable initiators for nucleic acid
synthesis by virtue of enzymes.

BACKGROUND

DNA synthesis methods, including
template-dependent and template-independent DNA
15 synthesis methods, require an initiator (i.e. a short
polynucleotide) that serves as a primer for nucleotide
additions. However, after DNA synthesis, such an
initiator is normally not reusable and discarded.
Therefore, a new initiator is required for each round
20 of new DNA synthesis, increasing the overall production
cost thereof and rendering such synthesis
inconvenient.

To facilitate the cost-efficient and robust DNA
synthesis process, there is a need to develop a novel
25 approach to synthesize DNA and render a reusable
initiator for new synthesis.

SUMMARY

Therefore, an object of the disclosure is to provide a method and a kit for nucleic acid synthesis and regeneration of a reusable initiator for such synthesis, which can alleviate at least one of the drawbacks of the prior art.

Such method includes:

exposing an initiator attached to a solid support for nucleic acid synthesis to a linking nucleotide in the presence of a polymerase so that the linking nucleotide is incorporated to the initiator, the linking nucleotide having a substrate base, a substrate sugar, and a 3' hydroxyl group;

exposing the initiator containing the linking nucleotide to nucleotide monomers in the presence of the polymerase, so that a nucleic acid is synthesized and is coupled to the initiator right after the linking nucleotide;

providing a mono-functional DNA glycosylase, the linking nucleotide with the substrate base being recognizable and excisable by the mono-functional DNA glycosylase;

subjecting the substrate base to an excision treatment with the mono-functional DNA glycosylase, so that the substrate base is excised by the mono-functional DNA glycosylase to generate an abasic site;

providing an abasic site endonuclease, the resulting abasic site being recognizable and the substrate sugar being cleavable by the abasic site endonuclease;

5 subjecting the abasic site to a cleavage treatment with the abasic site endonuclease, so that the substrate sugar and the backbone of the nucleic acid at the abasic site are both cleaved to release the newly synthesized nucleic acid from the
10 initiator, so that a 3'-terminal nucleotide of the initiator leaves a 3' phosphate group, and so that a 5'-terminal nucleotide of the newly synthesized nucleic acid has a 5' phosphate group;

15 providing a 3' phosphatase activity-possessing enzyme; and

20 subjecting the 3'-terminal nucleotide of the initiator to a dephosphorylation treatment with the 3' phosphatase activity-possessing enzyme, so that the 3' phosphate group of the 3'-terminal nucleotide of the initiator is converted back to the original 3' hydroxyl group for the initiator to be reusable for a new round of synthesis reaction.

25 The kit includes a polymerase, a linking nucleotide, a mono-functional DNA glycosylase, an abasic site endonuclease, and a 3' phosphatase activity-possessing enzyme. The kit is used according to the aforesaid method.

Another object of the disclosure is to provide a method of regenerating a reusable initiator for nucleic acid synthesis, which can alleviate at least one of the drawbacks of the prior art.

5 Such method includes:

 providing a mono-functional DNA glycosylase;

 providing an initiator and a newly synthesized nucleic acid, the initiator being linked to a solid support, the newly synthesized nucleic acid being
10 linked to the initiator right after a linking nucleotide having a substrate base and a substrate sugar, the linking nucleotide with the substrate base being recognizable and excisable by the mono-functional DNA glycosylase;

15 subjecting the substrate base to an excision treatment with the mono-functional DNA glycosylase, so that the substrate base is excised by the mono-functional DNA glycosylase to generate an abasic site;

20 providing an abasic site endonuclease, the resulting abasic site being recognizable and the substrate sugar being cleavable by the abasic site endonuclease;

25 subjecting the abasic site to a cleavage treatment with the abasic site endonuclease, so that the substrate sugar and the backbone of the nucleic acid at the abasic site are both cleaved to release

the newly synthesized nucleic acid from the initiator, so that a 3'-terminal nucleotide of the initiator leaves a 3' phosphate group, and so that a 5'-terminal nucleotide of the newly synthesized nucleic acid has a 5' phosphate group;

providing a 3' phosphatase activity-possessing enzyme; and

subjecting the 3'-terminal nucleotide of the initiator to a dephosphorylation treatment with the 3' phosphatase activity-possessing enzyme, so that the 3' phosphate group of the 3'-terminal nucleotide of the initiator is converted back to an original 3' hydroxyl group for the initiator to be reusable for a new round of synthesis reaction.

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:

FIG. 1 is a schematic diagram illustrating template-independent nucleic acid synthesis and reversion of an initiator back to its original form as applied in Example 1, *infra*, in which the symbol "U" represents a linking deoxyuridine, the symbol "N" represents an incorporated nucleoside monomer, the symbol "UDG" represents uracil-DNA glycosylase, the symbol "Nci" represents endonuclease VIII, and the

symbol "T4 PNKP" represents T4 polynucleotide kinase with 3' phosphatase activity;

FIG. 2 is a fluorescent image of urea-polyacrylamide gel showing the feasibility of template-independent nucleic acid synthesis validated in Example 1, *infra*;

FIG. 3 is a fluorescent image of urea-polyacrylamide gel showing results of Example 1, *infra*, in which the symbol "S" represents a polynucleotide containing an initiator and a newly synthesized nucleic acid with a linking deoxyuridine, the symbol "U" represents a treatment with UDG only, the symbol "N" represents a treatment with Nei only, the symbol "U+N" represents treatments with UDG and Nei, and the symbol "U+N+P" represents treatments with UDG, Nei, and T4 PNKP;

FIG. 4 is a schematic diagram illustrating template-independent nucleic acid synthesis and reversion of an initiator to its original form as applied in Example 2, *infra*, in which the symbol "I" represents a linking deoxyinosine, the symbol "N" represents an incorporated nucleoside monomer, the symbol "AAG" represents alkyladenine DNA glycosylase, the symbol "Nei" represents endonuclease VIII, and the symbol "T4 PNKP" represents T4 polynucleotide kinase with 3' phosphatase activity;

FIG. 5 is a fluorescent image of urea-polyacrylamide gel showing the feasibility of template-independent nucleic acid synthesis validated in Example 2, *infra*;

FIG. 6 is a fluorescent image of urea-polyacrylamide gel showing results of Example 2, *infra*, in which the symbol "S" represents a polynucleotide containing an initiator and a newly synthesized nucleic acid with a linking deoxyinosine, the symbol "A" represents a treatment with AAG only, the symbol "N" represents a treatment with Nei only, the symbol "A+N" represents treatments with AAG and Nei, and the symbol "A+N+P" represents treatments with AAG, Nei, and T4 PNKP;

FIG. 7 is a schematic diagram illustrating template-dependent nucleic acid synthesis and reversion of an initiator to its original form as applied in Example 3, *infra*, in which the symbol "U" represents a linking deoxyuridine, the symbol "N" represents a nucleoside, the symbol "UDG" represents uracil-DNA glycosylase, the symbol "Nei" represents endonuclease VIII, and the symbol "T4 PNKP" represents T4 polynucleotide kinase with 3' phosphatase activity;

FIG. 8 is a fluorescent image of urea-polyacrylamide gel showing results of Example 3, *infra*, in which the symbol "S" represents a duplex polynucleotide containing an initiator and a newly synthesized nucleic acid with a linking deoxyuridine, the symbol "U" represents a treatment with UDG only, the symbol "N" represents a treatment with Nei only, the symbol "U+N" represents treatments with UDG and Nei, and the symbol "U+N+P" represents treatments with UDG, Nei, and T4

PNKP;

FIG. 9 is a schematic diagram illustrating template-dependent nucleic acid synthesis and reversion of an initiator to its original form as applied in Example 4, *infra*, in which the symbol "I" represents a linking deoxyinosine, the symbol "N" represents an incorporated nucleoside monomer, the symbol "AAG" represents alkyladenine DNA glycosylase, the symbol "Nei" represents endonuclease VIII, and the symbol "T4 PNKP" represents T4 polynucleotide kinase with 3' phosphatase activity; and

FIG. 10 is a fluorescent image of urea-polyacrylamide gel showing results of Example 4, *infra*, in which the symbol "S" represents a duplex polynucleotide containing an initiator and a newly synthesized nucleic acid with a linking deoxyinosine, the symbol "A" represents a treatment with AAG only, the symbol "N" represents a treatment with Nei only, the symbol "A+N" represents treatments with AAG and Nei, and the symbol "A+N+P" represents treatments with AAG, Nei, and T4 PNKP.

DETAILED DESCRIPTION

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 "including but not limited to", and that the word "comprises" has a corresponding meaning.

5 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 present disclosure belongs. One skilled in the art will recognize many methods and materials similar or equivalent
10 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 described.

The present disclosure provides a method for nucleic
15 acid synthesis and regeneration of a reusable initiator for such synthesis, which includes:

exposing an initiator attached to a solid support
for nucleic acid synthesis to a linking nucleotide
in the presence of a polymerase so that the linking
20 nucleotide is incorporated to the initiator, the
linking nucleotide having a substrate base, a
substrate sugar, and a 3' hydroxyl group;

exposing the initiator containing the linking
nucleotide to nucleotide monomers in the presence
25 of the polymerase, so that a nucleic acid is
synthesized and is coupled to the initiator right
after the linking nucleotide;

providing a mono-functional DNA glycosylase, the linking nucleotide with the substrate base being recognizable and excisable by the mono-functional DNA glycosylase;

5 subjecting the substrate base to an excision treatment with the mono-functional DNA glycosylase, so that the substrate base is excised from the linking nucleotide by the mono-functional DNA glycosylase to generate an abasic site;

10 providing an abasic site endonuclease, the resulting abasic site being recognizable and the substrate sugar being cleavable by the abasic site endonuclease;

15 subjecting the abasic site to a cleavage treatment with the abasic site endonuclease, so that the substrate sugar and the backbone of the nucleic acid at the abasic site are both cleaved to release the newly synthesized nucleic acid from the initiator, so that a 3'-terminal nucleotide of the initiator leaves a 3' phosphate group, and so that
20 a 5'-terminal nucleotide of the synthesized nucleic acid has a 5' phosphate group;

providing a 3' phosphatase activity-possessing enzyme; and

25 subjecting the 3'-terminal nucleotide of the initiator to a dephosphorylation treatment with the 3' phosphatase activity-possessing enzyme, so that

the 3' phosphate group of the 3'-terminal nucleotide of the initiator is converted back to the original 3' hydroxyl group for the initiator to be reusable for a new round of synthesis reaction.

5 According to the present disclosure, the excision treatment, the cleavage treatment, and the dephosphorylation treatment may be conducted simultaneously or sequentially.

10 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 naturally occurring nucleotides or artificial chemical mimics. The term "nucleic acid" as used herein is
15 interchangeable with the terms "oligonucleotide", "polynucleotide", "gene", "DNA", "cDNA", "RNA", and "mRNA" in use.

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. The term "initiator" may also refer to a Xeno nucleic acid (XNA) or a peptide nucleic acid (PNA) having a 3'-hydroxyl group.

25 According to the present disclosure, the initiator may be in a template-independent form or a template-dependent form (namely, the initiator may not be annealed or hybridized to a complementary template,

or may be annealed to a template to form a duplex or a double strand).

When the initiator is in a template-independent form, the initiator may have a sequence selected from
5 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, a XNA, or a PNA sequence) folds back on itself
(i.e. a region of the sequence binds or hybridizes to
10 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
15 loops, junctions, bulges or internal loops. The term
"self complementarity forming" is used to describe a
sequence (e.g. a nucleotide sequence, a XNA, or a PNA
sequence) from which a complementary extended portion
is formed when such sequence serves as a template
20 (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
25 is formed from such sequence (i.e. a self-complementary
sequence "ATCCGGAT" is formed).

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 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.

According to the present disclosure, the initiator has a 5' end linked to the solid support, and the linking nucleotide is coupled to a 3'-terminal nucleotide of the initiator and a 5'-terminal nucleotide of the synthesized nucleic acid. The initiator may be directly attached to the support, or may be attached to the support via a linker.

Examples of the solid support include, but are not limited to, 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).

According to the present disclosure, depending on the form of the initiator, the synthesized nucleic acid and the linking nucleotide may each be in a template-independent form or a template-dependent form.

As used herein, 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.

According to the present disclosure, the

nucleotide monomers may be a natural nucleic acid nucleotide whose constituent elements are a sugar, a phosphate group and a nitrogen base. The sugar may be ribose in RNA or 2'-deoxyribose in DNA. Depending on
5 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 monomers may be a nucleotide which is modified in at least one of the three constituent
10 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 bromo-5-deoxyuridine, and any
15 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 derivatives).

20 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.

25 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 terminator, and the nucleotide monomer having a 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" 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 another nucleotide monomer by the polymerase. Such "reversible terminator" base and a 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]-tetrahydrofuranyl, O-[2-methyl,4-methoxy]-tetrahydropyranyl, O-[5-methyl]-tetrahydropyranyl, and O-tetrahydrothiofuranyl, 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,
5 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.

10 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
15 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
20 Biosciences Heliscope.

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
25 reversible terminators, applicable 3'-unblocked
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. (2011), *Nucleic Acids Research*, 39(6):e39, and Chen et al. (2013), *Genomics Proteomics Bioinformatics*, 11:34-40.

According to the present disclosure, the polymerase may be a template-dependent polymerase or a template-independent polymerase.

According to the present disclosure, the polymerase may be selected from the group consisting of a family-A DNA polymerase (e.g. T7 DNA polymerase, Pol I, Pol γ , θ , and ν), a family-B DNA polymerase (e.g. Pol II, Pol B, Pol ζ , Pol α , δ , and ϵ), a family-C DNA polymerase (e.g. Pol III), a family-D DNA polymerase (e.g. PolD), a family-X DNA polymerase (e.g. Pol β , Pol σ , Pol λ , Pol μ , and terminal deoxynucleotidyl transferase), a family-Y DNA polymerase (e.g. Pol ι , Pol κ , Pol η , DinB, Pol IV, and Pol V), a reverse transcriptase (e.g. telomerase and hepatitis B virus), and enzymatically active fragments thereof.

Non-limiting examples of widely employed template-dependent polymerases include T7 DNA polymerase of the phage T7 and T3 DNA polymerase of the phage T3 which are DNA-dependent DNA polymerases, T7 RNA polymerase of the phage T7 and T3 RNA polymerase of the phage T3 which are DNA-dependent RNA polymerases,

DNA polymerase I or its fragment known as Klenow fragment of *Escherichia coli* which is a DNA-dependent DNA polymerase, *Thermophilus aquaticus* DNA polymerase, Tth DNA polymerase and vent DNA polymerase, which are
5 thermostable DNA-dependent DNA polymerases, eukaryotic DNA polymerase β , which is a DNA-dependent DNA polymerase, telomerase which is a RNA-dependent DNA polymerase, and non-protein catalytic molecules such as modified RNA (ribozymes; Unrau & Bartel, 1998) and
10 DNA with template-dependent polymerase activity.

Non-limiting examples of the template-independent polymerases include reverse transcriptases, poly(A) polymerase, DNA polymerase theta (θ), DNA polymerase mu (μ), and terminal deoxynucleotidyl transferase.

15 Since polymerases suitable for nucleic acid synthesis, linking nucleotide addition, and nucleic acid synthesis are within the expertise and routine skills of those skilled in the art, further details thereof are omitted herein for the sake of brevity.

20 As used herein, the term "mono-functional DNA glycosylase" refers to naturally existing mono-functional glycosylases that originally have only glycosylase activity. The term "mono-functional DNA glycosylase" also refers to mono-functional
25 glycosylases that are derived from bi-functional DNA glycosylases originally having glycosylase activity and abasic-site lyase activity by removing or

inactivating the abasic-site lyase domain of the bi-functional DNA glycosylases.

According to the present disclosure, the mono-functional DNA glycosylase may be selected from the group consisting of uracil-DNA glycosylase (UDG or UNG), alkyladenine DNA glycosylase (AAG; also referred to as methylpurine DNA glycosylase (MPG)), single-strand-selective monofunctional uracil DNA glycosylase 1 (SMUG1), methyl-binding domain glycosylase 4 (MBD4), thymine DNA glycosylase (TDG), mutY homolog DNA glycosylase (MYH), alkylpurine glycosylase C (AlkC), alkylpurine glycosylase D (AlkD), 8-oxo-guanine glycosylase 1 (OGG1) without the abasic site lyase activity, endonuclease III-like 1 (NTHL1) without the abasic site lyase activity, endonuclease VIII-like glycosylase 1 (NEIL1) without the abasic site lyase activity, endonuclease VIII-like glycosylase 2 (NEIL2) without the abasic site lyase activity, endonuclease VIII-like glycosylase 3 (NEIL3) without the abasic site lyase activity, and enzymatically active fragments thereof.

Since removing or inactivating the abasic-site lyase domain of bi-functional DNA glycosylases to obtain mono-functional glycosylases are within the expertise and routine skill of those skilled in the art, details thereof are omitted herein for the sake of brevity.

As used herein, the term "enzymatically active fragment" refers to a fragment of a catalytically or enzymatically active protein or polypeptide which contains at least 10%, preferably at least 20%, even
5 more preferably at least 30%, even more preferably at least 40%, even more preferably at least 50%, even more preferably at least 60%, even more preferably at least 70%, even more preferably at least 80%, even more preferably at least 90%, or even more preferably at
10 least 95% of activity of the protein or polypeptide from which the fragment is derived.

In an exemplary embodiment of the present disclosure, the mono-functional DNA glycosylase is uracil-DNA glycosylase. In another exemplary
15 embodiment of the present disclosure, the mono-functional DNA glycosylase is alkyladenine DNA glycosylase.

The terms "abasic", "apurinic/apyrimidinic", and D-spacer can be interchangeably used to indicate a site
20 at which the base is not present, but the sugar phosphate backbone remains intact. Therefore, the abasic site endonuclease is also known as apurinic/apyrimidinic site endonuclease.

According to the present disclosure, the abasic
25 site endonuclease may be selected from the group consisting of endonuclease VIII (Nei), endonuclease III (EndoIII or Nth), and enzymatically active

fragments thereof. In an exemplary embodiment, the abasic site endonuclease is endonuclease VIII.

According to the present disclosure, the 3' phosphatase activity-possessing enzyme may be selected from the group consisting of a polynucleotide kinase 3'-phosphatase, a 3'-phosphoesterase, and enzymatically active fragments thereof. The 3' phosphatase activity-possessing enzyme may be T4 polynucleotide kinase (PNK) with 3' phosphatase activity (also referred to as T4 polynucleotide kinase/phosphatase (T4 PNKP)), as well as zinc finger DNA 3'-phosphoesterase (ZDP).

Since the applicable 3' phosphatase activity-possessing enzymes are within the expertise and routine skills of those skilled in the art, further details thereof are omitted herein for the sake of brevity. Nevertheless, the applicable 3' phosphatase activity-possessing enzymes can be found in, for instance, Blondal *et al.* (2005), *J. Bio. Chem.*, 280(7):5188-5194, Dobson *et al.* (2006), *Nucleic Acids Research*, 34(8):2230-2237, Blasius *et al.* (2007), *BMC Molecular Biology*, 8:69, Coquelle *et al.* (2011), *PNAS*, 108(52):21022-21027, Vance *et al.* (2001), *J. Bio. Chem.*, 276(18):15703-15781, and the NCBI website (<https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=11284#general-protein-info>).

According to the present disclosure, the substrate

base of the linking nucleotide coupled to the initiator may be selected from the group consisting of uracil, hypoxanthine, thymine, cytosine, guanine, 5-fluorouracil, 5-hydroxymethyluracil, 5-formylcytosine, 5-carboxylcytosine, 3-methyladenine, 3-methylguanine, 7-methyladenine, 7-methylguanine, N⁶-methyladenine, 8-oxo-7,8-dihydroguanine, 5-hydroxylcytosine, 5-hydroxyluracil, dihydroxyuracil, ethenocytosine, ethenoadenine, thymine glycol, cytosine glycol, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine, a formamidopyrimidine derivative of adenine, a formamidopyrimidine derivative of guanine, adenine opposite guanine, uracil opposite guanine, uracil opposite adenine, thymine opposite guanine, ethenocytosine opposite guanine, adenine opposite 8-oxo-7,8-dihydroguanine, and 2-hydroxyladenine opposite guanine. In an exemplary embodiment of the present disclosure, the substrate base of the linking nucleotide is uracil. In another exemplary embodiment of the present disclosure, the substrate base of the linking nucleotide is hypoxanthine.

Since the suitable mono-functional DNA glycosylases and their corresponding substrate bases are within the expertise and routine skill of those skilled in the art, details thereof are omitted herein for the sake of brevity. Nevertheless, the suitable

mono-functional DNA glycosylases and their corresponding substrate bases can be found in, for example, Jacobs et al. (2012), *Chromosoma*, 121:1-20, Krokan et al. (1997), *Biochem. J.*, 325:1-16, and Kim
5 et al. (2012), *Current Molecular Pharmacology*, 5:3-13.

The term "linking nucleotide" refers to the first nucleotide that is incorporated to the initiator with respect to the newly synthesized nucleic acid.

The term "substrate base" refers to the base of a
10 linking nucleotide that serves as a substrate for an enzyme. The term "substrate sugar" refers to a nucleoside sugar moiety of the linking nucleotide that serves as a substrate for an enzyme.

Furthermore, the present disclosure provides a kit
15 for nucleic acid synthesis and regeneration of a reusable initiator for such synthesis, which includes the aforesaid polymerase, the aforesaid mono-functional DNA glycosylase, the aforesaid linking nucleotide that serves as a substrate for
20 mono-functional DNA glycosylase, the aforesaid abasic site endonuclease, and the aforesaid 3' phosphatase activity-possessing enzyme. The kit is used according to the aforesaid method of the present disclosure.

In addition, the present disclosure provides a
25 method of regenerating a reusable initiator for nucleic acid synthesis, which includes:

providing a mono-functional DNA glycosylase as described above;

providing an initiator for nucleic acid synthesis as described above and a synthesized nucleic acid, the synthesized nucleic acid being
5 linked to the initiator right after a linking nucleotide as described above;

subjecting the substrate base to an excision treatment as described above with the
10 mono-functional DNA glycosylase;

providing an abasic site endonuclease as described above;

subjecting the abasic site to a cleavage treatment as described above with the abasic site
15 endonuclease;

providing a 3' phosphatase activity-possessing enzyme as described above; and

subjecting the 3'-terminal nucleotide of the initiator to a dephosphorylation treatment as
20 described above with the 3' phosphatase activity-possessing enzyme.

The disclosure will be further described by way of the following examples. However, it should be understood that the following examples are solely
25 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
and reversion of synthesis initiator back to
its original form by virtue of uracil-DNA
glycosylase (UDG), endonuclease VIII (Nei),
5 and T4 polynucleotide kinase with
3' phosphatase activity (T4 PNKP)

To test whether an initiator used for a
template-independent nucleic acid synthesis can be
converted back to its original form after nucleic acid
10 synthesis, the following experimental steps were
conducted. The detail scheme for the
template-independent nucleic acid synthesis using a
linking deoxyuridine nucleotide and the reversion of
the initiator to its original form utilizing enzymes
15 as applied in this example is illustrated in FIG. 1.

**A. Template-independent nucleic acid synthesis
initiated with the linking deoxyuridine
triphosphate (dUTP)**

An initiator (a single-stranded 21-mer
20 polynucleotide of SEQ ID NO: 1) with a
5'-hexachloro-fluorescein (HEX) label at the 5' end
thereof and a hydroxyl group at the 3' terminus thereof
was synthesized by Integrated DNA Technologies
(Coralville, Iowa, United States). The
25 template-independent nucleic acid synthesis reaction
was performed using a 3' to 5' exonuclease-deficient
Pfu DNA polymerase (Pfu^{exo-}) (200 nM) to incorporate a

linking deoxyuridine triphosphate (dUTP) (100 μ M) to the 3' end of the initiator.

Specifically, the Pfu^{exo-} DNA polymerase (having an amino acid sequence of SEQ ID NO: 8) was prepared as follows. The gene construct encoding an intein-free Pfu DNA polymerase was synthesized by Genomics BioSci and Tech Co. (New Taipei City, Taiwan). The Pfu^{exo-} DNA polymerase was created by changing the Asp¹⁴¹ thereof to Ala (D141A) and the Glu¹⁴³ thereof to Ala (E143A) on the gene backbone using the Q5 Site-directed Mutagenesis Kit from New England Biolabs (Ipswich, MA, United States). The Pfu^{exo-} DNA polymerase was expressed in *E. coli* BL21(DE3) cells and purified through Sepharose-Q and heparin columns using Akta FPLC system from GE Healthcare Life Sciences (Marlborough, MA, United States). As illustrated in FIG. 2, deoxyuridine monophosphate (dUMP) was efficiently incorporated by the Pfu^{exo-} DNA polymerase into the 3'-end of the initiator.

B. Template-independent nucleic acid synthesis right after the linking dUMP at the 3' end of the initiator

To demonstrate the template-independent nucleic acid synthesis right after the linking dUMP at the 3'-end of the synthesis initiator, the Pfu^{exo-} DNA polymerase (200 nM) was used to stepwise incorporate a 3'-O-azidomethyl-dATP and a 3'-O-azidomethyl-dTTP (100 μ M) (Jena Bioscience, Erfurt, Germany) to the

initiator containing the linking dUMP at the 3' terminus. The synthesis reaction was initiated by addition of 10 mM manganese cations and then incubated at 75°C for 30 minutes. The reaction was stopped by adding 10 µL of
5 a 2x quench solution (95% deionized formamide and 25 mM EDTA) and subjected to the heat denaturation at 98°C for 10 minutes. The reaction products were analyzed by a 15% denaturing urea-polyacrylamide gel, and were visualized by Amersham Typhoon Imager, GE Healthcare
10 Life Sciences (Marlborough, MA, United States).

As illustrated in FIG. 2, the template-independent nucleic acid synthesis using the Pfu^{exo-} DNA polymerase can incorporate dAMP and dTMP sequentially right after the linking dUMP at the 3' end of the initiator (the
15 resulting product containing the initiator, the linking dUMP, and dAMP and dTMP has SEQ ID NO: 2). Accordingly, the template-independent nucleic acid synthesis reaction can continue to synthesize a 16-mer polynucleotide of SEQ ID NO: 3 and therefore generate
20 a 38-mer nucleic acid (SEQ ID NO: 4) containing the initiator, the linking dUMP, and the newly synthesized 16-mer polynucleotide.

Please note that since the template-independent nucleic acid synthesis is within the expertise and
25 routine skills of those skilled in the art, the 16-mer nucleic acid of SEQ ID NO: 3 can be synthesized *de novo* by those skilled in the art with the information

provided herein. In this example, to simplify the experimental procedures, the 16-mer nucleic acid was synthesized by Integrated DNA Technologies (Coralville, Iowa, United States), and was linked to the initiator
5 with the linking dUMP as described in section C below to symbolize the template-independent nucleic acid synthesis of the 16-mer nucleic acid.

**C. The release of newly synthesized nucleic acid and the
reversion of synthesis initiator back to its original
10 form by the combined treatments of UDG, Nei, and T4
PNKP**

To demonstrate the feasibility of releasing the newly synthesized nucleic acid and regenerating the synthesis initiator by virtue of enzymes, the 38-mer
15 nucleic acid (SEQ ID NO: 4) containing the initiator, the linking dUMP, and the newly synthesized 16-mer polynucleotide was prepared. Specifically, the 16-mer polynucleotide was linked to the initiator with the linking dUMP using the Pfu^{exo-} DNA polymerase.

20 The 38-mer nucleic acid (25 nM) was subjected to the uracil-excision, the abasic site/nucleic acid backbone cleavage, and the dephosphorylation reaction by the addition of 10 units of UDG, Nei, and T4 PNKP purchased from New England Biolabs (Ipswich, MA, United
25 States), respectively. The reaction was conducted in the 1x Cleavage Buffer [10 mM MgCl₂, 50 mM KCl, 5 mM dithiothreitol (DTT), and 50 mM Tris-HCl, pH7.5] at

37°C for 15 minutes. The preparation of such the 38-mer nucleic acid (SEQ ID NO: 4) was confirmed by a 15% denaturing urea-polyacrylamide gel as described above.

5 In the control experiments, the 38-mer nucleic acid (SEQ ID NO: 4) was also subjected to the treatment with UDG, Nei or the mixture of UDG and Nei under the identical experimental conditions described above. Each reaction was then stopped by adding 10 µL of a 2x quench solution (95% formamide and 25 mM EDTA), and the
10 enzymes were inactivated by heating at 98°C for 10 minutes. The reaction products were analyzed by a 20% denaturing urea-polyacrylamide gel, and were visualized by Amersham Typhoon Imager, GE Healthcare Life Sciences (Marlborough, MA, United States).

15 As illustrated in FIG. 3, the treatments with the mixture of UDG, Nei, and T4 PNKP resulted in the removal of the linking dUMP from the single-stranded 38-mer nucleic acid, the release of the newly synthesized 16-mer polynucleotide (SEQ ID NO: 3), and the
20 regeneration of the initiator (SEQ ID NO: 1) with a hydroxyl group at the 3' terminus. None of UDG alone, Nei alone, or the combination of UDG and Nei can efficiently and completely release the newly synthesized nucleic acid and concurrently regenerate
25 the initiator with the hydroxyl group at the 3' end.

Example 2. Template-independent nucleic acid synthesis and reversion of synthesis initiator to its

original form by virtue of alkyladenine DNA
glycosylase (AAG), Nei, and T4 PNKP

To test whether an initiator used for a
template-independent nucleic acid synthesis can be
5 converted back to its original form after nucleic acid
synthesis, the following experimental procedures were
conducted. The detail scheme for the
template-independent nucleic acid synthesis using the
linking deoxyinosine triphosphate (dITP) and the
10 reversion of the initiator to its original form
utilizing the enzymes as applied in this example is
illustrated in FIG. 4.

**A. Template-independent nucleic acid synthesis
initiated with the linking dITP**

15 The initiator (SEQ ID NO: 1) with the
5'-hexachloro-fluorescein (HEX) label at the 5' end and
an unprotected hydroxyl group at the 3' terminus was
used. The template-independent nucleic acid synthesis
reaction was performed using the Pfu^{exo-} DNA polymerase
20 (200 nM) as described in Example 1 to incorporate a
linking dITP (100 μ M) to the 3' end of the initiator.
As illustrated in FIG. 5, the deoxyinosine
monophosphate (dIMP) was efficiently incorporated by
the Pfu^{exo-} DNA polymerase into the 3'-end of the
25 initiator.

**B. Template-independent nucleic acid synthesis right
after the linking dIMP at the 3' end of the initiator**

To demonstrate the template-independent nucleic acid synthesis right after the linking dIMP at the 3'-end of the synthesis initiator, the Pfu^{exo-} DNA polymerase (200 nM) was used to stepwise incorporate a 3'-O-azidomethyl-dATP and a 3'-O-azidomethyl-dTTP (100 μM) (Jena Bioscience, Erfurt, Germany) to the initiator containing the linking dIMP at the 3' terminus. The synthesis reaction was initiated by addition of 10 mM manganese cations and then incubated at 75°C for 30 minutes. The reaction was stopped by adding 10 μL of a 2x quench solution (95% deionized formamide and 25 mM EDTA) and subjected to the heat denaturation at 98°C for 10 minutes. The reaction products were analyzed by a 15% denaturing urea-polyacrylamide gel, and were visualized by Amersham Typhoon Imager, GE Healthcare Life Sciences (Marlborough, MA, United States).

As illustrated in FIG. 5, the template-independent nucleic acid synthesis using the Pfu^{exo-} DNA polymerase can incorporate dAMP and dTMP sequentially right after the linking dIMP at the 3' end of the initiator (the resulting product containing the initiator, the linking dIMP, and dAMP and dTMP has SEQ ID NO: 5). Accordingly, the template-independent nucleic acid synthesis reaction can continue to synthesize the 16-mer polynucleotide of SEQ ID NO: 3 and generate a 38-mer nucleic acid (SEQ ID NO: 6) containing the initiator, the linking dIMP, and the newly synthesized

16-mer polynucleotide. The preparation of such a 38-mer nucleic acid (SEQ ID NO: 6) was confirmed by a 15% denaturing urea-polyacrylamide gel as described above.

Please note that since the template-independent nucleic acid synthesis is within the expertise and routine skills of those skilled in the art, the 16-mer nucleic acid of SEQ ID NO: 3 can be synthesized *de novo* by those skilled in the art with the information provided herein. In this example, to simplify the experimental procedures, the 16-mer nucleic acid was linked to the initiator with the linking dIMP as described in section C below to symbolize the template-independent nucleic acid synthesis of the 16-mer nucleic acid.

C. The release of newly synthesized nucleic acid and the reversion of synthesis initiator back to its original form by the combined treatments of AAG, Nei, and T4 PNKP

To demonstrate the feasibility of releasing the newly synthesized nucleic acid and regenerating the synthesis initiator by virtue of enzymes, the single-stranded 38-mer nucleic acid (SEQ ID NO: 6) containing the initiator (SEQ ID NO: 1), the linking dIMP, and the newly synthesized 16-mer polynucleotide (SEQ ID NO: 3) was prepared. Specifically, the 16-mer polynucleotide was linked to the initiator with the linking dIMP using the Pfu^{exo-} DNA polymerase.

The single-stranded 38-mer nucleic acid (25 nM) was subjected to the inosine-excision, the abasic site/nucleic acid backbone cleavage, and the dephosphorylation reaction by the addition of 10 units
5 of AAG, Nei, and T4 PNKP purchased from New England Biolabs (Ipswich, MA, United States), respectively. The reaction was conducted in a 1x Cleavage Buffer [10 mM MgCl₂, 50 mM KCl, 5 mM dithiothreitol (DTT), and 50 mM Tris-HCl; pH 7.5] at 37°C for 15 minutes.

10 In the control experiments, the single-stranded 38-mer nucleic acid (SEQ ID NO: 6) was also subjected to the treatment with AAG, Nei or the mixture of AAG and Nei under the identical experimental conditions. Each reaction was then stopped by adding 10 µL of a 2x
15 quench solution (95% formamide and 25 mM EDTA), and the enzymes were inactivated by heating at 98°C for 10 minutes. The reaction products were analyzed by a 20% denaturing urea-polyacrylamide gel, and were visualized by Amersham Typhoon Imager, GE Healthcare
20 Life Sciences (Marlborough, MA, United States).

As illustrated in FIG. 6, the treatments with the mixture of AAG, Nei, and T4 PNKP resulted in the removal of the linking dIMP from the single-stranded 38-mer nucleic acid, the release of the newly synthesized
25 16-mer polynucleotide (SEQ ID NO: 3), and the regeneration of the initiator (SEQ ID NO: 1) with a hydroxyl group at the 3' terminus. None of AAG alone,

Nei alone, or the combination of AAG and Nei can efficiently and completely cut off the newly synthesized nucleic acid and concurrently regenerate the initiator with the hydroxyl group at the 3' end.

5 **Example 3. Template-dependent nucleic acid synthesis
and reversion of synthesis initiator back
to its original form by virtue of UDG, Nei,
and T4 PNKP**

10 To test whether an initiator used for a
template-dependent nucleic acid synthesis can be
converted back to its original form after nucleic acid
synthesis, the following experimental procedures were
conducted. The detail scheme for the
15 template-dependent nucleic acid synthesis using the
linking dUTP and the reversion of the initiator to its
original form utilizing the enzymes as applied in this
example is illustrated in FIG. 7.

20 **A. The release of newly synthesized nucleic acid and the
reversion of synthesis initiator back to its original
form by the combined treatments of UDG, Nei, and T4
PNKP**

25 To demonstrate the feasibility of releasing the newly
synthesized nucleic acid and regenerating the
synthesis initiator by virtue of enzymes, the
single-stranded 38-mer nucleic acid (SEQ ID NO: 4)
containing the initiator, the linking dUMP, and the
newly synthesized 16-mer polynucleotide was prepared

as described in Example 1. To exemplify the template-dependent nucleic acid synthesis, the single-stranded 38-mer nucleic acid (SEQ ID NO: 4) was hybridized with a complementary single-stranded 38-mer nucleic acid (SEQ ID NO: 7) by heating at 95°C for 10 minutes, followed by slowly cooling down to 4°C to form a duplex, blunt-end, double stranded 38-mer nucleic acid. The complementary single-stranded 38-mer nucleic acid (SEQ ID NO: 7) was obtained from Integrated DNA Technologies (Coralville, Iowa, United States).

25 nM of the duplex 38-mer nucleic acid was subjected to the uracil-excision, the abasic site/nucleic acid backbone cleavage, and the dephosphorylation reaction by the addition of 10 units of UDG, Nei, and T4 PNKP purchased from New England Biolabs (Ipswich, MA, United States), respectively. The reaction was conducted in a 1x Cleavage Buffer [10 mM MgCl₂, 50 mM KCl, 5 mM dithiothreitol (DTT), and 50 mM Tris-HCl; pH 7.5] at 37°C for 15 minutes.

In the control experiments, the duplex 38-mer nucleic acid was subjected to the treatment with UDG, Nei or the mixture of UDG and Nei under the identical experimental conditions. Each reaction was then stopped by adding 10 µL of a 2x quench solution (95% formamide and 25 mM EDTA), the enzymes were inactivated, and the duplex 38-mer nucleic acid was denatured by heating at 98°C for 10 minutes. The reaction products

were analyzed by a 20% denaturing urea-polyacrylamide gel, and were visualized by Amersham Typhoon Imager, GE Healthcare Life Sciences (Marlborough, MA, United States).

5 As shown in FIG. 8, the treatments with the mixture of UDG, Nei, and T4 PNKP resulted in the removal of the linking dUMP from the 38-mer nucleic acid, the release of the newly synthesized 16-mer polynucleotide (SEQ ID NO: 3) after the heat denaturation of the duplex 38-mer
10 nucleic acid, and the regeneration of the initiator (SEQ ID NO: 1) with the hydroxyl group at the 3' terminus. None of UDG alone, Nei alone, or the combination of UDG and Nei can efficiently and completely release the newly synthesized nucleic acid after the heat denaturation
15 of the duplex 38-mer nucleic acid and concurrently regenerate the initiator with the hydroxyl group at the 3' end.

**Example 4. Template-dependent nucleic acid synthesis
and reversion of synthesis initiator back
20 to its original form by virtue of AAG, Nei,
and T4 PNKP**

To test whether an initiator used for a template-dependent nucleic acid synthesis can be converted back to its original form after nucleic acid
25 synthesis, the following experimental procedures were conducted. The detail scheme for the template-dependent nucleic acid synthesis using the

linking dITP and the reversion of the initiator to its original form utilizing the enzymes as applied in this example is illustrated in FIG. 9.

A. The release of newly synthesized nucleic acid and the reversion of synthesis initiator back to its original form by the combined treatments of AAG, Nei, and T4 PNKP

To demonstrate the feasibility of releasing the newly synthesized nucleic acid and regenerating the synthesis initiator by virtue of enzymes, the single-stranded 38-mer nucleic acid (SEQ ID NO: 6), containing the initiator, the linking dIMP, and the newly synthesized 16-mer polynucleotide was prepared as described in Example 2. To exemplify the template-dependent nucleic acid synthesis, the single-stranded 38-mer nucleic acid (SEQ ID NO: 6) was hybridized with the complementary 38-mer nucleic acid (SEQ ID NO: 7) by heating at 95°C for 10 minutes, followed by slowly cooling down to 4°C to form a duplex, blunt-end, double stranded 38-mer nucleic acid. The complementary single-stranded 38-mer nucleic acid (SEQ ID NO: 7) was obtained from Integrated DNA Technologies (Coralville, Iowa, United States). 25 nM of the duplex 38-mer nucleic acid was subjected to the inosine-excision, the abasic site/nucleic acid backbone cleavage, and the dephosphorylation reaction by the addition of 10 units of AAG, Nei, and T4 PNKP

purchased from New England Biolabs (Ipswich, MA, United States), respectively. The reaction was conducted in a 1x Cleavage Buffer [10 mM MgCl₂, 50 mM KCl, 5 mM dithiothreitol (DTT), and 50 mM Tris-HCl; pH 7.5] at
5 37°C for 15 minutes.

In the control experiments, the duplex 38-mer nucleic acid was subjected to the treatment with AAG, Nei or the mixture of AAG and Nei under the identical experimental conditions. Each reaction was then
10 stopped by adding 10 µL of a 2x quench solution (95% formamide and 25 mM EDTA), the enzymes were inactivated, and the duplex nucleic acid was denatured by heating at 98°C for 10 minutes. The reaction products were analyzed by a 20% denaturing urea-polyacrylamide gel,
15 and were visualized by Amersham Typhoon Imager, GE Healthcare Life Sciences (Marlborough, MA, United States).

As shown in FIG. 10, the treatments with the mixture of AAG, Nei, and T4 PNKP resulted in the removal of the
20 linking dIMP from the duplex 38-mer nucleic acid, the release of the newly synthesized 16-mer polynucleotide (SEQ ID NO: 3) after the heat denaturation of the duplex 38-mer nucleic acid, and the regeneration of the initiator (SEQ ID NO: 1) with the hydroxyl group at the
25 3' terminus. None of AAG alone, Nei alone, or the combination of AAG and Nei can efficiently and completely release the newly synthesized nucleic acid

after the heat denaturation of the duplex 38-mer nucleic acid and concurrently regenerate the initiator with the hydroxyl group at the 3' end.

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 spirit and scope of the broadest interpretation so as
15 to encompass all such modifications and equivalent arrangements.

SEQUENCE LISTING

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<110>  Chen, Cheng-Yao
<120>  METHOD AND KIT FOR REGENERATING REUSABLE
5      INITIATORS FOR NUCLEIC ACID SYNTHESIS
<130>  PE-65691-WO
<160>  8
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20 25 30

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35 40 45

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50 55 60

Ile Val Asp Val Glu Lys Val Glu Lys Lys Phe Leu Gly Lys Pro Ile

20 65 70 75 80

Thr Val Trp Lys Leu Tyr Leu Glu His Pro Gln Asp Val Pro Thr Ile

85 90 95

Arg Glu Lys Val Arg Glu His Pro Ala Val Val Asp Ile Phe Glu Tyr

100 105 110

25 Asp Ile Pro Phe Ala Lys Arg Tyr Leu Ile Asp Lys Gly Leu Ile Pro

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130 135 140

Leu Tyr His Glu Gly Glu Glu Phe Gly Lys Gly Pro Ile Ile Met Ile

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45

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46

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			640

47

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 740 745 750
 15 Lys Glu Asp Leu Arg Tyr Gln Lys Thr Arg Gln Val Gly Leu Thr Ser
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 Trp Leu Asn Ile Lys Lys Ser
 770 775

20

WHAT IS CLAIMED IS:

1. A method for nucleic acid synthesis and regeneration of a reusable initiator for such synthesis, comprising:

5 exposing an initiator attached to a solid support for nucleic acid synthesis to a linking nucleotide in the presence of a polymerase so that the linking nucleotide is incorporated to the initiator, the linking nucleotide having a substrate base, a
10 substrate sugar, and a 3' hydroxyl group;

 exposing the initiator containing the linking nucleotide to nucleotide monomers in the presence of the polymerase, so that a nucleic acid is synthesized and is coupled to the initiator right
15 after the linking nucleotide;

 providing a mono-functional DNA glycosylase, the linking nucleotide with the substrate base being recognizable and excisable by the mono-functional DNA glycosylase;

20 subjecting the substrate base to an excision treatment with the mono-functional DNA glycosylase, so that the substrate base is excised by the mono-functional DNA glycosylase to generate an abasic site;

25 providing an abasic site endonuclease, the resulting abasic site being recognizable and the

substrate sugar being cleavable by the abasic site endonuclease;

subjecting the abasic site to a cleavage treatment with the abasic site endonuclease, so that
5 the substrate sugar and the backbone of the nucleic acid at the abasic site are both cleaved to release the nucleic acid from the initiator, so that a 3'-terminal nucleotide of the initiator has a 3' phosphate group, and so that a 5'-terminal
10 nucleotide of the synthesized nucleic acid has a 5' phosphate group;

providing a 3' phosphatase activity-possessing enzyme; and

subjecting the 3'-terminal nucleotide of the
15 initiator to a dephosphorylation treatment with the 3' phosphatase activity-possessing enzyme, so that the 3' phosphate group of the 3'-terminal nucleotide of the initiator is converted back to the original 3' hydroxyl group.

20 2. The method according to Claim 1, wherein the mono-functional DNA glycosylase is selected from the group consisting of uracil-DNA glycosylase, alkyladenine DNA glycosylase, single-strand-selective monofunctional uracil DNA
25 glycosylase 1, methyl-binding domain glycosylase 4, thymine DNA glycosylase, mutY homolog DNA glycosylase, alkylpurine glycosylase C, alkylpurine

- glycosylase D, 8-oxo-guanine glycosylase 1 without
abasic site lyase activity, endonuclease III-like 1
without abasic site lyase activity, endonuclease
VIII-like glycosylase 1 without abasic site lyase
5 activity, endonuclease VIII-like glycosylase 2
without abasic site lyase activity, endonuclease
VIII-like glycosylase 3 without abasic site lyase
activity, and enzymatically active fragments
thereof.
- 10 3. The method according to Claim 2, wherein the
mono-functional DNA glycosylase is one of uracil-DNA
glycosylase and alkyladenine DNA glycosylase.
4. The method according to Claim 1, wherein the abasic
site endonuclease is selected from the group
15 consisting of endonuclease VIII, endonuclease III,
and enzymatically active fragments thereof.
5. The method according to Claim 4, wherein the abasic
site endonuclease is endonuclease VIII.
6. The method according to Claim 1, wherein the 3'
20 phosphatase activity-possessing enzyme is selected
from the group consisting of a polynucleotide kinase
3'-phosphatase, a 3'-phosphoesterase, and
enzymatically active fragments thereof.
7. The method according to Claim 6, wherein the 3'
25 phosphatase activity-possessing enzyme is selected
from the group consisting of T4 polynucleotide kinase

with 3'phosphatase activity and zinc finger DNA
3'-phosphoesterase.

8. The method according to Claim 1, wherein the
substrate base of the linking nucleotide is selected
5 from the group consisting of uracil, hypoxanthine,
thymine, cytosine, guanine, 5-fluorouracil,
5-hydroxymethyluracil, 5-formylcytosine,
5-carboxylcytosine, 3-methyladenine,
3-methylguanine, 7-methyladenine, 7-methylguanine,
10 N⁶-methyladenine, 8-oxo-7,8-dihydroguanine,
5-hydroxyl cytosine, 5-hydroxyl uracil,
dihydroxyuracil, ethenocytosine, ethenoadenine,
thymine glycol, cytosine glycol,
2,6-diamino-4-hydroxy-5-N-methylformamidopyrimid-
15 ine, a formamidopyrimidine derivative of adenine, a
formamidopyrimidine derivative of guanine, adenine
opposite guanine, uracil opposite guanine, uracil
opposite adenine, thymine opposite guanine,
ethenocytosine opposite guanine, adenine opposite
20 8-oxo-7,8-dihydroguanine, and 2-hydroxyladenine
opposite guanine.

9. The method according to Claim 8, wherein the
substrate base of the linking nucleotide is one of
uracil and hypoxanthine.

25 10. The method according to Claim 1, wherein the
initiator, the synthesized nucleic acid, and the
linking nucleotide are each in one of a

template-independent form and a template-dependent form.

11. The method according to Claim 1, wherein the polymerase is selected from the group consisting of
- 5 a family-A DNA polymerase, a family-B DNA polymerase, a family-C DNA polymerase, a family-D DNA polymerase, a family-X DNA polymerase, a family-Y DNA polymerase, a reverse transcriptase, and enzymatically active fragments thereof.
- 10 12. A kit for nucleic acid synthesis and regeneration of a reusable nucleic acid for such synthesis, comprising:
- a polymerase and a linking nucleotide for nucleic acid synthesis;
- 15 a mono-functional DNA glycosylase;
- an abasic site endonuclease; and
- a 3' phosphatase activity-possessing enzyme;
- wherein the kit is used according to a method as described in Claim 1.
- 20 13. A method of regenerating a reusable initiator for nucleic acid synthesis, comprising:
- providing a mono-functional DNA glycosylase;
- providing an initiator for nucleic acid synthesis and a synthesized nucleic acid, the
- 25 initiator being attached to a solid support, the synthesized nucleic acid being linked to the initiator right after a linking nucleotide having

a substrate base and a substrate sugar, the linking nucleotide with the substrate base being recognizable and excisable by the mono-functional DNA glycosylase;

5 subjecting the substrate base to an excision treatment with the mono-functional DNA glycosylase, so that the substrate base is excised by the mono-functional DNA glycosylase to generate an abasic site;

10 providing an abasic site endonuclease, the resulting abasic site being recognizable and the substrate sugar being cleavable by the abasic site endonuclease;

15 subjecting the abasic site to a cleavage treatment with the abasic site endonuclease, so that the substrate sugar and the backbone of the nucleic acid at the abasic site are both cleaved to release the synthesized nucleic acid from the initiator, so that a 3'-terminal nucleotide of the initiator has a 3' phosphate group, and so that a 5'-terminal nucleotide of the synthesized nucleic acid has a 5' phosphate group;

20 providing a 3' phosphatase activity-possessing enzyme; and

25 subjecting the 3'-terminal nucleotide of the initiator to a dephosphorylation treatment with the 3' phosphatase activity-possessing enzyme, so that

the 3' phosphate group of the 3'-terminal nucleotide of the initiator is converted back to an original 3' hydroxyl group.

14. The method according to Claim 13, wherein the
5 mono-functional DNA glycosylase is selected from the group consisting of uracil-DNA glycosylase, alkyladenine DNA glycosylase, single-strand-selective monofunctional uracil DNA glycosylase 1, methyl-binding domain glycosylase 4,
10 thymine DNA glycosylase, mutY homolog DNA glycosylase, alkylpurine glycosylase C, alkylpurine glycosylase D, 8-oxo-guanine glycosylase 1 without abasic site lyase activity, endonuclease III-like 1 without abasic site lyase activity, endonuclease
15 VIII-like glycosylase 1 without abasic site lyase activity, endonuclease VIII-like glycosylase 2 without abasic site lyase activity, endonuclease VIII-like glycosylase 3 without abasic site lyase activity, and enzymatically active fragments
20 thereof.

15. The method according to Claim 14, wherein the mono-functional DNA glycosylase is one of uracil-DNA glycosylase and alkyladenine DNA glycosylase.

16. The method according to Claim 13, wherein the abasic
25 site endonuclease is selected from the group consisting of endonuclease VIII, endonuclease III, and enzymatically active fragments thereof.

17. The method according to Claim 16, wherein the abasic site endonuclease is endonuclease VIII.
18. The method according to Claim 13, wherein the 3' phosphatase activity-possessing enzyme is selected from the group consisting of a polynucleotide kinase 3'-phosphatase, a 3'-phosphoesterase, and enzymatically active fragments thereof.
19. The method according to Claim 18, wherein the 3' phosphatase activity-possessing enzyme is selected from the group consisting of T4 polynucleotide kinase with 3' phosphatase activity and zinc finger DNA 3'-phosphoesterase.
20. The method according to Claim 13, wherein the substrate base of the linking nucleotide is selected from the group consisting of uracil, hypoxanthine, thymine, cytosine, guanine, 5-fluorouracil, 5-hydroxymethyluracil, 5-formylcytosine, 5-carboxylcytosine, 3-methyladenine, 3-methylguanine, 7-methyladenine, 7-methylguanine, N⁶-methyladenine, 8-oxo-7,8-dihydroguanine, 5-hydroxyl cytosine, 5-hydroxyl uracil, dihydroxyuracil, ethenocytosine, ethenoadenine, thymine glycol, cytosine glycol, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine, a formamidopyrimidine derivative of adenine, a formamidopyrimidine derivative of guanine, adenine opposite guanine, uracil opposite guanine, uracil

opposite adenine, thymine opposite guanine, ethenocytosine opposite guanine, adenine opposite 8-oxo-7,8-dihydroguanine, and 2-hydroxyladenine opposite guanine.

- 5 21. The method according to Claim 13, wherein the initiator, the synthesized nucleic acid, and the linking nucleotide are each in one of a template-independent form and a template-dependent form.
- 10 22. A method of synthesizing a nucleic acid, the method comprising the following steps of:
- a) providing an initiator attached to a solid support, wherein the initiator is coupled with a linking nucleotide having a substrate base, a substrate sugar,
- 15 and a 3' hydroxyl group;
- b) exposing the initiator coupled with linking nucleotide to nucleotide monomers in the presence of a polymerase, so that the nucleic acid is synthesized and coupled to the initiator by reacting one of the
- 20 nucleotide monomers with the 3' hydroxyl group of the linking nucleotide;
- c) subjecting the substrate base to an excision treatment with a mono-functional DNA glycosylase, so that the substrate base is excised by the
- 25 mono-functional DNA glycosylase to generate an abasic site;

d) subjecting the abasic site to a cleavage treatment with an abasic site endonuclease, so that the substrate sugar and a sugar phosphate backbone at the abasic site are both cleaved to release the nucleic acid from the initiator, thereby forming a 3' phosphate group at a 3'-terminal nucleotide of the initiator; and

e) subjecting the 3'-terminal nucleotide having the 3' phosphate group to a dephosphorylation treatment with a 3' phosphatase activity-possessing enzyme, so that a hydroxyl group is formed at the 3'-terminal nucleotide to regenerate the initiator.

23. The method according to Claim 22, wherein the mono-functional DNA glycosylase is uracil-DNA glycosylase, alkyladenine DNA glycosylase, single-strand-selective monofunctional uracil DNA glycosylase 1, methyl-binding domain glycosylase 4, thymine DNA glycosylase, mutY homolog DNA glycosylase, alkylpurine glycosylase C, alkylpurine glycosylase D, 8-oxo-guanine glycosylase 1 without abasic site lyase activity, endonuclease III-like 1 without abasic site lyase activity, endonuclease VIII-like glycosylase 1 without abasic site lyase activity, endonuclease VIII-like glycosylase 2 without abasic site lyase activity, or endonuclease VIII-like glycosylase 3 without abasic site lyase activity.

24. The method according to Claim 22, wherein the mono-functional DNA glycosylase is uracil-DNA glycosylase or alkyladenine DNA glycosylase.
25. The method according to Claim 22, wherein the abasic
5 site endonuclease is endonuclease VIII, endonuclease III, a enzymatically active fragment of endonuclease VIII, or a enzymatically active fragment of endonuclease III.
26. The method according to Claim 22, wherein the abasic
10 site endonuclease is endonuclease VIII.
27. The method according to Claim 22, wherein the 3' phosphatase activity-possessing enzyme is a polynucleotide kinase 3'-phosphatase or a 3'-phosphoesterase.
- 15 28. The method according to Claim 22, wherein the 3' phosphatase activity-possessing enzyme is T4 polynucleotide kinase with 3' phosphatase activity or zinc finger DNA 3'-phosphoesterase.
29. The method according to Claim 22, wherein the
20 substrate base of the linking nucleotide is uracil, hypoxanthine, thymine, cytosine, guanine, 5-fluorouracil, 5-hydroxymethyluracil, 5-formylcytosine, 5-carboxylcytosine, 3-methyladenine, 3-methylguanine, 7-methyladenine,
25 7-methylguanine, N⁶-methyladenine, 8-oxo-7,8-dihydroguanine, 5-hydroxyl cytosine, 5-hydroxyl uracil, dihydroxyuracil, ethenocytosine,

- ethenoadenine, thymine glycol, cytosine glycol, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine, a formamidopyrimidine derivative of adenine, a formamidopyrimidine derivative of guanine, adenine
5 opposite guanine, uracil opposite guanine, uracil opposite adenine, thymine opposite guanine, ethenocytosine opposite guanine, adenine opposite 8-oxo-7,8-dihydroguanine, or 2-hydroxyladenine opposite guanine.
- 10 30. The method according to Claim 22, wherein the substrate base of the linking nucleotide is uracil or hypoxanthine.
31. The method according to Claim 22, wherein the substrate base is uracil thereby forming
15 deoxyuridine.
32. The method according to Claim 22, wherein the substrate base is hypoxanthine thereby forming deoxyinosine.
- 20 33. The method according to Claim 22, wherein the initiator is in a template-independent form or a template-dependent form.
34. The method according to Claim 22, wherein the polymerase is originated from family-A DNA polymerases, family-B DNA polymerases, family-C DNA polymerases, family-D DNA polymerases, family-X DNA
25 polymerases, family-Y DNA polymerases, or reverse transcriptases.

35. A kit for synthesizing a nucleic acid, comprising:

a polymerase;
an initiator attached to a solid support;
a mono-functional DNA glycosylase;
5 an abasic site endonuclease; and
a 3' phosphatase activity-possessing enzyme;
wherein the kit is used according to the method of
Claim 22.

36. A method of regenerating an initiator for nucleic
10 acid synthesis, the method comprising the following
steps of:

a) providing an initiator attached to a solid support,
wherein the initiator is coupled with a linking
nucleotide and a newly synthesized nucleic acid; and
15 the linking nucleotide has a substrate base and a
substrate sugar;

b) subjecting the substrate base to an excision
treatment with a mono-functional DNA glycosylase, so
that the substrate base is excised by the
20 mono-functional DNA glycosylase to generate an
abasic site;

c) subjecting the abasic site to a cleavage treatment
with an abasic site endonuclease, so that the
substrate sugar and a sugar phosphate backbone at the
25 abasic site are both cleaved to release the newly
synthesized nucleic acid from the initiator, thereby

forming a 3' phosphate group at a 3'-terminal nucleotide of the initiator; and

d) subjecting the 3'-terminal nucleotide having the 3' phosphate group to a dephosphorylation treatment with a 3' phosphatase activity-possessing enzyme, so that an hydroxyl group is formed at the 3'-terminal nucleotide to regenerate the initiator for being reused for further nucleic acid synthesis.

37. The method according to Claim 36, wherein the mono-functional DNA glycosylase is uracil-DNA glycosylase, alkyladenine DNA glycosylase, single-strand-selective monofunctional uracil DNA glycosylase 1, methyl-binding domain glycosylase 4, thymine DNA glycosylase, mutY homolog DNA glycosylase, alkylpurine glycosylase C, alkylpurine glycosylase D, 8-oxo-guanine glycosylase 1 without abasic site lyase activity, endonuclease III-like 1 without abasic site lyase activity, endonuclease VIII-like glycosylase 1 without abasic site lyase activity, endonuclease VIII-like glycosylase 2 without abasic site lyase activity, or endonuclease VIII-like glycosylase 3 without abasic site lyase activity.

38. The method according to Claim 36, wherein the mono-functional DNA glycosylase is uracil-DNA glycosylase or alkyladenine DNA glycosylase.

39. The method according to Claim 34, wherein the abasic
site endonuclease is endonuclease VIII,
endonuclease III, enzymatically active fragment of
endonuclease VIII, or enzymatically active fragment
5 of endonuclease III.
40. The method according to Claim 36, wherein the abasic
site endonuclease is endonuclease VIII.
41. The method according to Claim 36, wherein the 3'
phosphatase activity-possessing enzyme is a
10 polynucleotide kinase 3'-phosphatase or a
3'-phosphoesterase.
42. The method according to Claim 36, wherein the 3'
phosphatase activity-possessing enzyme is T4
polynucleotide kinase with 3' phosphatase activity or
15 zinc finger DNA 3'-phosphoesterase.
43. The method according to Claim 36, wherein the
substrate base of the linking nucleotide is uracil,
hypoxanthine, thymine, cytosine, guanine,
5-fluorouracil, 5-hydroxymethyluracil,
20 5-formylcytosine, 5-carboxylcytosine,
3-methyladenine, 3-methylguanine, 7-methyladenine,
7-methylguanine, N⁶-methyladenine,
8-oxo-7,8-dihydroguanine, 5-hydroxyl cytosine,
5-hydroxyl uracil, dihydroxyuracil, ethenocytosine,
25 ethenoadenine, thymine glycol, cytosine glycol,
2,6-diamino-4-hydroxy-5-N-methylformamidopyrimid-
ine, a formamidopyrimidine derivative of adenine, a

formamidopyrimidine derivative of guanine, adenine
opposite guanine, uracil opposite guanine, uracil
opposite adenine, thymine opposite guanine,
ethenocytosine opposite guanine, adenine opposite
5 8-oxo-7,8-dihydroguanine, or 2-hydroxyladenine
opposite guanine.

44. The method according to Claim 36, wherein the
substrate base of the linking nucleotide is uracil
or hypoxanthine.

10 45. The method according to Claim 36, wherein the
substrate base is uracil thereby forming
deoxyuridine.

46. The method according to Claim 36, wherein the
substrate base is hypoxanthine thereby forming
15 deoxyinosine.

47. The method according to Claim 36, wherein the
initiator is in a template-independent form or a
template-dependent form.

48. A kit for regenerating an initiator for nucleic acid
20 synthesis, comprising:
an initiator attached to a solid support;
a mono-functional DNA glycosylase;
an abasic site endonuclease; and
a 3' phosphatase activity-possessing enzyme;
25 wherein the kit is used according to the method of
Claim 36.