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(54) **Title:**
PCR REACTION MIXTURES WITH DECREASED NON-SPECIFIC ACTIVITY

(57) **Abstract:**
The present invention provides methods for improving the specificity of nucleic acid amplification comprising incubating a nucleic acid molecule with a polymerase-Sso7 DNA binding domain conjugate and arginine, spermidine, or spermine. The present invention also provides reaction mixtures and kits for improving the specificity of nucleic acid amplification.

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(54) **Title**: PCR REACTION MIXTURES WITH DECREASED NON-SPECIFIC ACTIVITY

(57) **Abstract**: The present invention provides methods for improving the specificity of nucleic acid amplification comprising incubating a nucleic acid molecule with a polymerase-Sso7 DNA binding domain conjugate and arginine, spermidine, or spermine. The present invention also provides reaction mixtures and kits for improving the specificity of nucleic acid amplification.

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PCR REACTION MIXTURES WITH DECREASED NON-SPECIFIC ACTIVITY

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

[0001] The present application claims benefit of priority to US Provisional Patent Application No. 61/473,710, filed April 8, 2011, which is incorporated by reference.

BACKGROUND OF THE INVENTION

[0002] Nucleic acid amplification reactions, such as polymerase chain reaction (PCR), are generally template-dependent reactions in which a desired nucleic acid sequence is amplified by treating separate complementary strands of a target nucleic acid with an excess of two oligonucleotide primers. The primers are extended to form complementary primer extension products which act as templates for synthesizing the desired nucleic acid sequence. In such processes, the nucleic acid sequence between the primers on the respective DNA strands is selectively amplified. However, the specificity of a nucleic acid amplification reaction can be negatively affected by a number of factors.

BRIEF SUMMARY OF THE INVENTION

[0003] The present invention provides methods of amplifying a nucleic acid molecule. In some embodiments, the method comprises:

(a) providing the nucleic acid in a composition sufficient for nucleic acid amplification, the composition comprising:

(i) one or more oligonucleotide primers;

(ii) a polymerase; and

(iii) a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises free arginine, spermidine, or spermine, or a salt thereof; and

(b) incubating the mixture under conditions sufficient for amplifying the nucleic acid, thereby amplifying the nucleic acid, if present.

[0004] In some embodiments, the polymerase is conjugated to a DNA binding domain. In some embodiments, the polymerase is conjugated to a Sso7 domain.

[0005] In some embodiments, the agent is present in an amount sufficient to increase by at least 10% the relative yield of a specific amplification product as compared to a non-specific amplification product. In some embodiments, the agent is free arginine or a salt thereof. In some embodiments, the concentration of the free arginine or the free arginine salt is from about 1 mM to about 500 mM.

[0006] In some embodiments, the polymerase substantially lacks a 3'-5' exonuclease activity. In some embodiments, the polymerase has the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2. In some embodiments, the Sso7 domain has at least 75% amino acid sequence identity to SEQ ID NO:3.

[0007] In some embodiments, the composition comprises a buffer (when measured at a concentration of 0.1 M) that has a change of no more than 0.027 pH units per degree C when between 20° and 37° C. In some embodiments, the buffer is selected from the group consisting of HEPES, ACES, PIPES, MOPSO, BES, MOPS, TES, TAPSO, POPSO, BICINE, TAPS, and AMPSO.

[0008] In some embodiments, the method comprises:

(a) providing the nucleic acid with a composition sufficient for nucleic acid amplification, the composition comprising:

(i) one or more oligonucleotide primers; and

(ii) a polymerase, wherein the polymerase is present at a concentration of at least 20 units/reaction; and

(b) incubating the nucleic acid in the composition under conditions sufficient for amplifying the nucleic acid, thereby amplifying the nucleic acid.

[0009] In some embodiments, the composition further comprises free arginine or a salt thereof. In some embodiments, the concentration of the free arginine or the free arginine salt is from about 1 mM to about 500 mM.

[0010] In some embodiments, the polymerase is conjugated to a DNA binding domain. In some embodiments, the polymerase is conjugated to a Sso7 domain.

[0011] In some embodiments, the polymerase substantially lacks a 3'-5' exonuclease activity. In some embodiments, the polymerase has the amino acid sequence of SEQ ID

NO:1 or SEQ ID NO:2. In some embodiments, the Sso7 domain has at least 75% amino acid sequence identity to SEQ ID NO:3.

[0012] In some embodiments, the composition comprises a buffer (when measured at a concentration of 0.1 M) that has a change of no more than 0.027 pH units per degree C when between 20° and 37° C. In some embodiments, the buffer is selected from the group consisting of HEPES, ACES, PIPES, MOPSO, BES, MOPS, TES, TAPSO, POPSO, BICINE, TAPS, and AMPSO.

[0013] In another aspect, the present invention provides reaction mixtures for amplifying a nucleic acid molecule. In some embodiments, the reaction mixture comprises:

a polymerase; and

a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises free arginine, spermidine, or spermine, or a salt thereof.

[0014] In yet another aspect, the present invention provides kits for amplifying a nucleic acid molecule. In some embodiments, the kit comprises:

a polymerase; and

a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises free arginine, spermidine, or spermine, or a salt thereof.

[0015] In some embodiments, the polymerase is conjugated to a DNA binding domain. In some embodiments, the polymerase is conjugated to a Sso7 domain.

[0016] In some embodiments, the agent is present in an amount sufficient to increase by at least 10% the relative yield of a specific amplification product as compared to a non-specific amplification product. In some embodiments, the agent is free arginine or a salt thereof. In some embodiments, the concentration of the free arginine or thefree arginine salt is from about 1 mM to about 500 mM.

[0017] In some embodiments, the polymerase substantially lacks a 3'-5' exonuclease activity. In some embodiments, the polymerase has the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2. In some embodiments, the Sso7 domain has at least 75% amino acid sequence identity to SEQ ID NO:3.

[0018] In some embodiments, the reaction mixture and/or kit further comprises one or more oligonucleotide primers. In some embodiments, the reaction mixture and/or kit further comprises at least one member selected from the group consisting of buffers, nucleotide triphosphates, salts, stabilizers, double stranded DNA binding dye, and nuclease-free water.

[0019] In some embodiments, the reaction mixture comprises a buffer (when measured at a concentration of 0.1 M) that has a change of no more than 0.027 pH units per degree C when between 20° and 37° C. In some embodiments, the buffer is selected from the group consisting of HEPES, ACES, PIPES, MOPSO, BES, MOPS, TES, TAPSO, POPSO, BICINE, TAPS, and AMPSO.

[0020] In another aspect, the invention provides for a reaction mixture for signal normalization in a real-time polymerase chain reaction (PCR) amplification of a target nucleic acid wherein the mixture is compatible for use in both (a) a real-time PCR amplification system employing a high passive reference dye concentration for the normalization and (b) a real-time PCR amplification system employing a low passive reference dye concentration for the normalization, wherein the mixture comprises: a plurality of passive reference dyes that produces fluorescent signals independent of the amplification reactions; and a buffer, wherein the buffer (when measured at a concentration of 0.1 M) has a change of no more than 0.027 pH units per degree C when between 20° and 37° C.

[0021] In some embodiments, the buffer is selected from the group consisting of HEPES, ACES, PIPES, MOPSO, BES, MOPS, TES, TAPSO, POPSO, BICINE, TAPS, and AMPSO.

[0022] In some embodiments, the reaction mixture further comprises a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises free arginine, spermidine, or spermine, or a salt thereof.

[0023] In some embodiments, the mixture comprises:

a first passive reference dye having a Stokes-shift, wherein the first passive reference dye is at a concentration sufficient for use in low concentration passive reference dye normalization, wherein the first passive reference dye has a first passive reference dye excitation wavelength maximum and a first passive reference dye emission wavelength maximum; and

a second passive reference dye having a Stokes-shift that is greater than the Stokes-shift of the first passive reference dye, wherein the second passive reference dye has an emission wavelength maximum approximately the same as the first passive reference dye emission

wavelength maximum, and an excitation wavelength maximum significantly different than the first passive reference dye excitation wavelength maximum.

[0024] In some embodiments, the second passive reference dye has a Stokes-shift of at least about 60 nm. In some embodiments, the first passive reference dye comprises 5- and/or 6-carboxy-X-rhodamine, or an analog thereof. In some embodiments, the second passive reference dye has a excitation wavelength maximum of 550 nm or less. In some embodiments, the concentration of the 5- and/or 6-carboxy-X-rhodamine dye is less than 100 nM.

[0025] In some embodiments, the reaction mixture further comprises one or more of an oligonucleotide primer, one or more deoxynucleoside triphosphates; a buffer, an intercalating dye, a reverse transcriptase, and a polymerase. In some embodiments, the reaction mixture comprises DNA polymerase. In some embodiments, the polymerase is complexed with an antibody. In some embodiments, the polymerase is chemically inactivated but is activated by heating.

[0026] In some embodiments, the second passive dye is a fluorescent dot.

[0027] In some embodiments, the second passive reference dye is conjugated to a moiety.

[0028] Also provided is a method of performing a real-time quantitative polymerase chain reaction. In some embodiments, the method comprises, performing a polymerase chain reaction (PCR) with a reaction mixture as described above, wherein the mixture further comprises a biological sample suspected of comprising a target nucleic acid.

[0029] Also provided is a method of making the reaction mixture as described above, the method comprising mixing the plurality of passive reference dyes, thereby generating the reaction mixture.

[0030] In another aspect, a kit is provided for performing a real-time quantitative polymerase chain reaction. In some embodiments, the kit comprises: a first passive reference dye having a Stokes-shift, wherein the first passive dye has a first passive reference dye excitation wavelength maximum and a first passive reference dye emission wavelength maximum; a second passive reference dye having a Stokes-shift that is greater than the Stokes-shift of the first passive reference dye, wherein the second passive reference dye has an emission wavelength maximum approximately the same as the first passive reference dye emission wavelength maximum, and an excitation wavelength maximum significantly different than the first passive reference dye excitation wavelength maximum and a buffer,

wherein the buffer (when measured at a concentration of 0.1 M) has a change of no more than 0.027 pH units per degree C when between 20° and 37° C.

[0031] In some embodiments, the buffer is selected from the group consisting of HEPES, ACES, PIPES, MOPSO, BES, MOPS, TES, TAPSO, POPSO, BICINE, TAPS, and AMPSO.

[0032] In some embodiments, the kit further comprises a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises free arginine, spermidine, or spermine, or a salt thereof.

[0033] In some embodiments, the second passive reference dye has a Stokes-shift of at least about 60 nm. In some embodiments, the first passive reference dye comprises 5- and/or 6-carboxy-X-rhodamine, or an analog thereof.

[0034] In some embodiments, the first passive reference dye comprises a 5- and/or 6-carboxy-X-rhodamine dye or an analog thereof, and the second passive reference dye is a fluorescent dye having a Stokes-shift of at least 60 nm, wherein the second passive reference dye has an emission wavelength maximum of about 620 nm.

[0035] In some embodiments, the mixture comprises a 5- and/or 6-carboxy-X-rhodamine dye; and a second passive reference dye having a Stokes-shift of at least about 60 nm, wherein the second passive reference dye has an emission wavelength maximum of about 590 nm.

[0036] In some embodiments, the kit further comprises one or more of: one or more deoxynucleoside triphosphates; one or more of an oligonucleotide primer, one or more deoxynucleoside triphosphates; a buffer, an intercalating dye, a reverse transcriptase, and a DNA polymerase.

[0037] In some embodiments, the first passive reference dye and the second passive reference dye are contained in different vessels in the kit. In some embodiments, the first passive reference dye and the second passive reference dye are contained in the same vessel in the kit.

DEFINITIONS

[0038] The term "polymerase" refers to an enzyme that performs template-directed synthesis of polynucleotides. The term encompasses both a full length polypeptide and a domain that has polymerase activity. DNA polymerases are well-known to those skilled in

the art, including but not limited to DNA polymerases isolated or derived from *Pyrococcus furiosus*, *Thermococcus litoralis*, and *Thermotoga maritime*, or modified versions there of. They include both DNA-dependent polymerases and RNA-dependent polymerases such as reverse transcriptase. At least five families of DNA-dependent DNA polymerases are known, although most fall into families A, B and C. There is little or no sequence similarity among the various families. Most family A polymerases are single chain proteins that can contain multiple enzymatic functions including polymerase, 3' to 5' exonuclease activity and 5' to 3' exonuclease activity. Family B polymerases typically have a single catalytic domain with polymerase and 3' to 5' exonuclease activity, as well as accessory factors. Family C polymerases are typically multi-subunit proteins with polymerizing and 3' to 5' exonuclease activity. In E. coli, three types of DNA polymerases have been found, DNA polymerases I (family A), II (family B), and III (family C). In eukaryotic cells, three different family B polymerases, DNA polymerases α, δ, and ε, are implicated in nuclear replication, and a family A polymerase, polymerase γ, is used for mitochondrial DNA replication. Other types of DNA polymerases include phage polymerases. Similarly, RNA polymerases typically include eukaryotic RNA polymerases I, II, and III, and bacterial RNA polymerases as well as phage and viral polymerases. RNA polymerases can be DNA-dependent and RNA-dependent. In some embodiments, a polymerase of the present invention is identical or substantially identical (*e.g.*, has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity) to an amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

[0039] "Thermally stable polymerase," as used herein, refers to any enzyme that catalyzes polynucleotide synthesis by addition of nucleotide units to a nucleotide chain using DNA or RNA as a template and has an optimal activity at a temperature above 45°C.

[0040] The term "Sso7-like protein" or "Sso7," as used herein, refers to nucleic acid and polypeptide polymorphic variants, alleles, mutants, and interspecies homologs that: (1) have an amino acid sequence that has greater than about 60% amino acid sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of at least about 15, 25, 35, 50, or more amino acids, to an Sso7 sequence of SEQ ID NO:3; (2) bind to antibodies, *e.g.*, polyclonal antibodies, raised against an immunogen comprising an amino acid sequence of SEQ ID NO:3 and conservatively modified variants thereof; (3) specifically hybridize under stringent hybridization conditions to a Sso7d nucleic acid sequence encoding the amino acid of SEQ ID NO:3 and conservatively modified variants thereof; or (4) have a nucleic acid

sequence that has greater than about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher nucleotide sequence identity, preferably over a region of at least about 50, 100, 150, or more nucleotides, to a nucleic acid sequence encoding SEQ ID NO:3. The term includes both full-length Sso7 polypeptides and fragments of the polypeptides that have sequence non-specific double-stranded binding activity. Sso7-like proteins include Sac7d, Sac7e, Ssh7b, and Sto7e.

[0041] "Wild-type Sso7" refers to a naturally occurring Sso7 protein. In some embodiments, a wild-type Sso7 has the amino acid sequence of SEQ ID NO:3. "Wild-type Sso7-like" refers to a naturally-occurring Sso7-like protein, *e.g.,* Sac7d, Sac7e, Ssh7b, and Sto7e.

[0042] A "domain" refers to a unit of a protein or protein complex, comprising a polypeptide subsequence, a complete polypeptide sequence, or a plurality of polypeptide sequences where that unit has a defined function. The function is understood to be broadly defined and can be ligand binding, catalytic activity, or can have a stabilizing effect on the structure of the protein.

[0043] The term "DNA binding domain" refers to a protein domain that binds DNA in a sequence non-specific manner. In some embodiments, the DNA binding domain is a protein domain which binds with significant affinity to DNA, for which there is no known nucleic acid which binds to the protein domain with more than 100-fold more affinity than another nucleic acid with the same nucleotide composition but a different nucleotide sequence.

[0044] The term "polymerase-Sso7 conjugate," as used herein, refers to a modified polymerase comprising at least one Sso7 DNA binding domain joined to a polymerase domain, or a catalytic subunit of the polymerase domain. A polymerase-Sso7 conjugate may comprise multiple Sso7 DNA binding domains.

[0045] "Join" refers to any method known in the art for functionally connecting protein domains, including without limitation recombinant fusion with or without intervening domains, intein-mediated fusion, non-covalent association, and covalent bonding, including disulfide bonding; hydrogen bonding; electrostatic bonding; and conformational bonding, *e.g.,* antibody-antigen, and biotin-avidin associations.

[0046] The term "nucleic acid amplification" or "amplification reaction" refers to any *in vitro* means for multiplying the copies of a target sequence of nucleic acid. Such methods include but are not limited to polymerase chain reaction (PCR), DNA ligase chain reaction

(*see* U.S. Pat. Nos. 4,683,195 and 4,683,202; PCR Protocols: A Guide to Methods and Applications (Innis *et al.*, eds, 1990)), (LCR), QBeta RNA replicase, and RNA transcription-based (such as TAS and 3SR) amplification reactions as well as others known to those of skill in the art.

[0047] "Amplifying" refers to a step of submitting a solution to conditions sufficient to allow for amplification of a polynucleotide if all of the components of the reaction are intact. Components of an amplification reaction include, *e.g.,* primers, a polynucleotide template, polymerase, nucleotides, and the like. The term amplifying typically refers to an "exponential" increase in target nucleic acid. However, amplifying as used herein can also refer to linear increases in the numbers of a select target sequence of nucleic acid, such as is obtained with cycle sequencing.

[0048] The term "amplification reaction mixture" or "amplification reaction composition" refers to an aqueous solution comprising the various reagents used to amplify a target nucleic acid. These include enzymes, aqueous buffers, salts, amplification primers, target nucleic acid, and nucleoside triphosphates. As discussed further herein, amplification reaction mixtures may also further include stabilizers and other additives to optimize efficiency and specificity. Depending upon the context, the mixture can be either a complete or incomplete amplification reaction mixture.

[0049] "Polymerase chain reaction" or "PCR" refers to a method whereby a specific segment or subsequence of a target double-stranded DNA, is amplified in a geometric progression. PCR is well known to those of skill in the art; *see, e.g.,* U.S. Pat. Nos. 4,683,195 and 4,683,202; and PCR Protocols: A Guide to Methods and Applications, Innis *et al.*, eds, 1990. Exemplary PCR reaction conditions typically comprise either two or three step cycles. Two step cycles have a denaturation step followed by a hybridization/elongation step. Three step cycles comprise a denaturation step followed by a hybridization step followed by a separate elongation step.

[0050] An "olignucleotide primer" or "primer" refers to an oligonucleotide sequence that hybridizes to a sequence on a target nucleic acid and serves as a point of initiation of nucleic acid synthesis. Primers can be of a variety of lengths and are often less than 50 nucleotides in length, for example 12-30 nucleotides in length. The length and sequences of primers for use in PCR can be designed based on principles known to those of skill in the art; *see, e.g.,* Innis *et al., supra.*

[0051] A "template" refers to a polynucleotide sequence that comprises the polynucleotide to be amplified, flanked by primer hybridization sites. Thus, a "target template" comprises the target polynucleotide sequence flanked by hybridization sites for a 5' primer and a 3' primer.

[0052] The term "specificity," as used with respect to nucleic acid amplification, refers to the likelihood of a nucleic acid amplification reaction producing specific amplification products as compared to non-specific amplification products. A "specific amplification product" refers to the polynucleotide produced by amplification with correctly matched primers and template (*i.e.*, the true target sequence). A "non-specific amplification product" refers to the polynucleotide produced during an amplification reaction that is other than the specific amplification product.

[0053] The phrase "improved specificity" or "improving specificity," as used with respect to nucleic acid amplification, refers to a detectable increase in the amount of specific amplification products produced in a nucleic acid amplification as compared to the amount of non-specific amplification products produced. Specificity of an amplification reaction can be measured according to any method, including but not limited to melt-curve analysis or gel analysis. In some embodiments, the specificity of an amplification reaction is increased when a reaction mixture comprising an agent that improves amplification specificity (*e.g.*, arginine, spermidine, or spermine, or a salt thereof) has a higher relative yield of the specific product than the non-specific product (*i.e.*, the ratio of the yield as measured by the height of the melting peak of the specific product over the height of the melting peak of the non-specific product) in an amplification reaction than the relative yield of the specific product over the non-specific product in a reaction mixture lacking the agent. In some embodiments, an agent that improves amplification specificity (*e.g.*, arginine, spermidine, or spermine, or a salt thereof) will exhibit at least 10%, 15%, 20%, 25%, 30%, 40%, 50%, 100%, 2-fold (200%), 2.5-fold (250%), 3-fold (300%) or greater increase in the ratio relative to reactions in which the agent that improves amplification specificity (*e.g.*, arginine, spermidine, or spermine, or a salt thereof) is not included.

[0054] The terms "nucleic acid" and "polynucleotide" are used interchangeably herein to refer to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples

of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, and peptide nucleic acids (PNAs).

[0055] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers.

[0056] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0057] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0058] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid

variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[0059] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0060] The term "promoter" refers to regions or sequences located upstream and/or downstream from the start of transcription and which are involved in recognition and binding of RNA polymerase and other proteins to initiate transcription.

[0061] A "vector" is a DNA construct for introducing a polynucleotide sequence into a cell. Vectors can comprise, e.g., transcription and translation terminators, transcription and translation initiation sequences, and promoters useful for regulation of the expression of the particular nucleic acid. In some embodiments, a vector is an expression vector having a promoter sequence operably linked to the polynucleotide sequence and capable of effecting the expression in a suitable host of the polypeptide encoded by the polynucleotide sequence.

[0062] "Recombinant" refers to a human manipulated polynucleotide or a copy or complement of a human manipulated polynucleotide. For instance, a recombinant expression cassette comprising a promoter operably linked to a second polynucleotide may include a promoter that is heterologous to the second polynucleotide as the result of human manipulation (*e.g.,* by methods described in Sambrook et al., Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998)) of an isolated nucleic acid comprising the expression cassette. As another example, a recombinant expression cassette may comprise polynucleotides combined in such a way that

the polynucleotides are extremely unlikely to be found in nature. For instance, human manipulated restriction sites or plasmid vector sequences may flank or separate the promoter from the second polynucleotide. One of skill will recognize that polynucleotides can be manipulated in many ways and are not limited to the examples above.

[0063] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same. Sequences are "substantially identical" to each other if they have a specified percentage of nucleotides or amino acid residues that are the same (*e.g.*, at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity) over a specified region where indicated, or across the entire reference sequence if not otherwise indicated, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. These definitions also refer to the complement of a test sequence.

[0064] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters are commonly used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities or similarities for the test sequences relative to the reference sequence, based on the program parameters.

[0065] A "comparison window," as used herein, includes reference to a segment of contiguous positions, for example from 20 to 600 contiguous positions, about 50 to about 200, or about 100 to about 150, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith and Waterman (*Adv. Appl. Math.* 2:482, 1970), by the homology alignment algorithm of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), by the search for similarity method of Pearson and Lipman (*Proc. Natl. Acad. Sci. USA* 85:2444, 1988), by computerized implementations of these algorithms (*e.g.*, GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by manual alignment and visual inspection (*see*, *e.g.*, Ausubel *et al.*, *Current Protocols in Molecular Biology* (1995 supplement)).

[0066] Algorithms suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (*Nuc. Acids Res.* 25:3389-402, 1977), and Altschul *et al.* (*J. Mol. Biol.* 215:403-10, 1990), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al, supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0067] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, Proc. Nat'l. Acad. Sci. USA 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0068] A "passive reference dye" refers to a fluorescent dye or dot that does not interact with other components of a polymerase chain reaction. For example, the passive reference dye does not significantly change its signal based on the presence or absence of nucleic acids and does not significantly interact in FRET interactions with another dye in the PCR reaction mixture or the detection of another dye by the real-time instrument. The passive reference dye can be, but in many embodiments, is not linked to a nucleic acid. The passive reference

dye can have a Stokes-shift, i.e., such that the excitation wavelength maximum and the emission maximum are different.

[0069] A "low" passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine dye such as ROX™) concentration, as used herein, is a concentration of a passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine dye or a 5- or 6-carboxy-X-rhodamine dye analog) suitable by itself for use in a low ROX concentration amplification (e.g., qPCR) system. Low 5- or 6-carboxy-X-rhodamine dye concentration instruments ("a qPCR instrument employing a low 5- or 6-carboxy-X-rhodamine dye concentration for normalization") include but are not limited to the Applied Biosystems ABI 7500 or Applied Biosystems ViiA7 or Stratagene MX series real-time PCR systems. Thus, low passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine dye) concentrations are generally less than 100 nM, e.g., 1-10 nM, 10-100 nM, etc.

[0070] A "high" passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine dye such as ROX™) concentration, as used herein, is a concentration of a passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine dye such as ROX™ or a 5- or 6-carboxy-X-rhodamine dye analog) suitable for use in a high 5- or 6-carboxy-X-rhodamine dye concentration amplification (e.g., qPCR) system. High 5- or 6-carboxy-X-rhodamine dye concentration instruments ("a qPCR instrument employing the use of a high 5- or 6-carboxy-X-rhodamine dye concentration for normalization") include but are not limited to the Applied Biosystems ABI PRISM 7000, 7700, or 7900 or the ABI 7300 Real-Time PCR Systems or the ABI GeneAmp 5700 Real-Time PCR System. Thus, high passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine dye) concentrations are generally more than 100 nM, e.g., 100-300 nM, 300-700nM, etc.

[0071] "An emission wavelength maximum approximately the same as the passive reference dye emission wavelength maximum" refers to an emission wavelength maximum that is within 30 nm, and optionally, 20, 10, 5, 3, or 1 nm of the passive reference dye emission wavelength maximum.

[0072] "Significantly different" in the context of excitation wavelengths of dyes, refers to wavelengths sufficiently different that one dye can be excited without exciting a second dye with a significantly different excitation wavelength maximum. In some embodiments, "significantly different" means the two dyes have excitation maxima at least 10, 20, 30, 40, 50, 60, 70 nm, or more apart.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0073] The present invention provides methods and compositions for improving the specificity of nucleic acid amplification and increasing the tolerance of an amplification reaction mixture to inhibitors in nucleic acid amplification. The inventors have surprisingly found that adding arginine mono-hydrochloride or a polyamine salt such as spermidine trihydrochloride or spermine tetrahydrochloride to an amplification reaction with a polymerase reduces the formation of non-specific PCR products and enhances the tolerance of amplification reagents to PCR inhibitors.

[0074] Thus, in one aspect, the present invention provides methods of amplifying a nucleic acid molecule. In some embodiments, the method comprises: (a) mixing the nucleic acid molecule with a composition sufficient for nucleic acid amplification, wherein the composition comprises a polymerase (*e.g.,* a polymerase conjugated to a DNA binding domain including but not limited to a Sso7 or Sso7-like protein) and a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises arginine (*i.e.,* "free" arginine not part of a polypeptide), spermidine and spermine, or a salt thereof; and (b) incubating the mixture under conditions sufficient for amplifying the nucleic acid molecule. In some embodiments, the agent is present in a sufficient amount to result in at least a 10% increase in the relative yield of the specific amplification product as compared to non-specific product. In some embodiments, the polymerase (*e.g.,* a polymerase-DNA binding domain conjugate, *e.g.,* a polymerase-Sso7 conjugate) comprises a polymerase domain lacking 3'-5' exonuclease activity and/or a Sso7 domain having one or more point mutations that decrease non-specific amplification activity.

[0075] In some embodiments, the method comprises: (a) mixing the nucleic acid molecule with a composition sufficient for nucleic acid amplification, wherein the composition comprises a polymerase (*e.g.,* a polymerase conjugated to a DNA binding domain including but not limited to a Sso7 or Sso7-like protein) at a concentration of at least 20 units/ml; and (b) incubating the mixture under conditions sufficient for amplifying the nucleic acid molecule. In some embodiments, the composition further comprises a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises arginine, spermidine or spermine, or a salt thereof.

[0076] In another aspect, the present invention provides reaction mixtures and kits for performing nucleic acid amplification reactions that use polymerases (*e.g.,* polymerase-Sso7

conjugates). In some embodiments, the reaction mixtures or kits comprise a polymerase (*e.g.*, a polymerase conjugated to a DNA binding domain including but not limited to a Sso7 or Sso7-like protein) and a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises arginine, spermidine or spermine.

[0077] It has also been discovered that certain buffers allow for improved stability for storage of passive (i.e., non-nucleic acid interacting) fluorescent dyes and also improve specificity of amplification reactions. Based on the discovery that HEPES and POPSO have these effects, it is believed that any buffer having (when measured at a concentration of 0.1 M) a change of no more than 0.027 pH units per degree C when between 20° and 37° C will improve stability of passive fluorescent dyes. While this discovery is separate from the discovery of the agents that improve specificity discussed above, agents that improve specificity can be combined in reaction mixtures having this particular type of buffer, for example in reaction mixtures.

II. Agents that Improve the Specificity of Nucleic Acid Amplification

[0078] The present invention provides for agents that improve the specificity of nucleic acid amplification when added to an amplification reaction mixture prior to amplification of the target nucleic acid molecule. In some embodiments, the agent is selected from free arginine (*e.g.*, L-arginine or D-arginine, not linked to another amino acid), spermidine, and spermine. In some embodiments, the agent is free arginine.

[0079] In some embodiments, the agent that improves the specificity of nucleic acid amplification is present in the amplification reaction mixture at a concentration of about 1 mM to about 500 mM. In some embodiments, the agent is present at a concentration of about 1 mM to about 100 mM, about 1 mM to about 75 mM, about 1 mM to about 50 mM, about 1 mM to about 25 mM, or about 5 mM to about 15 mM.

[0080] The arginine, spermidine, and/or spermine agents of the present invention may be provided as salts. Examples of applicable salt forms include hydrochlorides, hydrobromides, sulfates, methanesulfonates, nitrates, maleates, acetates, citrates, fumarates, tartrates (e.g., (+)-tartrates, (-)-tartrates or mixtures thereof including racemic mixtures), succinates, and benzoates. These salts may be prepared by methods known to those skilled in art. Also included are base addition salts such as sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When an agent of the present invention contains relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a

suitable inert solvent. Examples of acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. In some embodiments, arginine, spermidine, and/or spermine salts are monohydrochloride, dihydrochloride, trihydrochloride, or tetrahydrochloride salts.

[0081] Specificity of an amplification reaction can be measured, for example, using melt-curve analysis or gel analysis. In some embodiments, the specificity of a nucleic acid amplification reaction is determined by comparing the relative yield of two products, one of which is the specific product with the expected T_m and the other the non-specific product with a lower or higher T_m than that of the specific product. A reaction mixture comprising an agent that improves amplification specificity (*e.g.*, arginine, spermidine, or spermine, or a salt thereof) will have a higher relative yield in an amplification reaction of the specific product than the non-specific product (*i.e.*, the ratio of the yield as measured by the height of the melting peak of the specific product over the height of the melting peak of the non-specific product) than the relative yield of the specific product over the non-specific product in a reaction mixture lacking the agent. In some embodiments, an agent that improves amplification specificity (*e.g.*, arginine, spermidine, or spermine, or a salt thereof) will exhibit at least 10%, 15%, 20%, 25%, 30%, 40%, 50%, 100%, 2-fold (200%), 2.5-fold (250%), 3-fold (300%) or greater increase in the ratio relative to reactions in which the agent that improves amplification specificity (*e.g.*, arginine, spermidine, or spermine, or a salt thereof) is not included.

[0082] In some embodiments, an agent that improves amplification specificity (*e.g.*, arginine, spermidine, or spermine, or a salt thereof) also improves the tolerance of an amplification reaction mixture to amplification inhibitors. Inhibitor tolerance can be evaluated by performing an amplification reaction (*e.g.*, qPCR) in the presence or absence of different concentrations of known inhibitors (*e.g.*, chocolate, blood, soil, milk, or serum). A reaction mixture comprising an agent that improves reaction mixture tolerance to an amplification inhibitor (*e.g.*, arginine, spermidine, or spermine, or a salt thereof) will maintain reaction performance (*e.g.*, as measured by Ct value of amplification) in the presence of a higher concentration of inhibitor as compared to a reaction mixture lacking the agent.

III. Polymerase-Sso7 Conjugates

[0083] In some embodiments, the nucleic acid amplification methods of the present invention utilize a polymerase polypeptide or domain conjugated to a DNA binding domain or protein, *e.g.*, a Sso7 or Sso7-like domain. Such polymerase conjugates are known to show an increased processivity. *See, e.g.*, U.S. Patent Application No. 12/683,950, the contents of which are hereby incorporated by reference in its entirety for all purposes and in particular for all teachings related to polymerases, polymerase conjugates, as well as all methods for making and using such polymerases.

[0084] In some embodiments, the polymerase conjugates of the present invention comprise a polymerase domain or polypeptide lacking 3'-5' exonuclease activity. "Exonuclease deficient," as used herein, means that the polymerase has a substantially reduced exonuclease activity (*i.e.*, less than 10%, 5%, or 1% of 3'-5' exonuclease activity as compared to a wild-type polymerase) or no exonuclease activity). In some embodiments, said polymerase conjugates comprise one or more point mutations in the polymerase domain that provides this exonuclease deficiency.

[0085] In some embodiments, the polymerase conjugates of the present invention comprise a Sso7 DNA binding domain or protein. Polymerase conjugates comprising a Sso7 or Sso7-like DNA binding domain exhibit increased processivity and reduced reaction time in nucleic acid amplification as compared to polymerases lacking a Sso7-like domain. The inventors have surprisingly found that point mutations at one or more residues in the Sso7 DNA binding domain can decrease the formation of non-specific PCR products as compared to a polymerase comprising a Sso7 DNA binding domain lacking said point mutation(s). Thus, in some embodiments, the polymerase conjugates of the invention comprise one or more point mutations in the Sso7 DNA binding domain domain.

[0086] In some embodiments, a polymerase conjugate of the present invention is substantially identical (*e.g.*, has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity) to an amino acid sequence of SEQ ID NO:31-56. In some embodiments, a polymerase conjugate of the present invention has the amino acid sequence of SEQ ID NO:31-56.

A. Polymerases

[0087] A variety of polymerases can be used as the polymerases of the invention or as at least a portion of the polymerase domain of the polymerase-Sso7 conjugates of the present invention. At least five families of DNA-dependent DNA polymerases are known, although

most fall into families A, B and C. There is little or no sequence similarity among the various families. Most family A polymerases are single chain proteins that can contain multiple enzymatic functions including polymerase, 3' to 5' exonuclease activity and 5' to 3' exonuclease activity. Family B polymerases typically have a single catalytic domain with polymerase and 3' to 5' exonuclease activity, as well as accessory factors. Family C polymerases are typically multi-subunit proteins with polymerizing and 3' to 5' exonuclease activity. In *E. coli*, three types of DNA polymerases have been found, DNA polymerases I (family A), II (family B), and III (family C). In eukaryotic cells, three different family B polymerases, DNA polymerases α, δ, and ε, are implicated in nuclear replication, and a family A polymerase, polymerase γ, is used for mitochondrial DNA replication. Other types of DNA polymerases include phage polymerases. Any of these polymerases, combinations of all or portions of these polymerases, as well as chimeras or hybrids between two or more of such polymerases or their equivalents can be used to form a portion or all of the polymerase domain of polymerase conjugates of the invention.

[0088] Further, in some embodiments, non-thermostable polymerases may also be used in accordance with the invention. For example, the large fragment of *E. coli* DNA Polymerase I (Klenow) (the Klenow Fragment) with mutation (D355A, E357A) abolishes the 3'-5' exonuclease activity. This enzyme or equivalent enzymes can be used in embodiments where the amplification reaction is not performed at high temperatures.

[0089] In one exemplary embodiment, the polymerase conjugates of the invention have a polymerase domain derived from two parental polymerases, Pfu and DeepVent. Such polymerases are described for example in U.S. Application Publication Nos. 20040219558; 20040214194; 20040191825; 20030162173, each of which is hereby incorporated by reference in its entirety for all purposes and in particular for all teachings related to hybrid polymerases. In some embodiments, the polymerase is substantially (e.g., at least 60, 70, 80, 85, 90, or 95%) identical to SEQ ID NO:1 (optionally including a linker such as SEQ ID NO:4) or SEQ ID NO:2 or SEQ ID NO:61.

[0090] In some embodiments, a polymerase conjugate of the invention include a polymerase domain comprising mutations that reduce or abolish exonuclease activity of any hybrid polymerase comprising such a polymerase domain in comparison to a hybrid polymerase comprising a polymerase domain that does not have such mutations. A variety of mutations can be introduced into a native polymerase domain to reduce or eliminate 3'-5' exonuclease activity. For example, U.S. Pat. Nos. 6,015,668; 5,939,301 and 5,948,614 describe mutations of a metal-binding aspartate to an alanine residue in the 3'-5' exonuclease

domain of the Tma and Tne DNA polymerases. These mutations reduce the 3'-5' exonuclease activities of these enzymes to below detectable levels. Similarly, U.S. Pat. No. 5,882,904 describes an analogous aspartate-to-alanine mutation in *Thermococcus barossi*, and U.S. Pat. No. 5,489,523 teaches the double-mutant D141A E143A of the *Pyrococcus wosei* DNA polymerases. Both of these mutant polymerases have virtually no detectable 3'-5' exonuclease activity. Methods of assaying 3'-5' exonuclease activity are well-known in the art. *See, e.g.*, Freemont *et al.*, *Proteins* 1:66 (1986); Derbyshire et al., *EMBO J.* 16:17 (1991) and Derbyshire *et al.*, *Methods in Enzymology* 262:363 85 (1995). It will be understood that while the above-described mutations were originally identified in one polymerase, one can generally introduce such mutations into other polymerases to reduce or eliminate exonuclease activity.

[0091] In some embodiments, the polymerase conjugates of the present invention comprise a double point mutation (D141A/E143A) in the polymerase domain corresponding to the D141/E143 positions in SEQ ID NO:2. The phrase "corresponding to," in reference to polymerase amino acids, refers to an amino acid that aligns with the amino acid of interest (*e.g.*, D141 or E143) in a reference polymerase amino acid sequence (e.g., SEQ ID NO:2). Sequence comparisons can be performed using any BLAST including BLAST 2.2 algorithm with default parameters, described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389 3402 (1977) and Altschul *et al.*, *J. Mol. Biol.* 215:403 410 (1990), respectively. In some embodiments, the polymerase conjugates of the present invention comprise a polymerase polypeptide or domain that is substantially identical (*e.g.*, has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity) to an amino acid sequence of SEQ ID NO:2 (optionally including a linker such as SEQ ID NO:4) or SEQ ID NO:61.

B. Sso7-like proteins

[0092] Sso7d and Sac7d are small (about 7 kDa MW), basic chromosomal proteins from the hyperthermophilic archaeabacteria *Sulfolobus solfataricus* and *S. acidocaldarius*, respectively (*see, e.g.*, Choli et al., *Biochimica et Biophysica Acta* 950:193-203, 1988; Baumann et al., *Structural Biol.* 1:808-819, 1994; and Gao et al, *Nature Struc. Biol.* 5:782-786, 1998). These proteins bind DNA in a sequence-independent manner and when bound, increase the T_m of DNA by up to 40°C under some conditions (McAfee et al., Biochemistry 34:10063-10077, 1995). Sso7 proteins and their homologs are typically believed to be involved in stabilizing genomic DNA at elevated temperatures. Sso7d, Sac7d, Sac7e and related sequences (referred to herein as "Sso7 proteins" or "Sso7 domains") are known in the

art (*see, e.g.,* UniProt database accession numbers: P39476 (Sso7d); O59632 (Ssh7b); P13123 (Sac7d); P13125 (Sac7e); and Q96X56 (Sto7e)).

[0093] In some embodiments, the Sso7 domain or protein is a wild-type (*i.e.,* naturally occurring) Sso7 domain or protein having the amino acid sequence of SEQ ID NO:1. In some embodiments, Sso7 or Sso7-like domains or proteins are modified from the wild-type Sso7 by making one or more mutations in the Sso7 DNA binding domain.

[0094] In some embodiments, Sso7 or Sso7-like domains have amino acid changes from the native amino acid at the positions corresponding to K28 and/or R43 of SEQ ID NO:3. It should be understood that such position designations do not indicate the number of amino acids in the claimed molecule *per se*, but indicate where in the claimed molecule the residue occurs when the claimed molecule sequence is maximally aligned with SEQ ID NO:3. In the context of variant Sso7 domains, "correspondence" to a Sso7-like protein sequence is based on the convention of numbering according to amino acid position number of a particular sequence (i.e., SEQ ID NO:3) and then aligning the Sso7-like protein sequence in a manner that maximizes the percentage of sequence identity to SEQ ID NO:3. Alignment can be performed either manually or using a sequence comparison algorithm (e.g., using the NCBI BLAST program with default parameters (*see, e.g.,* Altschul *et al., Nucl. Acids Res.* 25:3389-3402, 1997). The corresponding sequences can be summarized as follows:

	Actual position of amino acid corresponding to K28 of SEQ ID NO:3	**Actual position of amino acid corresponding to R43 of SEQ ID NO:3**
Sso7	28	43
Ssh7b	28	43
Sto7e	28	42
Sac7d	28	42
Sac7e	28	42

[0095] Any Sso7 DNA binding protein domain can be substituted at the K28 and/or R43 position corresponding to SEQ ID NO:3. Thus, for example, in some embodiments, the variant Sso7 polypeptide sequence is substantially (*e.g.,* at least 60, 70, 80, 85, 90, or 95%) identical to any of, e.g., SEQ ID NOs:3, 57, 58, 59, or 60, and comprises an amino acid other than K at the amino acid position corresponding to K28. In some embodiments, the amino acid position corresponding to K28 is serine (S), threonine (T), cytosine (C), proline (P), aspartic acid (D), glutamic acid (E), asparagine (N), glutamine (Q), alanine (A),

phenylalanine (F), glycine (G), histidine (H), isoleucine (I), leucine (L), methionine (M), arginine (R), valine (V), tryptophan (W), or tyrosine (Y).

[0096] In some embodiments, the variant Sso7 polypeptide sequence is substantially (e.g., at least 60, 70, 80, 85, 90, or 95%) identical to any of, e.g., SEQ ID NOs:3, 57, 58, 59, or 60, and comprises an amino acid other than R at the amino acid position corresponding to R43. In some embodiments, the amino acid position corresponding to R43 is alanine (A), cytosine (C), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), valine (V), tryptophan (W), tyrosine (Y), or proline (P).

[0097] In some embodiments, the variant Sso7 polypeptide sequence is substantially (e.g., at least 60, 70, 80, 85, 90, or 95%) identical to any of, e.g., SEQ ID NOs:3, 57, 58, 59, or 60, and comprises an amino acid other than K at the amino acid position corresponding to K28 and an amino acid other than R at the amino acid position corresponding to R43. For example, in some embodiments, the amino acid at position K28 is selected from: serine (S), threonine (T), cytosine (C), proline (P), aspartic acid (D), glutamic acid (E), asparagine (N), glutamine (Q), alanine (A), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), leucine (L), valine (V), tryptophan (W), or tyrosine (Y) and the amino acid at position R43 is selected from: alanine (A), cytosine (C), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), valine (V), tryptophan (W), tyrosine (Y), or proline (P).

[0098] In some embodiments, the Sso7 domain or protein is identical or substantially identical (*e.g.,* has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity) to an amino acid sequence of any of SEQ ID NOs:5-30. In some embodiments, the Sso7 domain or protein has the amino acid sequence of any of SEQ ID NOs:5-30.

C. Conjugating the Sso7-like protein to the polymerase

[0099] Polymerase-Sso7 conjugates of the invention are generally produced by joining a polymerase domain to a Sso7 or Sso7-like DNA binding domain using chemical and/or recombinant methods.

[0100] Chemical methods of joining a DNA binding protein to a polymerase domain are described, e.g., in Bioconjugate Techniques, Hermanson, Ed., Academic Press (1996). These include, for example, derivitization for the purpose of linking the two proteins to each other,

either directly or through a linking compound, by methods that are well known in the art of protein chemistry. For example, in one chemical conjugation embodiment, the means of linking the catalytic domain and the DNA binding domain comprises a heterobifunctional-coupling reagent which ultimately contributes to formation of an intermolecular disulfide bond between the two moieties. Other types of coupling reagents that are useful in this capacity for the present invention are described, for example, in U.S. Patent No. 4,545,985. Alternatively, an intermolecular disulfide may conveniently be formed between cysteines in each moiety, which occur naturally or are inserted by genetic engineering. The means of linking moieties may also use thioether linkages between heterobifunctional crosslinking reagents or specific low pH cleavable crosslinkers or specific protease cleavable linkers or other cleavable or noncleavable chemical linkages.

[0101] The methods of linking a DNA binding domain, e.g., Sso7, and a polymerase domain may also comprise a peptidyl bond formed between moieties that are separately synthesized by standard peptide synthesis chemistry or recombinant means. The conjugate protein itself can also be produced using chemical methods to synthesize an amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, such as, e.g., the Merrifield solid phase synthesis method, in which amino acids are sequentially added to a growing chain of amino acids (see, Merrifield (1963) J. Am. Chem. Soc., 85:2149-2146). Equipment for automated synthesis of polypeptides is commercially available from suppliers such as PE Corp. (Foster City, Calif.), and may generally be operated according to the manufacturer's instructions. The synthesized peptides can then be cleaved from the resin, and purified, e.g., by preparative high performance liquid chromatography (see Creighton, Proteins Structures and Molecular Principles, 50-60 (1983)). The composition of the synthetic polypeptides or of subfragments of the polypeptide, may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, Proteins, Structures and Molecular Principles, pp. 34-49 (1983)).

[0102] In some embodiments, a DNA binding domain and polymerase domain can be joined via a linking group. The linking group can be a chemical crosslinking agent, including, for example, succinimidyl-(N-maleimidometh-yl)-cyclohexane-1-carboxylate (SMCC). The linking group can also be an additional amino acid sequence(s), including, for example, a polyalanine, polyglycine or similarly, linking group.

[0103] In some embodiments, the coding sequences of each polypeptide in a resulting fusion protein are directly joined at their amino- or carboxy-terminus via a peptide bond in any order. Alternatively, an amino acid linker sequence may be employed to separate the

first and second polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such an amino acid linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Typical peptide linker sequences contain Gly, Ser, Val and Thr residues. Other near neutral amino acids, such as Ala can also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea et al. (1985) Gene 40:39-46; Murphy et al. (1986) Proc. Natl. Acad. Sci. USA 83:8258-8262; U.S. Pat. Nos. 4,935,233 and 4,751,180, each of which is hereby incorporated by reference in its entirety for all purposes and in particular for all teachings related to linkers. The linker sequence may generally be from 1 to about 50 amino acids in length, e.g., 3, 4, 6, or 10 amino acids in length, but can be 100 or 200 amino acids in length. Linker sequences may not be required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference. In some embodiments, a linker sequence comprises the amino acid sequence of SEQ ID NO:4.

[0104] Other chemical linkers include carbohydrate linkers, lipid linkers, fatty acid linkers, polyether linkers, e.g., PEG, etc. For example, poly(ethylene glycol) linkers are available from Shearwater Polymers, Inc. Huntsville, Ala. These linkers optionally have amide linkages, sulfhydryl linkages, or heterobifunctional linkages.

[0105] Other methods of joining a DNA binding domain and polymerase domain include ionic binding by expressing negative and positive tails and indirect binding through antibodies and streptavidin-biotin interactions. (See, e.g., Bioconjugate Techniques, supra). The domains may also be joined together through an intermediate interacting sequence. For example, DNA binding domain-interacting sequence, i.e., a sequence that binds to a particular DNA binding domain (such as Sso7), can be joined to a polymerase. The resulting fusion protein can then be allowed to associate non-covalently with the DNA binding domain to generate a DNA-binding-domain-polymerase conjugate.

D. Producing polymerase-Sso7 conjugates

[0106] Polymerase-Sso7 conjugates of the invention can be produced using techniques known in the art. Methods for producing polymerases comprising a polymerase domain and a nucleic acid binding domain are described, for example, in U.S. Patent Application Publication Nos. 2006/005174; 2004/0219558; 2004/0214194; 2004/0191825; 2004/0081963; 2004/0002076; 2003/0162173; 2003/0148330; 2003/0138830 and U.S. Patent Nos. 6,627,424 and 7,445,898, each of which is hereby incorporated by reference in its entirety for all purposes and in particular for all teachings related to polymerases, hybrid/chimeric polymerases, as well as all methods for making and using such polymerases.

[0107] Nucleic acids encoding the polymerase or DNA binding domains can be obtained using routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook and Russell, Molecular Cloning, A Laboratory Manual (3rd ed. 2001); Kriegler, Gene Transfer and Expression: A Laboratory Manual (1990); and Current Protocols in Molecular Biology (Ausubel et al., eds., 1994-1999). Such nucleic acids may also be obtained through *in vitro* amplification methods such as those described herein and in Berger, Sambrook, and Ausubel, as well as Mullis et al., (1987) U.S. Pat. No. 4,683,202; PCR Protocols A Guide to Methods and Applications (Innis et al., eds) Academic Press Inc. San Diego, Calif. (1990) (Innis); Arnheim & Levinson (Oct. 1, 1990) C&EN 36-47; The Journal Of NIH Research (1991) 3: 81-94; Kwoh et al. (1989) Proc. Natl. Acad. Sci. USA 86: 1173; Guatelli et al. (1990) Proc. Natl. Acad. Sci. USA 87, 1874; Lomell et al. (1989) J. Clin. Chem., 35: 1826; Landegren et al., (1988) Science 241: 1077-1080; Van Brunt (1990) Biotechnology 8: 291-294; Wu and Wallace (1989) Gene 4: 560; and Barringer et al. (1990) Gene 89: 117, each of which is incorporated by reference in its entirety for all purposes and in particular for all teachings related to amplification methods.

[0108] One of skill will recognize that modifications can additionally be made to the polymerases of the present invention without diminishing their biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of a domain into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, the addition of codons at either terminus of the polynucleotide that encodes the binding domain to provide, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

[0109] The polymerases of the present invention can be expressed in a variety of host cells, including *E. coli*, other bacterial hosts, yeasts, filamentous fungi, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines. Techniques for gene expression in microorganisms are described in, for example, Smith, Gene Expression in Recombinant Microorganisms (Bioprocess Technology, Vol. 22), Marcel Dekker, 1994. Examples of bacteria that are useful for expression include, but are not limited to, *Escherichia*, *Enterobacter*, *Azotobacter*, *Erwinia*, *Bacillus*, *Pseudomonas*, *Klebsielia*, *Proteus*, *Salmonella*, *Serratia*, *Shigella*, *Rhizobia*, *Vitreoscilla*, and *Paracoccus*. Filamentous fungi that are useful as expression hosts include, for example, the following genera: *Aspergillus*, *Trichoderma*, *Neurospora*, *Penicillium*, *Cephalosporium*, *Achlya*, *Podospora*, *Mucor*, *Cochliobolus*, and *Pyricularia*. See, e.g., U.S. Pat. No. 5,679,543 and Stahl and Tudzynski, Eds., Molecular Biology in Filamentous Fungi, John Wiley & Sons, 1992. Synthesis of heterologous proteins in yeast is well known and described in the literature. Methods in Yeast Genetics, Sherman, F., et al., Cold Spring Harbor Laboratory, (1982) is a well recognized work describing the various methods available to produce the enzymes in yeast.

[0110] There are many expression systems for producing the polymerase polypeptides of the present invention that are well known to those of ordinary skill in the art. (See, e.g., Gene Expression Systems, Fernandex and Hoeffler, Eds. Academic Press, 1999; Sambrook and Russell, supra; and Ausubel et al, supra.) Typically, the polynucleotide that encodes the variant polypeptide is placed under the control of a promoter that is functional in the desired host cell. Many different promoters are available and known to one of skill in the art, and can be used in the expression vectors of the invention, depending on the particular application. Ordinarily, the promoter selected depends upon the cell in which the promoter is to be active. Other expression control sequences such as ribosome binding sites, transcription termination sites and the like are also optionally included. Constructs that include one or more of these control sequences are termed "expression cassettes." Accordingly, the nucleic acids that encode the joined polypeptides are incorporated for high level expression in a desired host cell.

[0111] Expression control sequences that are suitable for use in a particular host cell are often obtained by cloning a gene that is expressed in that cell. Commonly used prokaryotic control sequences, which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta-lactamase (penicillinase) and lactose (lac) promoter

systems (Change et al., Nature (1977) 198: 1056), the tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res. (1980) 8: 4057), the tac promoter (DeBoer, et al., Proc. Natl. Acad. Sci. U.S.A. (1983) 80:21-25); and the lambda-derived PL promoter and N-gene ribosome binding site (Shimatake et al., Nature (1981) 292: 128). The particular promoter system is not critical to the invention, any available promoter that functions in prokaryotes can be used. Standard bacterial expression vectors include plasmids such as pBR322-based plasmids, e.g., pBLUESCRIPT™, pSKF, pET23D, lambda-phage derived vectors, and fusion expression systems such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, e.g., c-myc, HA-tag, 6-His tag (SEQ ID NO:62), maltose binding protein, VSV-G tag, anti- DYKDDDDK (SEQ ID NO:63) tag, or any such tag, a large number of which are well known to those of skill in the art.

[0112] For expression in prokaryotic cells other than *E. coli*, a promoter that functions in the particular prokaryotic species is required. Such promoters can be obtained from genes that have been cloned from the species, or heterologous promoters can be used. For example, the hybrid trp-lac promoter functions in *Bacillus* sp. in addition to *E. coli*. These and other suitable bacterial promoters are well known in the art and are described, e.g., in Sambrook et al. and Ausubel et al. Bacterial expression systems for expressing the proteins of the invention are available in, e.g., *E. coli*, *Bacillus* sp., and *Salmonella* (Palva et al., Gene 22:229-235 (1983); Mosbach et al., Nature 302:543-545 (1983). Kits for such expression systems are commercially available.

[0113] Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. In yeast, vectors include Yeast Integrating plasmids (e.g., YIp5) and Yeast Replicating plasmids (the YRp series plasmids) and pGPD-2. Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, e.g., SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the CMV promoter, SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0114] Either constitutive or regulated promoters can be used in the present invention. Regulated promoters can be advantageous because the host cells can be grown to high densities before expression of the fusion polypeptides is induced. High level expression of

heterologous proteins slows cell growth in some situations. An inducible promoter is a promoter that directs expression of a gene where the level of expression is alterable by environmental or developmental factors such as, for example, temperature, pH, anaerobic or aerobic conditions, light, transcription factors and chemicals.

[0115] For *E. coli* and other bacterial host cells, inducible promoters are known to those of skill in the art. These include, for example, the lac promoter, the bacteriophage lambda P_L promoter, the hybrid trp-lac promoter (Amann et al. (1983) Gene 25: 167; de Boer et al. (1983) Proc. Nat'l. Acad. Sci. USA 80: 21), and the bacteriophage T7 promoter (Studier et al. (1986) J. Mol. Biol.; Tabor et al. (1985) Proc. Nat'l Acad. Sci. USA 82: 1074-8). These promoters and their use are also discussed in Sambrook et al., supra.

[0116] Translational coupling may be used to enhance expression. The strategy uses a short upstream open reading frame derived from a highly expressed gene native to the translational system, which is placed downstream of the promoter, and a ribosome binding site followed after a few amino acid codons by a termination codon. Just prior to the termination codon is a second ribosome binding site, and following the termination codon is a start codon for the initiation of translation. The system dissolves secondary structure in the RNA, allowing for the efficient initiation of translation. See Squires, et. al. (1988), J. Biol. Chem. 263: 16297-16302.

[0117] The construction of polynucleotide constructs generally requires the use of vectors able to replicate in bacteria. Such vectors are commonly used in the art. A plethora of kits are commercially available for the purification of plasmids from bacteria (for example, EasyPrep™, FlexiPrep™, from Pharmacia Biotech; StrataClean™, from Stratagene; and, QIAexpress® Expression System, Qiagen). The isolated and purified plasmids can then be further manipulated to produce other plasmids, and used to transform cells.

[0118] The polypeptides of the invention can be expressed intracellularly, or can be secreted from the cell. Intracellular expression often results in high yields. If necessary, the amount of soluble, active fusion polypeptide may be increased by performing refolding procedures (see, e.g., Sambrook et al., supra.; Marston et al., Bio/Technology (1984) 2: 800; Schoner et al., Bio/Technology (1985) 3: 151). Polypeptides of the invention can be expressed in a variety of host cells, including E. coli, other bacterial hosts, yeast, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines. The host cells can be mammalian cells, insect cells, or microorganisms, such as, for example, yeast cells, bacterial cells, or fungal cells.

[0119] Once expressed, the polypeptides can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see, generally, R. Scopes, Protein Purification, Springer-Verlag, N.Y. (1982), Deutscher, Methods in Enzymology Vol. 182: Guide to Protein Purification., Academic Press, Inc. N.Y. (1990)). Substantially pure compositions of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity are most preferred. Once purified, partially or to homogeneity as desired, the polypeptides may then be used (e.g., as immunogens for antibody production).

[0120] To facilitate purification of the polypeptides of the invention, the nucleic acids that encode the polypeptides can also include a coding sequence for an epitope or "tag" for which an affinity binding reagent is available. Examples of suitable epitopes include the myc and V-5 reporter genes; expression vectors useful for recombinant production of fusion polypeptides having these epitopes are commercially available (*e.g.,* Invitrogen (Carlsbad, CA) vectors pcDNA3.1/Myc-His and pcDNA3.1/V5-His are suitable for expression in mammalian cells). Additional expression vectors suitable for attaching a tag to the fusion proteins of the invention, and corresponding detection systems are known to those of skill in the art, and several are commercially available (*e.g.,* FLAG" (Kodak, Rochester, NY)). Another example of a suitable tag is a polyhistidine sequence, which is capable of binding to metal chelate affinity ligands. Typically, six adjacent histidines are used, although one can use more or less than six. Suitable metal chelate affinity ligands that can serve as the binding moiety for a polyhistidine tag include nitrilo-tri-acetic acid (NTA) (Hochuli, E. (1990) "Purification of recombinant proteins with metal chelating adsorbents" In Genetic Engineering: Principles and Methods, J. K. Setlow, Ed., Plenum Press, N.Y.; commercially available from Qiagen (Santa Clarita, CA)).

[0121] One of skill in the art will recognize that after biological expression or purification, the polymerase conjugates(s) may possess a conformation substantially different than the native conformations of the constituent polypeptides. In this case, it may be necessary or desirable to denature and reduce the polypeptide and then to cause the polypeptide to re-fold into the preferred conformation. Methods of reducing and denaturing proteins and inducing re-folding are well known to those of skill in the art (See, Debinski et al. (1993) J. Biol. Chem. 268: 14065-14070; Kreitman and Pastan (1993) Bioconjug. Chem. 4: 581-585; and Buchner et al. (1992) Anal. Biochem. 205: 263-270). Debinski et al., for example, describe the denaturation and reduction of inclusion body proteins in guanidine-DTE. The protein is then refolded in a redox buffer containing oxidized glutathione and L-arginine.

IV. Universal passive dyes for amplification controls

[0122] A universal pre-mix is provided that can be used for any type of real-time instrument or real-time amplification method without further addition of reagents aside from the sample to be tested and, in some embodiments, the primers to be used. Historically, different types of amplification instruments and methods used for quantitative PCR (qPCR) employed use of either high or low 5- or 6-carboxy-X-rhodamine dye concentrations as a passive reference dye for normalization and thus pre-mixes for use in qPCR were not "universal" because the pre-mixes either had low or high concentrations of 5- or 6-carboxy-X-rhodamine dye. If one wanted to use a high 5- or 6-carboxy-X-rhodamine dye concentration instrument with a low 5- or 6-carboxy-X-rhodamine dye concentration pre-mix, additional 5- or 6-carboxy-X-rhodamine dye had to be added to the pre-mix, thereby adding an additional step and possible introduction of error. However, it has been recently discovered (see, PCT/US2011/065617) that a single mix can be used for either instrument without addition of any reagents aside from the test sample itself and optionally, primers. Specifically, the amplification mixture comprises a fluorescent dye with a long Stokes-shift ("a second passive reference dye") as well as a low concentration of the first passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine dye). The fluorescent dye with a long Stokes-shift is selected such that the dye is excited at a wavelength significantly different than that of the first passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine dye has an excitation maximum at ~575 nm), but has an emission wavelength maximum substantially the same as the passive reference dye (5- or 6-carboxy-X-rhodamine dye has an emission wavelength maximum of ~620 nm). The concentration of the fluorescent dye with a long Stokes-shift is determined such that the combined signal of the fluorescent dye with a long Stokes-shift and the first passive reference dye (which can be, but is not limited to, 5- or 6-carboxy-X-rhodamine dye), in the mixture is sufficient for use in high concentration passive reference dye real-time amplification instruments and can be, for example, to subsequently normalize data. When used on a low concentration passive reference dye real-time amplification instrument, the fluorescent dye with a long Stokes-shift will not be excited in the passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine) channel, thus not generating any additional signal in the channel and so can be detected/used for passive reference dye normalization. The signal generated by the low concentration of passive reference dye present in the pre-mix is used for normalization instead. As a result, this pre-mix can be used on both "high-passive reference dye" and "low-passive reference dye" instruments.

[0123] It has now been discovered that POPSO, a buffer (when measured at a concentration of 0.1 M) having a change of no more than 0.027 pH units per degree C when between 20° and 37° C, is a useful buffer for stabilizing passive fluorescence dyes. For example, it was observed that POPSO provided the best stability at both low and high temperatures for dye DY-510XL (Dyomics, Jena, Germany), a fluorescent dye with a long Stokes-shift, and that other buffers Hepes, TAPS, Bicine and Tris provided less stability, in that order. Accordingly, reaction mixtures are provided that comprise a fluorescent dye with a long Stokes-shift as well as a low concentration of the first passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine dye), and a buffer that has a change of no more than 0.027 pH units per degree C when between 20° and 37° C (when measured at a concentration of 0.1 M). Buffers having a change of no more than 0.027 pH units per degree C when between 20° and 37° C are known. The pH change of buffers at different temperatures can be determined, for example, by measuring the pKa of the buffer at 20° and 37° C and determining the difference in the pKa values divided by the number of degrees difference (17 degrees). Exemplary buffers that have a change of no more than 0.027 pH units per degree C (when measured at a concentration of 0.1 M) include, but are not limited to, HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)), ACES (N-(2-Acetamido)-2-aminoethanesulfonic acid), PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid), MOPSO (3-(N-Morpholino)-2-hydroxypropanesulfonic Acid), BES (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic Acid), MOPS (3-(N-morpholino)propanesulfonic acid), TES (N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), TAPSO (3-[N-Tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic Acid), POPSO (Piperazine-N,N'-bis(2-hydroxypropanesulfonic acid)), BICINE (N,N-bis(2-hydroxyethyl)glycine), TAPS (N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid), and AMPSO (N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid).

[0124] The discovery that buffers having a change of no more than 0.027 pH units per degree C when between 20° and 37°C promote a particularly stable environment for fluorescent dyes with a long Stokes-shift is separate from the discovery that arginine and other agents improve PCR specificity. However, because the use of multiple dyes as a universal control find use in amplification reaction mixtures, in some embodiments, the reaction mixtures further comprises an agent that improves the specificity of nucleic acid amplification, including but not limited to, free arginine, spermidine, or spermine, in an amount to improve amplification specificity.

V. Amplification Methods

[0125] As described herein, the present invention provides compositions, including polymerase-Sso7 conjugates and agents selected from arginine, spermidine, and spermine, for use in nucleic acid amplification reactions. Such amplification reactions include without limitation polymerase chain reaction (PCR), DNA ligase chain reaction (LCR), QBeta RNA replicase, and RNA transcription-based (such as TAS and 3SR) amplification reactions as well as others known to those of skill in the art. Polymerase chain reactions that can be conducted using the compositions described herein include without limitation reverse-transcription PCR (rt-PCR) and quantitative PCR (qPCR).

[0126] The amplification methods of the present invention using polymerases (*e.g.*, polymerase-Sso7 conjugates) and agents selected from arginine, spermidine, and spermine are carried out using reaction mixtures that are sufficient for amplifying a nucleic acid molecule. In some embodiments, an amplification reaction mixture comprises, in addition to the polymerase-Sso7 conjugate and arginine, spermidine, or spermine, one or more of the following components: nucleotide triphosphates, one or more oligonucleotide primers, salt, buffer, water, stabilizer, and DNA-binding dye.

[0127] In some embodiments, an amplification reaction mixture of the present invention comprises: a polymerase (*e.g.*, a polymerase-Sso7 conjugate) as described herein at a concentration of about 1 U/ml to about 75 U/ml (*e.g.*, about 1 U/ml, 5 U/ml, 10 U/ml, 15 U/ml, 20 U/ml, 25 U/ml, 30 U/ml, 35 U/ml, 40 U/ml, 45 U/ml, 50 U/ml, 55 U/ml, 60 U/ml, 65 U/ml, 70 U/ml, or 75 U/ml); arginine, spermidine, or spermine or a salt thereof at a concentration of about 1 mM to about 100 mM (*e.g.*, about 1 mM, 5 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30mM, 35 mM, 40 mM, 45 mM, 50 mM, 55 mM, 60 mM, 65 mM, 70 mM, 75 mM, 80 mM, 85 mM, 90 mM, 95 mM, or 100 mM); dNTPs at a concentration of about 0.1 mM to about 10 mM (*e.g.*, about 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, or 10 mM); magnesium, *e.g.*, $MgCl_2$, at a concentration of about 1 mM to about 20 mM (*e.g.*, about 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, or 20 mM); $(NH_4)_2SO_4$ at a concentration of about 10 mM to about 100 mM (*e.g.*, about 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, or 100 mM); potassium, *e.g*, KCl, at a concentration of about 50 mM to about 200 mM (*e.g.*, about 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 110 mM, 120 mM, 130 mM, 140 mM, 150 mM, 160 mM, 170 mM, 180 mM, 190 mM, or 200 mM); a buffer, *e.g.*, Tris pH 8.5-9.5 at a concentration of about 50

mM to about 200 mM (*e.g.,* about 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 110 mM, 120 mM, 130 mM, 140 mM, 150 mM, 160 mM, 170 mM, 180 mM, 190 mM, or 200 mM) or a buffer (when measured at a concentration of 0.1 M) that has a change of no more than 0.027 pH units per degree C when between 20° and 37° C. at a concentration of about 5 mM to about 200 mM (*e.g.,* about 5 mM, 10 mM, 25 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 110 mM, 120 mM, 130 mM, 140 mM, 150 mM, 160 mM, 170 mM, 180 mM, 190 mM, or 200 mM); a disaccharide, *e.g.,* trehalose, at a concentration of about 100 mM to about 500 mM (*e.g.,* about 100 mM, 125 mM, 150 mM, 175 mM, 200 mM, 225 mM, 250 mM, 275 mM, 300 mM, 325 mM, 350 mM, 375 mM, 400 mM, 425 mM, 450 mM, 475 mM, or 500 mM); one or more osmolytes, *e.g,* sarcosine, trimethylamine N-oxide (TMAO), dimethylsulfoniopropionate, and trimethylglycine, at a concentration of about 50 mM to about 200 mM (*e.g.,* about 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 110 mM, 120 mM, 130 mM, 140 mM, 150 mM, 160 mM, 170 mM, 180 mM, 190 mM, or 200 mM); Tween-20 at a concentration of about 0.1% to about 0.5% (*e.g.,* about 0.1%, 0.2%, 0.3%, 0.4%, or 0.5%); glycerol at a concentration of about 1% to about 10% (*e.g.,* about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%); DMSO at a concentration of about 1% to about 10% (*e.g.,* about 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, 6%, 6.5%, 7%, 7.5%, 8%, 8.5%, 9%, 9.5%, or 10%); fluorescein at a concentration of about 0.001% to about 0.01% (*e.g.,* about 0.001%, 0.002%, 0.003%, 0.004%, 0.005%, 0.006%, 0.007%, 0.008%, 0.009%, or 0.01%); and DNA binding dye (e.g., cyanine dye) at a concentration of about 0.5X to about 5X (*e.g.,* about 0.5X, 0.6X, 0.7X, 0.8X, 0.9X, 1X, 1.5X, 2X, 2.5X, 3X, 3.5X, 4X, 4.5X, or 5X).

[0128] It has been discovered that inclusion of POPSO in an amplification reaction can improve amplification specificity, and based on this discovery, it is believed that any buffer (e.g., POPSO) having a change of no more than 0.027 pH units per degree C when between 20° and 37° C will have the same effect. Thus, the reaction mixtiures described above can include, e.g., HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)), ACES (N-(2-Acetamido)-2-aminoethanesulfonic acid), PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid), MOPSO (3-(N-Morpholino)-2-hydroxypropanesulfonic Acid), BES (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic Acid), MOPS (3-(N-morpholino)propanesulfonic acid), TES (N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), TAPSO (3-[N-Tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic Acid), POPSO (Piperazine-N,N'-bis(2-hydroxypropanesulfonic acid)), BICINE (N,N-bis(2-hydroxyethyl)glycine), TAPS

(N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid), or AMPSO (N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid).

[0129] Further, methods for using increased amount of polymerase with adverse effects have been discovered. Previously, one of skill in the art would not have added polymerase to an amplification reaction mixture at a concentration of more than about 20 U/ml due to the detrimental effects of non-specific binding that can result from high concentrations of polymerase. Surprisingly, the inventors have found that the addition of an agent selected from arginine, spermidine, and spermine, or a salt thereof, increases the specificity of polymerase-Sso7 conjugates even at high concentrations of polymerase. Thus, in some embodiments, a reaction mixture of the present invention comprises an agent selected from arginine, spermidine, and spermine, or a salt thereof, at a concentration of about 1 mM to about 100 mM and a polymerase-Sso7 conjugate at a concentration of more than 50 U/ml, more than 55 U/ml, more than 60 U/ml, more than 65 U/ml, more than 70 U/ml, or more than 75 U/ml.

[0130] Improvements in efficiency and specificity due to certain aspects of the present invention can be identified and quantified using assays known in the art and described in further detail below.

[0131] In some embodiments, dye-based qPCR detection methods are used to monitor amplification reactions utilizing components of the invention. Such detection methods generally rely on monitoring the increase in fluorescence signal due to the binding of DNA-binding dye to the amplified DNA. For example, SYBR Green I, a commonly used fluorescent DNA binding dye, binds all double–stranded DNA and detection is monitored by measuring the increase in fluorescence throughout the amplification cycles. SYBR Green I has an excitation and emission maxima of 494 nm and 521 nm, respectively.

[0132] In other embodiments, probe-based qPCR detection methods are used to monitor amplification reactions utilizing components of the invention. Such detection methods generally rely on the sequence-specific detection of a desired PCR product. Unlike dye-based qPCR methods that detect all double-stranded DNA, probe-based qPCR utilizes a fluorescent-labeled target-specific probe, which detects specific sequences in the amplified DNA.

Additives for improving efficiency

[0133] In certain aspects, it may be desirable to include an additional compound as an additive to improve efficiency in amplification reactions, including but not limited to qPCR. In some embodiments, inclusion of the additive is sufficient to increase efficiency of the

polymerase conjugate by at least 5, 10, 15, 20, 25, 35, 40, or 50% or more compared to a control mixture lacking the additive.

[0134] In some embodiments, a polymerase conjugate of the invention exhibits low efficiency for certain targets when used in a formulation that includes certain binding dyes (such as, for example, an EvaGreen DNA binding dye). Such low efficiency may in some embodiments result in a delay of Ct values associated with low input DNA concentrations. Methods for measuring efficiency of a particular reaction are known in the art and described in further detail below.

[0135] In some embodiments, the additive is an osmolyte included in an amplification reaction of the invention to improve efficiency. Members of the osmolyte family have been shown to improve the thermal stability of proteins (Santoro, Biochemistry, 1992) as well as decrease DNA double helix stability (Chadalavada, FEBS Letters, 1997). In some embodiments, osmolytes are small molecules or compounds which are produced by living organisms in response to environmental stresses such as extreme temperatures, dehydration, or salinity and which protect their cellular components and help to maintain optimal cytosolic conditions. Osmolytes of use in the present invention may include without limitation sarcosine, trimethylamine N-oxide (TMAO), dimethylsulfoniopropionate, and trimethylglycine. Sarcosine is chemically similar to betaine, a chemical which has been shown to improve conventional PCR (Henke, Nucleic Acids Research, 1997).

[0136] In conventional uses of osmolytes, the stabilizing effects of such compounds are generally observed at relatively high concentrations (> 1M). However, in methods of the present invention, millimolar concentrations of osmolytes have been found to be effective for improving the reaction efficiency of amplification reactions such as qPCR. Without being bound by a mechanism of action, it is possible that the improvement in efficiency is the result of improving the accessibility of the DNA polymerase to the targeted region of the DNA template for reactions that contain low concentrations of input DNA sample. In some embodiments, concentrations of about 100 to about 1000 mM of osmolytes are used in methods and kits of the present invention. In still further embodiments, concentrations of about 50 to about 700, about 100 to about 600, about 150 to about 500, about 200 to about 400 mM, and about 300 to about 350 mM osmolytes are used in methods and kits of the invention. In some embodiments, the osmolyte used in methods, reaction mixtures, and kits of the invention is sarcosine (optionally at the above-listed concentrations).

VI. Reaction Mixtures

[0137] In another aspect, the present invention provides reaction mixtures comprising a polymerase (*e.g.*, a polymerase-Sso7 conjugate) and a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent is selected from arginine, spermidine, and spermine, or a salt thereof. The reaction mixtures can optionally comprise a biological sample comprising a target nucleic acid, one or more oligonucleotides, buffers, nucleotide triphosphates, salts, stabilizers, one or more additives for improving efficiency, nuclease-free water, and/or a double stranded DNA binding dye. In some embodiments, the reaction mixtures comprise a buffer (e.g., POPSO) having a change of no more than 0.027 pH units per degree C when between 20° and 37° C. In some embodiments, the reaction mixtures comprise a fluorescent dye with a long Stokes-shift as well as a low concentration of the first passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine dye).

[0138] In some embodiments, a reaction mixture of the present invention comprises a polymerase-Sso7 conjugate at a concentration of about 1 U/ml to about 40 U/ml and an agent selected from arginine, spermidine, and spermine, or a salt thereof, at a concentration of about 1 mM to about 100 mM. In some embodiments, a reaction mixture of the present invention comprises a polymerase-Sso7 conjugate at a concentration of about 5 U/ml to about 40 U/ml and an agent selected from arginine, spermidine, and spermine, or a salt thereof, at a concentration of about 1 mM to about 50 mM. In some embodiments, a reaction mixture of the present invention comprises a polymerase-Sso7 conjugate at a concentration of about 10 U/ml to about 50 U/ml and an agent selected from arginine, spermidine, and spermine, or a salt thereof, at a concentration of about 3 mM to about 30 mM.

VII. Kits

[0139] In another aspect, the present invention provides kits for conducting nucleic acid amplification reactions. In some embodiments, the kits include a polymerase (*e.g.*, a polymerase-Sso7 conjugate) and a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent is selected from arginine, spermidine, and spermine, or a salt thereof. In some embodiments, kits of the invention include a polymerase conjugate having a polymerase domain that is substantially identical or identical to SEQ ID NO:1 or SEQ ID NO:2. In some embodiments, kits of the invention include a polymerase conjugate comprising a Sso7 domain that is substantially identical or identical to any of SEQ ID NOs:3 or 5-30. In some embodiments, kits of the invention include a polymerase conjugate that is substantially identical or identical to any of SEQ ID NOs:31-56.

[0140] Optionally, the kits comprise one or more dNTPs, at least one buffer (e.g., a buffer (e.g., POPSO) having a change of no more than 0.027 pH units per degree C when between 20° and 37° C), and/or a double stranded DNA binding dye. In some embodiments, the kits comprise a fluorescent dye with a long Stokes-shift as well as a low concentration of the first passive reference dye (e.g., 5- or 6-carboxy-X-rhodamine dye). Such kits may also include stabilizers and other additives (*e.g.,* sarcosine) to increase the efficiency of the amplification reactions. Such kits may also include one or more primers as well as instructions for conducting nucleic acid amplification reactions using the components of the kits.

EXAMPLES

[0141] The following examples are intended to illustrate, but not to limit, the claimed invention.

Example 1

[0142] A series of Sso7d-polymerase conjugates were generated based on SEQ ID NO:2 for the polymerase domain and SEQ ID NO:3 as the Sso7d domain. The Sso7d domain was mutated at positions 28 (wild-type K) or 43 (wild-type R). The resulting conjugates were tested by amplifying a template DNA molecule and detecting the resulting product with SYBR GREEN using melting temperature analysis. A two-step PCR was performed to amplify 18s amplicon (18s68) using 1 ng of HeLa cell derived cDNA as template. qPCR was performed on Bio-Rad CFX96 qPCR instrument using regular 2-step PCR protocol with 5s denaturation step at 95°C and followed by 30s anneal-extension step at 61°C in each amplification cycle. Forty cycles of PCR amplication were performed and followed by melt-curve analysis using 0.5°C temperature increments with 5s hold in each step. The melt-curve analysis was used to evaluate PCR specificity, which is a technique to characterize double-stranded DNA (dsDNA) based on their dissociation (melting) behavior as they transition from dsDNA to single-stranded DNA (ssDNA) with increasing temperature (Tm). In general, target sequence was amplified by PCR prior to melt-curve analysis. According to the nucleotide sequence, length, GC content, and strand complementarity, melting of PCR products will give a single peak of specific melting temperature. Therefore, single melt-peak indicates one specific PCR product. Multiple peaks indicates the presence of non-specific products in addition to the specific one.

[0143] While the wild-type conjugate generated considerable non-specific products (*i.e.,* two peaks in melt-curve analysis), a number of substitutions at the position corresponding to

K28 resulted in improved activity, *i.e.,* reduced non-specific polymerase activity. Mutants in which R28 was changed to any of S, T, C, P, D, E, N, or Q had improved activity (specificity) compared to the R28 wildtype. R28M and R28R were not significantly better than wildtype. The K28 variant Sso7d-polymerase conjugates tested included those having sequences according to SEQ ID NOs:31-38.

[0144] A two-step PCR was then performed to amplify beta-Actin amplicon (ActB86), using 1 ng of HeLa cell derived cDNA as template and under the same conditions as described above to evaluate PCR specificity for R43 variant Sso7d-polymerase conjugates. A number of substitutions resulted in decreased nonspecific polymerase activity. For example, mutants in which R43 was changed to any of G, A, S, T, C, V, L, I, M, F, Y, D, E, N, Q, H, K, or W had improved activity (specificity) compared to the R43 wildtype. R43M had a slight non-specific shoulder, but was still significantly improved compared to wildtype. The R43 variant Sso7-polymerase conjugates tested included those having sequences according to SEQ ID NOs:39-56.

[0145] In addition, it was discovered that non-specific polymerase activity can also be reduced by adding reagents to the PCR reaction mixture. PCR was performed to amplify 18s amplicon (18s68) using 10 ng, 1 ng, 100 pg, and 10 pg of HeLa cell derived cDNA as template. qPCR was performed using a regular 2-step PCR protocol with 5s denaturation step at 98°C and followed by 30s anneal-extension step at 60°C in each amplification cycle. Forty cycles of PCR amplifications were carried out and followed by melt-curve analysis as described above. The addition of L-Arginine monohydrochloride (10 mM), spermidine trihydrochloride (5 mM), or spermine tetrahydrochloride (5 or 10 mM) further reduced non-specific activity of the Sso7d-polymerase conjugate variants tested. This was further demonstrated in qPCR results using reaction mixtures containing 10 mM L-arginine monohydrochloride at different template concentrations (from 10 ng to 10 pg). Compared to the controls lacking free arginine, the corresponding reactions containing free arginine had more specific product and considerably less non-specific product at each template concentration.

[0146] Next, the effect of arginine in enhancing inhibitor tolerance of qPCR reagent mixture was tested. Amplification reaction mixtures contained an exonuclease deficient polymerase (SEQ ID NO:2) conjugated to a mutated Sso7d domain (SEQ ID NO:34) at a final concentration of about 24 U/ml and a polymerase inhibitor (one of two different chocolates (Enlveonet or Tanzanie), a common PCR inhibitor). Arginine was omitted from one set of samples and added at a concentration of 10 mM to another set of samples. The

final percentage concentration of chocolate in the qPCR reaction ranged from 0-2%. PCR was performed to amplify ADAR amplicon (ADAR_162) using 1 ng of HeLa cell derived cDNA as template. qPCR was performed using regular 2-step PCR protocol with 5s denaturation step at 95°C and followed by 30s anneal-extension step at 60°C in each amplification cycle. Forty cycles of PCR amplifications were carried out and followed by melt-curve analysis as described above. Success of PCR amplification (as reflected by an amplification and also evaluated by the Ct value) in the presence of different concentrations of inhibitor indicates how well the reaction mixture tolerates the PCR inhibitor. For both chocolates, in the presence of higher concentrations of inhibitor, poor or no amplification was observed for the reagent mixture in the absence of arginine. In contrast, reagent mixture supplemented with arginine still exhibited good PCR amplification even in the presence of 2% inhibitor, suggesting higher inhibitor tolerance.

Example 2

[0147] The effect of POPSO buffer in improving the specificity of a generic qPCR supermix in detecting miRNA was determined. Detection and quantification of microRNA (miR223) and the control snoRNA (RNU48) were determined by first converting mi/snoRNA into cDNAs, which were then quantified by real-time qPCR using a hybrid fusion polymerase that has a mutant Sso7d domain and does not have exo nuclease activity. In this experiment, pooled total human RNAs (purchased from Ambion) was first converted into cDNAs by a universal polyadenylation and reverse-transcription process. The synthesized cDNAs were then used as template for mi/snoRNA detection and quantification by using pre-designed target-specific primer-pairs, and qPCR supermix build with either Tris-buffer or POPSO-buffer (the rest of components of the qPCR supermix were the same). As demonstrated in the miR233 assay, the Tris-based formulation produced non-specific amplification and multiple peaks during the meltcurve analysis. In contrast, the POPSO-based formulation produced a PCR product with a clean single meltpeak, which shows that specific amplification occurred. Other than the specificity improvement, there was no significant performance difference between the POPSO-based formulation versus the Tris-based formulation. Both formulations showed comparable Cq value and clean meltpeak on the control assay, RNU48 snoRNA.

[0148] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be

suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

INFORMAL SEQUENCE LISTING

SEQ ID NO:1

Amino acid sequence of DNA polymerase having 3'-5' exo nuclease activity

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFDIETLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKS

SEQ ID NO:2

Amino acid sequence of DNA polymerase lacking 3'-5' exo nuclease activity

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKS

SEQ ID NO:3

Sso7d/Ssh7A/SsoP2

>gi |3891427|pdb|1BNZ|A Chain A, Hyperthermophile ProteinDNA COMPLEX
MATVKFKYKGEEKEVDISKIKKVWRVGKMISFTYDEGGGKTGRGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:4

Linker sequence

GTGGGG

SEQ ID NO:5

Sso7d-K28S

MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTGRGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:6
Sso7d-K28T
MATVKFKYKGEEKEVDISKIKKVWRVG**T**MISFTYDEGGGKTGRGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:7
Sso7d-K28C
MATVKFKYKGEEKEVDISKIKKVWRVG**C**MISFTYDEGGGKTGRGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:8
Sso7d-K28P
MATVKFKYKGEEKEVDISKIKKVWRVG**P**MISFTYDEGGGKTGRGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:9
Sso7d-K28D
MATVKFKYKGEEKEVDISKIKKVWRVG**D**MISFTYDEGGGKTGRGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:10
Sso7d-K28E
MATVKFKYKGEEKEVDISKIKKVWRVG**E**MISFTYDEGGGKTGRGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:11
Sso7d-K28N
MATVKFKYKGEEKEVDISKIKKVWRVG**N**MISFTYDEGGGKTGRGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:12
Sso7d-K28Q
MATVKFKYKGEEKEVDISKIKKVWRVG**Q**MISFTYDEGGGKTGRGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:13
Sso7d-R43G
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**G**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:14
Sso7d-R43A
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**A**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:15
Sso7d-R43S
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**S**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:16
Sso7d-R43T
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**T**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:17
Sso7d-R43C
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**C**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:18
Sso7d-R43V
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**V**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:19
Sso7d-R43L
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**L**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:20
Sso7d-R43I
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**I**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:21
Sso7d-R43M
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**M**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:22
Sso7d-R43F
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**F**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:23
Sso7d-R43Y
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**Y**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:24
Sso7d-R43W
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**W**GAVSEKDAPKEL
LQMLEKQKK

SEQ ID NO:25
Sso7d-R43D
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTG**D**GAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:26

Sso7d-R43E
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTGEGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:27
Sso7d-R43N
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTGNGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:28
Sso7d-R43Q
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTGQGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:29
Sso7d-R43H
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTGHGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:30
Sso7d-R43K
MATVKFKYKGEEKEVDISKIKKVWRVGSMISFTYDEGGGKTGKGAVSEKDAPKELL
QMLEKQKK

SEQ ID NO:31
Exo- polymerase + linker + Sso7d-K28S
MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGRGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:32
Exo- polymerase + linker + Sso7d-K28T
MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT

KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVG**T**M
ISFTYDEGGGKTGRGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:33

Exo- polymerase + linker + Sso7d-K28C

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVG**C**M
ISFTYDEGGGKTGRGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:34

Exo- polymerase + linker + Sso7d-K28P

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVG**P**MI
SFTYDEGGGKTGRGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:35

Exo- polymerase + linker + Sso7d-K28D

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG

NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVG**D**M
ISFTYDEGGGKTGRGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:36

Exo- polymerase + linker + Sso7d-K28E

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVG**E**M
ISFTYDEGGGKTGRGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:37

Exo- polymerase + linker + Sso7d-K28N

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVG**N**M
ISFTYDEGGGKTGRGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:38

Exo- polymerase + linker + Sso7d-K28Q

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS

EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGQM
ISFTYDEGGGKTGRGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:39

Exo- polymerase + linker + Sso7d-R43G

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTG[redacted]GAVSEKDAPKELLQMLEKQKK

SEQ ID NO:40

Exo- polymerase + linker + Sso7d-R43A

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTG[redacted]GAVSEKDAPKELLQMLEKQKK

SEQ ID NO:41

Exo- polymerase + linker + Sso7d-R43S

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK

RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGSGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:42
Exo- polymerase + linker + Sso7d-R43T
MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGTGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:43
Exo- polymerase + linker + Sso7d-R43C
MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGCGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:44
Exo- polymerase + linker + Sso7d-R43V

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGXGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:45

Exo- polymerase + linker + Sso7d-R43L

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGLGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:46

Exo- polymerase + linker + Sso7d-R43I

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGIGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:47

Exo- polymerase + linker + Sso7d-R43M

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGMGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:48

Exo- polymerase + linker + Sso7d-R43F

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGFGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:49

Exo- polymerase + linker + Sso7d-R43Y

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE

DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGWGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:50

Exo- polymerase + linker + Sso7d-R43W

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGWGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:51

Exo- polymerase + linker + Sso7d-R43D

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGDGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:52

Exo- polymerase + linker + Sso7d-R43E

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE

EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGIGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:53

Exo- polymerase + linker + Sso7d-R43N

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGNGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:54

Exo- polymerase + linker + Sso7d-R43Q

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGQGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:55

Exo- polymerase + linker + Sso7d-R43H

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
ALYPSIIITHNVSPDTLNREGCRNYDVAPEVGHKFCKDFPGFIPSLLKRLLDERQKIKT
KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI

EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGKGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:56
Exo- polymerase + linker + Sso7d-R43K

MILDADYITEEGKPVIRLFKKENGEFKIEHDRTFRPYIYALLKDDSKIEEVKKITAERH
GKIVRIVDAEKVEKKFLGRPITVWRLYFEHPQDVPTIREKIREHSAVVDIFEYDIPFAK
RYLIDKGLIPMEGDEELKLLAFAIATLYHEGEEFGKGPIIMISYADEEEAKVITWKKID
LPYVEVVSSEREMIKRFLKIIREKDPDIIITYNGDSFDLPYLAKRAEKLGIKLTIGRDGS
EPKMQRIGDMTAVEVKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADEI
AKAWETGEGLERVAKYSMEDAKATYELGKEFFPMEAQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNELAPNKPDEREYERRLRESYAGGFVKEPEKGLWENIVSLDFR
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KMKASQDPIEKIMLDYRQRAIKILANSYYGYYGYAKARWYCKECAESVTAWGREYI
EFVWKELEEKFGFKVLYIDTDGLYATIPGGKSEEIKKKALEFVDYINAKLPGLLELEY
EGFYKRGFFVTKKKYALIDEEGKIITRGLEIVRRDWSEIAKETQARVLEAILKHGNVE
EAVRIVKEVTQKLSKYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKRLAAKGVKIKP
GMVIGYIVLRGDGPISNRAILAEEYDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQTGLTSWLNIKKSGTGGGGATVKFKYKGEEKEVDISKIKKVWRVGSMI
SFTYDEGGGKTGKGAVSEKDAPKELLQMLEKQKK

SEQ ID NO:57
Ssh7b
>gi |3138797|dbj|BAA28275.1| [Sulfolobus shibatae]
MVTVKFKYKGEEKEVDTSKIKKVWRVGKMISFTYDEGGGKTGRGAVSEKDAPKEL
LQMLEKQKK

SEQ ID NO:58
Sac7d
>gi |152933|gb|AAA80315.1| DNA-binding protein [Sulfolobus sp.]
MVKVKFKYKGEEKEVDTSKIKKVWRVGKMVSFTYDDNGKTGRGAVSEKDAPKEL
LDMLARAEREKK

SEQ ID NO:59
Sac7e
>gi |70606201|ref|YP_255071.1| DNA-binding protein 7e [Sulfolobus acidocaldarius DSM 639]
MAKVRFKYKGEEKEVDTSKIKKVWRVGKMVSFTYDDNGKTGRGAVSEKDAPKEL
MDMLARAEKKK

SEQ ID NO:60
Sto7e
>gi |15920860|ref|NP_376529.1| DNA-binding protein 7e [Sulfolobus tokodaii str. 7]
MVTVKFKYKGEEKEVDISKIKKVWRVGKMISFTYDDNGKTGRGAVSEKDAPKELLQ
MLEKSGKK

SEQ ID NO:61 (polymerase sequence)

MILDADYITEEGKPVIRLFKKENGKFKIEHDRTFRPYIYALLKDDSKIDEVRKITGERH
GKIVRIVDAEKVEKKFLGRPIEVWKLYLEHPQDVPAIRDKVREHPAVVDIFEYDIPFA
KRYLIDKGLIPMEGEEELKILAFDIETLYHEGEEFGKGPIIMISYADENEAKVITWKNID
LPYVEVVSSEREMIKRFLRVIREKDPDVIITYNGDSFDFPYLVKRAEKLGIKLTIGRDG
SEPKMQRLGDMTAVEIKGRIHFDLYHVIRRTINLPTYTLEAVYEAIFGKPKEKVYADE
IAEAWESGEGLERVAKYSMEDAKVTYELGKEFLPMEIQLSRLVGQPLWDVSRSSTG
NLVEWFLLRKAYERNEVAPNKPSEEEYERRLRESYAGGYVKEPEKGLWENIVSLDF
RSLYPSIIITHNVSPDTLNREGCREYDIAPEVGHKFCKDFPGFIPSLLKHLLEERQKIKT
KMKESQDPIEKKMLDYRQRAIKILANSFYGYYGYAKARWYCKECAESVTAWGRKY
IEFVWKELEEKFGFKVLYIDTDGLYATIPGGEPEEIKKKALEFVKYINSKLPGLLELEY
EGFYVRGFFVTKKRYAVIDEEGKVITRGLEIVRRDWSEIAKETQARVLETILKHGNVE
EAVKIVKEVTQKLANYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKKLAARGVKIKP
GMVIGYIVLRGDGPISKRAILAEEFDPRKHKYDAEYYIENQVLPAVLRILEGFGYRKE
DLRWQKTKQVGLTSWLNIKKS

WHAT IS CLAIMED IS:

1. A method of amplifying a nucleic acid molecule, the method comprising:

(a) providing the nucleic acid in a composition sufficient for nucleic acid amplification, the composition comprising:

(i) one or more oligonucleotide primers;

(ii) a polymerase; and

(iii) a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises free arginine, spermidine, or spermine, or a salt thereof; and

(b) incubating the mixture under conditions sufficient for amplifying the nucleic acid, thereby amplifying the nucleic acid.

2. The method of claim 1, wherein the polymerase is conjugated to a DNA binding domain.

3. The method of claim 2, wherein the DNA binding domain is a Sso7 domain.

4. The method of claim 1, wherein the agent is present in an amount sufficient to increase by at least 10% the relative yield of a specific amplification product as compared to a non-specific amplification product.

5. The method of claim 1, wherein the agent is free arginine or a salt thereof.

6. The method of claim 5, wherein the concentration of the free arginine or the free arginine salt is from about 1 mM to about 500 mM.

7. The method of claim 1, wherein the polymerase substantially lacks a 3'-5' exonuclease activity.

8. The method of claim 1, wherein the polymerase has the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

9. The method of claim 3, wherein the Sso7 domain has at least 75% amino acid sequence identity to SEQ ID NO:3.

10. The method of claim 1, wherein the composition comprises a buffer (when measured at a concentration of 0.1 M) that has a change of no more than 0.027 pH units per degree C when between 20° and 37° C.

11. The method of claim 10, wherein the buffer is selected from the group consisting of HEPES, ACES, PIPES, MOPSO, BES, MOPS, TES, TAPSO, POPSO, BICINE, TAPS, and AMPSO.

12. A method of amplifying a nucleic acid, the method comprising:

(a) providing the nucleic acid with a composition sufficient for nucleic acid amplification, the composition comprising:

(i) one or more oligonucleotide primers; and

(ii) a polymerase, wherein the polymerase is present at a concentration of at least 20 units/reaction; and

(b) incubating the nucleic acid in the composition under conditions sufficient for amplifying the nucleic acid, thereby amplifying the nucleic acid.

13. The method of claim 12, wherein the polymerase is conjugated to a DNA binding domain.

14. The method of claim 13, wherein the DNA binding domain is a Sso7 domain.

15. The method of claim 12, wherein the composition further comprises free arginine or a salt thereof.

16. The method of claim 15, wherein the concentration of the free arginine or the free arginine salt is from about 1 mM to about 500 mM.

17. The method of claim 12, wherein the polymerase substantially lacks a 3'-5' exonuclease activity.

18. The method of claim 12, wherein the polymerase has the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

19. The method of claim 14, wherein the Sso7 domain has at least 75% amino acid sequence identity to SEQ ID NO:2.

20. The method of claim 12, wherein the composition comprises a buffer (when measured at a concentration of 0.1 M) that has a change of no more than 0.027 pH units per degree C when between 20° and 37° C.

21. The method of claim 20, wherein the buffer is selected from the group consisting of HEPES, ACES, PIPES, MOPSO, BES, MOPS, TES, TAPSO, POPSO, BICINE, TAPS, and AMPSO.

22. A reaction mixture comprising:

a polymerase; and

a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises free arginine, spermidine, or spermine, or a salt thereof.

23. The reaction mixture of claim 22, wherein the polymerase is conjugated to a DNA binding domain.

24. The reaction mixture of claim 23, wherein the DNA binding domain is a Sso7 domain.

25. The reaction mixture of claim 22, wherein the agent is present in an amount sufficient to increase by at least 10% the relative yield of a specific amplification product as compared to a non-specific amplification product.

26. The reaction mixture of claim 22, wherein the agent is free arginine or a salt thereof.

27. The reaction mixture of claim 26, wherein the concentration of the free arginine or the free arginine salt is from about 1 mM to about 500 mM.

28. The reaction mixture of claim 22, wherein the polymerase substantially lacks a 3'-5' exonuclease activity.

29. The reaction mixture of claim 22, wherein the polymerase has the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

30. The reaction mixture of claim 24, wherein the Sso7 domain has at least 75% amino acid sequence identity to SEQ ID NO:2.

31. The reaction mixture of claim 22, further comprising one or more oligonucleotide primers.

32. The reaction mixture of claim 22, further comprising at least one member selected from the group consisting of a buffer, nucleotide triphosphates, a salt, a stabilizer, a double stranded DNA binding dye, and nuclease-free water.

33. The method of claim 22, wherein the reaction mixture comprises a buffer (when measured at a concentration of 0.1 M) that has a change of no more than 0.027 pH units per degree C when between 20° and 37° C.

34. The method of claim 33, wherein the buffer is selected from the group consisting of HEPES, ACES, PIPES, MOPSO, BES, MOPS, TES, TAPSO, POPSO, BICINE, TAPS, and AMPSO.

35. A kit comprising:

a polymerase; and

a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises free arginine, spermidine, or spermine, or a salt thereof.

36. The kit of claim 35, wherein the polymerase is conjugated to a DNA binding domain.

37. The kit of claim 36, wherein the DNA binding domain is a Sso7 domain.

38. The kit of claim 35, wherein the agent is present in an amount sufficient to increase by at least 10% the relative yield of a specific amplification product as compared to a non-specific amplification product.

39. The kit of claim 35, wherein the agent is arginine.

40. The kit of claim 39, wherein the concentration of free arginine is from about 1 mM to about 500 mM.

41. The kit of claim 35, wherein the polymerase substantially lacks a 3'-5' exonuclease activity.

42. The kit of claim 35, wherein the polymerase has the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

43. The kit of claim 37, wherein the Sso7 domain has at least 75% amino acid sequence identity to SEQ ID NO:2.

44. The kit of claim 35, further comprising one or more oligonucleotide primers.

45. The kit of claim 35, further comprising at least one member selected from the group consisting of a buffer, nucleotide triphosphates, a salt, a stabilizer, a double stranded DNA binding dye, and nuclease-free water.

46. The kit of claim 35, wherein the polymerase and the agent are in a solution comprising a buffer, wherein the buffer (when measured at a concentration of 0.1 M) has a change of no more than 0.027 pH units per degree C when between 20° and 37° C.

47. The method of claim 46, wherein the buffer is selected from the group consisting of HEPES, ACES, PIPES, MOPSO, BES, MOPS, TES, TAPSO, POPSO, BICINE, TAPS, and AMPSO.

48. A reaction mixture for signal normalization in a real-time polymerase chain reaction (PCR) amplification of a target nucleic acid wherein the mixture is compatible for use in both (a) a real-time PCR amplification system employing a high passive reference dye concentration for the normalization and (b) a real-time PCR amplification system employing a low passive reference dye concentration for the normalization, wherein the mixture comprises:

a plurality of passive reference dyes that produces fluorescent signals independent of the amplification reactions; and

a buffer, wherein the buffer (when measured at a concentration of 0.1 M) has a change of no more than 0.027 pH units per degree C when between 20° and 37° C.

49. The mixture of claim 48,wherein the buffer is selected from the group consisting of HEPES, ACES, PIPES, MOPSO, BES, MOPS, TES, TAPSO, POPSO, BICINE, TAPS, and AMPSO.

50. The mixture of claim 48, further comprising a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises free arginine, spermidine, or spermine, or a salt thereof.

51. The mixture of claim 48, the mixture comprising

a first passive reference dye having a Stokes-shift, wherein the first passive reference dye is at a concentration sufficient for use in low concentration passive reference dye normalization, wherein the first passive reference dye has a first passive reference dye excitation wavelength maximum and a first passive reference dye emission wavelength maximum; and

a second passive reference dye having a Stokes-shift that is greater than the Stokes-shift of the first passive reference dye, wherein the second passive reference dye has an emission wavelength maximum approximately the same as the first passive reference dye emission wavelength maximum, and an excitation wavelength maximum significantly different than the first passive reference dye excitation wavelength maximum.

52. The mixture of claim 51, wherein the second passive reference dye has a Stokes-shift of at least about 60 nm.

53. The mixture of claim 51, wherein the first passive reference dye comprises 5- and/or 6-carboxy-X-rhodamine, or an analog thereof.

54. The mixture of claim 51, wherein the second passive reference dye has a excitation wavelength maximum of 550 nm or less.

55. The mixture of claim 51, wherein the concentration of the 5- and/or 6-carboxy-X-rhodamine dye is less than 100 nM.

56. The mixture of claims 51, further comprising one or more of an oligonucleotide primer, one or more deoxynucleoside triphosphates; a buffer, an intercalating dye, a reverse transcriptase, and a polymerase.

57. The mixture of claim 56, comprising DNA polymerase.

58. The mixture of claim 57, wherein the polymerase is complexed with an antibody.

59. The mixture of claim 57, wherein the polymerase is chemically inactivated but is activated by heating.

60. The mixture of claim 51, wherein said second passive dye is a fluorescent dot.

61. The mixture of claim 51, wherein the second passive reference dye is conjugated to a moiety.

62. A method of performing a real-time quantitative polymerase chain reaction, the method comprising,

performing a polymerase chain reaction (PCR) with the mixture of claim 51, wherein the mixture further comprises a biological sample suspected of comprising a target nucleic acid.

63. A method of making the reaction mixture of claim 51, the method comprising mixing the plurality of passive reference dyes, thereby generating the reaction mixture.

64. A kit for performing a real-time quantitative polymerase chain reaction, the kit comprising:

a first passive reference dye having a Stokes-shift, wherein the first passive dye has a first passive reference dye excitation wavelength maximum and a first passive reference dye emission wavelength maximum;

a second passive reference dye having a Stokes-shift that is greater than the Stokes-shift of the first passive reference dye, wherein the second passive reference dye has an emission wavelength maximum approximately the same as the first passive reference dye emission wavelength maximum, and an excitation wavelength maximum significantly different than the first passive reference dye excitation wavelength maximum and

a buffer, wherein the buffer (when measured at a concentration of 0.1 M) has a change of no more than 0.027 pH units per degree C when between 20° and 37° C.

65. The kit of claim 64,wherein the buffer is selected from the group consisting of HEPES, ACES, PIPES, MOPSO, BES, MOPS, TES, TAPSO, POPSO, BICINE, TAPS, and AMPSO.

66. The kit of claim 64, further comprising a sufficient amount of an agent to improve the specificity of nucleic acid amplification, wherein the agent comprises free arginine, spermidine, or spermine, or a salt thereof.

67. The kit of claim 64, wherein the second passive reference dye has a Stokes-shift of at least about 60 nm.

68. The kit of claim 64, wherein the first passive reference dye comprises 5- and/or 6-carboxy-X-rhodamine, or an analog thereof.

69. The kit of claim 64, wherein the first passive reference dye comprises

a 5- and/or 6-carboxy-X-rhodamine dye or an analog thereof, and

the second passive reference dye is a fluorescent dye having a Stokes-shift of at least 60 nm, wherein the second passive reference dye has an emission wavelength maximum of about 620 nm.

70. The kit of claim 64, the mixture comprising

a 5- and/or 6-carboxy-X-rhodamine dye; and

a second passive reference dye having a Stokes-shift of at least about 60 nm, wherein the second passive reference dye has an emission wavelength maximum of about 590 nm.

71. The kit of claim 64, further comprising one or more of: one or more deoxynucleoside triphosphates; one or more of an oligonucleotide primer, one or more deoxynucleoside triphosphates; a buffer, an intercalating dye, a reverse transcriptase, and a DNA polymerase.

72. The kit of claim 64, wherein the first passive reference dye and the second passive reference dye are contained in different vessels in the kit.

73. The kit of claim 64, wherein the first passive reference dye and the second passive reference dye are contained in the same vessel in the kit.