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(54) **Title:** COMPOSITIONS AND METHODS FOR ENHANCING AND/OR PREDICTING DNA AMPLIFICATION

(57) **Abstract:** The present invention features compositions and methods for amplifying a target oligonucleotide in a sample, including detection of the target oligonucleotide in real time.



COMPOSITIONS AND METHODS FOR ENHANCING AND/OR PREDICTING DNA AMPLIFICATION

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to and the benefit of U.S. Provisional Application No. 61/982,784, filed April 22, 2014, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

10 While methods of nucleic acid amplification are common and well-known, nucleic acid amplification reactions still suffer from unpredictable and/or poor performance due in large part to the difficulties associated with primer design. The unpredictability of nucleic acid amplification reactions is due in large part to a lack of sequence-predictable design parameters for primer and probes that allow a quantitative assessment of target-specific primer and probe hybridization versus off-target binding, self/self and self/non-self dimerization.

15 Indeed, variability in the selection of possible primer sequences and sequence constraints imposed by target nucleic acid sequences, has made it difficult to empirically and systematically interrogate the landscape of primer combinations and their outcomes.

 PCR assay-based design parameters have focused on primer and probe melting temperatures, GC (guanine and cytosine) content, primer length, and minimizing interactions

20 of the primer with all but the target nucleic acid (see e.g., www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html). In particular, use of a primer having intermolecular interactions with itself (e.g., primer-dimers) is highly discouraged, as such primers are believed to undesirably affect the interaction of the primer with the target nucleic acid molecule. Additionally, it is proposed that the optimal length of nucleic acid primers is

25 18-22 nucleotides, which is considered long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature. Regarding GC content, a GC content of 40-60% is recommended. Erroneously, common assay design practice assumes that when all the above mentioned parameters are designed equally for a forward and reverse primer of an amplification assay the hybridization kinetics of these two

30 primers will turn out similar as well. None of these commonly used design parameters adequately correlate with complex thermodynamics of two stage oligonucleotide hybridization, which involves changes in enthalpy, entropy, solvation, nearest neighbor effects, and hydrogen bonding in the transition from the single stranded form to the double

stranded hybrid stage. Moreover, even the use of the two stage model of oligonucleotide hybridization as the foundation of thermodynamic algorithms is overly simplified and does not reflect the real situation accurately. Therefore, the performance of primers meeting all of the recommended criteria remains unpredictable.

5 Progress in determining the effectiveness of a set of primers to predictably amplify a target nucleic acid under essentially isothermal conditions has the potential to reduce the expenditure of resources, including the effort, time, and expense of synthesizing and empirically testing a large multitude of primer sets (e.g., designing multiple primers and pairwise screening of multiple primer combinations). New parameters and methods are
10 required to enhance and better predict the performance and yield of nucleic acid amplification reactions.

SUMMARY OF THE INVENTION

As described herein, the present invention provides improved methods for designing
15 primers capable of predictably amplifying a target nucleic acid molecule, and methods of using these primers for nucleic acid amplification, including enhancing amplification of a target nucleic acid molecule and/or reducing or eliminating the generation of background products. Further, the invention provides compositions and methods for the amplification and detection of a sample target oligonucleotide. In particular embodiments, compositions
20 and methods of the invention are compatible with isothermal nucleic acid amplification reactions.

In one aspect, the invention provides an isolated primer oligonucleotide containing from 5' to 3', a first region having a self-complementary sequence containing from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence,
25 and said nicking enzyme recognition sequence, and a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid having a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure involving or interacting with the second region, where the second region has at the 3' end one or more 2' modified nucleotides.

30 In another aspect, the invention provides an isolated primer-dimer having two oligonucleotide monomers containing from 5' to 3', a first region having a self-complementary sequence containing from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition

sequence, and a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid having a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure involving or interacting with the second region, where the second region has at the 3' end one or more 2' modified nucleotides.

In another aspect, the invention provides an isolated oligonucleotide probe for detecting a target nucleic acid molecule containing: an oligonucleotide; a fluorescent reporter; and a quenching molecule capable of absorbing the excitation energy from the fluorescent reporter; where the fluorescent reporter and quenching molecule are covalently attached to opposite 5' and 3' ends of the oligonucleotide, where the oligonucleotide contains a first region having a nucleic acid sequence that is substantially complementary to a target nucleic acid sequence and a second region 5' upstream of the first region, and a third region 3' downstream of the first region that has a nucleic acid sequence complementary to the second region, where the oligonucleotide is capable of forming a stem-loop hairpin structure by hybridization of the second and third regions when the oligonucleotide is not bound to the target nucleic acid molecule, and where the ΔG of the double-stranded hybrid between the target sequence and the first sequence of the oligonucleotide probe is at least 15 kcal/mol lower than the ΔG of any alternate structure involving or interacting with the oligonucleotide probe.

In another aspect, the invention provides a method of amplifying a specific product in a nicking and extension amplification reaction, the method involving: contacting a target nucleic acid molecule under substantially isothermal conditions with a polymerase, two or more primers, each of which specifically binds to a complementary sequence on the target nucleic acid molecule, a nicking enzyme, and a detectable polynucleotide probe, where at least one primer contains from 5' to 3', a first region having a self-complementary sequence containing from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition sequence, and a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid having a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure involving or interacting with the second region, where the second region has at the 3' end one or more 2' modified nucleotides; and generating amplicons containing at least a portion of the target nucleic acid molecule.

In another aspect, the invention provides a method of detecting a specific product in a nicking and extension amplification reaction, the method involving: contacting a target nucleic acid molecule under substantially isothermal conditions with a polymerase, two or more primers, each of which specifically binds to a complementary sequence on the target nucleic acid molecule, a nicking enzyme, and a detectable oligonucleotide probe, where at least one primer contains from 5' to 3', a first region having a self-complementary sequence containing from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition sequence, and a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid having a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure involving or interacting with the second region, where the second region has at the 3' end one or more 2' modified nucleotides; generating amplicons containing at least a portion of said target nucleic acid molecule; and detecting a signal specific for oligonucleotide probe hybridization to the target nucleic acid molecule or amplicon thereof, where the signal indicates the presence of the target nucleic acid molecule present in the sample or an amplicon thereof.

In another aspect, the invention provides a kit for amplifying a target sequence in a nicking amplification reaction, the kit containing one or more primer oligonucleotides containing from 5' to 3', a first region having a self-complementary sequence containing from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition sequence, and a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid having a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure involving or interacting with the second region, where the second region has at the 3' end one or more 2' modified nucleotides; and directions for use of the primer oligonucleotides in the methods of the invention.

In a related aspect, the invention provides a method of selecting a primer oligonucleotide according to any aspect of the invention delineated herein, including providing computer program instructions on a tangible, non-transitory computer readable medium for implementing such methods of selecting a primer oligonucleotide.

In various embodiments of the above aspects, the first region is 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides in length. In various embodiments, the first region is fully self-complementary. In various embodiments of the above aspects, the palindromic

sequence in the first region is 2, 4, or 6 nucleotides long. In various embodiments of the above aspects, the second region is 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

In various embodiments of the above aspects, the alternate structure is one or more of a partially double-stranded hybrid that the second region can form with itself (e.g., self-self dimers), with a partial sequence of the first region (e.g., heterodimers), with other oligonucleotides, primers, or probes in an amplification reaction, or with partially complementary nucleic acid sequences outside of the target sequence region (e.g., off-target hybrids).

In various embodiments of the above aspects, the oligonucleotide (e.g., primer oligonucleotide, oligonucleotide monomer) is in the form of a homodimer (e.g., primer-dimer) formed by hybridization of the self-complementary first region sequences of two primer oligonucleotide molecules. In various embodiments, the homodimer has a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure involving or interacting with the primer oligonucleotide, including the most stable alternate structure comprising the primer oligonucleotide. In various embodiments of the above aspects, the free energy (ΔG) is determined or calculated for a nucleic acid at 25°C ($\Delta G_{25^\circ\text{C}}$) and/or at 1 atmosphere (atm) of pressure (~101.3 kPa). In various embodiments of the above aspects, the free energy (ΔG) is determined or calculated by the mFold algorithm.

In various embodiments of the above aspects, the second region of the oligonucleotide (e.g., primer oligonucleotide, oligonucleotide monomer) includes one or more 2' modified nucleotides, where the 2' modification is selected from the group consisting of 2'-O-methyl, 2'-methoxyethoxy, 2'-fluoro, 2'-hydroxyl, 2'-alkyl, 2'-allyl, 2'-O-[2-(methylamino)-2-oxoethyl], 4'-thio, 4'-CH₂-O-2'-bridge, 4'-(CH₂)₂-O-2'-bridge, and 2'-O-(N-methylcarbamate) or those containing base analogs. In particular embodiments, the one or more 2' modified nucleotides are positioned at the 3' terminus of the second region. In other embodiments, the two or more 2' modified nucleotides are contiguous. In still other embodiments, the number of contiguous 2' modified nucleotides is 2, 3, 4, 5, or 6. In various embodiments of any aspect delineated herein, the nicking enzyme is one or more of Nt.BspD6I and Nt.BstNBI. In various embodiments of any aspect delineated herein, the nicking enzyme recognition sequence within the first region is 5'-GAGTC-3' (and the reverse complement of the nicking enzyme recognition sequence is 5'-GACTC-3'). In various embodiments of any aspect

delineated herein, the oligonucleotide (e.g., primer oligonucleotide, oligonucleotide monomer) has recognition sequence of the nicking agent is positioned to cleave the phosphodiester bond between the first and second regions. In various embodiments of the above aspects, the polymerase is a *Geobacillus* spp. or *Bacillus stearothermophilus* DNA
 5 polymerase I, or active fragments and derivatives thereof. In certain embodiments, the polymerase is one or more of Bst DNA polymerase I, Gst DNA polymerase I, or Gka DNA polymerase I.

In various embodiments of any aspect delineated herein, the oligonucleotide (e.g., primer oligonucleotide, oligonucleotide monomer) has a first region including a nucleic acid
 10 sequence set forth below:

5'- GACTCN₁N₁'GAGTC -3';

5'- GACTCN₁N₁'GAGTCN -3';

5'- N₂GACTCN₁N₁'GAGTCN₂' -3';

5'- N₂GACTCN₁N₁'GAGTCN₂'N -3';

15 5'- N₃N₂GACTCN₁N₁'GAGTCN₂'N₃' -3';

5'- N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N -3';

5'- N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄' -3';

5'- N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄'N -3';

5'- N₅N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄'N₅' -3';

20 5'- GACTCN₂N₁N₁'N₂'GAGTC -3';

5'- GACTCN₂N₁N₁'N₂'GAGTCN -3';

5'- N₃GACTCN₂N₁N₁'N₂'GAGTCN₃' -3';

5'- N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N -3';

5'- N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄' -3';

25 5'- N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N -3';

5'- N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅' -3';

5'-N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅'N -3'; and

5'-N₆N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅'N₆' -3',

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTC -3';

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN -3';

5 5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNN -3';

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNNN -3';

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNNNN -3';

5'-N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄' -3';

5'-N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N -3';

10 5'-N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'NN -3';

5'-N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'NNN -3';

5'-N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅' -3';

5'-N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N -3';

5'-N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆' -3';

15 5'-N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆'N -3'; and

5'-N₇N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆'N₇' -3',

where "N" is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C), or guanine (G) nucleobase), and N₁ is complementary to N₁', N₂ to N₂', N₃ to N₃', N₄ to N₄', N₅ to N₅', N₆ to N₆', and N₇ to N₇'. In certain embodiments, the oligonucleotide (e.g., primer
20 oligonucleotide, oligonucleotide monomer) comprises a 5' tail region comprising a sequence set forth in the Sequence Listing submitted herewith.

In various embodiments of any aspect delineated herein, the oligonucleotide probe for detecting a target nucleic acid molecule contains: an oligonucleotide; a fluorescent reporter; and a quenching molecule capable of absorbing the excitation energy from the fluorescent
25 reporter; where the fluorescent reporter and quenching molecule are covalently attached to opposite 5' and 3' ends of the oligonucleotide, where the oligonucleotide contains a first

region having a nucleic acid sequence that is substantially complementary to a target nucleic acid sequence and a second region 5' upstream of the first region, and a third region 3' downstream of the first region that has a nucleic acid sequence complementary to the second region, where the oligonucleotide is capable of forming a stem-loop hairpin structure by hybridization of the second and third regions when the oligonucleotide is not bound to the target nucleic acid molecule. In various embodiments of any aspect delineated herein, the ΔG of the double-stranded hybrid between the target sequence and the first sequence of the oligonucleotide probe is at least 15 kcal/mol lower than the ΔG of any alternate structure involving or interacting with the oligonucleotide probe. In various aspects, the second and the third regions of the oligonucleotide probe are 4-8 nucleotides in length.

In various embodiments of any aspect delineated herein, directions are provided for use of the primer oligonucleotides and/or oligonucleotide probes of the invention. In various embodiments of any aspect delineated herein, the primers and probes in a reaction are designed and/or selected to perform together in an amplification and detection reaction.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Definitions

By "amplicon" is meant a polynucleotide generated during the amplification of a polynucleotide of interest. In one example, an amplicon is generated during a polymerase chain reaction.

By "amplification rate modifiers" is meant an agent capable of affecting the rate of polymerase extension.

By "base substitution" is meant a substituent of a nucleobase polymer that does not cause significant disruption of the hybridization between complementary nucleotide strands.

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

By “complementary” or “complementarity” is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or Hoogsteen base pairing. Complementary base pairing includes not only G-C and A-T base pairing, but also includes base pairing involving universal bases, such as inosine. A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, or 10 nucleotides out of a total of 10 nucleotides in the first oligonucleotide being based paired to a second nucleic acid sequence having 10 nucleotides represents 50%, 60%, 70%, 80%, 90%, and 100% complementary respectively). To determine that a percent complementarity is of at least a certain percentage, the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence is calculated and rounded to the nearest whole number (e.g., 12, 13, 14, 15, 16, or 17 nucleotides out of a total of 23 nucleotides in the first oligonucleotide being based paired to a second nucleic acid sequence having 23 nucleotides represents 52%, 57%, 61%, 65%, 70%, and 74%, respectively; and has at least 50%, 50%, 60%, 60%, 70%, and 70% complementarity, respectively). As used herein, “substantially complementary” refers to complementarity between the strands such that they are capable of hybridizing under biological conditions. Substantially complementary sequences have 60%, 70%, 80%, 90%, 95%, or even 100% complementarity. Additionally, techniques to determine if two strands are capable of hybridizing under biological conditions by examining their nucleotide sequences are well known in the art.

As used herein, “duplex” refers to a double helical structure formed by the interaction of two single stranded nucleic acids. A duplex is typically formed by the pairwise hydrogen bonding of bases, i.e., “base pairing”, between two single stranded nucleic acids which are oriented antiparallel with respect to each other. Base pairing in duplexes generally occurs by Watson-Crick base pairing, e.g., guanine (G) forms a base pair with cytosine (C) in DNA and RNA, adenine (A) forms a base pair with thymine (T) in DNA, and adenine (A) forms a base pair with uracil (U) in RNA. Conditions under which base pairs can form include physiological or biologically relevant conditions (e.g., intracellular: pH 7.2, 140 mM potassium ion; extracellular pH 7.4, 145 mM sodium ion). Furthermore, duplexes are stabilized by stacking interactions between adjacent nucleotides. As used herein, a duplex may be established or maintained by base pairing or by stacking interactions. A duplex is formed by two complementary nucleic acid strands, which may be substantially

complementary or fully complementary. Single-stranded nucleic acids that base pair over a number of bases are said to “hybridize.”

“Detect” refers to identifying the presence, absence or amount of the analyte to be detected.

5 By “detectable moiety” is meant a composition that when linked to a molecule of interest renders the latter detectable, via spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radioactive isotopes, magnetic beads, metallic beads, colloidal particles, fluorescent dyes, electron-dense reagents, enzymes (for example, as commonly used in an ELISA), biotin, digoxigenin, or
10 haptens.

By “fragment” is meant a portion of a nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 nucleotides.

15 By “free energy (ΔG)” is meant the net exchange of energy between the system and its environment at a constant temperature and pressure described by the formula: $\Delta G = \Delta H - T\Delta S$. Free energy represents how favorable the formation of a structure is, where the formation of a structure having a more negative ΔG (e.g., expressed in kcal/mol) is thermodynamically more favored in a dynamic equilibrium of multiple alternate structures.

20 Accordingly, $\Delta\Delta G$ measures the difference in favorability between the formation of two different structures having two different ΔG s. In one aspect, $\Delta\Delta G$ provides a numerical score measuring the favorability of formation of a desired oligonucleotide structure over the possible formation of other undesirable structures in dynamic equilibrium. In one embodiment, the first structure is the duplex hybrid formed between the first region (5’ tail region) of one oligonucleotide and the first region (5’ tail region) of a second oligonucleotide having the same nucleic acid sequence as the first oligonucleotide; and the second structure is
25 any other alternate structure comprising the oligonucleotide.

By “hybridize” is meant to form a double-stranded molecule between complementary polynucleotide sequences (e.g., a gene described herein), or portions thereof, under various
30 conditions of stringency. (See, e.g., Wahl, G. M. and S. L. Berger (1987) *Methods Enzymol.* 152:399; Kimmel, A. R. (1987) *Methods Enzymol.* 152:507). Hybridization occurs by hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen

bonding, between complementary nucleobases. For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds.

By "nucleic acid hybrid" is meant a substantially double-stranded molecule formed by the binding of complementary nucleic acid molecules. In one embodiment, the double-
5 stranded molecule is partially or fully double-stranded.

By "isolated polynucleotide" is meant a nucleic acid (e.g., a DNA) that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid molecule of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously
10 replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In addition, the term includes an RNA molecule that is transcribed from a DNA molecule, as well as a recombinant DNA that is part of a hybrid gene encoding additional polypeptide
15 sequence.

The terms "isolated," "purified," or "biologically pure" refer to material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings. "Purify" denotes a degree of separation that is higher than isolation. A "purified" or "biologically
20 pure" protein is sufficiently free of other materials such that any impurities do not materially affect the biological properties of the protein or cause other adverse consequences. That is, a nucleic acid or peptide of this invention is purified if it is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Purity and
25 homogeneity are typically determined using analytical chemistry techniques, for example, polyacrylamide gel electrophoresis or high performance liquid chromatography. The term "purified" can denote that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. For a protein that can be subjected to modifications, for example, phosphorylation or glycosylation, different modifications may give rise to different isolated
30 proteins, which can be separately purified.

By "melting temperature (T_m)" is meant the temperature of a system in equilibrium where 50% of the molecular population is in one state and 50% of the population is in another state. With regard to the nucleic acids of the invention, T_m is the temperature at

which 50% of the population is single-stranded and 50% is double-stranded (e.g., intramolecularly or intermolecularly).

By “monitoring a reaction” is meant detecting the progress of a reaction. In one embodiment, monitoring reaction progression involves detecting polymerase extension
5 and/or detecting the completion of an amplification reaction.

As used herein, “obtaining” as in “obtaining an agent” includes synthesizing, purchasing, or otherwise acquiring the agent.

As used herein, the term “nucleic acid” refers to deoxyribonucleotides, ribonucleotides, or modified nucleotides, and polymers thereof in single- or double-stranded
10 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, 2’ modified nucleotides (e.g., 2’-O-methyl ribonucleotides, 2’-F
15 nucleotides).

As used herein, “modified nucleotide” refers to a nucleotide that has one or more modifications to the nucleoside, the nucleobase, pentose ring, or phosphate group. For example, modified nucleotides exclude ribonucleotides containing adenosine monophosphate, guanosine monophosphate, uridine monophosphate, and cytidine
20 monophosphate and deoxyribonucleotides containing deoxyadenosine monophosphate, deoxyguanosine monophosphate, deoxythymidine monophosphate, and deoxycytidine monophosphate. Modifications include those naturally occurring that result from modification by enzymes that modify nucleotides, such as methyltransferases.. Modified nucleotides also include synthetic or non-naturally occurring nucleotides. Synthetic or non-
25 naturally occurring modifications in nucleotides include those with 2’ modifications, e.g., 2’-O-methyl, 2’-methoxyethoxy, 2’-fluoro, , 2’-hydroxyl (RNA), 2’-allyl, 2’-O-[2-(methylamino)-2-oxoethyl], 4’-thio, 4’-CH₂-O-2’-bridge, 4’-(CH₂)₂-O-2’-bridge, and 2’-O-(N-methylcarbamate) or those comprising base analogs.

By “nucleotide adduct” is meant a moiety that is bound covalently or otherwise fixed
30 to a standard nucleotide base.

By “nicking agent” is meant a chemical entity capable of recognizing and binding to a specific structure in double stranded nucleic acid molecules and breaking a phosphodiester bond between adjoining nucleotides on a single strand upon binding to its recognized specific

structure, thereby creating a free 3'-hydroxyl group on the terminal nucleotide preceding the nick site. In preferred embodiments, the 3' end can be extended by an exonuclease deficient polymerase. Exemplary nicking agents include nicking enzymes, RNAzymes, DNAzymes, and transition metal chelators.

5 By "palindromic" is meant nucleic acid sequences that are identical when read from 5' to 3' on one strand or 5' to 3' on the complementary strand. A perfect palindrome refers to a sequence having two adjacent subsequences, such that when one subsequence is read from the 5' to 3' direction, it is identical to the other subsequence read from the 3' to 5' direction.

10 By "polymerase-arresting molecule" is meant a moiety associated with a polynucleotide template or primer that prevents or significantly reduces the progression of a polymerase on the polynucleotide template. Preferably, the moiety is incorporated into the polynucleotide. In one preferred embodiment, the moiety prevents the polymerase from progressing on the template.

15 By "polymerase extension" is meant the forward progression of a polymerase that matches incoming monomers to their binding partners on a template polynucleotide.

As used herein, "primer-dimer" is meant a dimer of two monomer oligonucleotide primers. In the oligonucleotide primers of the invention, the 5' tail regions of monomer primers dimerize.

20 By "specific product" is meant a polynucleotide product resulting from the hybridization of primer oligonucleotides to a complementary target sequence and subsequent polymerase mediated extension of the target sequence.

25 By "substantially isothermal condition" is meant at a single temperature or within a narrow range of temperatures that does not vary significantly. In one embodiment, a reaction carried out under substantially isothermal conditions is carried out at a temperature that varies by only about 1-5° C (e.g., varying by 1, 2, 3, 4, or 5 degrees). In another embodiment, the reaction is carried out at a single temperature within the operating parameters of the instrument utilized.

30 By "reference" is meant a standard or control condition. As is apparent to one skilled in the art, an appropriate reference is where an element is changed in order to determine the effect of the element.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

By "target nucleic acid molecule" is meant a polynucleotide to be analyzed. Such polynucleotide may be a sense or antisense strand of the target sequence. The term "target nucleic acid molecule" also refers to amplicons of the original target sequence.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a mechanism describing a strand displacement nucleic acid amplification reaction of a target nucleic acid molecule having efficient, exponential amplification. The interaction of the 3' end of a target nucleic acid molecule (gray) with a corresponding, sequence-specific primer having a 3' recognition region (red) and "5' tail" region capable of dimerization (black) is shown. For target specific amplification, the 3' recognition region (red) is complementary in sequence to and capable of specifically hybridizing to the target nucleic acid. The primer in an active configuration is a stable homodimer (termed a "primer-dimer") formed by the dimerization of the 5' tail regions of two monomer primers. In one specific embodiment, the primer has a self-complementary,

symmetrically inverted sequence. The resulting primer-dimer has a double-stranded or substantially double-stranded dimerization region (black) that is 5' with respect to the single-stranded recognition regions (red). That is, the target specific portion of the primer (capable of annealing to the target nucleic acid molecule) is exposed in the primer-dimer as the 3'

5 single-stranded regions. At the start of the reaction, a 3' single-strand portion of the primer-dimer anneals to a target nucleic acid molecule, and initiates replication of the target nucleic acid molecule. During the course of the reaction, monomer primers are released from extended primer-dimers by polymerase strand displacement action from synthesis of the complementary strand. To enhance the specificity and/or efficiency of the amplification

10 reaction, monomer primers are designed to have (1) a 5' tail region having a sequence that is self-complementary, symmetrical or substantially symmetrical, and capable of base pairing to itself and (2) a 3' recognition region perfectly or substantially complementary in sequence to and capable of specifically hybridizing to the target nucleic acid molecule. When ΔG for primer-dimer formation is low, the time a primer is present as a monomer with an open

15 configuration is minimized. Monomer primers are undesirable because they can serve as either a template or primer in non-target-specific primer extension reactions, thereby generating and/or amplifying background. Preferably, the transition from monomer to dimer is "virtually instantaneous" or as brief as kinetically possible. Thermodynamic factors (e.g., very low ΔG of primer-dimer formation, high primer concentration, high dimer T_m) are used

20 to favor primer-dimer formation.

Figure 2 depicts a mechanism describing a strand displacement nucleic acid amplification reaction of a target nucleic acid having inefficient amplification. The interaction of the 3' end of a target nucleic acid molecule (gray) with a corresponding, sequence-specific primer (red and black) is shown. Isothermal reactions where monomer

25 primers in an open configuration persist throughout the reaction lead to linear non-specific background amplification. Primer monomers that do not favor primer-dimer formation have, for example, minimal or no self-complementary sequences in the 5' portion of the monomer primer (black), resulting in a high ΔG for primer-dimer formation. Because interaction of the primer-dimer with the target nucleic acid molecule drives the annealing and extension

30 reactions, formation of primer-dimers affects the efficiency of target nucleic acid synthesis and/or amplification. In the fully or partially open configuration, a primer monomer can serve either as a template or a primer in non-target-specific primer extension reactions (see inset), which leads to additional reaction inefficiencies. Where the open configuration of

monomer primers is not minimized (e.g., by sequence design), the transition from monomer to dimer is slow, because it is not favored by thermodynamic factors (e.g., multiple dimer and partial dimer configurations co-having similar ΔG 's and low dimer T_m 's).

Figure 3 depicts primer structures that inhibit primer-dimer formation including, for example, structures (A) to (C) which lead to amplification of non-specific by-products (structures (H) to (J)) or structures (D) and (K), which are incapable of target hybridization. Interactions between primers (red and black) nucleic acid fragments (gray) are shown. Structure (A) depicts a primer monomer having a discontinuous inverted sequence in the target specific region that is capable of "looping" back to create an extendable 3'-end for "self-replication." Structure (A) also has a 5' region that loops back on itself to form a hairpin structure. Structure (B) depicts a primer-dimer having discontinuous inverted sequences in the target specific region of the primer that are capable of "looping" back to create extendable 3'-ends for "self-replication. Structure (C) depicts a false hybrid structure between a primer monomer in "open" configuration and a single stranded DNA fragment (gray) displaced by DNA polymerase nick-extension reactions from closely positioned random and/or sequence-specific nicks in template DNA. Structure (D) depicts a partially double-stranded dimer structure, where a significant part of the target-specific region is not accessible for target hybridization. Structure (D) may be formed by inverted sequence repeats in the primer monomer. Structure (E) depicts an intermediate, false primer extension/strand displacement reaction product generated from structure (A). Structure (G) depicts an intermediate, false primer extension/strand displacement reaction product generated from structure (B). Structure (H) depicts a mature, false reaction product in "nick & kick" mode, resulting in continuous recycling and amplification of single stranded by-products. Structure (J) depicts mature, false reaction products in "nick & kick" mode, resulting in continuous recycling and amplification of single stranded by-products. Structure (K) depicts the persistence of the partially double-stranded dimer of structure (D) in the reaction. Because a significant part of the target-specific region is not accessible for target hybridization, target nucleic acid replication and/or amplification is inhibited or fails to initiate. Primer design in accordance with the invention promotes optimal primer-dimer formation, which minimizes the formation of undesirable primer structures and their inhibitory effects on target specific nucleic acid amplification.

Figure 4 shows desirable (right) and undesirable (left) primer structures. The primers of the invention may adopt a structure favorable to the amplification reaction, including

primer-dimers and/or a hairpin, due to the presence of self-complementary sequences. Without being bound to theory, primer dimers are more stable and form when primers are present at high concentration in the reaction (e.g., at the start of the reaction) and hairpins form when primer concentration is reduced (e.g., during the course of the reaction).

5 Figure 5 depicts an undesirable primer configuration where a dynamic equilibrium exists between configurations of non-optimal primers. Where the ΔG 's corresponding to the formation of each structure are about the same (i.e., $\Delta G_1 \approx \Delta G_2 \approx \Delta G_3 \approx \Delta G_4$), there is no favored primer configuration or structure. When a primer exists in such multiple configurations, it is not optimally configured to promote the nucleic acid amplification
10 reaction.

Figure 6 shows that an optimal primer is one in which the dimer configuration is highly favored (i.e., $\Delta G_1 \gg \Delta G_2$), especially over the next favored configuration (e.g., where the primer interacts with itself intramolecularly). This configuration is in contrast to Figure 5, where a dynamic equilibrium exists between configurations. Primers can be designed to
15 have stable configurations and structures favorable for nucleic acid amplification reactions, while reducing or eliminating the formation of undesirable primer configurations and structures. For primers having a 5' tail dimerization region, the 5' tail sequence is selected to favor and be more stable than other structures ($\Delta G_1 \leq -30$ to -62 kcal/mol). For primers in nicking amplification reactions, the 5' tail sequence is substantially self-complementary and
20 symmetrically inverted. Such primers may have the potential to form intramolecular interactions (e.g., a hair-pin loop). However, such configurations are minimized when the symmetrically inverted sequences are fully self-complementary, as indicated by the stability of the primer-dimer over the hair-pin loop structure (a very low ΔG_1 in comparison to ΔG_2). Thus, when the primer is at a high enough concentration, the hair-pin is not effectively
25 formed.

Figure 7 depicts sequences of stable primer-dimer structures of primers useful in nicking amplification reactions. Figure 7 depicts a homodimer of a primer dimer having a 5' tail region with a nicking enzyme recognition sequence for a top strand nicking enzyme (Nt.BstNBI). The nick site is designed to be positioned between the nucleotide at the 3' end
30 of the 5' tail region and the nucleotide at the 5' end of the 3' recognition region. Without being bound to theory, the nicking enzyme does not cleave at the nick site when the 3' recognition is single-stranded. However, cleavage at the nick site occurs when the 3' recognition region is double stranded (e.g., when the primer is incorporated into a double-

stranded target nucleic acid molecule during the course of the nucleic acid amplification reaction).

Figures 8A-8C depict exemplary 5' tail region sequences. Figure 8A depicts exemplary 5' tail region sequences 24 nucleotides in length having a Nt.BstNBI recognition sequence and based on the following formula: 5'-NNNNGACTCNNNNNNGAGTCNNNN-3'. Based on this formula, there are 537,824 5' tail sequences having the following properties: $\Delta G = -34.2$ Kcal/mole to -65 kcal/mole; $\Delta\Delta G < -40$ kcal/mole; and GC content 68% to 84%. Of these, 1050 selected sequences are provided, representing 0.2% of the entire sequence space (248,832). Figure 8B depicts exemplary 5' tail region sequences 22 nucleotides in length having a Nt.BstNBI recognition sequence and based on the following formula: 5'-NNNNGACTCNNNNNGAGTCNNNN-3'. Based on this formula, there are 248,832 5' tail sequences having the following properties: $\Delta G = -47$ Kcal/mole to -55 kcal/mole; $\Delta\Delta G < -40$ kcal/mole; and GC content 72% to 82%. Of these, 200 selected sequences are provided, representing 0.08% of the entire sequence space (248,832). Figure 8C depicts exemplary 5' tail region sequences having a 6 nucleotide spacer region.

Figure 9 are graphs showing target specific nucleic acid amplification using primers of the invention having 5' tail and 3' recognition regions as detected by dual channel detection of DNA amplification. Target-specific products are detected by a molecular beacon probe (top panel). Non-specific nucleic acid amplification is detected by SYBRGreen. As seen in both graphs, exponential amplification begins at around 6 minutes, indicating that amplification is target specific. Control reactions having no input did not produce substantial background.

Figure 10 is a graph showing that nucleic acid amplification using primers of the invention having 5' tail and 3' recognition regions have characteristics useful for rapid detection of target nucleic acids. Such reactions are characterized by a high signal-to-noise ratio ($[RFU_{MAX} - RFU_{BL}]$), steep slope (high value of $[RFU_{MAX} - RFU_{BL}]/[T_{OSEA} - T_{RFU_{max}}]$), early onset of the exponential amplification phase ($T_{OSEA} < 10$ min) and low signal variance between replicated assay reactions of the same sample.

Figure 11 is a graph of working and failed assay primers plotted according to their relative stabilities. Primers having a 3' recognition region having a $\Delta\Delta G \leq$ about -16 kcal/mole were observed to work in nicking amplification assays, while primers having a $\Delta\Delta G >$ about -16 were prone to failure. Primers having a 3' recognition region having a

$\Delta\Delta G \leq$ about -16 kcal/mole and a 5' tail having a $\Delta\Delta G \leq$ about -30 kcal/mole were all observed to work.

Figure 12 depicts the design of an isothermal DNA amplification assay for Soy Lectin from a single pair of predicted optimal primer structures and a single predicted optimal probe without screening a multitude of candidate primer combinations of unknown performance.

Figure 13 is a graph depicting results of the first experimental trial of the isothermal DNA amplification assay for Soy Lectin, which empirically confirm assay performance prediction. Target specific amplification was observed in reactions containing soy genomic DNA. No amplification was observed in no target control reactions.

Figure 14 depicts the design of an isothermal DNA amplification assay for pathogenic *Salmonella enterica invA* gene from a single pair of predicted optimal primer structures and a single predicted optimal probe without screening a multitude of candidate primer combinations of unknown performance.

Figure 15 is a graph depicting results of the first experimental trial of the isothermal DNA amplification assay for pathogenic *Salmonella enterica invA* gene, which empirically confirm assay performance prediction. Target specific amplification was observed in reactions containing soy genomic DNA. No amplification was observed in no target control reactions.

Figure 16 includes two graphs showing that the isothermal DNA amplification assay for *Salmonella enterica invA* gene is readily optimized.

Figures 17A-17I shows Primer Structure Parameter (T_M , %GC, bp Length, $\Delta G_{25^\circ C}$, $\Delta\Delta G_{25^\circ C}$) Cluster Analysis. Figure 17A is a graph plotting $\Delta\Delta G$ in kcal/mol of first primer region (5' -tail) self-self dimer of working assay primers and failed assay primers against $\Delta\Delta G$ in kcal/mol of second primer region/target hybrid duplex. Clustering of working assay primers and failed assay primers showed correlation with function/non-function. Figure 17B is a graph plotting length in bases of second primer target-complementary region of working assay primers and failed assay primers against %GC content of second primer target-complementary region. Working assay primers and failed assay primers showed a random distribution. Figure 17C is a graph plotting length in bases of second primer target-complementary region of working assay primers and failed assay primers against ΔG in kcal/mol of second primer region/target hybrid duplex. Working assay primers and failed assay primers showed a random distribution. Figure 17D is a graph plotting T_M in $^\circ C$ of second primer region/target hybrid duplex of working assay primers and failed assay primers

against ΔG in kcal/mol of second primer region/target hybrid duplex. Working assay primers and failed assay primers showed no correlation with function/non-function. Figure 17E is a graph plotting ΔG in kcal/mol of most favored second primer region/off-target hybrid duplex of working assay primers and failed assay primers against ΔG in kcal/mol of primer region/target hybrid duplex. Working assay primers and failed assay primers showed a random distribution. Figure 17F is a graph plotting %GC content of second primer region of working assay primers and failed assay primers against T_M in $^{\circ}\text{C}$ of second primer region/target hybrid duplex. Working assay primers and failed assay primers showed a random distribution. Figure 17G is a graph plotting %GC content of second primer region of working assay primers and failed assay primers against ΔG in kcal/mol of second primer region/target hybrid duplex. Working assay primers and failed assay primers showed a random distribution. Figure 17H is a graph plotting %GC content of second primer region of working assay primers and failed assay primers against T_M in $^{\circ}\text{C}$ of second primer region/target hybrid duplex. Working assay primers and failed assay primers showed a random distribution. Figure 17I is a graph plotting length in bases of primer (3' target recognition region) of working assay primers and failed assay primers against T_M in $^{\circ}\text{C}$ of primer-target hybrid duplex. Working assay primers and failed assay primers showed a random distribution.

DETAILED DESCRIPTION OF THE INVENTION

The invention methods that are useful for the predictable design of primers, and methods of using such primers for the amplification of a target nucleic acid molecule in a nucleic acid amplification reaction. The compositions and methods are also useful for the amplification and detection of a target nucleic acid molecule in a nucleic acid amplification reaction, including in a nicking amplification reaction.

The invention is based, at least in part, on several discoveries relating to oligonucleotides used in nucleic acid amplification reactions, including primers and probes, and their effect on enhancing or enabling nucleic acid amplification reactions. Applicants were the first to appreciate that one or more of these discoveries in combination could be applied to design primers and probes for nucleic acid amplification reactions and/or detection assays.

The invention is based, at least in part, on the discovery that oligonucleotides (e.g., primers and probes) in certain configurations have the potential to enhance nucleic acid amplification reaction performance, and that the ability of the oligonucleotides to adopt

favorable configurations can be determined by examining the difference in free energy change (ΔG) between desired specific oligonucleotide-target sequence hybrids and various non-specific intramolecular (partially double-stranded monomers) and intermolecular complexes (primer-primer and primer-probe dimers, off-target hybrids) of the oligonucleotide. To date, primer and probe design has not employed the difference in free energy change between target-specific and non-specific primer and probe structures as a score ranking tool to identify the most suitable primer/probe set for a target sequence amplification assay. Application of the foregoing considerations to primer design results in the design of primers that function predictably, minimizing or eliminating the need for empirical testing. Accordingly, the invention provides methods for primer sequence selection, primer modification, and/or probe design that facilitates operational nucleic acid amplification and detection reactions.

Applicants have discovered that a primer having a 3' recognition sequence whose primer-target formation is stable (e.g., $\Delta G_{25^\circ\text{C}} \leq$ about -20 kcal/mol or more) has the potential to enhance nucleic acid amplification reaction performance. In particular embodiments, the 3' target recognition sequence comprises 12-24, 12-17, or 12-14 bases. In particular embodiments, the primer-target formation is more stable than self dimer formation (e.g., $\Delta\Delta G \leq$ about -15, -16, -17, -18, -19, -20 kcal/mol or more). Indeed, it has been found that a difference between a working and non-working assay is when the $\Delta\Delta G <$ about -15 kcal/mol (see, e.g., Figure 11).

Additionally, Applicants have discovered that a primer having a 5' tail region capable of self-dimerization enhances nucleic acid amplification reaction performance. Without being bound to theory, in a nucleic acid amplification reaction the primer anneals to the target nucleic acid as a primer-dimer (see e.g., Figure 1). Surprisingly and unexpectedly, this primer-dimer configuration does not detectably interfere with polymerase activity, and reduces or prevents non-specific interaction of the 5' portion of the primer with itself or other nucleic acid molecules. The 5' tail region comprises a perfect palindrome sequence, through which monomer primers can dimerize. For example, primers have a nicking agent recognition site present at the 5' end that is unrelated to the binding specificity of the primer for the target recognition sequence. Non-specific background products from non-specific primer interactions have the potential to sequester reaction components that would otherwise have been utilized for the amplification of the specific product. In various embodiments,

homodimer formation is stable (e.g., $\Delta G_{25^{\circ}\text{C}} \leq$ about -30, -35, -40, -45, -50, -55, -60 kcal/mol or more). In various embodiments, the homodimer has a melting temperature higher than the extension reaction temperature. In particular embodiments, the 5' tail region has a sequence that is a palindrome. In further embodiments, the 5' tail region is at least 20 bases (e.g., 20, 21, 22, 23, 24 bases) in length. In additional embodiments, the 5' tail region has a GC content of 80-90%. In certain embodiments, homodimer formation is more stable than formation of other less stable primer dimer conformations ($\Delta\Delta G \leq$ about -15, -20, -25, -30, -35, -40 kcal/mol or more).

Further, Applicants have discovered that primers comprising a 2' modified nucleotide (e.g., 2'-O-methyl, 2'-Fluoro, 2'-alkyl) at the 3' end of the 3' recognition region reduce or eliminate non-specific background products. This increases the generation of specific products in nucleic acid amplification reactions without the generation of background products. In various embodiments, one or more of the nucleotides in the 3' recognition region comprises a modified nucleobase that destabilizes mismatched base pairs. In particular embodiments, a modified nucleobase is in two or more continuous nucleotides starting at position 1, 2, 3, 4, 5, or 6 from the 3' terminus, where position 1 is the nucleotide at the 3' terminus. In specific embodiments, no fewer than 8 and no more than 10 unmodified nucleotides are positioned between the first 5' nucleotide of the 3' recognition region and the nearest 3' modified nucleotide.

In specific embodiments, the primer comprises a 3' recognition region and a 5' tail dimerization region. Under reaction conditions, monomer primers dimerize to form primer-dimers by binding of their 5' tail dimerization regions. Without being bound to theory, a single-stranded 3' recognition region of the primer-dimer anneals the target nucleic acid molecule and synthesizes the complementary target strand. A monomer primer is released from the extended strand by polymerase strand displacement action from synthesis of the complementary strand. The released monomer primer dimerizes with another free monomer by 5' tail region binding. Multiple cycles result in exponential target nucleic acid amplification.

Primer Design

Conventional methods for primer design have focused on primer melting temperature, primer annealing temperature, GC (guanine and cytosine) content, primer length, and

minimizing interactions of the primer with all but the target nucleic acid (see e.g., www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html). Contrary to these methods, it has been found that primers that form stable primer/dimers, expressed in terms of free energy of formation (ΔG), function predictably in nucleic acid amplification reactions. While

5 Free Energy (ΔG) and Melting Temperature (T_m) share primary components Enthalpy (ΔH) and Entropy (ΔS), ΔG and T_m values are derived differently and have no correlative relationship, and the only way to relate a given ΔG with a given T_m value is to explicitly know the value of ΔH and ΔS from which they are derived (Manthey, "mFold, Delta G, and Melting Temperature" ©2005 and 2011 Integrated DNA Technologies). Figures 1-11 relate

10 to the design of optimal primers.

The free energy of formation (ΔG) for intermolecular primer structures may be calculated using formulas known in the art. A number of programs are available for determining the formation of various intramolecular and intermolecular primer structures and calculating their ΔG 's, including for example mfold and UNAFold prediction algorithms (see

15 e.g., Markham and Zuker. UNAFold: Software for Nucleic Acid Folding and Hybridization. Bioinformatics: Volume 2, Chapter 1, pp 3-31, Humana Press Inc., 2008; Zuker et al. Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide In RNA Biochemistry and Biotechnology, 11-43, NATO ASI Series, Kluwer Academic Publishers, 1999; M. Zuker. Prediction of RNA Secondary Structure by Energy

20 Minimization. Methods in Molecular Biology, 267-294, 1994; Jaeger et al. Predicting Optimal and Suboptimal Secondary Structure for RNA. In Molecular Evolution: Computer Analysis of Protein and Nucleic Acid Sequences, Methods in Enzymology 183, 281-306, 1990; Zuker. On Finding All Suboptimal Foldings of an RNA Molecule. Science 244, 48-52, 1989). OligoAnalyzer 3.1 is one such implementation of mfold for primer design

25 (www.idtdna.com/analyzer/Applications/OligoAnalyzer/). For example with reference to OligoAnalyzer 3.1, ΔG calculations may be performed using the following parameters: Target Type: DNA; Oligo Concentration 0.25 μM ; Na^+ Concentration: 60mM; Mg^{++} Concentration: 15mM; and dNTPs Concentration: 0.3 mM.

30 3' Recognition region

The invention provides a primer having a 3' recognition sequence whose primer-target formation is stable ($\Delta G \leq$ about -20 kcal/mol or more) has the potential to enhance nucleic acid amplification reaction performance. The 3' recognition region specifically binds

to the a target nucleic acid, for example a complementary sequence of the target nucleic acid. In certain embodiments, the 3' recognition region has a sequence that is complementary to 12, 13, 14, 15, 16, 17, 18, 19, or 20 bases or more of a target nucleic acid sequence. In various embodiments, the primer-target melting temperature is equal to or greater than 8° or 6
5 °C below the reaction or extension temperature of the assay ($T_m \geq \text{assay temperature} - 8^\circ$). In particular embodiments, the 3' recognition sequence comprises 12-20, 12-17, or 12-14 nucleotides. In particular embodiments, the primer-target formation is more stable than self dimer formation (e.g., $\Delta\Delta G \leq$ about -15, -16, -17, -18, -19, -20 kcal/mol or more). Indeed, it has been found that a difference between a working and non-working assay is when the $\Delta\Delta G$
10 $<$ about -15 kcal/mol (see, e.g., Figure 11). Preferably, the 3' recognition sequence does not contain self-complementary sequences, short inverted repeats (e.g., >4 bases/repeat), or sequences that otherwise promote intramolecular interactions, which have the potential to interfere with primer-target annealing.

In particular, a primer of the invention having a 3' recognition sequence is useful in
15 nicking amplification assays. Additionally, the target specific 3' recognition region comprises one or more 2' modified nucleotides (e.g., 2'-O-methyl, 2'-methoxyethoxy, 2'-fluoro, , 2'-alkyl, 2'-allyl, 2'-O-[2-(methylamino)-2-oxoethyl], 2'-hydroxyl (RNA), 4'-thio, 4'-CH₂-O-2'-bridge, 4'-(CH₂)₂-O-2'-bridge, and 2'-O-(N-methylcarbamate)). Without being bound to theory, it is hypothesized that incorporating one or more 2' modified nucleotides in
20 the recognition regions reduces or eliminates intermolecular and/or intramolecular interactions of primers (e.g., primer-dimer formation), and, thereby, reduces or eliminates the background signal in isothermal amplification. The 2' modified nucleotide preferably has a base that base pairs with the target sequence. In particular embodiments, two or more 2' modified nucleotides (e.g., 2, 3, 4, 5 or more 2' modified nucleotides) in the target specific
25 recognition region are contiguous (e.g., a block of modified nucleotides). In some embodiments, the block of 2' modified nucleotides is positioned at the 3' end of the target specific recognition region. In other embodiments, the block of 2' modified nucleotides is positioned at the 5' end of the target specific recognition region. When the block of 2' modified nucleotides is positioned at the 5' end of the target specific recognition region, the
30 2' modified nucleotides may be separated from the nick site by one or more non-modified nucleotides (e.g., 2, 3, 4, 5 or more 2' unmodified nucleotides). Applicants have found that positioning of one or more 2' modified nucleotides or of a block of 2' modified nucleotides

alters the kinetics of amplification. When the one or more 2' modified nucleotides or block of 2' modified nucleotides are positioned at or near the 5' end of the recognition region or proximal to the nick site, real-time amplification reactions showed decreased time to detection. Additionally, the signal curve is contracted and the slope of the curve shifted.

5 In a related embodiment, ratios of a primer having one or more 2' modified nucleotides can be used to alter the time-to-detection and/or the efficiency of the reaction for the 'tuning' of reactions, resulting in a predictable control over reaction kinetics. Increasing the ratio of primer having one or more 2' modified nucleotides at the 3' end of the recognition sequence to primer having one or more 2' modified nucleotides at the 5' end of the
10 the recognition sequence contracted the signal curve and shifted the slope of the curve. It is advantageous to be able to "tune" a reaction providing a means to manipulate both the time-to-detection as well as the efficiency of the reaction. Tuning the reactions can be used to match efficiencies of target nucleic acid amplification and reference nucleic amplification (e.g., internal standard) in quantitative PCR (qPCR). Additionally, amplification curves of
15 the target nucleic acid and the internal standard may be altered so time of detection of their amplification products are separated, while providing the same efficiency for target nucleic acid amplification and internal standard amplification. Through the use of specific combinations and ratios of oligonucleotide structures within a reaction it is possible to create conditions which enable tuned reaction performance.

20 **5' Tail Dimerization Region**

The invention provides a primer having a 5' tail region capable of self-dimerization that enhances nucleic acid amplification reaction performance. Without being bound to theory, in a nucleic acid amplification reaction the primer anneals to the target nucleic acid as
25 a primer-dimer (see e.g., Figure 1). For example, nicking amplification primers have a nicking agent recognition site present at the 5' end that is unrelated to the binding specificity of the primer for the target recognition sequence. Non-specific background products from non-specific primer interactions have the potential to sequester reaction components that would otherwise have been utilized for the amplification of the specific product. In various
30 embodiments, homodimer formation is stable (e.g., $\Delta G \leq$ about -30, -35, -40, -45, -50, -55, -60 kcal/mol or more). In various embodiments, the homodimer has a melting temperature higher than the extension reaction temperature. In particular embodiments, the 5' tail region has a sequence that is a palindrome. In further embodiments, the 5' tail region is at least 12

bases (e.g., 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 bases) in length. In additional embodiments, the 5' tail region has a GC content of 80-90%. In certain embodiments, homodimer formation is more stable than formation of other less stable primer dimer conformations formation (e.g., $\Delta\Delta G \leq$ about -12, -13, -14, -15, -16, -17, -18, -19, -20, -25, -30, -35, -40 kcal/mol or more).

In particular, a primer of the invention having a 5' tail sequence is useful in nicking amplification reactions. For use in nicking amplification reactions, the 5' tail region comprises one or more nicking agent recognition sites and the 5' tail region has a symmetrically inverted sequence. In some embodiments, the nick site is designed to be positioned between the nucleotide at the 3' end of the 5' tail region and the nucleotide at the 5' end of the 3' recognition region. Without being bound to theory, the nicking enzyme does not cleave at the nick site when the 3' recognition is single-stranded. However, cleavage at the nick site occurs when the 3' recognition region is double stranded (e.g., when the primer is incorporated into a double-stranded target nucleic acid molecule during the course of the nucleic acid amplification reaction).

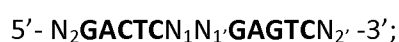
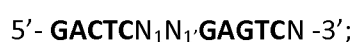
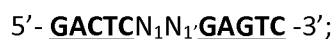
In various embodiments, the 5' tail sequence comprises from 5' to 3' an inverted nicking enzyme recognition sequence that is operatively linked to a palindromic sequence (or self-complementary sequence) that is operatively linked to a nicking enzyme recognition sequence. In certain embodiments, the spacer region is an even number of nucleotides (e.g., 2, 4, 6, etc.).

An exemplary 5' tail based on the Nt.BstNBI nicking enzyme recognition sequence (5'-GAGTC-3') having a 2 nucleotide spacer comprises a nucleic acid sequence according to the formula below:



where "N" is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C), or guanine (G) nucleobase), and N_1 is complementary to N_1' .

Exemplary sequences for such 2 base spacer tail sequences comprise, for example, the nucleic acid sequences according to the formulas below:



5'-N₂GACTCN₁N₁'GAGTCN₂'N₁-3';

5'-N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'-3';

5'-N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₁-3';

5'-N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄'-3';

5 5'-N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄'N₁-3'; and

5'-N₅N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄'N₅'-3',

where "N" is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C), or guanine (G) nucleobase), and N₁ is complementary to N₁', N₂ to N₂', N₃ to N₃', N₄ to N₄', and N₅ to N₅', etc.

10 In some embodiments, 2 base spacer tails exclude those comprising the nucleic acid sequences according to the formulas below:

5'-TCGACTCN₁N₁'GAGTCGA₁-3';

5'-TCGACTCN₁N₁'GAGTCGAN₁-3';

5'-N₂TCGACTCN₁N₁'GAGTCGAN₂'-3';

15 5'-N₂TCGACTCN₁N₁'GAGTCGAN₂'N₁-3';

5'-GTCGACTCN₁N₁'GAGTCGAC₁-3';

5'-GTCGACTCN₁N₁'GAGTCGACN₁-3';

5'-N₃N₂TCGACTCN₁N₁'GAGTCGAN₂'N₃'-3';

5'-N₃GTCGACTCN₁N₁'GAGTCGACN₃'-3'; and

20 5'-AGTCGACTCN₁N₁'GAGTCGACT₁-3',

where "N" is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C), or guanine (G) nucleobase), and N₁ is complementary to N₁', N₂ to N₂', N₃ to N₃', etc.

An exemplary 5' tail based on the Nt.BstNBI nicking enzyme recognition sequence (5'-GAGTC-3') having a 4 nucleotide spacer comprises a nucleic acid sequence according to
25 the formula below:

5'-GACTCN₂N₁N₁'N₂'GAGTC-3'

where “N” is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C), or guanine (G) nucleobase), and N₁ is complementary to N₁′, and N₂ is complementary to N₂′.

Exemplary sequences for such 4 base spacer tail sequences include, for example, the nucleic acid sequences according to the formulas below:

- 5 5′- GACTCN₂N₁N₁′N₂′GAGTC -3′;
- 5′- GACTCN₂N₁N₁′N₂′GAGTCN -3′;
- 5′- N₃GACTCN₂N₁N₁′N₂′GAGTCN₃′ -3′;
- 5′- N₃GACTCN₂N₁N₁′N₂′GAGTCN₃′N -3′;
- 5′- N₄N₃GACTCN₂N₁N₁′N₂′GAGTCN₃′N₄′ -3′;
- 10 5′- N₄N₃GACTCN₂N₁N₁′N₂′GAGTCN₃′N₄′N -3′;
- 5′- N₅N₄N₃GACTCN₂N₁N₁′N₂′GAGTCN₃′N₄′N₅′ -3′;
- 5′- N₅N₄N₃GACTCN₂N₁N₁′N₂′GAGTCN₃′N₄′N₅′N -3′; and
- 5′- N₆N₅N₄N₃GACTCN₂N₁N₁′N₂′GAGTCN₃′N₄′N₅′N₆′ -3′,

- where “N” is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C),
15 or guanine (G) nucleobase), and N₁ is complementary to N₁′, N₂ to N₂′, N₃ to N₃′, N₄ to N₄′,
 N₅ to N₅′, and N₆ to N₆′, etc.

In some embodiments, 4 base spacer tails exclude those comprising the nucleic acid sequences according to the formulas below:

- 5′- GACTCGATCGAGTC -3′;
- 20 5′- GACTCGATCGAGTCN -3′;
- 5′- N₁GACTCGATCGAGTCN₁′ -3′;
- 5′- N₁GACTCGATCGAGTCN₁′N -3′;
- 5′- N₂N₁GACTCGATCGAGTCN₁′N₂′ -3′;
- 5′- N₂N₁GACTCGATCGAGTCN₁′N₂′N -3′;
- 25 5′- TCGACTCN₂N₁N₁′N₂′GAGTCGA -3′;
- 5′- TCGACTCN₂N₁N₁′N₂′GAGTCGAN -3′;

5'-N₃N₂N₁GACTCGATCGAGTCN₁'N₂'N₃'-3';

5'-N₃N₂N₁GACTCGATCGAGTCN₁'N₂'N₃'N-3';

5'-N₃TCGACTCN₂N₁N₁'N₂'GAGTCGAN₃'-3';

5'-N₃TCGACTCN₂N₁N₁'N₂'GAGTCGAN₃'N-3';

5 5'-GTCGACTCN₂N₁N₁'N₂'GAGTCGAC-3';

5'-GTCGACTCN₂N₁N₁'N₂'GAGTCGACN-3';

5'-N₄N₃N₂N₁GACTCGATCGAGTCN₁'N₂'N₃'N₄'-3';

5'-N₄N₃TCGACTCN₂N₁N₁'N₂'GAGTCGAN₃'N₄'-3';

5'-N₃GTCGACTCN₂N₁N₁'N₂'GAGTCGACN₃'-3'; and

10 5'-AGTCGACTCN₂N₁N₁'N₂'GAGTCGACT-3',

where "N" is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C), or guanine (G) nucleobase), and N₁ is complementary to N₁', N₂ to N₂', N₃ to N₃', etc.

An exemplary 5' tail based on the Nt.BstNBI nicking enzyme recognition sequence (5'-GAGTC-3') having a 6 nucleotide spacer comprises a nucleic acid sequence according to

15 the formula below:

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTC-3'

where "N" is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C), or guanine (G) nucleobase), and N₁ is complementary to N₁', N₂ is complementary to N₂', and N₃ is complementary to N₃'.

20 Exemplary sequences for such 6 base spacer tail sequences comprise, for example, the nucleic acid sequences according to the formulas below:

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTC-3';

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN-3';

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNN-3';

25 5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNNN-3';

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNNNN-3';

5'- N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄' -3';

5'- N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N -3';

5'- N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'NN -3';

5'- N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'NNN -3';

5 5'- N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅' -3';

5'- N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N -3';

5'- N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆' -3';

5'- N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆'N -3'; and

5'- N₇N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆'N₇' -3',

10 where "N" is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C), or guanine (G) nucleobase), and N₁ is complementary to N₁', N₂ to N₂', N₃ to N₃', N₄ to N₄', N₅ to N₅', N₆ to N₆', and N₇ to N₇', etc.

In some embodiments, 6 base spacer tails exclude those having the nucleic acid sequences according to the formulas below:

15 5'- GACTCGAN₁N₁'TCGAGTC -3';

5'- GACTCGAN₁N₁'TCGAGTCN -3';

5'- N₂GACTCGAN₁N₁'TCGAGTCN₂' -3';

5'- N₂GACTCGAN₁N₁'TCGAGTCN₂'N -3';

5'- N₃N₂GACTCGAN₁N₁'TCGAGTCN₂'N₃' -3';

20 5'- N₃N₂GACTCGAN₁N₁'TCGAGTCN₂'N₃'N -3';

5'- TCGACTCN₃N₂N₁N₁'N₂'N₃'GAGTCGA -3';

5'- TCGACTCN₃N₂N₁N₁'N₂'N₃'GAGTCGAN -3';

5'- N₄N₃N₂GACTCGAN₁N₁'TCGAGTCN₂'N₃'N₄' -3';

5'- N₄N₃N₂GACTCGAN₁N₁'TCGAGTCN₂'N₃'N₄'N -3';

25 5'- N₄GTCGACTCN₃N₂N₁N₁'N₂'N₃'GAGTCGACN₄' -3';

5'-GTCGACTCN₃N₂N₁N₁'N₂'N₃'GAGTCGAC-3';

5'-GTCGACTCN₃N₂N₁N₁'N₂'N₃'GAGTCGACN-3';

5'-N₅N₄N₃N₂GACTCGAN₁N₁'TCGAGTCN₂'N₃'N₄'N₅'-3';

5'-N₅N₄TCGACTCN₃N₂N₁N₁'N₂'N₃'GAGTCGAN₄'N₅'-3';

5'-N₄GTCGACTCN₃N₂N₁N₁'N₂'N₃'GAGTCGACN₄'-3'; and

5'-AGTCGACTCN₃N₂N₁N₁'N₂'N₃'GAGTCGACT-3',

where "N" is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C), or guanine (G) nucleobase), and N₁ is complementary to N₁', N₂ to N₂', N₃ to N₃', N₄ to N₄', N₅ to N₅', etc.

Exemplary 5' tail region sequences are provided at Figures 8A-8E. Exemplary 5' tail region sequences 24 nucleotides in length having a Nt.BstNBI recognition sequence can be generated based on the following template 5'-NNNNGACTCNNNNNNGAGTCNNNN-3' (Figure 8A). Based on this template, there are 537,824 5' tail sequences having the following properties: $\Delta G = -48$ Kcal/mole to -62 kcal/mole; $\Delta\Delta G < -40$ kcal/mole; and GC content 68% to 84%. Of these, 1050 selected sequences are provided, representing 0.2% of the entire sequence space (248,832). Exemplary 5' tail region sequences 22 nucleotides in length having a Nt.BstNBI recognition sequence and based on the following template 5'-NNNNGACTCNNNNNGAGTCNNNN-3' (Figure 8B). Based on this template, there are 248,832 5' tail sequences having the following properties: $\Delta G = -47$ Kcal/mole to -55 kcal/mole; $\Delta\Delta G < -40$ kcal/mole; and GC content 72% to 82%. Of these, 200 selected sequences are provided, representing 0.08% of the entire sequence space (248,832).

Nucleic Amplification Methods

Nucleic acid amplification technologies have provided a means of understanding complex biological processes, detection, and identification, of pathogenic and non-pathogenic organisms, forensic criminology analysis, disease association studies, and detection of events in genetically modified organisms, etc. The polymerase chain reaction (PCR) is a common thermal cycling dependent nucleic acid amplification technology used to amplify DNA consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA using a DNA polymerase. Real-Time quantitative PCR

(qPCR) is a technique used to quantify the number of copies of a given nucleic acid sequence in a biological sample. Currently, qPCR utilizes the detection of reaction products in real-time throughout the reaction and compares the amplification profile to the amplification of controls which contain a known quantity of nucleic acids at the beginning of each reaction (or
5 a known relative ratio of nucleic acids to the unknown tested nucleic acid). The results of the controls are used to construct standard curves, typically based on the logarithmic portion of the standard reaction amplification curves. These values are used to interpolate the quantity of the unknowns based on where their amplification curves compared to the standard control quantities.

10 In addition to PCR, non-thermal cycling dependent amplification systems or isothermal nucleic acid amplification technologies exist including, without limitation: Nicking Amplification Reaction, Rolling Circle Amplification (RCA), Helicase-Dependent Amplification (HDA), Loop-Mediated Amplification (LAMP), Strand Displacement Amplification (SDA), Transcription-Mediated Amplification (TMA), Self-Sustained
15 Sequence Replication (3SR), Nucleic Acid Sequence Based Amplification (NASBA), Single Primer Isothermal Amplification (SPIA), Q- β Replicase System, and Recombinase Polymerase Amplification (RPA).

Isothermal nicking amplification reactions have similarities to PCR thermocycling. Like PCR, nicking amplification reactions employ oligonucleotide sequences which are
20 complementary to a target sequences referred to as primers. In addition, nicking amplification reactions of target sequences results in a logarithmic increase in the target sequence, just as it does in standard PCR. Unlike standard PCR, the nicking amplification reactions progress isothermally. In standard PCR, the temperature is increased to allow the two strands of DNA to separate. In nicking amplification reactions, the target nucleic acid
25 sequence is nicked at specific nicking sites present in a test sample. The polymerase infiltrates the nick site and begins complementary strand synthesis of the nicked target nucleotide sequence (the added exogenous DNA) along with displacement of the existing complimentary DNA strand. The strand displacement replication process obviates the need for thermal strand separation. At this point, primer molecules anneal to the displaced
30 complementary sequence from the added exogenous DNA. The polymerase now extends from the 3' end of the primer, creating a complementary strand to the previously displaced strand. The second primer oligonucleotide then anneals to the newly synthesized complementary strand and is extended making a duplex of DNA which includes the nicking

agent recognition sequence. This strand is then liable to be nicked with subsequent strand displacement extension by the polymerase, which leads to the production of an interim amplicon of duplex of DNA which has nick sites on either side of the original target DNA. Once this interim amplicon is synthesized, the molecule continues to be amplified

5 exponentially through replication of the displaced strands with new primer molecules. In addition, amplification also proceeds linearly from each product molecule through the repeated action of the nick translation synthesis at the template introduced nick sites. The result is a very rapid increase in target signal amplification; much more rapid than as produced by PCR thermocycling, with amplification results in less than ten minutes.

10

Nicking Agent-Dependent Isothermal Nucleic Acid Amplification Assays

The invention provides for the detection of target nucleic acid molecules amplified in an isothermal nicking amplification assay. . Polymerases useful in the methods described herein are capable of catalyzing the incorporation of nucleotides to extend a 3' hydroxyl

15 terminus of an oligonucleotide (e.g., a primer) bound to a target nucleic acid molecule and/or a 3' hydroxyl terminus at a nick site in a double-stranded DNA molecule in conjunction with strand displacement activity. Such polymerases also lack or have substantially reduced 5'-3' exonuclease activity and may include those that are thermophilic. DNA polymerases useful in methods involving primers having 2'-modified nucleotides in the primer region comprising

20 the six 3'-terminal nucleotides include derivatives and variants of the DNA polymerase I isolated from *Bacillus stearothermophilus*, also classified as *Geobacillus stearothermophilus*, and from closely related bacterial strains, isolates and species comprising the genus *Geobacillus*, which lack or have substantially reduced 5'-3' exonuclease activity and have strand-displacement activity. Exemplary polymerases include, but are not limited to the large

25 fragments of Bst DNA polymerase I, Gst DNA polymerase I, Gka DNA polymerase I.

A nicking agent useful in methods described herein is a chemical entity capable of recognizing and binding to a specific structure in double stranded nucleic acid molecules and cleaving a phosphodiester bond between adjoining nucleotides on the top strand with a substantially higher rate than cleaving the phosphodiester bond between adjoining

30 nucleotides on the bottom strand upon binding to its recognized specific structure, thereby creating a free 3'-hydroxyl group on the terminal nucleotide preceding the nick site that can be extended by a 5'-3'-exonuclease deficient strand displacement polymerase. In a preferred embodiment of the methods disclosed herein, the top strand phosphodiester bond cleavage

rate of the “nicking agent” approaches 100%, while the cleavage rate of the bottom strand phosphodiester bond approaches 0%. Nicking agents useful in methods described herein, can either be enzymes, i.e. self-regenerating catalysts turning over multiple substrate molecules, or non-regenerating catalysts turning over just a single substrate molecule at an equimolar ratio.

A nicking enzyme binds double-stranded DNA and cleaves one strand of a double-stranded duplex. In the methods of the invention, the nicking enzyme cleaves the top strand (the strand comprising the 5'-3' sequence of the nicking agent recognition site). The nicking enzyme may cleave either upstream or downstream of the binding site or nicking enzyme recognition site. In a particular embodiment of the invention disclosed herein, the nicking enzyme cleaves the top strand only and 3' downstream of the recognition site. In exemplary embodiments, the reaction comprises the use of a nicking enzyme that cleaves or nicks downstream of the binding site such that the product sequence does not contain the nicking site. Using an enzyme that cleaves downstream of the binding site allows the polymerase to more easily extend without having to displace the nicking enzyme. Ideally, the nicking enzyme is functional under the same reaction conditions as the polymerase. Exemplary nicking enzymes include, but are not limited to, N.Bst9I, N.BstSEI, Nb.BbvCI(NEB), Nb.Bpu10I(Fermentas), Nb.BsmI(NEB), Nb.BsrDI(NEB), Nb.BtsI(NEB), Nt.AlwI(NEB), Nt.BbvCI(NEB), Nt.Bpu10I(Fermentas), Nt.BsmAI, Nt.BspD6I, Nt.BspQI(NEB), Nt.BstNBI(NEB), and Nt.CviPII(NEB). Sequences of nicking enzyme recognition sites are provided at Table 1.

Table 1. Nicking enzyme recognition sequences

N.Bst9I	5' -GAGTCNNNNN↓NN-3' 3' -CTCAGNNNNN•NN-5'
N.BstSEI	5' -GAGTNCNNNNN↓NN-3' 3' -CTCAGNNNNN•NN-5'
Nb.BbvCI(NEB)	5' -CCTCA•GC-3' 3' -GGAGT↑CG-5'
Nb.Bpu10I(Fermentas)	5' -CCTNA•GC-3' 3' -GGANT↑CG-5'
Nb.BsmI(NEB)	5' -GAATG•CN-3'

	3' -CTTAC↑GN-5'
Nb.BsrDI(NEB)	5' -GCAATG•NN-3' 3' -CGTTAC↑NN-5'
Nb.BtsI(NEB)	5' -GCAGTG•NN-3' 3' -CGTCAC↑NN-5'
Nt.AIwI(NEB)	5' -GGATCNNNN↓N-3' 3' -CCTAGNNNN•N-5'
Nt.BbvCI(NEB)	5' -CC↓TCAGC-3' 3' -GG•AGTCG-5'
Nt.Bpu10I(Fermentas)	5' -CC↓TNAGC-3' 3' -GG•ANTCG-5'
Nt.BsmAI	5' -GTCTCN↓N-3' 3' -CAGAGN•N-5'
Nt.BspD6I	5' -GAGTCNNNN↓N-3' 3' -CTCAGNNNN•N-5'
Nt.BspQI(NEB)	5' -GCTCTTCN↓-3' 3' -CGAGAAGN -5'
Nt.BstNBI(NEB)	5' -GAGTCNNNN↓N-3' 3' -CTCAGNNNN•N-5'
Nt.CviPII(NEB)	5' -↓CCD-3' 3' - GGH-5'

Nicking enzymes also include engineered nicking enzymes created by modifying the cleavage activity of restriction endonucleases (NEB expressions July 2006, vol 1.2). when restriction endonucleases bind to their recognition sequences in DNA, two catalytic sites within each enzyme for hydrolyzing each strand drive two independent hydrolytic reactions which proceed in parallel. Altered restriction enzymes can be engineered that hydrolyze only one strand of the duplex, to produce DNA molecules that are “nicked” (3'-hydroxyl, 5'-phosphate), rather than cleaved. Nicking enzymes may also include modified CRISPR/Cas proteins, Transcription activator-like effector nucleases (TALENs), and Zinc-finger nucleases having nickase activity.

A nicking amplification reaction typically comprises nucleotides, such as, for example, dideoxyribonucleoside triphosphates (dNTPs). The reaction may also be carried out in the presence of dNTPs that comprise a detectable moiety including but not limited to a radiolabel (e.g., ^{32}P , ^{33}P , ^{125}I , ^{35}S) an enzyme (e.g., alkaline phosphatase), a fluorescent label (e.g., fluorescein isothiocyanate (FITC)), biotin, avidin, digoxigenin, antigens, haptens, or fluorochromes. The reaction further comprises certain salts and buffers that provide for the activity of the nicking enzyme and polymerase.

Advantageously, the nicking amplification reaction is carried out under substantially isothermal conditions where the temperature of the reaction is more or less constant during the course of the amplification reaction. Because the temperature does not need to be cycled between an upper temperature and a lower temperature, the nicking amplification reaction can be carried out under conditions where it would be difficult to carry out conventional PCR. Typically, the reaction is carried out at about between 35 °C and 90 °C (e.g., about 35, 37, 42, 55, 60, 65, 70, 75, 80, or 85 °C). Advantageously, it is not essential that the temperature be maintained with a great degree of precision. Some variability in temperature is acceptable.

Sets of primers for amplification reactions are selected having $\Delta\Delta G$'s ≤ -15 , -16 , 17 , -18 , -19 , -20 , -25 , -30 kcal/mole or more. The performance characteristics of amplification reactions may be altered by increasing the concentration of one or more oligonucleotides (e.g., one or more primers and/or probes) and/or their ratios. High concentrations of primers also favor primer-dimer formation. In various embodiments, concentration of a primers is 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 nM or more. Melt temperature (T_m) and reaction rate modifiers may also be used to lower the melting temperature of the oligonucleotides, such as (but not limited to) ethylene glycol and glycerol. In addition, DNA polymerase reaction rate modifiers (such as dNTP and magnesium concentration) may be used to alter the reaction rate. In particular embodiments, the 5' tail sequences of the forward and reverse primers have the same nucleic acid sequence.

The invention also provides a method of designing a nicking agent-dependent isothermal strand-displacement amplification assay without experimental screening of a multitude of combinations of candidate forward primers and/or candidate reverse primers. A 35 to 70 bp long region within the target sequence is identified having a 12 to 20 bp sequence in the central portion with a $T_m \geq$ the assay temperature (e.g., $\sim 55^\circ\text{C}$). Adjacent sequences

12 bp to 20 bp long immediately downstream and upstream of the 15 to 20 bp long central region are identified, according to the above criteria. The T_m of the chosen double stranded downstream and upstream adjacent sequences deviate from each other by less than $\pm 3^\circ\text{C}$. A target-specific pair of forward and reverse primers are created by attaching a 5'-tail region for a stable dimer-forming primer to the 5'-terminus of the 12-20 base upstream adjacent sequence and to the 5'-terminus of the complementary strand of the 12-20 base downstream adjacent sequence. When combining the forward primer, reverse primer, and a probe, the primer driving the synthesis of the strand complementary to the probe is in excess over the other primer at a molar ratio of about 1.1:1 to 10:1. The combined concentration of a primer in the assay is no higher than 1000 nM. The assay design method can also be used to convert a pre-validated PCR assay for an amplicon ≤ 70 bp to an nicking agent-dependent isothermal strand-displacement amplification assay.

Target Nucleic Acid Molecules

Methods and compositions of the invention are useful for the amplification and/or identification of a target nucleic acid molecule in a test sample. The target sequences is amplified from virtually any samples that comprises a target nucleic acid molecule, including but not limited to samples comprising fungi, spores, viruses, or cells (e.g., prokaryotes, eukaryotes). In specific embodiments, compositions and methods of the invention detect *Clavibacter michiganensis subsp. michiganensis*, *Clavibacter michiganensis subsp. sepedonicus*, *Pseudomonas syringae pv Tomato*, *Xanthomonas campestris pv Vesicatoria*, *Alternaria spp*, *Cladosporium spp*, *Fusarium oxysporum*, *Verticilium dahlia*, *Pseudomonas currugata*, *Erwina carotovora*, and *Ralstonia solanacearum*. Exemplary test samples include body fluids (e.g. blood, serum, plasma, amniotic fluid, sputum, urine, cerebrospinal fluid, lymph, tear fluid, feces, or gastric fluid), tissue extracts, culture media (e.g., a liquid in which a cell, such as a pathogen cell, has been grown), environmental samples, agricultural products or other foodstuffs, and their extracts, and DNA identification tags. If desired, the sample is purified prior to inclusion in a nicking amplification reaction using any standard method typically used for isolating a nucleic acid molecule from a biological sample.

In one embodiment, primers amplify a target nucleic acid of a pathogen to detect the presence of a pathogen in a sample. Exemplary pathogens include fungi, bacteria, viruses and yeast. Such pathogens may be detected by identifying a nucleic acid molecule encoding a pathogen protein, such as a toxin, in a test sample. Exemplary toxins include, but are not

limited to aflatoxin, cholera toxin, diphtheria toxin, Salmonella toxin, Shiga toxin, *Clostridium botulinum* toxin, endotoxin, and mycotoxin. For environmental applications, test samples may include water, liquid extracts of air filters, soil samples, building materials (e.g., drywall, ceiling tiles, wall board, fabrics, wall paper, and floor coverings), environmental swabs, or any other sample. In one embodiment, primer oligonucleotides amplify a target nucleic acid of a plant used as an internal control in molecular breeding experiments geared towards improving, for example, the plant's resistance to drought, the plant's resistance to herbicides, or to predation by harmful insects.

Target nucleic acid molecules include double-stranded and single-stranded nucleic acid molecules (e.g., DNA, and other nucleobase polymers known in the art capable of hybridizing with a nucleic acid molecule described herein). DNA molecules suitable for detection with a detectable oligonucleotide probe or primer of the invention include, but are not limited to, double stranded DNA (e.g., genomic DNA, plasmid DNA, mitochondrial DNA, viral DNA, and synthetic double stranded DNA). Single-stranded DNA target nucleic acid molecules include, for example, viral DNA, cDNA, and synthetic single-stranded DNA, or other types of DNA known in the art.

In general, a target sequence for detection is between 10 and 100 nucleotides in length (e.g., 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 nucleotides. The GC content of the target nucleic acid molecule is selected to be less than about 45, 50, 55, or 60%.

Desirably, the target sequence and nicking enzymes are selected such that the target sequence does not contain nicking sites for any nicking enzymes that will be included in the reaction mix.

Applications

Target nucleic acid amplification using primers of the invention have characteristics useful for rapid detection of target nucleic acids (Figure 10). Such reactions are characterized by a high signal-to-noise ratio ($[RFU_{MAX} - RFU_{BL}]$), steep slope (high value of $[RFU_{MAX} - RFU_{BL}]/[T_{OSEA} - T_{RFU_{max}}]$), early onset of the exponential amplification phase ($T_{OSEA} < 10$ min) and low signal variance between replicated assay reactions of the same sample. In certain embodiments, $T_{OSEA} - T_{RFU_{max}} \approx 3$ min.

The present invention provides for the real-time monitoring of the isothermal amplification reaction. Compositions and methods of the invention are useful in human diagnostics, where rapid detection is desired (e.g., detectable amplification in under 20, 15,

10, 9, 8, 7, 6, 5 min. or less). In particular embodiments, the invention provides for the use of nicking amplification reaction assays in human diagnostics in clinical settings. In other embodiments, the invention provides for the use of nicking amplification reaction assays in diagnostic field work, where access to thermocycling equipment is unavailable or would be prohibitively expensive. In still other embodiments, the invention provides for the use of nicking amplification reaction assays in an academic setting where rapid nucleic acid amplification and detection are desired.

Detectable Oligonucleotide Probes

10 The present invention provides for the detection of target nucleic acid molecules or amplicons thereof in a nicking amplification reaction using non-amplifiable detectable polynucleotide probes comprising at least one polymerase-arresting molecule (e.g., nucleotide modification or other moiety that renders the oligonucleotide capable of binding a target nucleic acid molecule, but incapable of supporting polymerase extension utilizing the detectable oligonucleotide probe as a target). Without wishing to be bound by theory, the presence of one or more moieties which does not allow polymerase progression likely causes polymerase arrest in non-nucleic acid backbone additions to the oligonucleotide or through stalling of a replicative polymerase (i.e. C3-spacer, damaged DNA bases, other spacer moiety, O-2-Me bases). These constructs thus prevent or reduce illegitimate amplification of the probe during the course of a nicking amplification reaction. This distinguishes them from conventional detection probes, which must be added at the end of the nicking amplification reaction to prevent their amplification.

25 Conventional detection probes have proven impractical for detecting a nicking amplification reaction in real time. If conventional detection probes are incorporated into the nicking amplification reaction, these conventional detection probes are amplified concurrently with the target. The amplification of these detection molecules masks the detection of legitimate target amplicons due to the number of starting molecules of the detection probe at the start of the reaction.

30 The invention provides non-amplifiable detectable polynucleotide probe that comprise at least one polymerase-arresting molecule. A polymerase-arresting molecule of the invention includes, but is not limited to, a nucleotide modification or other moiety that blocks extension by replicative DNA polymerases, thereby preventing the amplification of detection molecules; but can allow proper hybridization or nucleotide spacing to the target molecule or

amplified copies of the target molecule. In one embodiment, a detectable oligonucleotide probe of the invention comprises a 3 carbon spacer (C3-spacer) that prevents or reduces the illegitimate amplification of a detection molecule.

In one embodiment, a detectable oligonucleotide probe comprises one or more modified nucleotide bases having enhanced binding affinity to a complementary nucleotide. Examples of modified bases include, but are not limited to 2' Fluoro amidites, and 2'OMe RNA amidites (also functioning as a polymerase arresting molecule). Detectable oligonucleotide probes of the invention can be synthesized with different colored fluorophores and may be designed to hybridize with virtually any target sequence. In view of their remarkable specificity, a non-amplifiable detectable polynucleotide probe of the invention is used to detect a single target nucleic acid molecule in a sample, or is used in combination with detectable oligonucleotide probes each of which binds a different target nucleic acid molecule. Accordingly, the non-amplifiable detectable polynucleotide probes of the invention may be used to detect one or more target nucleic acid molecules in the same reaction, allowing these targets to be detected simultaneously. The present invention encompasses the use of such fluorophores in conjunction with the detectable oligonucleotide probes described herein.

Use of Non-Amplifiable Detectable Polynucleotide Probes

Non-amplifiable detectable polynucleotide probe are useful in methods for amplifying and detecting a target nucleic acid molecule in a nicking amplification reaction. The method involves contacting a target nucleic acid molecule under substantially isothermal conditions with a polymerase, two primers, each of which specifically binds to a complementary sequence on the target nucleotide molecule, a nicking enzyme, and the detectable oligonucleotide probe in the presence of a suitable buffer and dNTPs, generating amplicons comprising at least a portion of said target nucleic acid molecule; and detecting the target nucleic acid molecule present in the reaction by detecting the oligonucleotide probe that hybridizes to the target nucleic acid molecule in real time during the reaction based on fluorescent intensity from the probe molecules in the reaction. Advantageously, such methods are useful for monitoring nicking amplification reactions in real time.

In general, non-amplifiable detectable polynucleotide probes of the invention are included in a nicking amplification reaction that comprises (1) a target nucleic acid molecule; (2) two primers comprising some number of oligonucleotides that are complementary to the

target nucleic acid molecule and a site that can be cleaved by a nicking enzyme; (3) dNTPs; (4) a strand displacing polymerase; and (5) a nicking enzyme. Accordingly, the invention provides a method of using these components to detect a target nucleic acid molecule.

5 **Implementation in Hardware and/or Software**

The methods described herein can be implemented on general-purpose or specially programmed hardware or software. For example, the methods can be implemented by a computer readable medium. Accordingly, the present invention also provides a software and/or a computer program product configured to perform the algorithms and/or methods according to any embodiment of the present invention. It is well-known to a skilled person in the art how to configure software which can perform the algorithms and/or methods provided in the present invention. The computer-readable medium can be non-transitory and/or tangible. For example, the computer readable medium can be volatile memory (*e.g.* , random access memory and the like) or non-volatile memory (*e.g.* , read-only memory, hard disks, floppy discs, magnetic tape, optical discs, paper table, punch cards, and the like). The computer executable instructions may be written in a suitable computer language or combination of several languages. Basic computational biology methods are described in, for example Setubal and Meidanis et al., Introduction to Computational Biology Methods (PWS Publishing Company, Boston, 1997); Salzberg, Searles, Kasif, (Ed.), Computational Methods in Molecular Biology, (Elsevier, Amsterdam, 1998); Rashidi and Buehler, Bioinformatics Basics: Application in Biological Science and Medicine (CRC Press, London, 2000) and Ouelette and Bzevanis Bioinformatics: A Practical Guide for Analysis of Gene and Proteins (Wiley & Sons, Inc., 2nd ed., 2001).

The present invention may also make use of various computer program products and software for a variety of purposes, such as probe design, management of data, analysis, and instrument operation. (See, US Patent Nos 5,593,839, 5,795,716, 5,733,729, 5,974,164, 6,066,454, 6,090,555, 6,185,561, 6,188,783, 6,223,127, 6,229,911 and 6,308,170.) Additionally, the present invention may have preferred embodiments that include methods for providing genetic information over networks such as the Internet as shown in US Ser Nos 10/197,621, 10/063,559 (US Pub No 20020183936), 10/065,856, 10/065,868, 10/328,818, 10/328,872, 10/423,403, and 60/482,389.

Kits

The invention also provides kits for the amplification of a target nucleic acid molecule. Such kits are useful for the amplification and detection of a target nucleic acid in a biological sample obtained from a subject. Kits of the present invention may comprise, for example, one or more polymerases, forward and reverse primers, and one or more nicking enzymes, as described herein. Where one target is to be amplified, one or two nicking enzymes may be included in the kit. Where multiple target sequences are to be amplified, and the primers designed for those target sequences comprise the nicking enzyme sites for the same nicking enzyme, then one or two nicking enzymes may be included. Where the primers are recognized by different nicking enzymes, more nicking enzymes may be included in the kit, such as, for example, 3 or more.

In one aspect, the invention provides a kit for nucleic acid amplification comprising a DNA polymerase; a primary primer, a secondary primer, a nicking enzyme with specificity to a nicking enzyme binding site within the primers, and deoxynucleotide triphosphates (dNTP's) (e.g., in a buffered solution containing components sufficient for amplification. In various embodiments, the primary primer and secondary primer, each have a 3'-end specific recognition region sequence complementary or substantially complementary to the target sequence, where the end specific recognition region comprises one or more 2' modified nucleotides; a 5'-end tail region containing a nicking enzyme binding site upstream of the 3'-end specific recognition region sequences that is able to dimerize with itself (e.g., self-complementary). In particular embodiments, one or more primers are in a primer-dimer configuration (e.g., produced by heating about T_m and slow cooling).

The kits of the present invention may also comprise one or more of the components in any number of separate containers, packets, tubes (e.g., <0.2 ml, 0.2 ml, 0.6 ml, 1.5 ml, 5.0 ml, >5.0 ml), vials, microtiter plates (e.g., <96-well, 96-well, 384-well, 1536-well, >1536-well), ArrayTape, and the like, or the components may be combined in various combinations in such containers. In various embodiments, the kit further comprises a pair of primers capable of binding to and amplifying a reference sequence. In particular embodiments, the kit comprises one or more primers in a primer-dimer configuration (e.g., produced by heating about T_m and slow cooling). In yet other embodiments, the kit comprises a sterile container which contains the primers; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container form known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding nucleic acids.

The components of the kit may, for example, be present in one or more containers, for example, all of the components may be in one container, or, for example, the enzymes may be in a separate container from the primers. The components may, for example, be dried (e.g., powder) or in a stable buffer (e.g., chemically stabilized, thermally stabilized). Dry components may, for example, be prepared by lyophilization, vacuum and centrifugal assisted drying and/or ambient drying. In various embodiments, the polymerase and nicking enzymes are in lyophilized form in a single container, and the primers are either lyophilized, freeze dried, or in buffer, in a different container. In some embodiments, the polymerase, nicking enzymes, and the primers are, in lyophilized form, in a single container. In other embodiments, the polymerase and the nicking enzyme may be separated into different containers.

Kits may further comprise, for example, dNTPs used in the reaction, or modified nucleotides, cuvettes or other containers used for the reaction, or a vial of water or buffer for re-hydrating lyophilized components. The buffer used may, for example, be appropriate for both polymerase and nicking enzyme activity.

The kits of the present invention may also comprise instructions for performing one or more methods described herein and/or a description of one or more compositions or reagents described herein. Instructions and/or descriptions may be in printed form and may be included in a kit insert. A kit also may include a written description of an Internet location that provides such instructions or descriptions.

Kits may further comprise reagents used for detection methods (e.g., real-time or endpoint), such as, for example, hybridization probes or DNA binding dyes. Kits may further comprise reagents used for detection methods, such as, for example, reagents used for FRET, lateral flow devices, dipsticks, fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, or polystyrene beads. Detection components may be incorporated into a lateral flow device. The lateral flow device may be used at a point of care.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook, 1989); "Oligonucleotide Synthesis" (Gait, 1984); "Animal Cell Culture" (Freshney, 1987);

“Methods in Enzymology” “Handbook of Experimental Immunology” (Weir, 1996); “Gene Transfer Vectors for Mammalian Cells” (Miller and Calos, 1987); “Current Protocols in Molecular Biology” (Ausubel, 1987); “PCR: The Polymerase Chain Reaction”, (Mullis, 1994); “Current Protocols in Immunology” (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

EXAMPLES

Example 1. Design of a System for Isothermal DNA Amplification of Soy Lectin by Determining Stability of Primer Target Binding.

A successful working isothermal DNA amplification assay for detection of Soy Lectin was created using no more than the design principles in accordance with the invention and without the aid of experimental testing of the primers (Figure 12).

An assay target sequence in the Soy Lectin gene was selected based on the absence of single nucleotide polymorphisms (SNP's) in a 30 to 60 bp sequence window:

5'-ccaaggttct**cattac**ctatgatg**cc**ctccaccagcctctt**gg**ttgcttctt**gg**tctacccttcacagagaa-3'

Shown in bold are the sequence regions selected for design of the forward and reverse primers, respectively.

For selecting the target binding sequence of the Forward primer (5'-ATTACCTATGATGCC-3'), the stability of Forward primer binding to target was compared to the stability of Forward primer self-dimerization, as follows:

$\Delta G_{25^{\circ}\text{C}}$ of Forward Primer-Target Hybrid (PrTH) = -25.99 kcal/mole;

$\Delta G_{25^{\circ}\text{C}}$ of Primer-Target binding region self dimer (PrTBRHD) = -3.14 kcal/mole;

$\Delta\Delta G_{25^{\circ}\text{C}} = \Delta G_{\text{PTH}} - \Delta G_{\text{PTBRHD}} = -22.85 \text{ kcal/mole.}$

A low $\Delta\Delta G$ ($\leq$ about -16 kcal/mole) indicates that the Forward primer is capable of annealing to and initiating synthesis of the complementary strand of the target nucleic acid molecule.

For selecting the target binding sequence of the Reverse primer (5'-ACCAAAGAAGCAAC-3'), the stability of Reverse primer binding to target was compared to the stability of Reverse primer self-dimerization, as follows:

The stability (ΔG) and relative stability to nearest ($\Delta\Delta G$) of the target binding region of the Reverse primer 5'-ACCAAAGAAGCAAC-3' was determined as follows:

$\Delta G_{25^\circ\text{C}}$ of rev Primer-Target Hybrid (PrTH) = -25.35 kcal/mole;

$\Delta G_{25^\circ\text{C}}$ of Primer-Target binding region self dimer (PrTBRHD) = -3.14 kcal/mole;

$\Delta\Delta G_{25^\circ\text{C}} = \Delta G_{\text{PTH}} - \Delta G_{\text{PTBRHD}} = \mathbf{-22.21 \text{ kcal/mole.}}$

A low $\Delta\Delta G$ ($\leq$ about -16 kcal/mole) indicates that the Reverse primer is capable of annealing to and initiating synthesis of the complementary strand of the target nucleic acid molecule.

Nicking amplification reactions (50 μl) contained 0.3mM dNTPs, 0.38 U/ml Bst 2.0 WarmStart DNA Polymerase (New England Biolabs), 0.15 units Nt.BstNBI (New England Biolabs), 250 nM forward primer, 250nM reverse primer, 200 nM molecular beacon probe labeled at the 5'-terminus with CalRed (BioSearch) and at the 3'-terminus with BHQ2 (BioSearch), and *Glycine max* genomic DNA (10,000 copies). Primer and probe sequences (synthesized by BioSearch Inc., Novato, CA) were as follows:

Forward primer ("m" = 2'-methoxy nucleotide)

5'-ACGCGACTCGTCGACGAGTCGCGTATTACCTATGAmUmGmCmC-3'

Reverse primer ("m" = 2'-methoxy nucleotide)

5'-ACGCGACTCGTCGACGAGTCGCGTACCAAAGAAmGmCmAmAmC-3'

Molecular beacon probe ("I" stands for universal inosine nucleotide)

5'-CalRed₆₁₀-CGCGCTCCACCAICCTCTTGCGCG-BHQ2-3'

Reactions were incubated at 56°C for approximately 10 minutes on an IQ5 thermo cycler (BioRad). Fluorescent signal was recorded on ROX channel (excitation: 575 nm; emission: 602 nm). Reactions containing soy gDNA (n=3) generated sigmoid-shaped curves (Figure 13). In contrast control reactions containing no target DNA (n=3) generated no signal. Time to onset of exponential amplification was about 3 minutes and time to onset of maximum relative fluorescence units was about 6 minutes. The Δ slope of the exponential amplification phase of the reactions was determined to be 660 RFU/min.

Example 2. Design of a System for Isothermal DNA Amplification of *Salmonella enterica invA* by Determining Stability of Primer Target Binding.

A readily, operational isothermal DNA amplification assay for detection of *Salmonella enterica invA* was created using no more than the design principles in accordance with the invention and without the aid of further experimental testing of the primers.

An assay target sequence in the *Salmonella enterica invA* gene was selected based on the absence of single nucleotide polymorphisms (SNP's) in a 30 to 60 bp sequence window:

5'-ATACTCATCTGTTTACCGGGCATACCATCCAGAGAAAA-3'

Shown in bold are the sequence regions selected for design of the forward and reverse primers, respectively.

For selecting the target binding sequence of the Forward primers (5'-ATACTCATCTGTTTACC-3'), the stability of Forward primer binding to target was compared to the stability of Forward primer self-dimerization, as follows:

$\Delta G_{25^{\circ}\text{C}}$ of fwd Primer-Target Hybrid (PTH) = -26.11 kcal/mole;

$\Delta G_{25^{\circ}\text{C}}$ of Primer-Target binding region self dimer (PTBRHD) = -1.95 kcal/mole;

$\Delta\Delta G_{25^{\circ}\text{C}} = \Delta G_{\text{PTH}} - \Delta G_{\text{PTBRHD}} = \mathbf{-24.16 \text{ kcal/mole}}$

A low $\Delta\Delta G$ ($\leq$ about -16 kcal/mole) indicates that the Forward primers are capable of annealing to and initiating synthesis of the complementary strand of the target nucleic acid molecule.

For selecting the target binding sequence of the Reverse primer (5'-TTTTCTCTGGATGG-3'), the stability of Reverse primer binding to target was compared to the stability of Reverse primer self-dimerization, as follows:

$\Delta G_{25^{\circ}\text{C}}$ of rev Primer-Target Hybrid (PTH) = -25.28 kcal/mole;

$\Delta G_{25^{\circ}\text{C}}$ of Primer-Target binding region self dimer (PTBRHD) = -1.57 kcal/mole;

$\Delta\Delta G_{25^{\circ}\text{C}} = \Delta G_{\text{PTH}} - \Delta G_{\text{PTBRHD}} = \mathbf{-23.71 \text{ kcal/mole.}}$

A low $\Delta\Delta G$ ($\leq$ about -16 kcal/mole) indicates that the Reverse primer is capable of annealing to and initiating synthesis of the complementary strand of the target nucleic acid molecule.

Nucleic Acid Amplification Reactions (26 μ l) contained 0.3mM dNTPs, 0.38 U/ml Bst 2.0 WarmStart DNA Polymerase (New England Biolabs), 0.15 units Nt.BstNBI (New England Biolabs), 250 nM forward primer, 250nM reverse primer, 300 nM molecular beacon probe labeled at the 5'-terminus with CalRed (BioSearch) and at the 3'-terminus with BHQ2 (BioSearch), and *Salomonella* genomic DNA (10,000 copies). Primer and probe sequences (synthesized by BioSearch Inc., Novato, CA) are as follows:

Forward primer 1 ("m" = 2'-methoxy nucleotide)

5'-GGCTGACTCCTGCAGGAGTCAGCCATACTCATCTGmUmUmUmAmCmC-3'

Forward primer 2 ("m" = 2'-methoxy nucleotide)

10 5'-GGCTGACTCCTGCAGGAGTCAGCCATACTCATCTGTmUmUmAmCmC-3'

Reverse primer ("m" = 2'-methoxy nucleotide)

5'-GGCTGACTCCTGCAGGAGTCAGCCTTTTCTCTGmGmAmUmGmG-3'

Molecular beacon probe ("I" stands for universal inosine nucleotide)

5'-CalRed₆₁₀-ACCTGTTTACCGGGCATACAAACAGGT-BHQ2-3'

15 Reactions were incubated at 56°C for approximately 10 minutes on an IQ5 thermo cycler (BioRad). Fluorescent signal was recorded on ROX channel (excitation: 575 nm; emission: 602 nm). Reactions containing soy gDNA (n=3) generated sigmoid-shaped curves (Figure 15). In contrast control reactions containing no target DNA (n=3) generated no signal. Time to onset of exponential amplification was about 3 minutes and time to onset of maximum relative fluorescenec units was about 6 minutes. The Ø slope of the exponential amplification phase of the reactions was determined to be 660 RFU/min.

The results of assay optimization are shown in Figures 16A and 16B.

25 **Example 3. Identification of $\Delta\Delta G$ as a Relative Thermodynamic Parameter Correlating with Performance of Candidate Primer/Probe Sets Designed for Isothermal Amplification Assays. Determination of a Threshold Values Predicting Success or Failure of a Primer Oligonucleotide in a Primer/Probe Set Designed *in silico*.**

30 A set of 34 oligonucleotide primers comprising in 5'-3' direction a self-complementary first region (5'-tail with the nicking enzyme recognition sequence) and a target sequence complementary second region were analyzed for a multitude of parameters commonly used in primer design algorithms, such as melting temperature (T_M) of the hybrid duplex, % GC content, sequence length and ΔG . All parameters were determined separately for the first and second primer regions, respectively. A subset of 25 primers belonged to

verified functional isothermal DNABle® assays. A subset of 9 primers belonged to candidate primer/probe sets that failed to produce a probe-detectable amplification product in assay screening experiments.

5 A number of two-dimensional matrix plots (Figures 17A-17H) were generated using different combinations of design parameters for the X- and Y-axis, respectively, with the intent to identify parameter combinations at which the data points of failing assay primers would cluster separately from the data points of functional assay primers. As demonstrated by the plots at Figures 17B-17H, no combination of standard design parameters used in prior art for primer design (T_M , ΔG , % GC, sequence length in bases) produced a discernable
10 separation of functional from non-functional primers in a two dimensional plot matrix. The data points of both primer populations widely overlap in these plots, as was expected due to the wide overlapping ranges of standard parameter values among functional and non-functional oligonucleotide primers.

Surprisingly, only the novel relative thermodynamic parameter of $\Delta\Delta G$ as defined by
15 the inventors of this application produced a matrix in which the data points of functional and non-functional primers clustered separately in correlation with the experimental outcome of primer set screening tests. The plot at Figure 17A shows a matrix in which the X-axis provides the scale for plotting the $\Delta\Delta G$ values of the self-complementary first primer region and the Y-axis provides the scale for plotting the $\Delta\Delta G$ values of the target-complementary
20 second primer region, respectively. All failing assay primers possessed $\Delta\Delta G$ values for either the second region/target duplex (in most cases), or the first region self-complementary duplex (in 1 case) that were less than -15 kcal/mol. Therefore, the $\Delta\Delta G$ value of about -15 kcal/mol turned to be the experimental threshold for this oligonucleotide primer data set that separates functional from non-functional primers.

25 $\Delta\Delta G$ is a relative parameter that measures the difference, i.e. relative advantage, of thermodynamic favorability of formation of the desired oligonucleotide structure (specific primer/target hybrid and first region self dimer) over the favorability of the formation of a multitude of predicted alternative structures at room temperature and atmospheric pressure in a dynamic equilibrium. Surprisingly, this ΔG advantage ($\Delta\Delta G$) turned out to be the predictive
30 parameter for assay success when candidate primer oligonucleotides are designed *in silico*. The use of calculated $\Delta\Delta G$ values as a ranking score and predictor for functional isothermal assays was reduced to praxis in the following examples describing the design and test of isothermal DNABle® assays for the detection of the soy lectin gene and the *Salmonella*

enterica invA gene. For the very first time, the screening of candidate primers and probes was performed exclusively *in silico* using calculated $\Delta\Delta G$ values. In each case, only a single primer/probe set was selected and tested experimentally. Two functional assays were established at first trial without time-consuming and costly screening of a multitude of candidate primer/probe sets.

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions.

Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

This application may be related to International Patent Application No. PCT/US2013/035750, filed April 9, 2013, which claims priority to and benefit of U.S. Provisional Application No.: 61/621,975, filed April 9, 2012, the entire contents of which are incorporated herein by reference.

This application may be related to International Patent Application No. PCT/US2011/047049, filed August 9, 2011, which claims priority to and benefit of U.S. Provisional Application No.: 61/373,695, filed August 13, 2010, the entire contents of which are incorporated herein by reference.

All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. An isolated primer oligonucleotide comprising from 5' to 3',
 - i) a first region comprising a self-complementary sequence comprising from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition sequence, and
 - ii) a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid having a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure comprising the second region, wherein the second region comprises at the 3' end one or more 2' modified nucleotides.
2. The isolated primer oligonucleotide of claim 1, wherein said palindromic sequence in the first region is 2, 4, or 6 nucleotides long.
3. The isolated primer oligonucleotide of claim 1 or 2, wherein the first region is 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides in length.
4. The isolated primer oligonucleotide of any one of claims 1-3, wherein the second region is 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.
5. The isolated primer oligonucleotide of any one of claims 1-4, wherein the first region is fully self-complementary.
6. The isolated primer oligonucleotide of any one of claims 1-5, wherein the alternate structure is one or more of a partially double-stranded hybrid that the second region can form with itself, with a partial sequence of the first region, with other oligonucleotides, primers, or probes in an amplification reaction, or with partially complementary nucleic acid sequences outside of the target sequence region.
7. The isolated primer oligonucleotide of any one of claims 1-6, in the form of a homodimer formed by hybridization of the self-complementary first region sequences of two primer oligonucleotide molecules.

8. The isolated primer oligonucleotide of claim 7, wherein the homodimer has a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure comprising the primer oligonucleotide.

5

9. The isolated primer oligonucleotide of any one of claims 1-8, wherein the one or more 2' modified nucleotides has a 2' modification selected from the group consisting of 2'-O-methyl, 2'-methoxyethoxy, 2'-fluoro, 2'-hydroxyl, 2'-alkyl, 2'-allyl, 2'-O-[2-(methylamino)-2-oxoethyl], 4'-thio, 4'-CH₂-O-2'-bridge, 4'-(CH₂)₂-O-2'-bridge, 2'-LNA, and 2'-O-(N-methylcarbamate) or those comprising base analogs.

10

10. The isolated primer oligonucleotide of any one of claims 1-9, wherein the one or more 2' modified nucleotides are positioned at the 3' terminus of the second region.

15

11. The isolated primer oligonucleotide of claim 9 or 10, wherein two or more 2' modified nucleotides are contiguous.

12. The isolated primer oligonucleotide of claim 11, wherein the number of contiguous 2' modified nucleotides is 4, 5, or 6.

20

13. The isolated primer oligonucleotide of any of claims 1-12, wherein the nicking enzyme is one or more of Nt.BspD6I and Nt.BstNBI.

14. The isolated primer oligonucleotide of claim 13, wherein the nicking enzyme recognition sequence within the first region is 5'-GAGTC-3'.

25

15. The isolated primer oligonucleotide of any one of claims 1-14, wherein the recognition sequence of the nicking agent is positioned to cleave the phosphodiester bond between the first and second regions.

30

16. The isolated primer oligonucleotide of any one of claims 1-15, having a first region comprising a nucleic acid sequence set forth below:

5'- GACTCN₁N₁GAGTC -3';

- 5'- GACTCN₁N₁'GAGTCN -3';
- 5'- N₂GACTCN₁N₁'GAGTCN₂' -3';
- 5'- N₂GACTCN₁N₁'GAGTCN₂'N -3';
- 5'- N₃N₂GACTCN₁N₁'GAGTCN₂'N₃' -3';
- 5 5'- N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N -3';
- 5'- N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄' -3';
- 5'- N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄'N -3';
- 5'- N₅N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄'N₅' -3';
- 5'- GACTCN₂N₁N₁'N₂'GAGTC -3';
- 10 5'- GACTCN₂N₁N₁'N₂'GAGTCN -3';
- 5'- N₃GACTCN₂N₁N₁'N₂'GAGTCN₃' -3';
- 5'- N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N -3';
- 5'- N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄' -3';
- 5'- N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N -3';
- 15 5'- N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅' -3';
- 5'- N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅'N -3'; and
- 5'- N₆N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅'N₆' -3',
- 5'- GACTCN₃N₂N₁N₁'N₂'N₃'GAGTC -3';
- 5'- GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN -3';
- 20 5'- GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNN -3';
- 5'- GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNNN -3';
- 5'- GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNNNN -3';
- 5'- N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄' -3';

5'-N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N -3';

5'-N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'NN -3';

5'-N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'NNN -3';

5'-N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅' -3';

5 5'-N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N -3';

5'-N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆' -3';

5'-N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆'N -3'; and

5'-N₇N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆'N₇' -3',

10 where "N" is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C),
or guanine (G) nucleobase), and N₁ is complementary to N₁', N₂ to N₂', N₃ to N₃', N₄ to N₄',
N₅ to N₅', N₆ to N₆', and N₇ to N₇'.

17. The isolated primer oligonucleotide of any one of claims 1-16, wherein the first
region comprises a sequence set forth in the Sequence Listing.

15

18. An isolated primer-dimer comprising two oligonucleotide monomers, the
oligonucleotide monomer comprising from 5' to 3',

20 i) a first region comprising a self-complementary sequence comprising from 5' to 3'
the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence,
and said nicking enzyme recognition sequence, and

25 ii) a second region at least 16 nucleotides long that specifically binds to a
complementary region on a target nucleic acid molecule to form a double-stranded hybrid
having a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure
comprising the second region, wherein the second region comprises at the 3' end one or more
2' modified nucleotides.

19. The isolated primer-dimer of claim 18, wherein said palindromic sequence in the first
region is 2, 4, or 6 nucleotides long.

20. The isolated primer-dimer of claim 18 or 19, wherein the first region is 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides in length.
21. The isolated primer-dimer of any one of claims 18-20, wherein the second region is 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.
22. The isolated primer-dimer of any one of claims 18-21, wherein the first region is fully self-complementary.
23. The isolated primer-dimer of any one of claims 18-22, wherein the alternate structure is one or more of a partially double-stranded hybrid that the second region can form with itself, with a partial sequence of the first region, with other oligonucleotides, primers, or probes in an amplification reaction, or with partially complementary nucleic acid sequences outside of the target sequence region.
24. The isolated primer-dimer of any one of claims 18-23, in the form of a homodimer formed by hybridization of the self-complementary first region sequences of two oligonucleotide monomers.
25. The isolated primer-dimer of claim 24, wherein the homodimer has a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure comprising the oligonucleotide monomer.
26. The isolated primer-dimer of any one of claims 18-25, wherein the one or more 2' modified nucleotides has a 2' modification selected from the group consisting of 2'-O-methyl, 2'-methoxyethoxy, 2'-fluoro, 2'-hydroxyl, 2'-alkyl, 2'-allyl, 2'-O-[2-(methylamino)-2-oxoethyl], 4'-thio, 4'-CH₂-O-2'-bridge, 4'-(CH₂)₂-O-2'-bridge, 2'-LNA, and 2'-O-(N-methylcarbamate) or those comprising base analogs.
27. The isolated primer-dimer of any one of claims 18-26, wherein the one or more 2' modified nucleotides are positioned at the 3' terminus of the second region.

28. The isolated primer-dimer of claim 26 or 27, wherein two or more 2' modified nucleotides are contiguous.

29. The isolated primer-dimer of claim 28, wherein the number of contiguous 2' modified nucleotides is 4, 5, or 6.

30. The isolated primer-dimer of any of claims 18-29, wherein the nicking enzyme is one or more of Nt.BspD6I and Nt.BstNBI.

31. The isolated primer-dimer of claim 30, wherein the nicking enzyme recognition sequence within the first region is 5'-GAGTC-3'.

32. The isolated primer-dimer of any one of claims 18-31, wherein the recognition sequence of the nicking agent is positioned to cleave the phosphodiester bond between the first and second regions.

33. The isolated primer-dimer of any one of claims 18-32, wherein the oligonucleotide monomer has a first region comprising a nucleic acid sequence set forth below:

5'- GACTCN₁N₁'GAGTC -3';

5'- GACTCN₁N₁'GAGTCN -3';

5'- N₂GACTCN₁N₁'GAGTCN₂' -3';

5'- N₂GACTCN₁N₁'GAGTCN₂'N -3';

5'- N₃N₂GACTCN₁N₁'GAGTCN₂'N₃' -3';

5'- N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N -3';

5'- N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄' -3';

5'- N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄'N -3';

5'- N₅N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄'N₅' -3';

5'- GACTCN₂N₁N₁'N₂'GAGTC -3';

- 5'- GACTCN₂N₁N₁'N₂'GAGTCN -3';
- 5'- N₃GACTCN₂N₁N₁'N₂'GAGTCN₃' -3';
- 5'- N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N -3';
- 5'- N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄' -3';
- 5 5'- N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N -3';
- 5'- N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅' -3';
- 5'- N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅'N -3'; and
- 5'- N₆N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅'N₆' -3',
- 5'- GACTCN₃N₂N₁N₁'N₂'N₃'GAGTC -3';
- 10 5'- GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN -3';
- 5'- GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNN -3';
- 5'- GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNNN -3';
- 5'- GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNNNN -3';
- 5'- N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄' -3';
- 15 5'- N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N -3';
- 5'- N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'NN -3';
- 5'- N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'NNN -3';
- 5'- N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅' -3';
- 5'- N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N -3';
- 20 5'- N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆' -3';
- 5'- N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆'N -3'; and
- 5'- N₇N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆'N₇' -3',

where “N” is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C), or guanine (G) nucleobase), and N_1 is complementary to N_1 , N_2 to N_2 , N_3 to N_3 , N_4 to N_4 , N_5 to N_5 , N_6 to N_6 , and N_7 to N_7 .

5 34. The isolated primer-dimer of any one of claims 18-33, wherein the oligonucleotide monomer comprises a first region comprising a sequence set forth in the Sequence Listing.

35. An isolated oligonucleotide probe for detecting a target nucleic acid molecule comprising:

- 10 i) an oligonucleotide;
ii) a fluorescent reporter; and
iii) a quenching molecule capable of absorbing the excitation energy from the fluorescent reporter;

wherein the fluorescent reporter and quenching molecule are covalently attached to
15 opposite 5' and 3' ends of the oligonucleotide,

wherein the oligonucleotide comprises a first region having a nucleic acid sequence that is substantially complementary to a target nucleic acid sequence and

- a) a second region 5' upstream of the first region, and
b) a third region 3' downstream of the first region that has a nucleic acid sequence
20 complementary to the second region,

wherein the oligonucleotide is capable of forming a stem-loop hairpin structure by hybridization of the second and third regions when the oligonucleotide is not bound to the target nucleic acid molecule, and

wherein the ΔG of the double-stranded hybrid between the target sequence and the
25 first sequence of the oligonucleotide probe is at least 15 kcal/mol lower than the ΔG of any alternate structure comprising the oligonucleotide probe.

36. The isolated oligonucleotide probe of claim 35, wherein the alternate structure is one or more of a partially double-stranded hybrid that the first region can form with itself, with a
30 partial sequence of the first region, with other oligonucleotides, primers, or probes in an amplification reaction, or with partially complementary nucleic acid sequences outside of the target sequence region.

37. The isolated oligonucleotide probe claim 35 or 36, wherein the second and the third regions are 4-8 nucleotides in length.

38. A method of amplifying a specific product in a nicking and extension amplification reaction, the method comprising:

(a) contacting a target nucleic acid molecule under substantially isothermal conditions with a polymerase, two or more primers, each of which specifically binds to a complementary sequence on the target nucleic acid molecule, a nicking enzyme, and a detectable polynucleotide probe, wherein at least one primer comprises from 5' to 3',

i) a first region comprising a self-complementary sequence comprising from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition sequence, and

ii) a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid having a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure comprising the second region, wherein the second region comprises at the 3' end one or more 2' modified nucleotides; and

(b) generating amplicons comprising at least a portion of said target nucleic acid molecule.

39. A method of detecting a specific product in a nicking and extension amplification reaction, the method comprising:

(a) contacting a target nucleic acid molecule under substantially isothermal conditions with a polymerase, two or more primers, each of which specifically binds to a complementary sequence on the target nucleic acid molecule, a nicking enzyme, and a detectable oligonucleotide probe, wherein at least one primer comprises from 5' to 3',

i) a first region comprising a self-complementary sequence comprising from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition sequence, and

ii) a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid having a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate

structure comprising the second region, wherein the second region comprises at the 3' end one or more 2' modified nucleotides;

(b) generating amplicons comprising at least a portion of said target nucleic acid molecule; and

5 (c) detecting a signal specific for oligonucleotide probe hybridization to the target nucleic acid molecule or amplicon thereof, wherein the signal indicates the presence of the target nucleic acid molecule present in the sample or an amplicon thereof.

40. The method of claim 39, wherein the oligonucleotide probe comprises:

- 10 i) an oligonucleotide;
ii) a fluorescent reporter; and
iii) a quenching molecule capable of absorbing the excitation energy from the fluorescent reporter;

wherein the fluorescent reporter and quenching molecule are covalently attached to
15 opposite 5' and 3' ends of the oligonucleotide,

wherein the oligonucleotide comprises a first region having a nucleic acid sequence that is substantially complementary to a target nucleic acid sequence and

- a) a second region 5' upstream of the first region, and
b) a third region 3' downstream of the first region that has a nucleic acid
20 sequence complementary to the second region,
wherein the oligonucleotide is capable of forming a stem-loop hairpin structure by hybridization of the second and third regions when the oligonucleotide is not bound to the target nucleic acid molecule.

25 41. The method of any one of claims 38-40, wherein the polymerase is a *Geobacillus* spp. or *Bacillus stearothermophilus* DNA polymerase I, or active fragments and derivatives thereof.

42. The method of claim 41, wherein the polymerase is one or more of Bst DNA
30 polymerase I, Gst DNA polymerase I, or Gka DNA polymerase I.

43. The method of claim 42, wherein said palindromic sequence in the first region is 2, 4, or 6 nucleotides long.

44. The method of any one of claims 38-43, wherein the first region is 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides in length.

5 45. The method of any one of claims 38-44, wherein the second region is 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

46. The method of any one of claims 38-45, wherein the first region is fully self-complementary.

10

47. The isolated primer oligonucleotide of any one of claims 38-46, wherein the alternate structure is one or more of a partially double-stranded hybrid that the second region can form with itself, with a partial sequence of the first region, with other oligonucleotides, primers, or probes in an amplification reaction, or with partially complementary nucleic acid sequences
15 outside of the target sequence region.

48. The isolated primer oligonucleotide of any one of claims 38-47, in the form of a homodimer formed by hybridization of the self-complementary first region sequences of two primer oligonucleotide molecules.

20

49. The isolated primer oligonucleotide of claim 48, wherein the homodimer has a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure comprising the primer oligonucleotide.

25 50. The method of any one of claims 38-49, wherein the one or more 2' modified nucleotides has a 2' modification selected from the group consisting of 2'-O-methyl, 2'-methoxyethoxy, 2'-fluoro, 2'-hydroxyl, 2'-alkyl, 2'-allyl, 2'-O-[2-(methylamino)-2-oxoethyl], 4'-thio, 4'-CH₂-O-2'-bridge, 4'-(CH₂)₂-O-2'-bridge, 2'-LNA, and 2'-O-(N-methylcarbamate) or those comprising base analogs.

30

51. The method of any one of claims 38-50, wherein the one or more 2' modified nucleotides are positioned at the 3' terminus of the second region.

52. The method of claim 38 or 51, wherein two or more 2' modified nucleotides are contiguous.

53. The method of claim 52, wherein the number of contiguous 2' modified nucleotides is
5 4, 5, or 6.

54. The method of any of claims 38-53, wherein the nicking enzyme is one or more of Nt.BspD6I and Nt.BstNBI.

10 55. The method of claim 54, wherein the nicking enzyme recognition sequence within the first region is 5'-GAGTC-3'.

56. The method of any one of claims 38-55, wherein the recognition sequence of the nicking agent is positioned to cleave the phosphodiester bond between the first and second
15 regions.

57. The method of any one of claims 38-56, wherein the primer has a first region comprising a nucleic acid sequence set forth below:

5'- GACTCN₁N₁'GAGTC -3';

20 5'- GACTCN₁N₁'GAGTCN -3';

5'- N₂GACTCN₁N₁'GAGTCN₂' -3';

5'- N₂GACTCN₁N₁'GAGTCN₂'N -3';

5'- N₃N₂GACTCN₁N₁'GAGTCN₂'N₃' -3';

5'- N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N -3';

25 5'- N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄' -3';

5'- N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄'N -3';

5'- N₅N₄N₃N₂GACTCN₁N₁'GAGTCN₂'N₃'N₄'N₅' -3';

5'- GACTCN₂N₁N₁'N₂'GAGTC -3';

5'-GACTCN₂N₁N₁'N₂'GAGTCN -3';

5'-N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'-3';

5'-N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N -3';

5'-N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'-3';

5 5'-N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N -3';

5'-N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅'-3';

5'-N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅'N -3'; and

5'-N₆N₅N₄N₃GACTCN₂N₁N₁'N₂'GAGTCN₃'N₄'N₅'N₆'-3',

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTC -3';

10 5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN -3';

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNN -3';

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNNN -3';

5'-GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCNNNN -3';

5'-N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'-3';

15 5'-N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N -3';

5'-N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'NN -3';

5'-N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'NNN -3';

5'-N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'-3';

5'-N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N -3';

20 5'-N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆'-3';

5'-N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆'N -3'; and

5'-N₇N₆N₅N₄GACTCN₃N₂N₁N₁'N₂'N₃'GAGTCN₄'N₅'N₆'N₇'-3',

where "N" is any nucleotide (e.g., having an adenine (A), thymine (T), cytosine (C), or guanine (G) nucleobase), and N_1 is complementary to N_1' , N_2 to N_2' , N_3 to N_3' , N_4 to N_4' , N_5 to N_5' , N_6 to N_6' , and N_7 to N_7' .

5 58. The method of any one of claims 38-57, wherein the primer comprises a first region comprising a sequence set forth in the Sequence Listing.

59. A kit for amplifying a target sequence in a nicking amplification reaction, the kit comprising one or more primer oligonucleotides comprising from 5' to 3',

10 i) a first region comprising a self-complementary sequence comprising from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition sequence, and

ii) a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid
15 having a ΔG that is at least 15 kcal/mol lower than the ΔG of any alternate structure comprising the second region, wherein the second region comprises at the 3' end one or more 2' modified nucleotides;

and directions for use of the primer oligonucleotides in the method of any one of claims 38-58.

20

60. The kit of claim 59, further comprising an oligonucleotide probe comprising:

i) an oligonucleotide;

ii) a fluorescent reporter; and

iii) a quenching molecule capable of absorbing the excitation energy from the

25 fluorescent reporter;

wherein the fluorescent reporter and quenching molecule are covalently attached to opposite 5' and 3' ends of the oligonucleotide,

wherein the oligonucleotide comprises a first region having a nucleic acid sequence that is substantially complementary to a target nucleic acid sequence and

30 a) a second region 5' upstream of the first region, and

b) a third region 3' downstream of the first region that has a nucleic acid sequence complementary to the second region,

wherein the oligonucleotide is capable of forming a stem-loop hairpin structure by hybridization of the second and third regions when the oligonucleotide is not bound to the target nucleic acid molecule;

5 and directions for use of the primer oligonucleotides in the method of any one of claims 38-58.

61. A tangible, non-transitory computer readable medium comprising: computer program instructions for implementing a method of selecting a primer oligonucleotide, the primer oligonucleotide comprising:

10 i) a first region comprising a self-complementary sequence comprising from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition sequence, and

ii) a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule;

15 the method comprising:

a) selecting a second region of said primer oligonucleotide, wherein the ΔG for the double-stranded hybrid formed by the hybridization of the second region and the target nucleic acid molecule that is at least 15 kcal/mol lower than the ΔG of any alternate structure comprising the second region;

20 b) selecting a first region of said primer oligonucleotide, wherein the ΔG for the homodimer formed by hybridization of the self-complementary first region sequences of two primer oligonucleotide molecules is at least 15 kcal/mol lower than the ΔG of any alternate structure comprising the primer oligonucleotide.

25

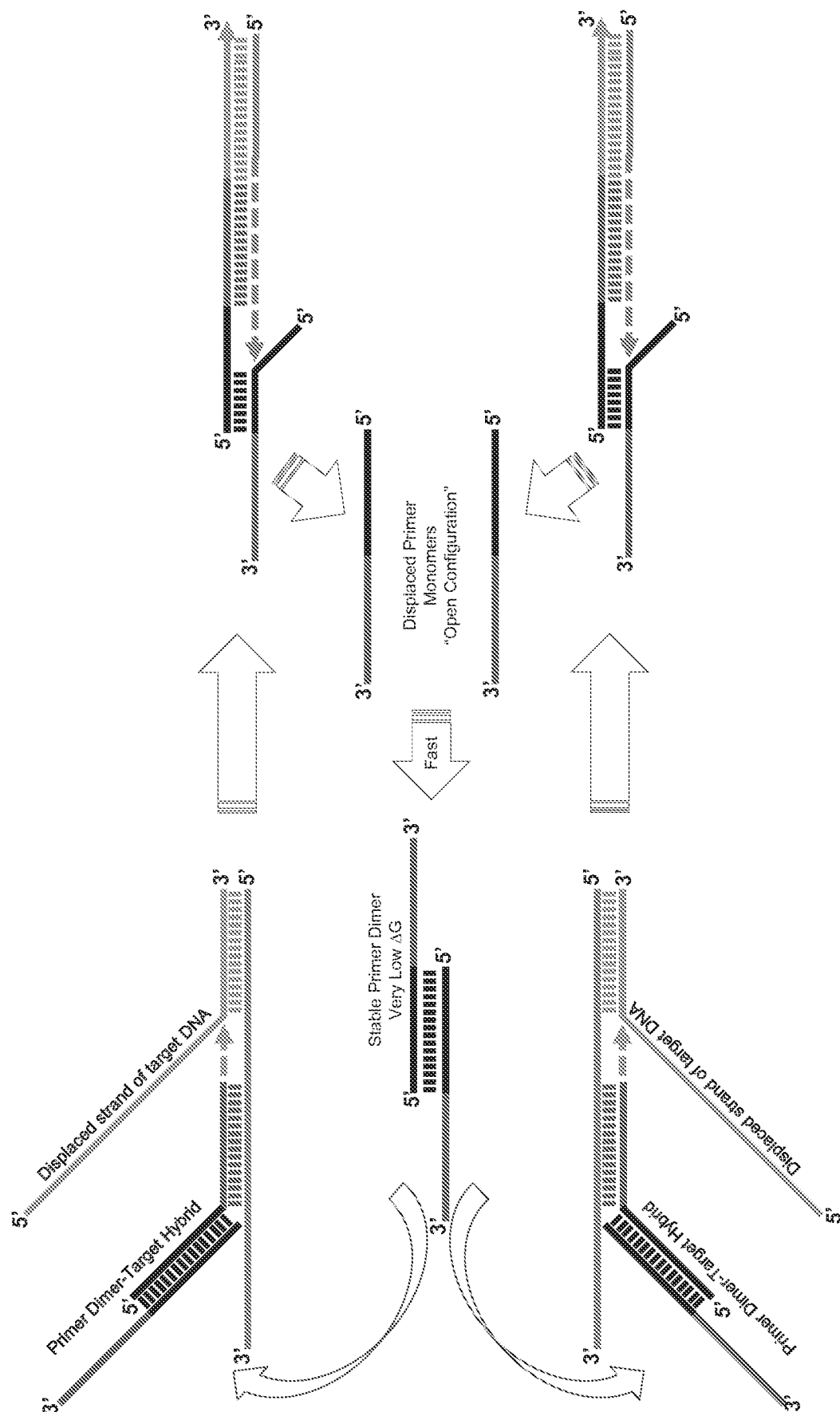


Fig. 1 Mechanism of an efficient isothermal reaction with exponential amplification, where at all times the active primer configuration is a dimer that is "recycled" to form new dimers from monomers released as result of polymerase strand displacement action. Ideally, at no point during the reaction the primers are present as an monomer with an open configuration, which could either serve as a template or primer for non-target-specific primer extension reactions amplifying background. The transition from monomer to dimer is "instantaneous" driven by thermodynamic factors (very low ΔG of dimer, high primer concentration, high dimer T_m) favoring the dimer formation.

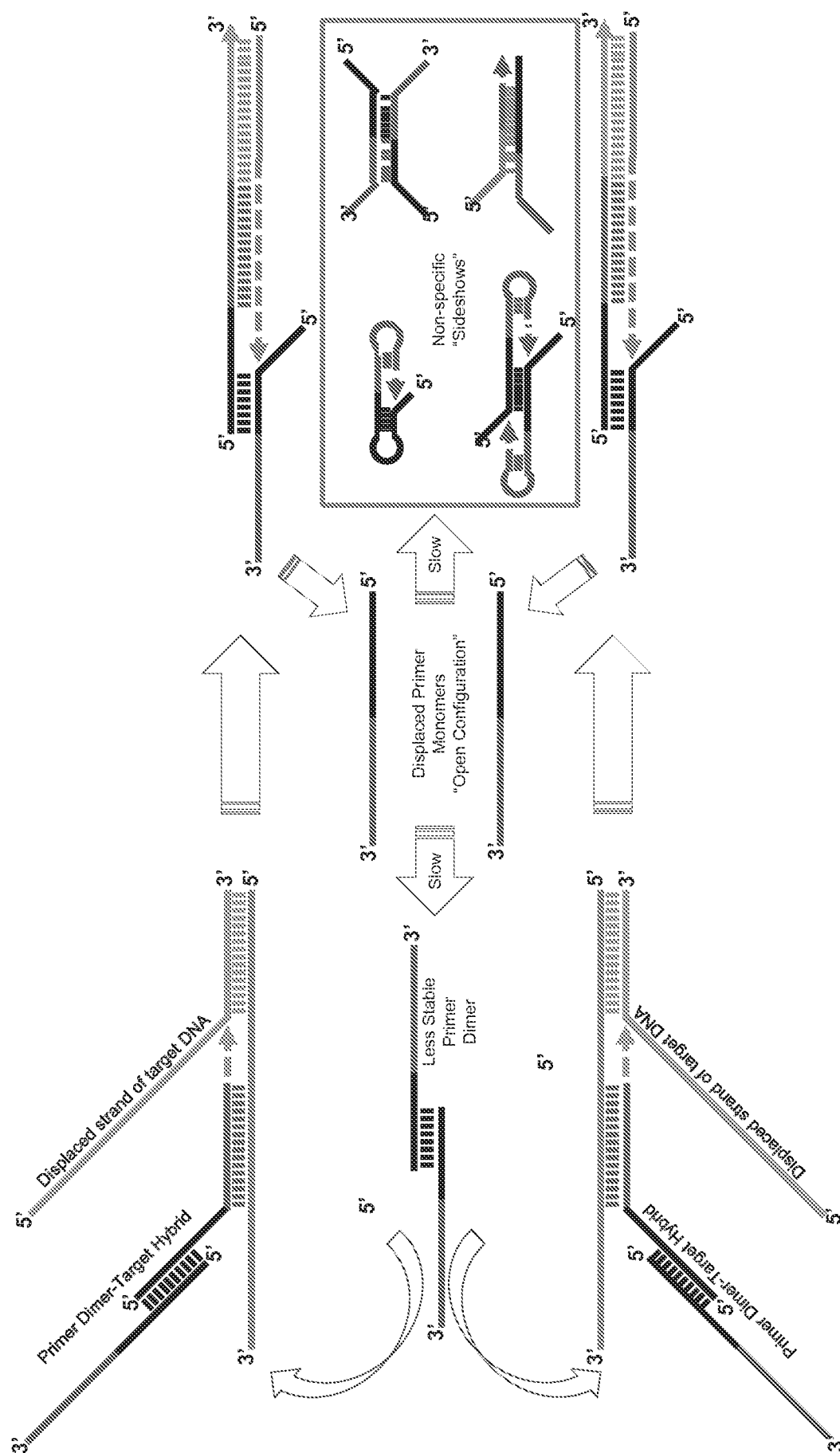


Fig 2. Mechanism of an inefficient isothermal reaction with background amplification, where monomer primers with an open configuration persists throughout the reaction leading to linear non-specific background amplification. A primer monomer with a fully or partially open configuration can serve either as a template or primer for non-target-specific primer extension reactions. The transition from monomer to dimer is slow, because it is not favored by thermodynamic factors (many different dimer and partial dimer configurations co-exist with similar ΔG 's and low dimer T_m 's).

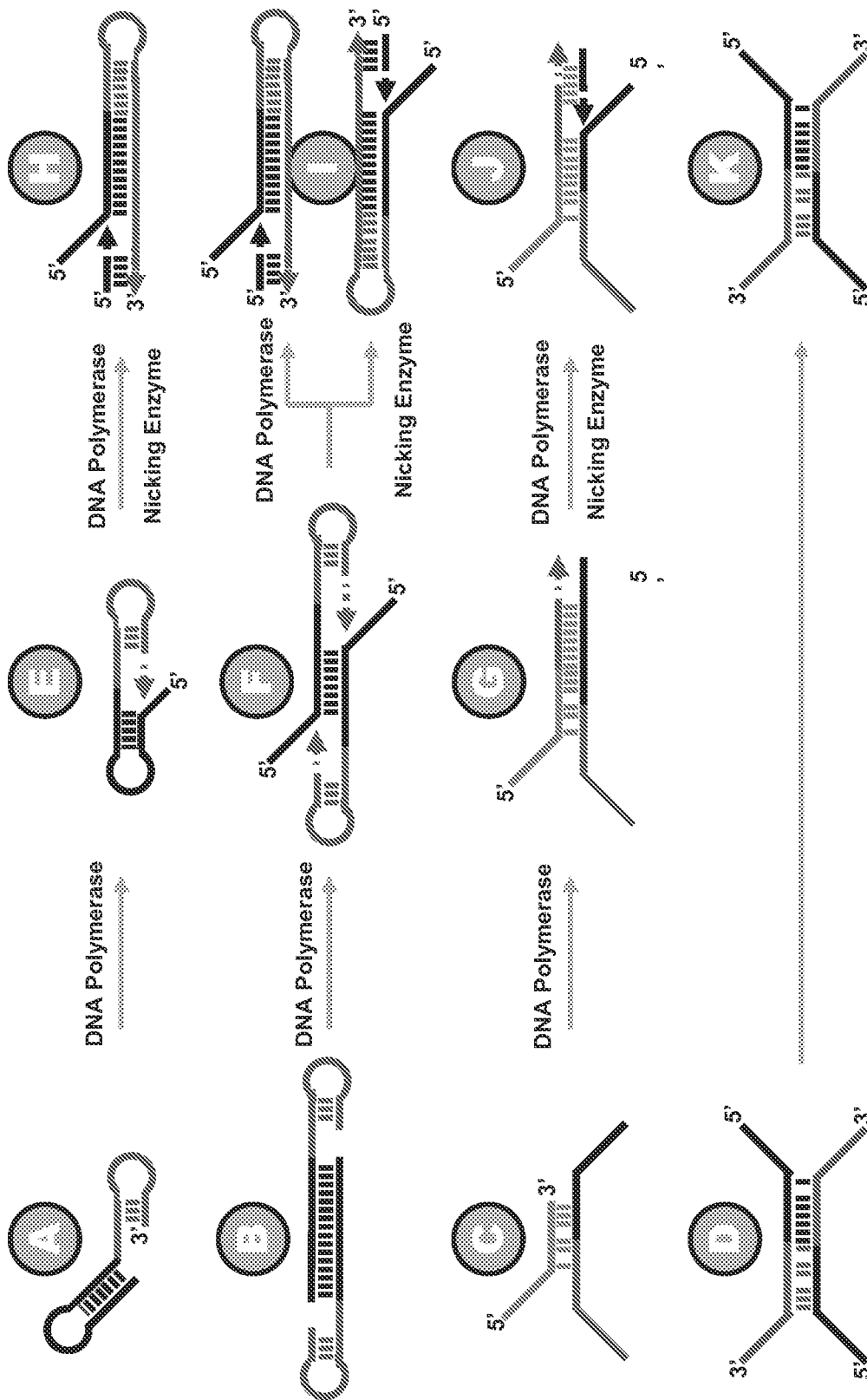
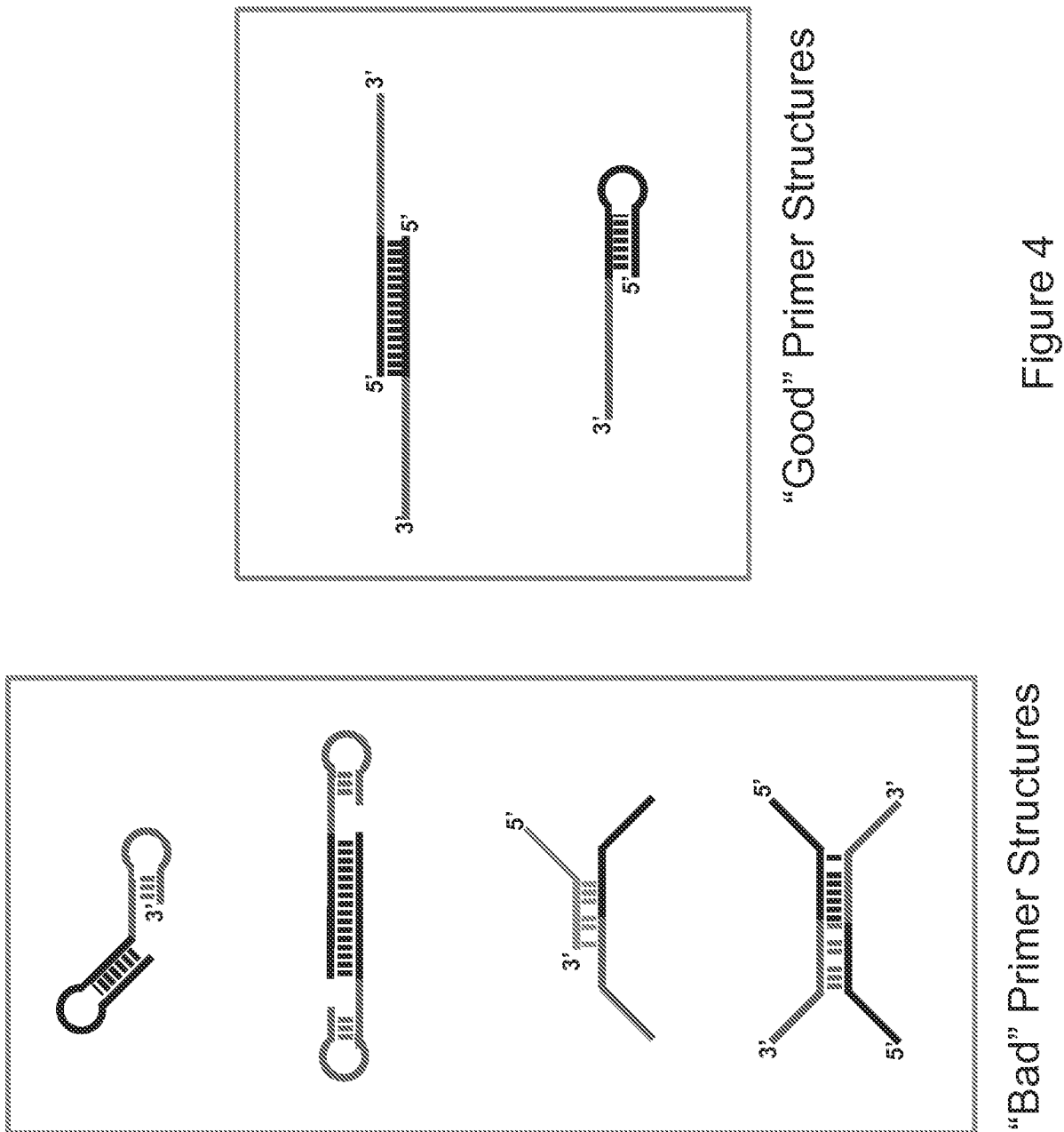


Fig 3. Primer structures leading to amplification of non-specific by-products (A to C) or incapable of target hybridization (D, K): A) Primer monomer with discontinuous inverted sequence in target specific region "looping" back to create an extendable 3'-end for "self-replication". B) Like A) in primer dimer structure. C) False hybrid structures between primer monomer in "open" configuration and single stranded DNA fragments (gray) displaced by DNA polymerase nick-extension reactions from closely positioned random and/or sequence-specific nicks in template DNA. D) Partially double-stranded primer displaced by template DNA. E) Intermediate, false primer extension/strand displacement reaction for target hybridization, formed by inverted sequence repeats. F) Mature, false reaction product in "nick & kick" mode, continuously recycling and amplifying single stranded by-products. G) False reaction product. H) Mature, false reaction product in "nick & kick" mode, continuously recycling and amplifying single stranded by-products. I) Mature, false reaction product in "nick & kick" mode, continuously recycling and amplifying single stranded by-products. J) Mature, false reaction product in "nick & kick" mode, continuously recycling and amplifying single stranded by-products. K) Mature, false reaction product in "nick & kick" mode, continuously recycling and amplifying single stranded by-products.



Dynamic equilibrium between configurations of non-optimal primers

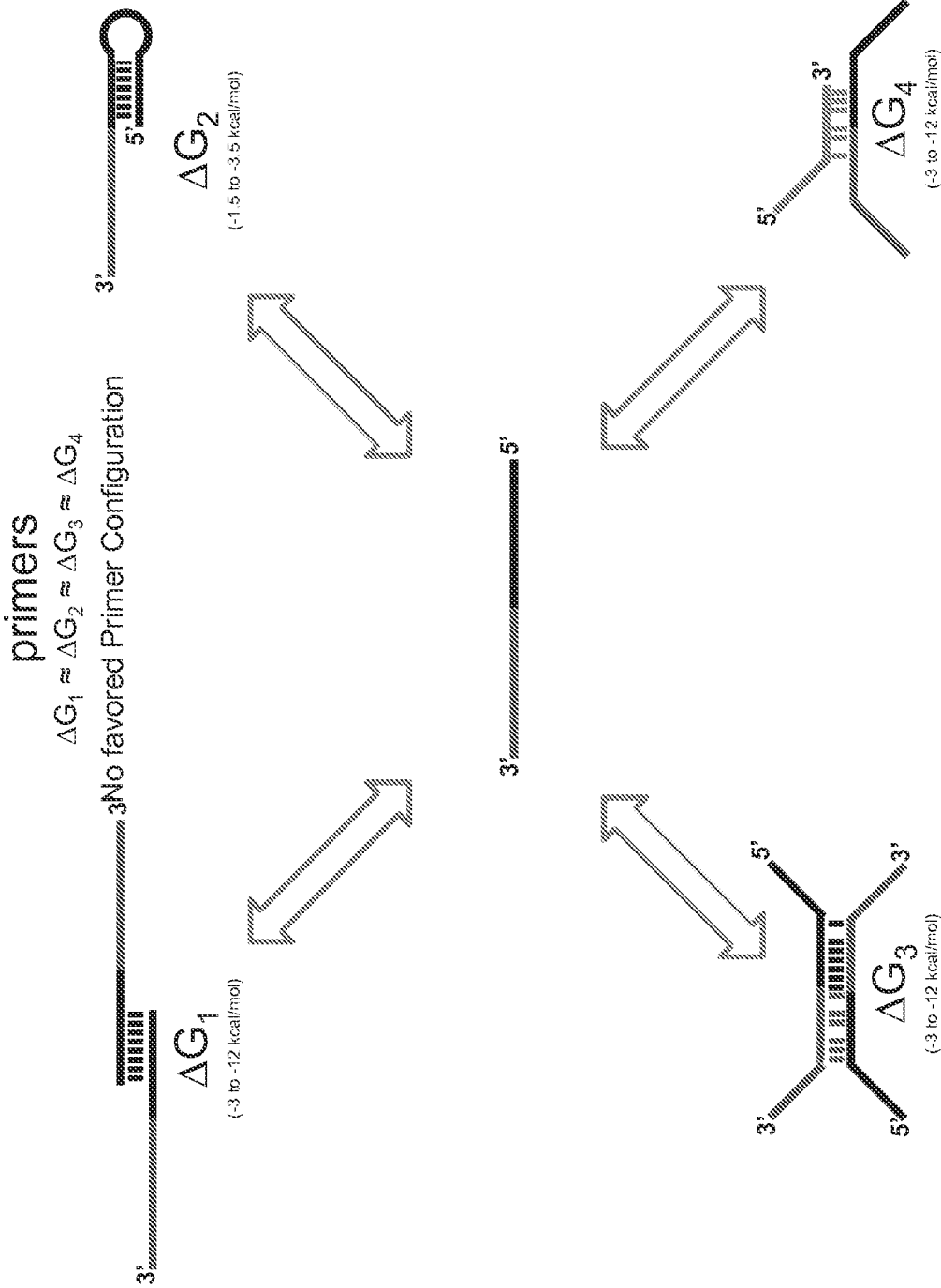


Figure 5

Highly favored dimer configuration of optimal primers

$\Delta G_1 \gg \Delta G_2$

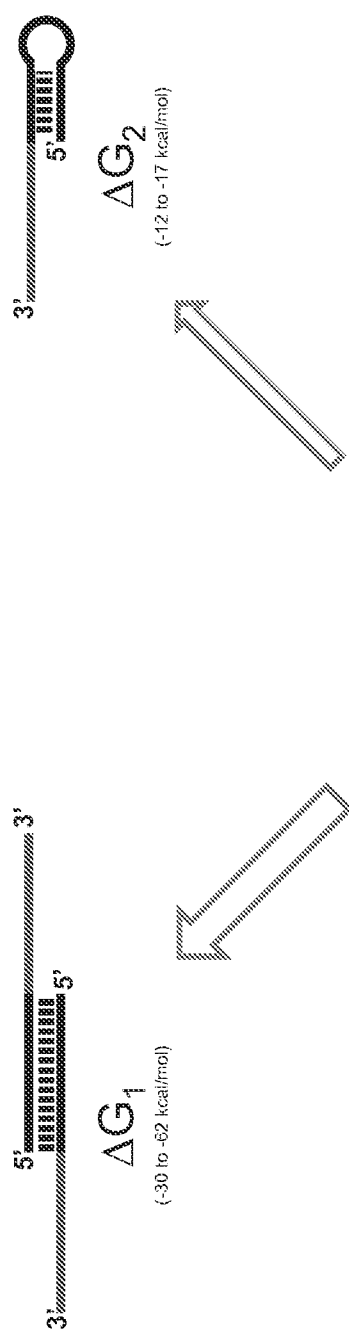


Figure 6

Example of primer dimer structure with the nicking enzyme *Nt.BstNBI* recognition sequence positioned in the 3'-terminal half of the 5'-terminal inverted region:

- X** -- any base comprising self-complementary, symmetrically inverted sequence of 5'-terminal region
- Red** -- Nicking enzyme recognition/binding sequence
- N** -- any base comprising 3'-terminal target-complementary primer region
- Purple** -- modified base destabilizing mismatched base pair
- -- Watson-Crick base pair between complementary nucleobases

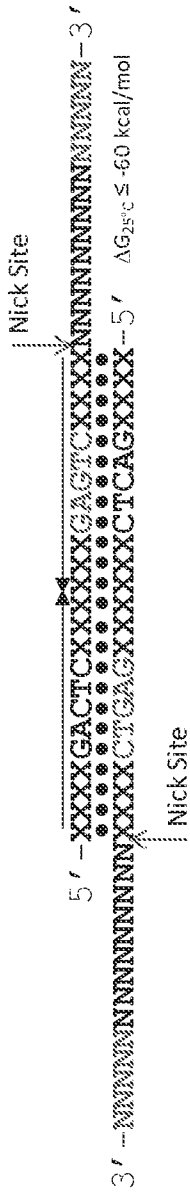


Figure 7

Figure 8A

Sequences of 5'-tails

1050 Selected Sequences = 0.2% of entire sequence space (537824)

 $\Delta G = -48$ Kcal/mole to -62 kcal/mole

GC content 68% to 84%

NNNNGACTCNNNNNNGAGTCNNNN

 $\Delta\Delta G < -40$ kcal/mole

GGCGGACTCGCGCGCGAGTCCGCC
 GCGCGACTCGCGCGCGAGTCGGGC
 GGGCGACTCGCGCGCGAGTCGCCC
 GGGAGACTCGCGCGCGAGTCTCCC
 GGGTGAAGTCGCGCGCGAGTCACCC
 GGACGACTCGCGCGCGAGTCGTCC
 GCACGACTCGCGCGCGAGTCGTGC
 GACCGACTCGCGCGCGAGTCGGTC
 GTCCGACTCGCGCGCGAGTCGGAC
 ACCCGACTCGCGCGCGAGTCGGGT
 TCCCGACTCGCGCGCGAGTCGGGA
 GCCAGACTCGCGCGCGAGTCTGGC
 GCCTGACTCGCGCGCGAGTCAGGC
 GACGGACTCGCGCGCGAGTCCGTC
 GTCGGACTCGCGCGCGAGTCCGAC
 AGCGGACTCGCGCGCGAGTCCGCT
 TGCGGACTCGCGCGCGAGTCCGCA
 GGAGGACTCGCGCGCGAGTCCCTCC
 GGTGGACTCGCGCGCGAGTCCACC
 GGCAGACTCGCGCGCGAGTCTGCC
 GGCTGACTCGCGCGCGAGTCAGCC
 CACGGACTCGCGCGCGAGTCCGTG
 CTCGGACTCGCGCGCGAGTCCGAG
 CCAGGACTCGCGCGCGAGTCCCTGG
 CCTGGACTCGCGCGCGAGTCCAGG
 CCCAGACTCGCGCGCGAGTCTGGG
 CCCTGACTCGCGCGCGAGTCAGGG
 ACGGGACTCGCGCGCGAGTCCCGT
 TCGGGACTCGCGCGCGAGTCCCGA
 CAGGGACTCGCGCGCGAGTCCCTG
 CTGGGACTCGCGCGCGAGTCCAG

GGCGGACTCTCGCGAGAGTCCGCC
 GCGCGACTCTCGCGAGAGTCGGGC
 GGGCGACTCTCGCGAGAGTCGCCC
 GGGAGACTCTCGCGAGAGTCTCCC
 GGGTGAAGTCCTCGCGAGAGTCACCC
 GGACGACTCTCGCGAGAGTCGTCC
 GCACGACTCTCGCGAGAGTCGTGC
 GACCGACTCTCGCGAGAGTCGGTC
 GTCCGACTCTCGCGAGAGTCGGAC
 ACCCGACTCTCGCGAGAGTCGGGT
 TCCCGACTCTCGCGAGAGTCGGGA
 GCCAGACTCTCGCGAGAGTCTGGC
 GCCTGACTCTCGCGAGAGTCAGGC
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 CCCAGACTCTCGCGAGAGTCTGGG
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 TCGGGACTCTCGCGAGAGTCCCGA
 CAGGGACTCTCGCGAGAGTCCCTG
 CTGGGACTCTCGCGAGAGTCCAG

CCGGGACTCCGCGCGGAGTCCCGG
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 GCGGGACTCCGCGCGGAGTCCGCC
 GCCGGACTCCGCGCGGAGTCCGGC
 GCGCGACTCCGCGCGGAGTCGGGC
 GGCCGACTCCGCGCGGAGTCGGCC
 GGGCGACTCCGCGCGGAGTCGCCC
 GGGAGACTCCGCGCGGAGTCTCCC

CCGGGACTCTGCGCAGAGTCCCGG
 CCCGGACTCTGCGCAGAGTCCGGG
 GCGGGACTCTGCGCAGAGTCCGCC
 GCCGGACTCTGCGCAGAGTCCGGC
 GCGCGACTCTGCGCAGAGTCGGGC
 GGCCGACTCTGCGCAGAGTCGGCC
 GGGCGACTCTGCGCAGAGTCGCCC
 GGGAGACTCTGCGCAGAGTCTCCC

Figure 8A

GGGTGACTCCGCGCGGAGTCACCC
 GGACGACTCCGCGCGGAGTCGTCC
 GGTCGACTCCGCGCGGAGTCGACC
 GCACGACTCCGCGCGGAGTCGTGC
 GCTCGACTCCGCGCGGAGTCGAGC
 GACCGACTCCGCGCGGAGTCGGTC
 GTCCGACTCCGCGCGGAGTCGGAC
 ACCCGACTCCGCGCGGAGTCGGGT
 TCCCGACTCCGCGCGGAGTCGGGA
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 GACGGACTCCGCGCGGAGTCCGTC
 GTCGGACTCCGCGCGGAGTCCGAC
 ACCGGACTCCGCGCGGAGTCCGGT
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 CTCGGACTCCGCGCGGAGTCCGAG
 CCAGGACTCCGCGCGGAGTCCTGG
 CCTGGACTCCGCGCGGAGTCCAGG
 CCCAGACTCCGCGCGGAGTCTGGG
 CCCTGACTCCGCGCGGAGTCAGGG
 ACGGGACTCCGCGCGGAGTCCCGT
 TCGGGACTCCGCGCGGAGTCCCGA
 CAGGGACTCCGCGCGGAGTCCCTG
 CTGGGACTCCGCGCGGAGTCCCAG

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 GCACGACTCGGGCCCGAGTCGTGC
 GACCGACTCGGGCCCGAGTCGGTC
 GTCCGACTCGGGCCCGAGTCGGAC
 ACCCGACTCGGGCCCGAGTCGGGT
 TCCCGACTCGGGCCCGAGTCGGGA
 GCCAGACTCGGGCCCGAGTCTGGC

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 GGACGACTCTGCGCAGAGTCGTCC
 GGTCGACTCTGCGCAGAGTCGACC
 GCACGACTCTGCGCAGAGTCGTGC
 GCTCGACTCTGCGCAGAGTCGAGC
 GACCGACTCTGCGCAGAGTCGGTC
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 TGCGGACTCTGCGCAGAGTCCGCA
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 GGTGGACTCTGCGCAGAGTCCACC
 GGCAGACTCTGCGCAGAGTCTGCC
 GGCTGACTCTGCGCAGAGTCAGCC
 CACGGACTCTGCGCAGAGTCCGTG
 CTCGGACTCTGCGCAGAGTCCGAG
 CCAGGACTCTGCGCAGAGTCCTGG
 CCTGGACTCTGCGCAGAGTCCAGG
 CCCAGACTCTGCGCAGAGTCTGGG
 CCCTGACTCTGCGCAGAGTCAGGG
 ACGGGACTCTGCGCAGAGTCCCGT
 TCGGGACTCTGCGCAGAGTCCCGA
 CAGGGACTCTGCGCAGAGTCCCTG
 CTGGGACTCTGCGCAGAGTCCCAG

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 GCCCGACTCTGGCCAGAGTCGGGC
 GGGCGACTCTGGCCAGAGTCGCCC
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 GGACGACTCTGGCCAGAGTCGTCC
 GCACGACTCTGGCCAGAGTCGTGC
 GACCGACTCTGGCCAGAGTCGGTC
 GTCCGACTCTGGCCAGAGTCGGAC
 ACCCGACTCTGGCCAGAGTCGGGT
 TCCCGACTCTGGCCAGAGTCGGGA
 GCCAGACTCTGGCCAGAGTCTGGC

Figure 8A

GCCTGACTCGGGCCCGAGTCAGGC
GACGGACTCGGGCCCGAGTCCGTC
GTCGGACTCGGGCCCGAGTCCGAC
AGCGGACTCGGGCCCGAGTCCGCT
TGCGGACTCGGGCCCGAGTCCGCA
GGAGGACTCGGGCCCGAGTCCTCC
GGTGGACTCGGGCCCGAGTCCACC
GGCAGACTCGGGCCCGAGTCTGCC
GGCTGACTCGGGCCCGAGTCAGCC
CACGGACTCGGGCCCGAGTCCGTG
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CCTGGACTCGGGCCCGAGTCCAGG
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TGCGGACTCTCGCGAGAGTCCGCA
GGAGGACTCTCGCGAGAGTCCTCC
GGTGGACTCTCGCGAGAGTCCACC
GGCAGACTCTCGCGAGAGTCTGCC
GGCTGACTCTCGCGAGAGTCAGCC
CACGGACTCTCGCGAGAGTCCGTG
CTCGGACTCTCGCGAGAGTCCGAG
CCAGGACTCTCGCGAGAGTCCTGG

Figure 8A

CCTGGACTCCCGCGGGAGTCCAGG
 CCCAGACTCCCGCGGGAGTCTGGG
 CCCTGACTCCCGCGGGAGTCAGGG
 ACGGGACTCCCGCGGGAGTCCCGT
 TCGGGACTCCCGCGGGAGTCCCGA
 CAGGGACTCCCGCGGGAGTCCCTG
 CTGGGACTCCCGCGGGAGTCCCAG

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 TCGGGACTCGGCGCCGAGTCCCGA
 CAGGGACTCGGCGCCGAGTCCCTG
 CTGGGACTCGGCGCCGAGTCCCAG

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 TCGGGACTCTGCGCAGAGTCCCGA
 CAGGGACTCTGCGCAGAGTCCCTG
 CTGGGACTCTGCGCAGAGTCCCAG

GGCGGACTCTCCGGAGAGTCCGCC
 GCGCGACTCTCCGGAGAGTCCGGC
 GGGCGACTCTCCGGAGAGTCCGCC
 GGGAGACTCTCCGGAGAGTCTCCC

Figure 8A

GGGTGACTCGCCGGCGAGTCACCC
GGACGACTCGCCGGCGAGTCGTCC
GCACGACTCGCCGGCGAGTCGTGC
GACCGACTCGCCGGCGAGTCGGTC
GTCCGACTCGCCGGCGAGTCGGAC
ACCCGACTCGCCGGCGAGTCGGGT
TCCCGACTCGCCGGCGAGTCGGGA
GCCAGACTCGCCGGCGAGTCTGGC
GCCTGACTCGCCGGCGAGTCAGGC
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GTCGGACTCGCCGGCGAGTCCGAC
AGCGGACTCGCCGGCGAGTCCGCT
TGCGGACTCGCCGGCGAGTCCGCA
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 GTCGGACTCACGCGTGAGTCCGAC
 AGCGGACTCACGCGTGAGTCCGCT
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 GGTGGACTCACGCGTGAGTCCACC
 GGCAGACTCACGCGTGAGTCTGCC
 GGCTGACTCACGCGTGAGTCAGCC
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 CTCGGACTCACGCGTGAGTCCGAG
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 CCTGGACTCACGCGTGAGTCCAGG
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 TCGGGACTCACGCGTGAGTCCCGA
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 GGGCGACTCCTCGAGGAGTCGCCC
 GGGAGACTCCTCGAGGAGTCTCCC

Figure 8A

GGGTGACTCAGCGCTGAGTCACCC
GGACGACTCAGCGCTGAGTCGTCC
GGTCGACTCAGCGCTGAGTCGACC
GCACGACTCAGCGCTGAGTCGTGC
GCTCGACTCAGCGCTGAGTCGAGC
GACCGACTCAGCGCTGAGTCGGTC
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CCCTGACTCAGCGCTGAGTCAGGG
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TCGGGACTCAGCGCTGAGTCCCGA
CAGGGACTCAGCGCTGAGTCCCTG
CTGGGACTCAGCGCTGAGTCCCAG

GGCGGACTCAGGCCTGAGTCCGCC
GCCCCGACTCAGGCCTGAGTCGGGC
GGGCGACTCAGGCCTGAGTCGCCC
GGGAGACTCAGGCCTGAGTCTCCC
GGGTGACTCAGGCCTGAGTCACCC
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GCACGACTCAGGCCTGAGTCGTGC
GACCGACTCAGGCCTGAGTCGGTC
GTCCGACTCAGGCCTGAGTCGGAC
ACCCGACTCAGGCCTGAGTCGGGT
TCCCGACTCAGGCCTGAGTCGGGA
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GGACGACTCCTCGAGGAGTCGTCC
GGTCGACTCCTCGAGGAGTCGACC
GCACGACTCCTCGAGGAGTCGTGC
GCTCGACTCCTCGAGGAGTCGAGC
GACCGACTCCTCGAGGAGTCGGTC
GTCCGACTCCTCGAGGAGTCGGAC
ACCCGACTCCTCGAGGAGTCGGGT
TCCCGACTCCTCGAGGAGTCGGGA
GCCAGACTCCTCGAGGAGTCTGGC
GCCTGACTCCTCGAGGAGTCAGGC
GACGGACTCCTCGAGGAGTCCGTC
GTCGGACTCCTCGAGGAGTCCGAC
ACCGGACTCCTCGAGGAGTCCGGT
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CCCAGACTCCTCGAGGAGTCTGGG
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TCGGGACTCCTCGAGGAGTCCCGA
CAGGGACTCCTCGAGGAGTCCCTG
CTGGGACTCCTCGAGGAGTCCCAG

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GGGAGACTCGGTACCGAGTCTCCC
GGGTGACTCGGTACCGAGTCACCC
GGACGACTCGGTACCGAGTCGTCC
GCACGACTCGGTACCGAGTCGTGC
GACCGACTCGGTACCGAGTCGGTC
GTCCGACTCGGTACCGAGTCGGAC
ACCCGACTCGGTACCGAGTCGGGT
TCCCGACTCGGTACCGAGTCGGGA
GCCAGACTCGGTACCGAGTCTGGC

Figure 8A

GCCTGACTCAGGCCTGAGTCAGGC
GACGGACTCAGGCCTGAGTCCGTC
GTCGGACTCAGGCCTGAGTCCGAC
AGCGGACTCAGGCCTGAGTCCGCT
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GGAGGACTCAGGCCTGAGTCCTCC
GGTGGACTCAGGCCTGAGTCCACC
GGCAGACTCAGGCCTGAGTCTGCC
GGCTGACTCAGGCCTGAGTCAGCC
CACGGACTCAGGCCTGAGTCCGTG
CTCGGACTCAGGCCTGAGTCCGAG
CCAGGACTCAGGCCTGAGTCCTGG
CCTGGACTCAGGCCTGAGTCCAGG
CCCAGACTCAGGCCTGAGTCTGGG
CCCTGACTCAGGCCTGAGTCAGGG
ACGGGACTCAGGCCTGAGTCCCGT
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CAGGGACTCAGGCCTGAGTCCCTG
CTGGGACTCAGGCCTGAGTCCCAG

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GGGTGACTCACGCGTGAGTCACCC
GGACGACTCACGCGTGAGTCGTCC
GCACGACTCACGCGTGAGTCGTGC
GACCGACTCACGCGTGAGTCGGTC
GTCCGACTCACGCGTGAGTCGGAC
ACCCGACTCACGCGTGAGTCGGGT
TCCCGACTCACGCGTGAGTCGGGA
GCCAGACTCACGCGTGAGTCTGGC
GCCTGACTCACGCGTGAGTCAGGC
GACGGACTCACGCGTGAGTCCGTC
GTCGGACTCACGCGTGAGTCCGAC
AGCGGACTCACGCGTGAGTCCGCT
TGCGGACTCACGCGTGAGTCCGCA
GGAGGACTCACGCGTGAGTCCTCC
GGTGGACTCACGCGTGAGTCCACC
GGCAGACTCACGCGTGAGTCTGCC
GGCTGACTCACGCGTGAGTCAGCC
CACGGACTCACGCGTGAGTCCGTG
CTCGGACTCACGCGTGAGTCCGAG
CCAGGACTCACGCGTGAGTCCTGG

GCCTGACTCGGTACCGAGTCAGGC
GACGGACTCGGTACCGAGTCCGTC
GTCGGACTCGGTACCGAGTCCGAC
AGCGGACTCGGTACCGAGTCCGCT
TGCGGACTCGGTACCGAGTCCGCA
GGAGGACTCGGTACCGAGTCCTCC
GGTGGACTCGGTACCGAGTCCACC
GGCAGACTCGGTACCGAGTCTGCC
GGCTGACTCGGTACCGAGTCAGCC
CACGGACTCGGTACCGAGTCCGTG
CTCGGACTCGGTACCGAGTCCGAG
CCAGGACTCGGTACCGAGTCCGG
CCTGGACTCGGTACCGAGTCCAGG
CCCAGACTCGGTACCGAGTCTGGG
CCCTGACTCGGTACCGAGTCAGGG
ACGGGACTCGGTACCGAGTCCCGT
TCGGGACTCGGTACCGAGTCCCGA
CAGGGACTCGGTACCGAGTCCCTG
CTGGGACTCGGTACCGAGTCCCAG

GGCGGACTCCTGCAGGAGTCCGCC
GCCCCACTCCTGCAGGAGTCGGGC
GGGCGACTCCTGCAGGAGTCGCCC
GGGAGACTCCTGCAGGAGTCTCCC
GGGTGACTCCTGCAGGAGTCACCC
GGACGACTCCTGCAGGAGTCGTCC
GCACGACTCCTGCAGGAGTCGTGC
GACCGACTCCTGCAGGAGTCGGTC
GTCCGACTCCTGCAGGAGTCGGAC
ACCCGACTCCTGCAGGAGTCGGGT
TCCCGACTCCTGCAGGAGTCGGGA
GCCAGACTCCTGCAGGAGTCTGGC
GCCTGACTCCTGCAGGAGTCAGGC
GACGGACTCCTGCAGGAGTCCGTC
GTCGGACTCCTGCAGGAGTCCGAC
AGCGGACTCCTGCAGGAGTCCGCT
TGCGGACTCCTGCAGGAGTCCGCA
GGAGGACTCCTGCAGGAGTCCTCC
GGTGGACTCCTGCAGGAGTCCACC
GGCAGACTCCTGCAGGAGTCTGCC
GGCTGACTCCTGCAGGAGTCAGCC
CACGGACTCCTGCAGGAGTCCGTG
CTCGGACTCCTGCAGGAGTCCGAG
CCAGGACTCCTGCAGGAGTCCGG

Figure 8A

CCTGGACTCACGCGTGAGTCCAGG
CCCAGACTCACGCGTGAGTCTGGG
CCCTGACTCACGCGTGAGTCAGGG
ACGGGACTCACGCGTGAGTCCCGT
TCGGGACTCACGCGTGAGTCCCGA
CAGGGACTCACGCGTGAGTCCCTG
CTGGGACTCACGCGTGAGTCCCAG

GGCGGACTCAGCGCTGAGTCCGCC
GCCCCGACTCAGCGCTGAGTCGGGC
GGGCGACTCAGCGCTGAGTCGCCC
GGGAGACTCAGCGCTGAGTCTCCC
GGGTGACTCAGCGCTGAGTCACCC
GGACGACTCAGCGCTGAGTCGTCC
GCACGACTCAGCGCTGAGTCGTGC
GACCGACTCAGCGCTGAGTCGGTC
GTCCGACTCAGCGCTGAGTCGGAC
ACCCGACTCAGCGCTGAGTCGGGT
TCCCCGACTCAGCGCTGAGTCGGGA
GCCAGACTCAGCGCTGAGTCTGGC
GCCTGACTCAGCGCTGAGTCAGGC
GACGGACTCAGCGCTGAGTCCGTC
GTCGGACTCAGCGCTGAGTCCGAC
AGCGGACTCAGCGCTGAGTCCGCT
TGCGGACTCAGCGCTGAGTCCGCA
GGAGGACTCAGCGCTGAGTCCCTCC
GGTGGACTCAGCGCTGAGTCCACC
GGCAGACTCAGCGCTGAGTCTGCC
GGCTGACTCAGCGCTGAGTCAGCC
CACGGACTCAGCGCTGAGTCCGTG
CTCGGACTCAGCGCTGAGTCCGAG
CCAGGACTCAGCGCTGAGTCCCTGG
CCTGGACTCAGCGCTGAGTCCAGG
CCCAGACTCAGCGCTGAGTCTGGG
CCCTGACTCAGCGCTGAGTCAGGG
ACGGGACTCAGCGCTGAGTCCCGT
TCGGGACTCAGCGCTGAGTCCCGA
CAGGGACTCAGCGCTGAGTCCCTG
CTGGGACTCAGCGCTGAGTCCCAG

GGCGGACTCACCGGTGAGTCCGCC
GCCCCGACTCACCGGTGAGTCGGGC
GGGCGACTCACCGGTGAGTCGCCC
GGGAGACTCACCGGTGAGTCTCCC

CCTGGACTCCTGCAGGAGTCCAGG
CCCAGACTCCTGCAGGAGTCTGGG
CCCTGACTCCTGCAGGAGTCAGGG
ACGGGACTCCTGCAGGAGTCCCGT
TCGGGACTCCTGCAGGAGTCCCGA
CAGGGACTCCTGCAGGAGTCCCTG
CTGGGACTCCTGCAGGAGTCCCAG

GGCGGACTCGTCGACGAGTCCGCC
GCCCCGACTCGTCGACGAGTCGGGC
GGGCGACTCGTCGACGAGTCGCCC
GGGAGACTCGTCGACGAGTCTCCC
GGGTGACTCGTCGACGAGTCACCC
GGACGACTCGTCGACGAGTCGTCC
GCACGACTCGTCGACGAGTCGTGC
GACCGACTCGTCGACGAGTCGGTC
GTCCGACTCGTCGACGAGTCGGAC
ACCCGACTCGTCGACGAGTCGGGT
TCCCCGACTCGTCGACGAGTCGGGA
GCCAGACTCGTCGACGAGTCTGGC
GCCTGACTCGTCGACGAGTCAGGC
GACGGACTCGTCGACGAGTCCGTC
GTCGGACTCGTCGACGAGTCCGAC
AGCGGACTCGTCGACGAGTCCGCT
TGCGGACTCGTCGACGAGTCCGCA
GGAGGACTCGTCGACGAGTCCCTCC
GGTGGACTCGTCGACGAGTCCACC
GGCAGACTCGTCGACGAGTCTGCC
GGCTGACTCGTCGACGAGTCAGCC
CACGGACTCGTCGACGAGTCCGTG
CTCGGACTCGTCGACGAGTCCGAG
CCAGGACTCGTCGACGAGTCCCTGG
CCTGGACTCGTCGACGAGTCCAGG
CCCAGACTCGTCGACGAGTCTGGG
CCCTGACTCGTCGACGAGTCAGGG
ACGGGACTCGTCGACGAGTCCCGT
TCGGGACTCGTCGACGAGTCCCGA
CAGGGACTCGTCGACGAGTCCCTG
CTGGGACTCGTCGACGAGTCCCAG

GGCGGACTCGTCGACGAGTCCGCC
GCCCCGACTCGTCGACGAGTCGGGC
GGGCGACTCGTCGACGAGTCGCCC
GGGAGACTCGTCGACGAGTCTCCC

Figure 8A

GGGTGACTCACCAGGTGAGTCACCC
GGACGACTCACCAGGTGAGTCGTCC
GCACGACTCACCAGGTGAGTCGTGC
GACCGACTCACCAGGTGAGTCGGTC
GTCCGACTCACCAGGTGAGTCGGAC
ACCCGACTCACCAGGTGAGTCGGGT
TCCCGACTCACCAGGTGAGTCGGGA
GCCAGACTCACCAGGTGAGTCTGGC
GCCTGACTCACCAGGTGAGTCAGGC
GACGGACTCACCAGGTGAGTCCGTC
GTCGGACTCACCAGGTGAGTCCGAC
AGCGGACTCACCAGGTGAGTCCGCT
TGCGGACTCACCAGGTGAGTCCGCA
GGAGGACTCACCAGGTGAGTCCTCC
GGTGGACTCACCAGGTGAGTCCACC
GGCAGACTCACCAGGTGAGTCTGCC
GGCTGACTCACCAGGTGAGTCAGCC
CACGGACTCACCAGGTGAGTCCGTG
CTCGGACTCACCAGGTGAGTCCGAG
CCAGGACTCACCAGGTGAGTCCTGG
CCTGGACTACCGGTGAGTCCAGG
CCCAGACTCACCAGGTGAGTCTGGG
CCCTGACTCACCAGGTGAGTCAGGG
ACGGGACTCACCAGGTGAGTCCCGT
TCGGGACTCACCAGGTGAGTCCCGA
CAGGGACTCACCAGGTGAGTCCCTG
CTGGGACTCACCAGGTGAGTCCAG

GGGTGACTCGTCGACGAGTCACCC
GGACGACTCGTCGACGAGTCGTCC
GCACGACTCGTCGACGAGTCGTGC
GACCGACTCGTCGACGAGTCGGTC
GTCCGACTCGTCGACGAGTCGGAC
ACCCGACTCGTCGACGAGTCGGGT
TCCCGACTCGTCGACGAGTCGGGA
GCCAGACTCGTCGACGAGTCTGGC
GCCTGACTCGTCGACGAGTCAGGC
GACGGACTCGTCGACGAGTCCGTC
GTCGGACTCGTCGACGAGTCCGAC
AGCGGACTCGTCGACGAGTCCGCT
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GGAGGACTCGTCGACGAGTCCTCC
GGTGGACTCGTCGACGAGTCCACC
GGCAGACTCGTCGACGAGTCTGCC
GGCTGACTCGTCGACGAGTCAGCC
CACGGACTCGTCGACGAGTCCGTG
CTCGGACTCGTCGACGAGTCCGAG
CCAGGACTCGTCGACGAGTCCTGG
CCTGGACTCGTCGACGAGTCCAGG
CCCAGACTCGTCGACGAGTCTGGG
CCCTGACTCGTCGACGAGTCAGGG
ACGGGACTCGTCGACGAGTCCCGT
TCGGGACTCGTCGACGAGTCCCGA
CAGGGACTCGTCGACGAGTCCCTG
CTGGGACTCGTCGACGAGTCCAG

Figure 8A

GGCGGACTCGCTAGCGAGTCCGCC
GCCCCGACTCGCTAGCGAGTCGGGC
GGGCGACTCGCTAGCGAGTCGCCC
GGGAGACTCGCTAGCGAGTCTCCC
GGGTGACTCGCTAGCGAGTCACCC
GGACGACTCGCTAGCGAGTCGTCC
GCACGACTCGCTAGCGAGTCGTGC
GACCGACTCGCTAGCGAGTCGGTC
GTCCGACTCGCTAGCGAGTCGGAC
ACCCGACTCGCTAGCGAGTCGGGT
TCCCCGACTCGCTAGCGAGTCGGGA
GCCAGACTCGCTAGCGAGTCTGGC
GCCTGACTCGCTAGCGAGTCAGGC
GACGGACTCGCTAGCGAGTCCGTC
GTCGGACTCGCTAGCGAGTCCGAC
AGCGGACTCGCTAGCGAGTCCGCT
TGCGGACTCGCTAGCGAGTCCGCA
GGAGGACTCGCTAGCGAGTCCTCC
GGTGGACTCGCTAGCGAGTCCACC
GGCAGACTCGCTAGCGAGTCTGCC
GGCTGACTCGCTAGCGAGTCAGCC
CACGGACTCGCTAGCGAGTCCGTG
CTCGGACTCGCTAGCGAGTCCGAG
CCAGGACTCGCTAGCGAGTCCTGG
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CCCAGACTCGCTAGCGAGTCTGGG
CCCTGACTCGCTAGCGAGTCAGGG
ACGGGACTCGCTAGCGAGTCCCGT
TCGGGACTCGCTAGCGAGTCCCGA
CAGGGACTCGCTAGCGAGTCCCTG
CTGGGACTCGCTAGCGAGTCCCAG

CCGGGACTCCACGTGGAGTCCCGG
CCCCGACTCCACGTGGAGTCCGGG
GGCGGACTCCACGTGGAGTCCGCC
GCCGGACTCCACGTGGAGTCCGGC
GCCCCGACTCCACGTGGAGTCGGGC
GGCCGACTCCACGTGGAGTCGGCC
GGGCGACTCCACGTGGAGTCGCCC
GGGAGACTCCACGTGGAGTCTCCC

GGCGGACTCGCATGCGAGTCCGCC
GCCCCGACTCGCATGCGAGTCGGGC
GGGCGACTCGCATGCGAGTCGCCC
GGGAGACTCGCATGCGAGTCTCCC
GGGTGACTCGCATGCGAGTCACCC
GGACGACTCGCATGCGAGTCGTCC
GCACGACTCGCATGCGAGTCGTGC
GACCGACTCGCATGCGAGTCGGTC
GTCCGACTCGCATGCGAGTCGGAC
ACCCGACTCGCATGCGAGTCGGGT
TCCCCGACTCGCATGCGAGTCGGGA
GCCAGACTCGCATGCGAGTCTGGC
GCCTGACTCGCATGCGAGTCAGGC
GACGGACTCGCATGCGAGTCCGTC
GTCGGACTCGCATGCGAGTCCGAC
AGCGGACTCGCATGCGAGTCCGCT
TGCGGACTCGCATGCGAGTCCGCA
GGAGGACTCGCATGCGAGTCCTCC
GGTGGACTCGCATGCGAGTCCACC
GGCAGACTCGCATGCGAGTCTGCC
GGCTGACTCGCATGCGAGTCAGCC
CACGGACTCGCATGCGAGTCCGTG
CTCGGACTCGCATGCGAGTCCGAG
CCAGGACTCGCATGCGAGTCCTGG
CCTGGACTCGCATGCGAGTCCAGG
CCCAGACTCGCATGCGAGTCTGGG
CCCTGACTCGCATGCGAGTCAGGG
ACGGGACTCGCATGCGAGTCCCGT
TCGGGACTCGCATGCGAGTCCCGA
CAGGGACTCGCATGCGAGTCCCTG
CTGGGACTCGCATGCGAGTCCCAG

CCGGGACTCCGTACGGAGTCCCGG
CCCCGACTCCGTACGGAGTCCGGG
GGCGGACTCCGTACGGAGTCCGCC
GCCGGACTCCGTACGGAGTCCGGC
GCCCCGACTCCGTACGGAGTCGGGC
GGCCGACTCCGTACGGAGTCGGCC
GGGCGACTCCGTACGGAGTCGCCC
GGGAGACTCCGTACGGAGTCTCCC

Figure 8A

GGGTGACTCCACGTGGAGTCACCC
 GGACGACTCCACGTGGAGTCGTCC
 GGTCGACTCCACGTGGAGTCGACC
 GCACGACTCCACGTGGAGTCGTGC
 GCTCGACTCCACGTGGAGTCGAGC
 GACCGACTCCACGTGGAGTCGGTC
 GTCCGACTCCACGTGGAGTCGGAC
 ACCCGACTCCACGTGGAGTCGGGT
 TCCCGACTCCACGTGGAGTCGGGA
 GCCAGACTCCACGTGGAGTCTGGC
 GCCTGACTCCACGTGGAGTCAGGC
 GACGGACTCCACGTGGAGTCCGTC
 GTCGGACTCCACGTGGAGTCCGAC
 ACCGGACTCCACGTGGAGTCCGGT
 TCCGGACTCCACGTGGAGTCCGGA
 AGCGGACTCCACGTGGAGTCCGCT
 TGCGGACTCCACGTGGAGTCCGCA
 GGAGGACTCCACGTGGAGTCCTCC
 GGTGGACTCCACGTGGAGTCCACC
 GGCAGACTCCACGTGGAGTCTGCC
 GGCTGACTCCACGTGGAGTCAGCC
 CACGGACTCCACGTGGAGTCCGTG
 CTCGGACTCCACGTGGAGTCCGAG
 CCAGGACTCCACGTGGAGTCCTGG
 CCTGGACTCCACGTGGAGTCCAGG
 CCCAGACTCCACGTGGAGTCTGGG
 CCCTGACTCCACGTGGAGTCAGGG
 ACGGGACTCCACGTGGAGTCCCGT
 TCGGGACTCCACGTGGAGTCCCGA
 CAGGGACTCCACGTGGAGTCCCTG
 CTGGGACTCCACGTGGAGTCCCAG

GGCGGACTCCCATGGGAGTCCGCC
 GCCCGACTCCCATGGGAGTCGGGC
 GGGCGACTCCCATGGGAGTCGCCC
 GGGAGACTCCCATGGGAGTCTCCC
 GGGTGACTCCCATGGGAGTCACCC
 GGACGACTCCCATGGGAGTCGTCC
 GCACGACTCCCATGGGAGTCGTGC
 GACCGACTCCCATGGGAGTCGGTC
 GTCCGACTCCCATGGGAGTCGGAC
 ACCCGACTCCCATGGGAGTCGGGT
 TCCCGACTCCCATGGGAGTCGGGA
 GCCAGACTCCCATGGGAGTCTGGC

GGGTGACTCCGTACGGAGTCACCC
 GGACGACTCCGTACGGAGTCGTCC
 GGTCGACTCCGTACGGAGTCGACC
 GCACGACTCCGTACGGAGTCGTGC
 GCTCGACTCCGTACGGAGTCGAGC
 GACCGACTCCGTACGGAGTCGGTC
 GTCCGACTCCGTACGGAGTCGGAC
 ACCCGACTCCGTACGGAGTCGGGT
 TCCCGACTCCGTACGGAGTCGGGA
 GCCAGACTCCGTACGGAGTCTGGC
 GCCTGACTCCGTACGGAGTCAGGC
 GACGGACTCCGTACGGAGTCCGTC
 GTCGGACTCCGTACGGAGTCCGAC
 ACCGGACTCCGTACGGAGTCCGGT
 TCCGGACTCCGTACGGAGTCCGGA
 AGCGGACTCCGTACGGAGTCCGCT
 TGCGGACTCCGTACGGAGTCCGCA
 GGAGGACTCCGTACGGAGTCCTCC
 GGTGGACTCCGTACGGAGTCCACC
 GGCAGACTCCGTACGGAGTCTGCC
 GGCTGACTCCGTACGGAGTCAGCC
 CACGGACTCCGTACGGAGTCCGTG
 CTCGGACTCCGTACGGAGTCCGAG
 CCAGGACTCCGTACGGAGTCCTGG
 CCTGGACTCCGTACGGAGTCCAGG
 CCCAGACTCCGTACGGAGTCTGGG
 CCCTGACTCCGTACGGAGTCAGGG
 ACGGGACTCCGTACGGAGTCCCGT
 TCGGGACTCCGTACGGAGTCCCGA
 CAGGGACTCCGTACGGAGTCCCTG
 CTGGGACTCC**CGTACG**GAGTCCCAG

CCGGGACTCCGATCGGAGTCCCGG
 CCCGGACTCCGATCGGAGTCCGGG
 GGCGGACTCCGATCGGAGTCCGCC
 GCCGGACTCCGATCGGAGTCCGGC
 GCCCGACTCCGATCGGAGTCGGGC
 GGCCGACTCCGATCGGAGTCGGCC
 GGGCGACTCCGATCGGAGTCGCC
 GGGAGACTCCGATCGGAGTCTCCC
 GGGTGACTCCGATCGGAGTCACCC
 GGACGACTCCGATCGGAGTCGTCC
 GGTCGACTCCGATCGGAGTCGACC
 GCACGACTCCGATCGGAGTCGTGC

Figure 8A

GCCTGACTCCCATGGGAGTCAGGC
 GACGGACTCCCATGGGAGTCCGTC
 GTCGGACTCCCATGGGAGTCCGAC
 AGCGGACTCCCATGGGAGTCCGCT
 TCGGGACTCCCATGGGAGTCCGCA
 GGAGGACTCCCATGGGAGTCCTCC
 GGTGGACTCCCATGGGAGTCCACC
 GGCAGACTCCCATGGGAGTCTGCC
 GGCTGACTCCCATGGGAGTCAGCC
 CACGGACTCCCATGGGAGTCCGTG
 CTCGGACTCCCATGGGAGTCCGAG
 CCAGGACTCCCATGGGAGTCCTGG
 CCTGGACTCCCATGGGAGTCCAGG
 CCCAGACTCCCATGGGAGTCTGGG
 CCCTGACTCCCATGGGAGTCAGGG
 ACGGGACTCCCATGGGAGTCCCGT
 TCGGGACTCCCATGGGAGTCCCGA
 CAGGGACTCCCATGGGAGTCCCTG
 CTGGGACTCCCATGGGAGTCCCAG

GCCCGACTCGGATCCGAGTCGGGC
 GCCCGACTCGGATCCGAGTCGGGC
 GGGCGACTCGGATCCGAGTCGCCC
 GGGAGACTCGGATCCGAGTCTCCC
 GGGTGACTCGGATCCGAGTCACCC
 GGACGACTCGGATCCGAGTCGTCC
 GCACGACTCGGATCCGAGTCGTGC
 GACCGACTCGGATCCGAGTCGGTC
 GTCCGACTCGGATCCGAGTCGGAC
 ACCCGACTCGGATCCGAGTCGGGT
 TCCCGACTCGGATCCGAGTCGGGA
 GCCAGACTCGGATCCGAGTCTGGC
 GCCTGACTCGGATCCGAGTCAGGC
 GACGGACTCGGATCCGAGTCCGTC
 GTCGGACTCGGATCCGAGTCCGAC
 AGCGGACTCGGATCCGAGTCCGCT
 TCGGGACTCGGATCCGAGTCCGCA
 GGAGGACTCGGATCCGAGTCCTCC
 GGTGGACTCGGATCCGAGTCCACC
 GGCAGACTCGGATCCGAGTCTGCC
 GGCTGACTCGGATCCGAGTCAGCC
 CACGGACTCGGATCCGAGTCCGTG
 CTCGGACTCGGATCCGAGTCCGAG
 CCAGGACTCGGATCCGAGTCCTGG

GCTCGACTCCGATCGGAGTCGAGC
 GACCGACTCCGATCGGAGTCGGTC
 GTCCGACTCCGATCGGAGTCGGAC
 ACCCGACTCCGATCGGAGTCGGGT
 TCCCGACTCCGATCGGAGTCGGGA
 GCCAGACTCCGATCGGAGTCTGGC
 GCCTGACTCCGATCGGAGTCAGGC
 GACGGACTCCGATCGGAGTCCGTC
 GTCGGACTCCGATCGGAGTCCGAC
 ACCCGACTCCGATCGGAGTCCGGT
 TCCCGACTCCGATCGGAGTCCGGA
 AGCGGACTCCGATCGGAGTCCGCT
 TCGGGACTCCGATCGGAGTCCGCA
 GGAGGACTCCGATCGGAGTCCTCC
 GGTGGACTCCGATCGGAGTCCACC
 GGCAGACTCCGATCGGAGTCTGCC
 GGCTGACTCCGATCGGAGTCAGCC
 CACGGACTCCGATCGGAGTCCGTG
 CTCGGACTCCGATCGGAGTCCGAG
 CCAGGACTCCGATCGGAGTCCTGG
 CCTGGACTCCGATCGGAGTCCAGG
 CCCAGACTCCGATCGGAGTCTGGG
 CCCTGACTCCGATCGGAGTCAGGG
 ACGGGACTCCGATCGGAGTCCCGT
 TCGGGACTCCGATCGGAGTCCCGA
 CAGGGACTCCGATCGGAGTCCCTG
 CTGGGACTCC**GATCGG**AGTCCCAG

GGCGGACTCGCATGCGAGTCCGCC
 GCCCGACTCGCATGCGAGTCGGGC
 GGGCGACTCGCATGCGAGTCGCCC
 GGGAGACTCGCATGCGAGTCTCCC
 GGGTGACTCGCATGCGAGTCACCC
 GGACGACTCGCATGCGAGTCGTCC
 GCACGACTCGCATGCGAGTCGTGC
 GACCGACTCGCATGCGAGTCGGTC
 GTCCGACTCGCATGCGAGTCGGAC
 ACCCGACTCGCATGCGAGTCGGGT
 TCCCGACTCGCATGCGAGTCGGGA
 GCCAGACTCGCATGCGAGTCTGGC
 GCCTGACTCGCATGCGAGTCAGGC
 GACGGACTCGCATGCGAGTCCGTC
 GTCGGACTCGCATGCGAGTCCGAC
 AGCGGACTCGCATGCGAGTCCGCT

Figure 8A

CCTGGACTCGGATCCGAGTCCAGG
CCCAGACTCGGATCCGAGTCTGGG
CCCTGACTCGGATCCGAGTCAGGG
ACGGGACTCGGATCCGAGTCCCGT
TCGGGACTCGGATCCGAGTCCCGA
CAGGGACTCGGATCCGAGTCCCTG
CTGGGACTCGGATCCGAGTCCCAG

GGCGGACTCGCTAGCGAGTCCGCC
GCCCCGACTCGCTAGCGAGTCGGGC
GGGCGACTCGCTAGCGAGTCGCCC
GGGAGACTCGCTAGCGAGTCTCCC
GGGTGACTCGCTAGCGAGTCACCC
GGACGACTCGCTAGCGAGTCGTCC
GCACGACTCGCTAGCGAGTCGTGC
GACCGACTCGCTAGCGAGTCGGTC
GTCCGACTCGCTAGCGAGTCGGAC
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GTCGGACTCGCTAGCGAGTCCGAC
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TCGGGACTCGCTAGCGAGTCCCGA
CAGGGACTCGCTAGCGAGTCCCTG
CTGGGACTCGCTAGCGAGTCCCAG
ACGGGACTCCAGCTGGAGTCCCGT
TCGGGACTCCAGCTGGAGTCCCGA
CAGGGACTCCAGCTGGAGTCCCTG
CTGGGACTCCAGCTGGAGTCCCAG

TGCGGACTCGCATGCGAGTCCGCA
GGAGGACTCGCATGCGAGTCCCTCC
GGTGGACTCGCATGCGAGTCCACC
GGCAGACTCGCATGCGAGTCTGCC
GGCTGACTCGCATGCGAGTCAGCC
CACGGACTCGCATGCGAGTCCGTG
CTCGGACTCGCATGCGAGTCCGAG
CCAGGACTCGCATGCGAGTCCCTGG
CCTGGACTCGCATGCGAGTCCAGG
CCCAGACTCGCATGCGAGTCTGGG
CCCTGACTCGCATGCGAGTCAGGG
ACGGGACTCGCATGCGAGTCCCGT
TCGGGACTCGCATGCGAGTCCCGA
CAGGGACTCGCATGCGAGTCCCTG
CTGGGACTCGCATGCGAGTCCCAG

GGCGGACTCCAGCTGGAGTCCGCC
GCCCCGACTCCAGCTGGAGTCGGGC
GGGCGACTCCAGCTGGAGTCGCCC
GGGAGACTCCAGCTGGAGTCTCCC
GGGTGACTCCAGCTGGAGTCACCC
GGACGACTCCAGCTGGAGTCGTCC
GCACGACTCCAGCTGGAGTCGTGC
GACCGACTCCAGCTGGAGTCGGTC
GTCCGACTCCAGCTGGAGTCGGAC
ACCCGACTCCAGCTGGAGTCGGGT
TCCCCGACTCCAGCTGGAGTCGGGA
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GCCTGACTCCAGCTGGAGTCAGGC
GACGGACTCCAGCTGGAGTCCGTC
GTCGGACTCCAGCTGGAGTCCGAC
AGCGGACTCCAGCTGGAGTCCGCT
TGCGGACTCCAGCTGGAGTCCGCA
GGAGGACTCCAGCTGGAGTCCCTCC
GGTGGACTCCAGCTGGAGTCCACC
GGCAGACTCCAGCTGGAGTCTGCC
GGCTGACTCCAGCTGGAGTCAGCC
CACGGACTCCAGCTGGAGTCCGTG
CTCGGACTCCAGCTGGAGTCCGAG
CCAGGACTCCAGCTGGAGTCCCTGG
CCTGGACTCCAGCTGGAGTCCAGG
CCCAGACTCCAGCTGGAGTCTGGG
CCCTGACTCCAGCTGGAGTCAGGG

Figure 8B

Sequences of 5'-tails

NNNNGACTCNNNNGAGTCNNNN

200 Selected Sequences = 0.08% of entire sequence space (248832)

 $\Delta G = -47$ Kcal/mole to -55 kcal/mole $\Delta\Delta G < -40$ kcal/mole

GC content 72% to 82%

GGCGGACTCCGCGGAGTCCGCC
 GCGCGACTCCGCGGAGTCCGGC
 GGGCGACTCCGCGGAGTCCGCC
 CCGGGACTCCGCGGAGTCCCGG
 CCCGGACTCCGCGGAGTCCGGG
 GCCGGACTCCGCGGAGTCCGGC
 GGCCGACTCCGCGGAGTCCGCC
 GGGAGACTCGCGCGAGTCTCCC
 GGGTGACTCCGCGGAGTCACCC
 GGACGACTCCGCGGAGTCGTCC
 GCACGACTCCGCGGAGTCGTGC
 GACCGACTCCGCGGAGTCGGTC
 GTCCGACTCCGCGGAGTCGGAC
 ACCCGACTCCGCGGAGTCGGGT
 TCCCGACTCCGCGGAGTCGGGA
 GCCAGACTCCGCGGAGTCTGGC
 GCCTGACTCCGCGGAGTCAGGC
 GACGGACTCCGCGGAGTCCGTC
 GTCGGACTCCGCGGAGTCCGAC
 AGCGGACTCCGCGGAGTCCGCT
 TGCGGACTCCGCGGAGTCCGCA
 GGAGGACTCCGCGGAGTCCCTC
 GGTGGACTCCGCGGAGTCCACC
 GGCAGACTCCGCGGAGTCTGCC
 GGCTGACTCCGCGGAGTCAGCC
 CACGGACTCCGCGGAGTCCGTG
 CTCGGACTCCGCGGAGTCCGAG
 CCAGGACTCCGCGGAGTCCCTGG
 CCTGGACTCCGCGGAGTCCAGG
 CCCAGACTCCGCGGAGTCTGGG
 CCCTGACTCCGCGGAGTCAGGG
 ACGGGACTCCGCGGAGTCCCGT
 TCGGGACTCCGCGGAGTCCCGA
 CAGGGACTCCGCGGAGTCCCTG
 CTGGGACTCCGCGGAGTCCCAG
 ACCGGACTCCGCGGAGTCCGGT

GGCGGACTCTGCAGAGTCCGCC
 GCGCGACTCTGCAGAGTCCGGC
 GGGCGACTCTGCAGAGTCCGCC
 CCGGGACTCTGCAGAGTCCCGG
 CCCGGACTCTGCAGAGTCCGGG
 GCCGGACTCTGCAGAGTCCGGC
 GGCCGACTCTGCAGAGTCCGCC
 GGGAGACTCAGCTGAGTCCGCC
 GCGCGACTCAGCTGAGTCCGGC
 GGGCGACTCAGCTGAGTCCGCC
 CCGGGACTCAGCTGAGTCCCGG
 CCCGGACTCAGCTGAGTCCGGG
 GCCGGACTCAGCTGAGTCCGGC
 GGCCGACTCAGCTGAGTCCGCC
 GGCGGACTCCTAGGAGTCCGCC
 GCGCGACTCCTAGGAGTCCGGC
 GGGCGACTCCTAGGAGTCCGCC
 CCGGGACTCCTAGGAGTCCCGG
 CCCGGACTCCTAGGAGTCCGGG
 GCCGGACTCCTAGGAGTCCGGC
 GGCCGACTCCTAGGAGTCCGCC
 GGCGGACTCCATGGAGTCCGCC
 GCGCGACTCCATGGAGTCCGGC
 GGGCGACTCCATGGAGTCCGCC
 CCGGGACTCCATGGAGTCCCGG
 CCCGGACTCCATGGAGTCCGGG
 GCCGGACTCCATGGAGTCCGGC
 GGCCGACTCCATGGAGTCCGCC
 CCGGGACTCGCGCGAGTCCCGG
 CCCGGACTCGCGCGAGTCCGGG
 GGCGGACTCGCGCGAGTCCGCC
 GCGCGACTCGCGCGAGTCCGGC
 GCGCGACTCGCGCGAGTCCGGC
 GGCCGACTCGCGCGAGTCCGCC
 GGGCGACTCGCGCGAGTCCGCC
 GGGAGACTCGCGCGAGTCTCCC

Figure 8B

GGGTGACTCGCGCGAGTCACCC	CCCGGACTCACGTGAGTCCGGG
GGACGACTCGCGCGAGTCGTCC	GGCGGACTCACGTGAGTCCGCC
GCACGACTCGCGCGAGTCGTGC	GCCGGACTCACGTGAGTCCGGC
GACCGACTCGCGCGAGTCGGTC	GCCCGACTCACGTGAGTCCGGC
GTCCGACTCGCGCGAGTCGGAC	GGCCGACTCACGTGAGTCCGGC
ACCCGACTCGCGCGAGTCGGGT	GGGCGACTCACGTGAGTCCGCC
TCCCGACTCGCGCGAGTCGGGA	CCGGGACTCGTACGAGTCCCGG
GCCAGACTCGCGCGAGTCTGGC	CCCGGACTCGTACGAGTCCGGG
GCCTGACTCGCGCGAGTCAGGC	GGCGGACTCGTACGAGTCCGCC
GACGGACTCGCGCGAGTCCGTC	GCCGGACTCGTACGAGTCCGGC
GTCGGACTCGCGCGAGTCCGAC	GCCCGACTCGTACGAGTCCGGC
ACCGGACTCGCGCGAGTCCGGT	GGCCGACTCGTACGAGTCCGGC
AGCGGACTCGCGCGAGTCCGCT	GGGCGACTCGTACGAGTCCGCC
TGCGGACTCGCGCGAGTCCGCA	CCGGGACTCGATCGAGTCCCGG
GGAGGACTCGCGCGAGTCCCTCC	CCCGGACTCGATCGAGTCCGGG
GGTGGACTCGCGCGAGTCCACC	GGCGGACTCGATCGAGTCCGCC
GGCAGACTCGCGCGAGTCTGCC	GCCGGACTCGATCGAGTCCGGC
GGCTGACTCGCGCGAGTCAGCC	GCCCGACTCGATCGAGTCCGGC
CACGGACTCGCGCGAGTCCGTG	GGCCGACTCGATCGAGTCCGGC
CTCGGACTCGCGCGAGTCCGAG	GGGCGACTCGATCGAGTCCGCC
CCAGGACTCGCGCGAGTCCCTGG	GGCGGACTCGGCCGAGTCCGCC
CCTGGACTCGCGCGAGTCCAGG	GCCCGACTCGGCCGAGTCCGGC
CCCAGACTCGCGCGAGTCTGGG	GGGCGACTCGGCCGAGTCCGCC
CCCTGACTCGCGCGAGTCAGGG	CCGGGACTCGGCCGAGTCCCGG
ACGGGACTCGCGCGAGTCCCGT	CCCGGACTCGGCCGAGTCCGGG
TCGGGACTCGCGCGAGTCCCGA	GCCGGACTCGGCCGAGTCCGGC
CAGGGACTCGCGCGAGTCCCTG	GGCCGACTCGGCCGAGTCCGGC
CTGGGACTCGCGCGAGTCCCAG	GGGAGACTCGGCCGAGTCTCCC
CCGGGACTCTCGAGAGTCCCGG	GGGTGACTCGGCCGAGTCACCC
CCCGGACTCTCGAGAGTCCGGG	GGACGACTCGGCCGAGTCGTCC
GGCGGACTCTCGAGAGTCCGCC	GCACGACTCGGCCGAGTCGTGC
GCCGGACTCTCGAGAGTCCGGC	GACCGACTCGGCCGAGTCGGTC
GCCCGACTCTCGAGAGTCCGGC	GTCCGACTCGGCCGAGTCGGAC
GGCCGACTCTCGAGAGTCCGGC	ACCCGACTCGGCCGAGTCGGGT
GGGCGACTCTCGAGAGTCCGCC	TCCCGACTCGGCCGAGTCGGGA
CCGGGACTCACGTGAGTCCCGG	GCCAGACTCGGCCGAGTCTGGC

Figure 8B

GCCTGACTCGGCCGAGTCAGGC
GACGGACTCGGCCGAGTCCGTC
GTCGGACTCGGCCGAGTCCGAC
AGCGGACTCGGCCGAGTCCGCT
TGCGGACTCGGCCGAGTCCGCA
GGAGGACTCGGCCGAGTCCCTCC
GGTGGACTCGGCCGAGTCCACC
GGCAGACTCGGCCGAGTCTGCC
GGCTGACTCGGCCGAGTCAGCC
CACGGACTCGGCCGAGTCCGTG
CTCGGACTCGGCCGAGTCCGAG
CCAGGACTCGGCCGAGTCCCTGG
CCTGGACTCGGCCGAGTCCAGG
CCCAGACTCGGCCGAGTCTGGG
CCCTGACTCGGCCGAGTCAGGG
ACGGGACTCGGCCGAGTCCCGT
TCGGGACTCGGCCGAGTCCCGA
CAGGGACTCGGCCGAGTCCCTG
CTGGGACTCGGCCGAGTCCCAG
ACCGGACTCGGCCGAGTCCGGT
GGCGGACTCCCGGGAGTCCGCC
GCCCCGACTCCCGGGAGTCGGGC
GGGCGACTCCCGGGAGTCGCCC
CCGGGACTCCCGGGAGTCCCGG
CCCGGACTCCCGGGAGTCCGGG
GCCGGACTCCCGGGAGTCCGGC
GGCCGACTCCCGGGAGTCGGCC
GGGAGACTCCCGGGAGTCTCCC
GGGTGACTCCCGGGAGTCACCC
GGACGACTCCCGGGAGTCGTCC
GCACGACTCCCGGGAGTCGTGC
GACCGACTCCCGGGAGTCGGTC
GTCCGACTCCCGGGAGTCGGAC
ACCCGACTCCCGGGAGTCGGGT
TCCCGACTCCCGGGAGTCGGGA
GCCAGACTCCCGGGAGTCTGGC

GCCTGACTCCCGGGAGTCAGGC
GACGGACTCCCGGGAGTCCGTC
GTCGGACTCCCGGGAGTCCGAC
AGCGGACTCCCGGGAGTCCGCT
TGCGGACTCCCGGGAGTCCGCA
GGAGGACTCCCGGGAGTCCCTCC
GGTGGACTCCCGGGAGTCCACC
GGCAGACTCCCGGGAGTCTGCC
GGCTGACTCCCGGGAGTCAGCC
CACGGACTCCCGGGAGTCCGTG
CTCGGACTCCCGGGAGTCCGAG
CCAGGACTCCCGGGAGTCCCTGG
CCTGGACTCCCGGGAGTCCAGG
CCCAGACTCCCGGGAGTCTGGG
CCCTGACTCCCGGGAGTCAGGG
ACGGGACTCCCGGGAGTCCCGT
TCGGGACTCCCGGGAGTCCCGA
CAGGGACTCCCGGGAGTCCCTG
CTGGGACT**CCCGGG**AGTCCCAG
ACCGGACT**CCCGGG**AGTCCGGT

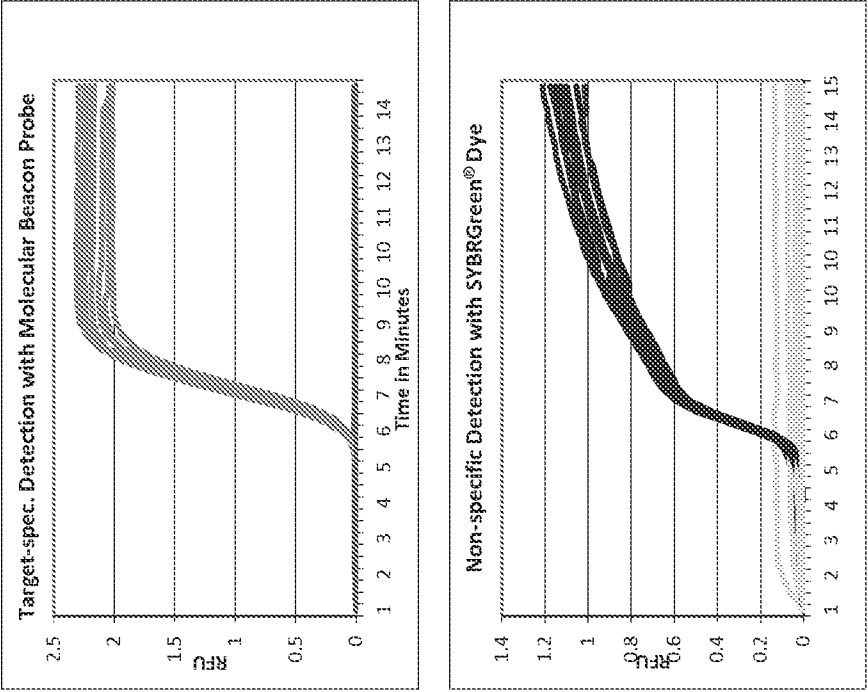


Figure 9

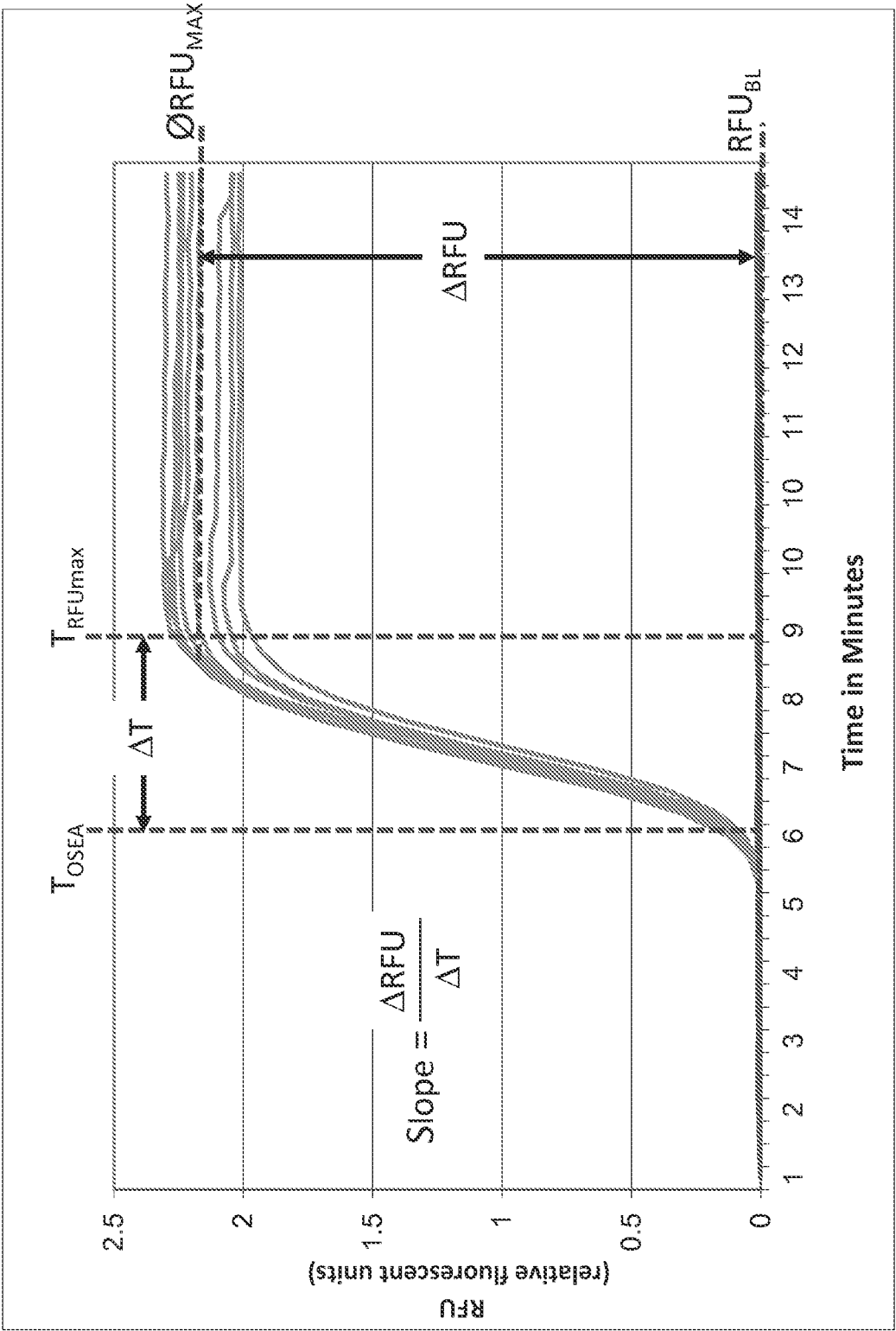


Figure 10

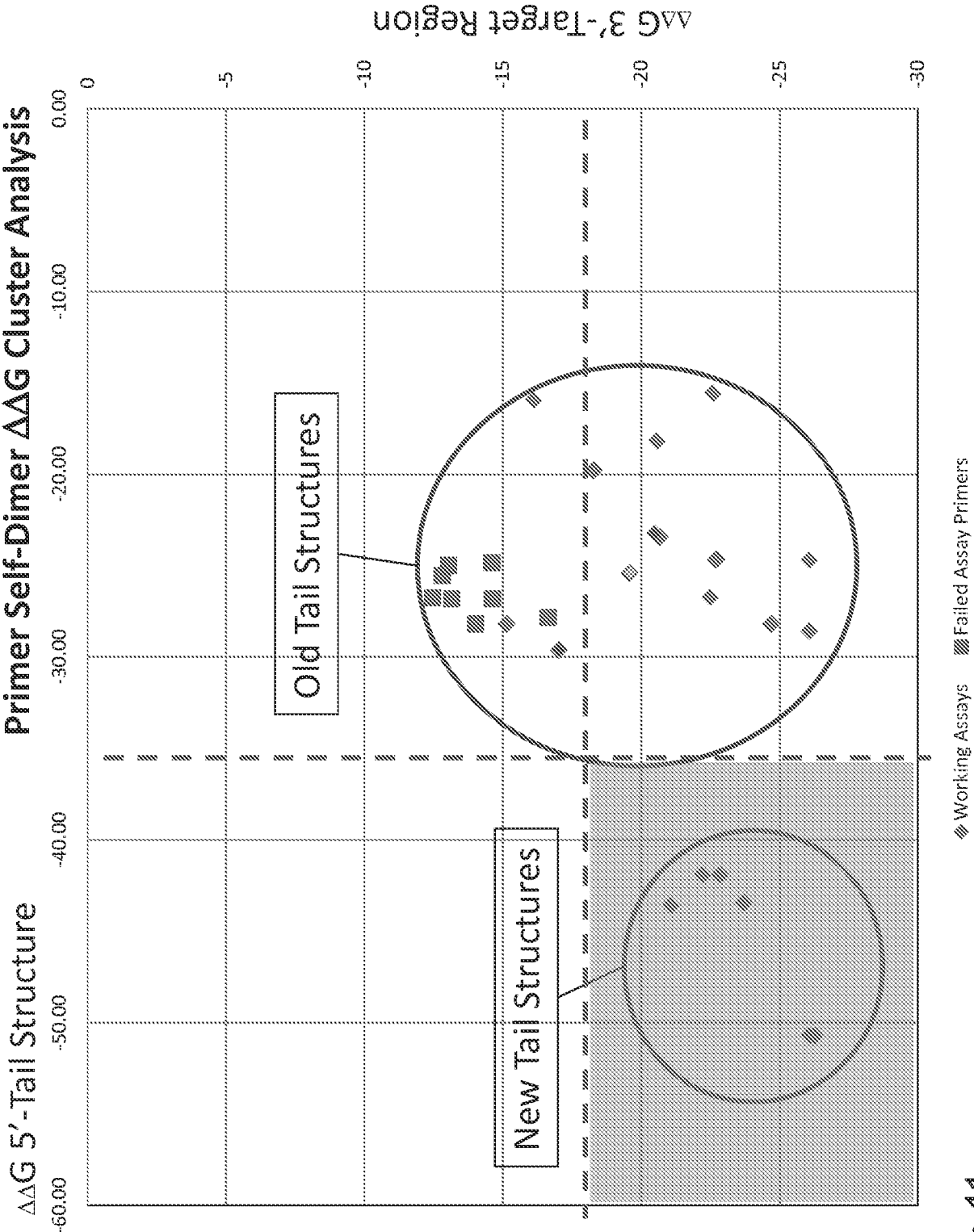


Figure 11

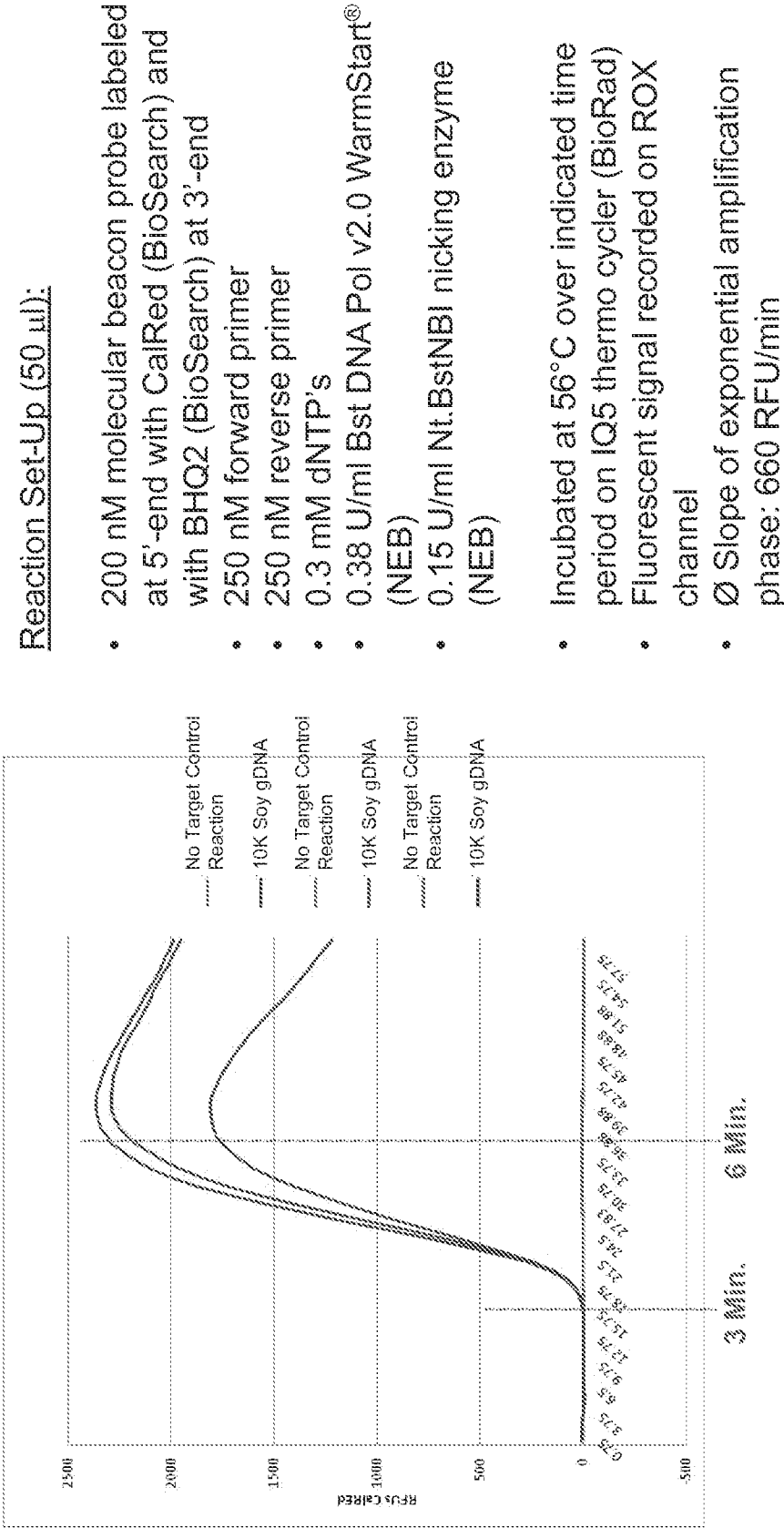
Design of an Isothermal DNA Amplification Assay for Soy Lectin From a Single Pair of Predicted Optimal Primer Structures without Screening a Multitude of Candidate Primer Combinations of Unknown Performance

- Assay target sequence in Soy Lectin gene selected based on absence of SNP's a 30 to 60 bp sequence window:
- 5'-ccaaggtctcattacctatgatgcctccaccagcctcttggtgcttcttggtctacccttcacagagaa-3'
- Underlined are the sequence regions selected for design of the forward and reverse primers, respectively.
- Target binding region of Fwd primer: 5' -ATTACCTATGATGCC-3'
- $\Delta G_{25^{\circ}\text{C}}$ of fwd Primer-Target Hybrid (PTH) = -25.99 kcal/mole; $\Delta G_{25^{\circ}\text{C}}$ of Primer-Target binding region self dimer (PTBRHD) = -3.14 kcal/mole; $\Delta\Delta G_{25^{\circ}\text{C}} = \Delta G_{\text{PTH}} - \Delta G_{\text{PTBRHD}} = -22.85$ kcal/mole
- Target binding region of Rev primer: 5' -ACCAAAGAGCAAC-3'
- $\Delta G_{25^{\circ}\text{C}}$ of rev Primer-Target Hybrid (PTH) = -25.35 kcal/mole; $\Delta G_{25^{\circ}\text{C}}$ of Primer-Target binding region self dimer (PTBRHD) = -3.14 kcal/mole; $\Delta\Delta G_{25^{\circ}\text{C}} = \Delta G_{\text{PTH}} - \Delta G_{\text{PTBRHD}} = -22.21$ kcal/mole

Figure 12

Figure 13

First Trial Experimental Results
Empirical Confirmation of Assay Performance Prediction



Primer and probe sequences used in the assay (all synthesized by BioSearch Inc., Novato, CA.)

5'-ACGCGACTCGTCGACGAGTCGCGTATTACCTATGAmUmGmCmC-3' fwd primer ("m" stands for 2'-methoxy nucleotide)

5'-ACGCGACTCGTCGACGAGTCGCGTACCAAGAAmGmCmAmC-3' rev primer

5'-CalRed₆₁₀-CGCGCTCCACCAICCTCTTCGCGG-BHQ2 molecular beacon probe ("I" stands for universal inosine nucleotide)

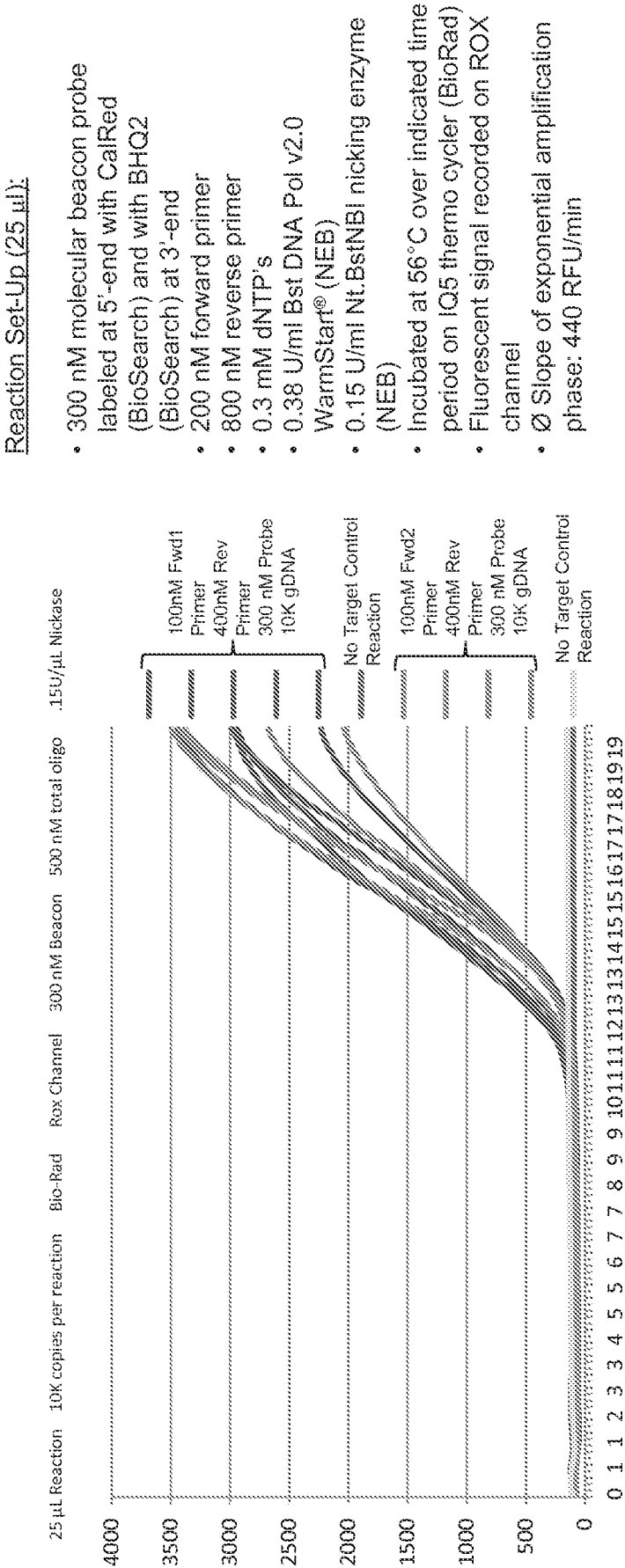
Design of an Isothermal DNA Amplification Assay for Pathogenic *Salmonella enterica* *invA* Gene From a Single Pair of Predicted Optimal Primer Structures without Screening a Multitude of Candidate Primer Combinations of Unknown Performance

- *Salmonella enterica* *invA* assay target sequence selected based on absence of SNP's a 30 to 60 bp sequence window:
- 5'-ATACTCATCTGTTTACCGGCATACCATCCAGAGAAAA-3'
- Underlined are the sequence regions selected for design of the forward and reverse primers, respectively.
- Target binding region of fwd1 and fwd2 primers: 5' -ATACTCATCTGTTTACC-3'
- $\Delta G_{25^{\circ}\text{C}}$ of fwd Primer-Target Hybrid (PTH) = -26.11 kcal/mole; $\Delta G_{25^{\circ}\text{C}}$ of Primer-Target binding region self dimer (PTBRHD) = -1.95 kcal/mole; $\Delta\Delta G_{25^{\circ}\text{C}} = \Delta G_{\text{PTH}} - \Delta G_{\text{PTBRHD}} = -24.16$ kcal/mole
- Target binding region of Rev primer: 5' -TTTCTCTCTGGATGG-3'
- $\Delta G_{25^{\circ}\text{C}}$ of rev Primer-Target Hybrid (PTH) = -25.28 kcal/mole; $\Delta G_{25^{\circ}\text{C}}$ of Primer-Target binding region self dimer (PTBRHD) = -1.57 kcal/mole; $\Delta\Delta G_{25^{\circ}\text{C}} = \Delta G_{\text{PTH}} - \Delta G_{\text{PTBRHD}} = -23.71$ kcal/mole

Figure 14

Figure 15

Salmonella invA Assay - First Trial Experimental Results
Empirical Confirmation of Assay Performance Prediction



Primer and probe sequences used in the assay (all synthesized by BioSearch Inc., Novato, CA.)

5'-GGCTGACTCCTGCAGGAGTCAGCCATCACTCATCTGmUmUmAmCmC-3' fwd1 primer ("m" stands for 2'-methoxy nucleotide)

5'-GGCTGACTCCTGCAGGAGTCAGCCATCACTCATCTGmUmUmAmCmC-3' fwd2 primer

5'-GGCTGACTCCTGCAGGAGTCAGCCCTTTCTCTGmGmAmUmGmG-3' rev primer

5'-CalRed₆₁₀-ACCTGTTACCGGCATACAAACAGT-BHQ2 molecular beacon probe ("l" stands for universal inosine nucleotide)

Figure 16
Salmonella invA Assay – Assay Optimization

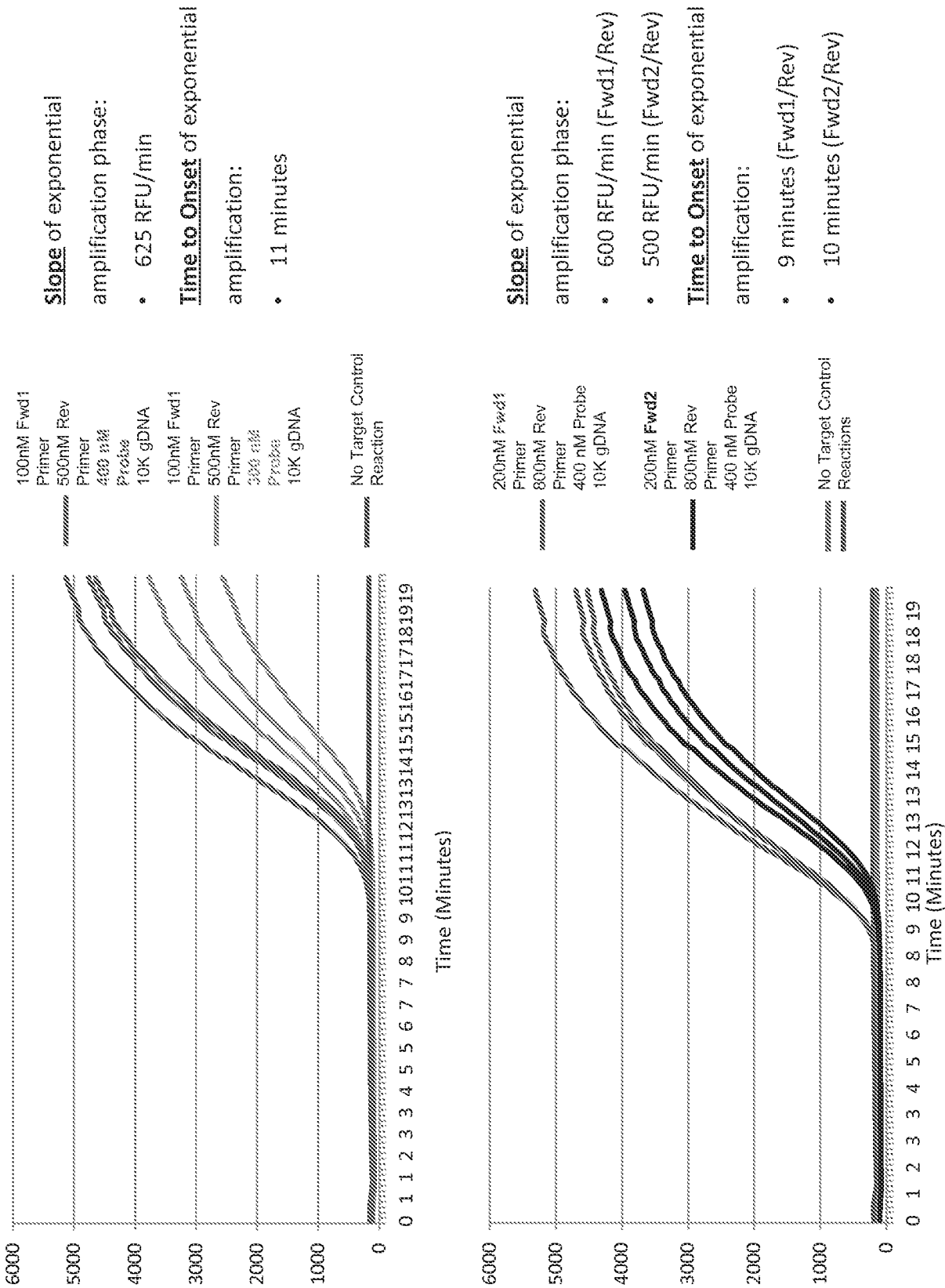


Figure 17
Primer Structure Parameter (T_M , %GC, bp Length, $\Delta G_{25^\circ C}$, $\Delta\Delta G_{25^\circ C}$) Cluster Analysis
To Identify Structural Parameter Predicting Function & Performance in Isothermal Amplification

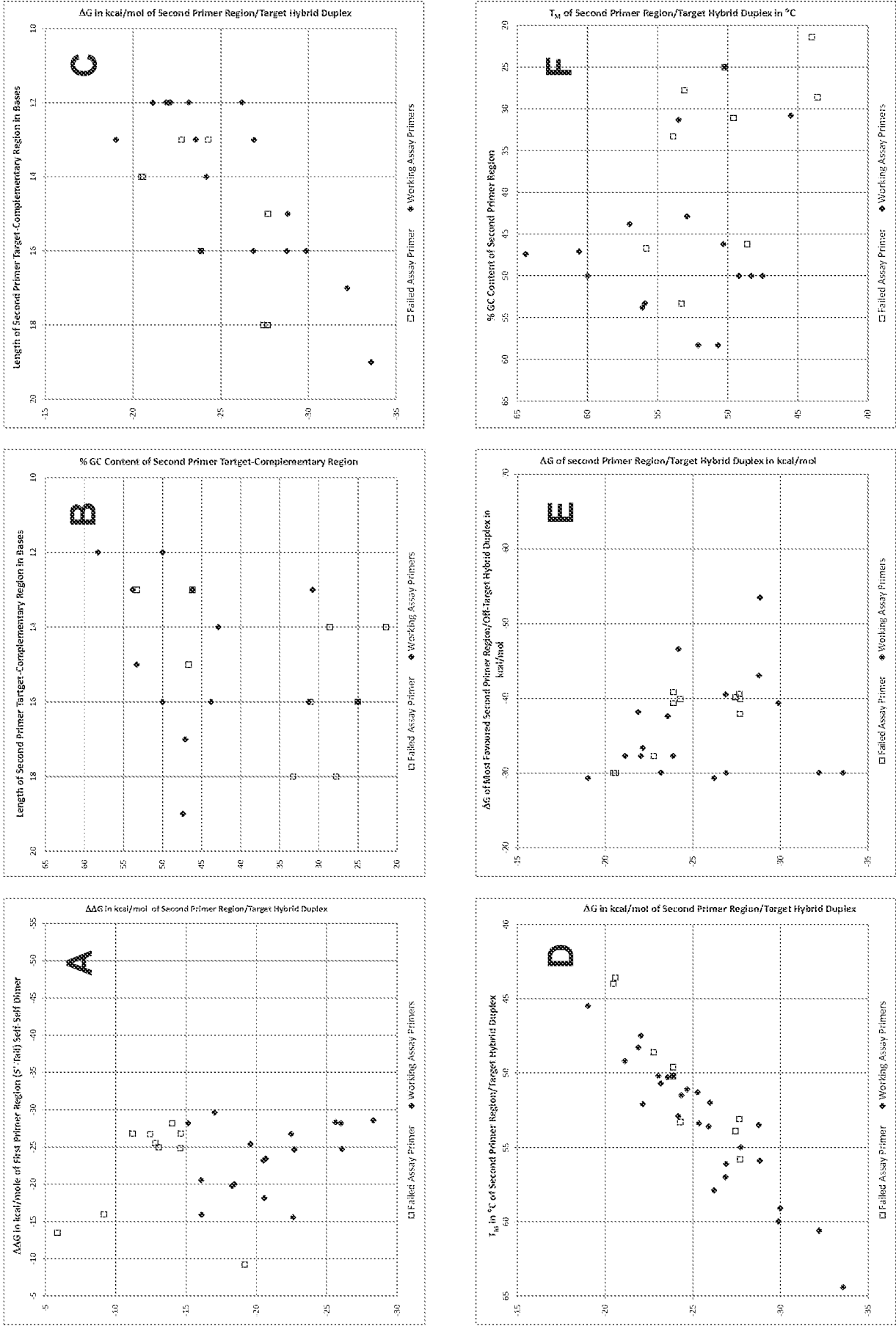
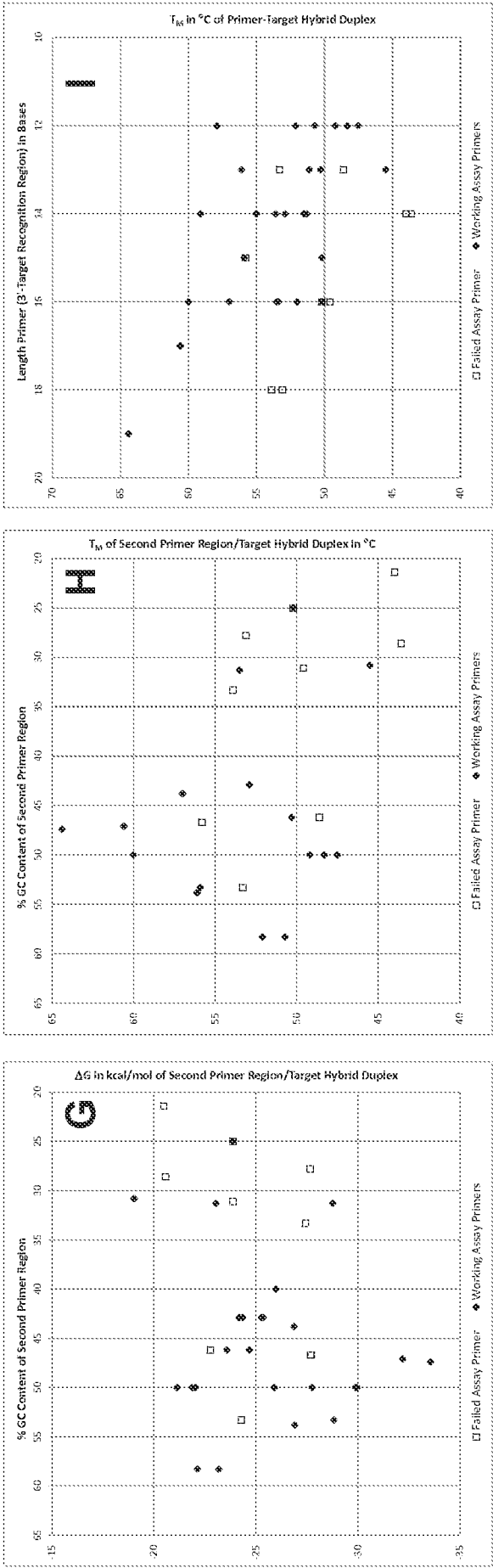


Figure 17 continued
Primer Structure Parameter (T_M , %GC, bp Length, $\Delta G_{25^\circ C}$, $\Delta \Delta G_{25^\circ C}$) Cluster Analysis
To Identify Structural Parameter Predicting Function & Performance in Isothermal Amplification



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/27074

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68; C07H 21/04 (2015.01)

CPC - C12Q 1/6844, 1/6876, 1/6853

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
CPC: C12Q 1/6844, 1/6876, 1/6853Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: C12Q 1/6844, 1/6876, 1/6853 (text search)
USPC: 435/6.11, 6.12; 536/24.3, 24.33 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Electronic data bases: PatBase; Google Patents; Google Scholar

Search terms: Nicking and extension amplification reaction (NEAR); isothermal, primer-dimer, probe, hairpin loop, fluorescent moiety, quencher

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/155056 A1 (SHAFFER et al.) 17 October 2013 (17.10.2013). Especially pg 6 ln 9-14, pg 6 ln 33-pg 7 ln 1, pg 17 ln 21-24; pg 19 ln 7-11, pg 22 ln 5-pg 23 ln 27, sheet 3 fig 1.	1-3, 18-20, 35-37
A	US 2013/0280706 A1 (JUDICE). 23 October 2013 (23.10.2013); Especially para [0006]-[0021].	1-3, 18-20, 35-37

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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

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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

17 August 2015 (17.08.2015)

Date of mailing of the international search report

08 SEP 2015

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/27074

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-17, 21-34, 44-60
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
-----go to Extra Sheet for continuation-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1-3, 18-20, 35-37

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/27074

-----continuation of Box III (Lack of Unity of Invention)-----

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-3, 18-20, 35-37, drawn to a composition comprising an isolated primer oligonucleotide, primer-dimer thereof or probe comprising from 5' to 3',

i) a first region comprising a self-complementary sequence comprising from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition sequence, and

ii) a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid having a delta G that is at least 15 kcal/mol lower than the delta G of any alternate structure comprising the second region, wherein the second region comprises at the 3' end one or more 2' modified nucleotides.

Group II: Claims 38-43, drawn to a method of amplifying a specific product in a nicking and extension amplification reaction (NEAR), or detecting a specific product in a nicking and extension amplification reaction.

Group III: Claim 61, drawn to a method of selecting [i.e. designing] a primer oligonucleotide.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I has the special technical feature of being a composition, not required by Groups II or III.

Groups II and III have the special technical feature of being a method, not required by Groups I.

Group II has the special technical feature of a method of USING a primer in a nicking and extension amplification reaction (NEAR), not required by Group III.

Group III has the special technical feature of a method of DESIGNING a primer for use in a NEAR amplification reaction using a computer-implemented method, not required by Groups II.

Common Technical Feature:

Groups I-III share the common technical feature of an primer oligonucleotide or primer-dimer thereof comprising i) a first region comprising a self-complementary sequence comprising from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition sequence, and

ii) a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid having a delta G that is at least 15 kcal/mol lower than the delta G of any alternate structure comprising the second region, wherein the second region comprises at the 3' end one or more 2' modified nucleotides.

However, said common technical feature does not represent a contribution over the prior art, and is obvious over WO 2013/155056 A1 to SHAFFER et al. (hereinafter "Shaffer").

Concerning the common technical feature, Shaffer generally teaches (pg 6 ln 9-14; In one aspect, the invention provides an isolated oligonucleotide having, from 5' to 3', a first region, and a second region, where the first region has a nicking enzyme recognition where the second region has at least 9 or more nucleotides (e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more contiguous nucleotides) that specifically bind a complementary sequence on a target nucleic acid molecule; and where the second region has one or more 2' modified nucleotides"). Concerning the first region, Shaffer further generally teaches that (pg 19 ln 7-11; "In various embodiments, primer/template pairs are constructed with a stem and loop configuration. The 5' end of the primer/template oligonucleotide comprises a self-complementary region that forms at least part of the stem. In some embodiments of the invention the stem further encompasses at least a portion or all of the nicking enzyme recognition sequence"). Concerning the claim limitation i) a first region comprising a self-complementary sequence comprising from 5' to 3' the reverse complement of a nicking enzyme recognition sequence, a palindromic sequence, and said nicking enzyme recognition sequence, Shaffer teaches an exemplary sequence in Fig 1 (sheet 3 last oligo of fig 1: full sequence: 5'-TGACTCCATATGGAGTCACATGGTTCATTTCGTmG-3', where TGACTC is the reverse complement of the nicking enzyme recognition site, the palindrome follows with the sequence CATATG, the nicking enzyme recognition site follows as GAGTCA [note: the actual recognition site is GAGTC--see Shaffer pg 6 col 33 -pg 7 col 1; "the nicking enzyme recognition sequence is 5' -GAGTC-3' [but there is still perfect complementarity between the extension of one nucleotide "A" matched with T]) and claim limitation ii) a second region at least 16 nucleotides long that specifically binds to a complementary region on a target nucleic acid molecule to form a double-stranded hybrid [...] wherein the second region comprises at the 3' end one or more 2' modified nucleotides (continuing sheet 3 Fig 1 last oligo: ACATGGTTCATTTCGTmG-3 [note: Shaffer specifically teaches (pg 6 ln 9-14; "where the second region has at least 9 or more nucleotides (e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more contiguous nucleotides) that specifically bind a complementary sequence on a target nucleic acid molecule; and where the second region has one or more 2' modified nucleotides"). -----[continued on next sheet]----

-----continued on next sheet-----

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/27074

-----continued from previous sheet-----

Shaffer does not specifically teach a double-stranded hybrid having a delta G that is at least 15 kcal/mol lower than the delta G of any alternate structure comprising the second region. However, it would have been obvious that with a specific designed primer having LNA nucleotides as possible at the 3' terminus would have bound very tightly, or about 15 kcal/mol lower (See Shaffer pg 17 In 21-24; "the target specific recognition sequence comprising one or more 2' modified nucleotides [...] 2'-LNA [...] In some embodiments, the block of 2' modified nucleotides is positioned at the 3' end of the target specific recognition region).

As the common technical feature was known in the art at the time of the invention, this cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I-III lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note concerning item 4: Claims 4-17, 21-34, 44-60 are multiple dependent claims and are not drafted according to the second and third sentences of PCT Rule 6.4(a).