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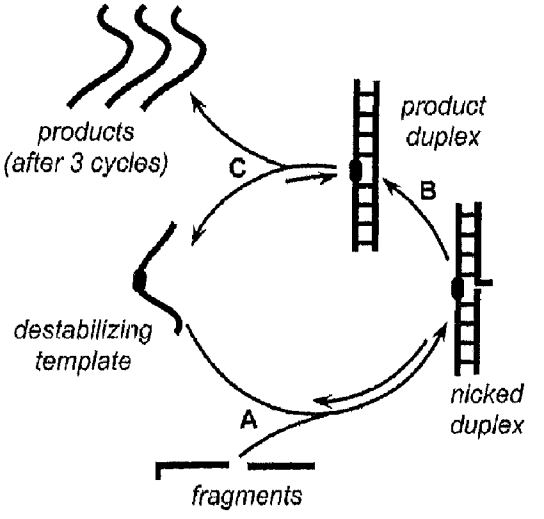

(57) Abrégé/Abstract:
The invention is directed to a method for isothermally amplifying a DNA sequence involving hybridizing a destabilizing DNA template to complementary nucleotide fragments to form a first nicked duplex; ligating the first nicked duplex to form a product duplex comprising the DNA sequence and the template, wherein the product duplex is capable of dissociating to release the DNA sequence and the template; and repeating these steps to generate multiple copies of the template and the DNA sequence. Further, the method may also involve hybridizing the DNA sequence to complementary destabilizing fragments or probes to form a second nicked duplex; ligating the second nicked duplex to form the product duplex comprising the DNA sequence and the template, wherein the product duplex dissociates to release the DNA sequence and the template; and repeating these steps to generate multiple copies of the template and the DNA sequence.

Abstract

The invention is directed to a method for isothermally amplifying a DNA sequence involving hybridizing a destabilizing DNA template to complementary nucleotide fragments to form a first nicked duplex; ligating the first nicked duplex to form a product duplex comprising the DNA sequence and the template, wherein the product duplex is capable of dissociating to release the DNA sequence and the template; and repeating these steps to generate multiple copies of the template and the DNA sequence. Further, the method may also involve hybridizing the DNA sequence to complementary destabilizing fragments or probes to form a second nicked duplex; ligating the second nicked duplex to form the product duplex comprising the DNA sequence and the template, wherein the product duplex dissociates to release the DNA sequence and the template; and repeating these steps to generate multiple copies of the template and the DNA sequence.

ISOTHERMAL DNA AMPLIFICATION

Field of the Invention

[0001] The invention relates to methods for isothermally amplifying a DNA sequence using a destabilizing DNA template.

Background of the Invention

[0002] DNA-based systems have been developed that are capable of performing sophisticated functions initiated by molecular recognition. Key examples are the DNA walkers where directional motion or load pick-up, transfer, and release are achieved with molecular and spatial selectivity (Gu *et al.*, 2010; Lund *et al.*, 2010). However, attempts to develop autonomous DNA self-replicating systems without specific sequence requirements have in recent years lagged behind (Lincoln, 2009; Patzke *et al.*, 2007; Ye *et al.*, 2000; Zielinkski *et al.*, 1987). As one of the hallmarks of organisms is their ability to amplify information and materials through biocatalysis and self-replication, the development of truly biomimetic systems capable of integrated functions requires incorporating amplification into self assembly and nanotechnology (Aldaye *et al.*, 2008; Patzke *et al.*, 2007; Paul *et al.*, 2004). Replicating DNA systems not only provide tools for DNA-based nanotechnology and insights into the origins of life, but also can be used to isothermally amplify signal in DNA detection, which can simplify the requirements for point-of-care diagnostics (Aldaye *et al.*, 2008; Connolly *et al.*, 2010; Orgel, 1992; Patzke *et al.*, 2007; Paul *et al.*, 2004).

[0003] One method for introducing amplification into DNA-based systems involves generating turnover in DNA-templated processes (Grossman *et al.*, 2008). To achieve turnover, the DNA template that facilitates the reaction of two complementary fragment strands (Figure 1, steps A-B) must dissociate from the product after it has formed (Figure 1, step C). However, for ligation reactions, turnover is minimal under isothermal conditions owing to the enhanced affinity of the template for the ligated product (Grossman *et al.*, 2008). Many detection strategies have thus focused on template-triggered scission and transfer reactions rather than ligations to avoid this product inhibition (Grossman *et al.*, 2008). One strategy for introducing turnover into ligation reactions exploits the sensitivity of DNA to

destabilizing modifications present in the middle of a duplex (Silverman *et al.*, 2006; Ye *et al.*, 2000; Zhan *et al.*, 1997). By ligating strands at a destabilizing site, the stability of the hybridization complex can be modified without changing the temperature or any other reaction condition (Li *et al.*, 2003). If the stabilities of the complexes before and after ligation are properly balanced, isothermal turnover should be achieved.

Summary of the Invention

[0004] The present invention relates to methods for isothermally amplifying a DNA sequence using a destabilizing DNA template. In one aspect, the invention comprises a method for isothermally amplifying a DNA sequence of interest (target sequence), comprising the steps of:

(a) preparing probes complementary to the target sequence wherein at least one probe comprises a destabilizing moiety;

(b) allowing the probes to hybridize to the target sequence and ligating the probes together to form a product duplex comprising the target sequence and a destabilizing template;

(c) allowing the product duplex to dissociate to release the target sequence and the destabilizing template; and

(d) repeating steps (a) to (c) to generate multiple copies of the target sequence and the destabilizing template.

[0005] In one embodiment, the probe destabilizing moiety comprises an abasic, butyl, cis-butenyl, or ethyl group. In one embodiment, the probes comprise one or more mismatches which are preferably spaced away from the ligation site.

[0006] In one embodiment, the method comprises the further step of detecting the target sequence or the destabilizing template. The detection step may comprise the steps of fluorescently labeling the probes and detecting fluorescence after electrophoretic separation. Alternatively, the probes may be separately labeled with a fluorescent donor and a fluorescent acceptor, which when ligated together produce detectable Forster resonant energy transfer. In a further alternative embodiment, one probe may be immobilized to a surface while another probe is labeled with a gold nanoparticle, which is covalently bound to the surface following ligation of the two probes. The gold nanoparticle may then be detected by catalytic silver

reduction plating.

[0007] In one embodiment, ligation is conducted at a temperature ranging between about 13 °C to about 30 °C. In one embodiment, ligation is conducted using a T4 DNA ligase. In one embodiment, the concentration of the ligase ranges from about 1 to about about 5 units of enzyme per reaction. In one embodiment, the equivalents of template ranges from about 0.001 to about 0.01.

[0008] The isothermal amplification of DNA may be used to detect target sequences to detect and identify genomic DNA, or to detect gene mutations, including single point mutations.

[0009] Additional aspects and advantages of the present invention will be apparent in view of the description, which follows. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

[00010] The invention will now be described by way of an exemplary embodiment with reference to the accompanying simplified, diagrammatic, not-to-scale drawings:

[00011] Figure 1 is a schematic diagram showing isothermal turnover in DNA-templated ligation reactions using a destabilizing DNA template.

[00012] Figure 2 is a schematic diagram comparing the product/copy yield of amplification using the destabilizing template (left) and self replication using the target as template (right).

[00013] Figure 3 is a schematic diagram showing the nicked site prior to ligation. The destabilizing templates contain a modification (D) in place of a thymidine. The perfect

template contains the complementary thymidine (T).

[00014] Figures 4A-B show the thermal dissociation profiles of nicked duplexes (template:fragments) (Figure 4A) and product duplexes (template:product) (Figure 4B) with DNA = 1.3 μM per strand (pH 7.0, 10 mM PBS, 10 mM $MgCl_2$). Figure 4C shows the sequences of strands where D is thymidine or a destabilizing group. Figure 4D is a table of dissociation (melting) temperatures (Tm).

[00015] Figure 5A shows fluorescent images of denaturing polyacrylamide gels for ligation mixtures using fluorescein-labelled thymine (T_F) with one equiv template. Figure 5B is a graph showing turnover number (TON) versus temperature for ligations with 0.01 equivalents of template. Figure 5C is a graph showing percent yield versus time with one equivalent template. Figure 5D is a graph showing TON versus enzyme concentration (1 unit vs 5 unit) with 0.01 equivalent template at 24 °C. (Template Labels: -- no template; *T* thymidine; *del* deletion; *Et* ethyl; *Bu* butyl; *cB* cis-butenyl; *Xy* xylyl; *Ab* abasic. Conditions unless noted: 1 equiv (1.4 μM) T_F-labeled reactant, 1 Unit T4 DNA ligase, 20 h, 16 °C).

[00016] Figure 6 is a schematic diagram showing cross catalytic cycles using destabilizing fragments.

[00017] Figure 7 is a graph comparing the turnover numbers for a single catalyst cycle using an abasic destabilizing template and fragments (SC), and a cross catalytic cycle using target as template and fragments, one of which contained an abasic group (CC) (Conditions: 0.01 equiv template (14 nM); *0.001 equiv (1.4 nM); 1 Unit T4 DNA ligase; 24 °C; 20 h).

[00018] Figure 8 is a schematic diagram showing cross catalytic cycles using destabilizing probes, preferably containing an abasic destabilizing group.

[00019] Figures 9A-B are graphs showing the measured target formed over time (Figure 9A) and the difference in target formed over time (Figure 9B).

[00020] Figure 10 is a graph showing the difference in target formed over time using

the indicated sequences, including a mutant target sequence having a mutation (G instead of T) at the ligation site.

[00021] Figure 11 is a graph showing the difference in target formed over time using the indicated sequences, including a probe containing one abasic and one mismatch (G, not at the ligation site).

Detailed Description of Preferred Embodiments

[00022] When describing the present invention, all terms not defined herein have their common art-recognized meanings. To the extent that the following description is of a specific embodiment or a particular use of the invention, it is intended to be illustrative only, and not limiting of the claimed invention. The following description is intended to cover all alternatives, modifications and equivalents that are included in the spirit and scope of the invention, as defined in the appended claims.

[00023] To facilitate understanding of the invention, the following definitions are provided.

[00024] "Amplification" means the production of multiple copies of a DNA sequence.

[00025] A "complementary sequence" is a sequence of nucleotides which forms a duplex with another sequence of nucleotides according to Watson-Crick base pairing rules where "A" pairs with "T" and "C" pairs with "G."

[00026] "Destabilizing" means the ability of templates, fragments or probes to impart a destabilizing site to a duplex such that its component strands dissociate without the need to vary any reaction condition.

[00027] A "duplex" means at least two polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or most of their nucleotides so that a complex is formed.

[00028] "Hybridization" is used to mean the formation of a duplex.

[00029] "Isothermal" means a process which takes place at a substantially constant temperature.

[00030] "Ligating" means forming a covalent bond or linkage between the termini of two or more nucleic acids, e.g. polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary. As used herein, ligation may be carried out enzymatically.

[00031] "Mismatch" means a base pair between any two of the bases A, T (or U for RNA), G, and C other than the Watson-Crick base pairs G-C and A-T. The eight possible mismatches are A-A, T-T, G-G, C-C, T-G, C-A, T-C, and A-G.

[00032] "Nicked duplex" means a duplex having a single-stranded cut or break.

[00033] "Nucleic acid" means polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA.

[00034] A "polynucleotide" is a linear sequence of ribonucleotides (RNA) or deoxyribonucleotides (DNA) in which the 3' carbon of the pentose sugar of one nucleotide is linked to the 5' carbon of the pentose sugar of another nucleotide. The deoxyribonucleotide bases are abbreviated as "A" deoxyadenine; "C" deoxycytidine; "G" deoxyguanine; "T" deoxythymidine; "I" deoxyinosine. Some oligonucleotides described herein are produced synthetically and contain different deoxyribonucleotides occupying the same position in the sequence. The blends of deoxyribonucleotides are abbreviated as "W" A or T; "Y" C or T; "H" A, C or T; "K" G or T; "D" A, G or T; "B" C, G or T; "N" A, C, G or T.

[00035] The present invention relates to methods for isothermally amplifying a DNA sequence using a destabilizing DNA probes which are ligated to form a destabilizing template. Introducing destabilizing modifications into a DNA template leads to turnover in a DNA-templated ligation reaction. Utilizing destabilizing DNA template, the product duplex is destabilized after ligation and releases the DNA template which is free to template further ligation reactions. Introducing turnover into simple, enzymatic ligation reactions provides an avenue for amplifying DNA-based assemblies constructed by enzymatic ligation. Isothermal ligation strategies also have potential applications in cross-catalytic replication of DNA, which represents a general method for amplifying any DNA sequence. By incorporating a cross-catalytic cycle, the present invention also achieves self replication (Figure 2).

[00036] In one embodiment, the invention comprises a method for isothermally amplifying a DNA sequence of interest (target sequence), comprising the steps of:

(a) preparing probes complementary to the target sequence wherein at least one probe comprises a destabilizing moiety;

(b) allowing the probes to hybridize to the target sequence and ligating the probes together to form a product duplex comprising the target sequence and a destabilizing template;

(c) allowing the product duplex to dissociate to release the target sequence and the destabilizing template; and

(d) repeating steps (a) to (c) to generate multiple copies of the target sequence and the destabilizing template.

[00037] The following is a specific example of one embodiment of the present invention. This example is offered by way of illustration and is not intended to limit the invention in any manner.

[00038] Isothermal turnover in nonenzymatic, chemical ligation systems has been useful in DNA and mRNA detection. Destabilization may be introduced in a ligating fragment strand by adding an alkyl group between the terminal nucleotide and the electrophilic end (Abe *et al.*, 2004). After ligation with a nucleophilic fragment using target DNA as a template, the resulting alkyl bridge causes the product duplex to dissociate. The present invention involves creating a DNA template containing a destabilizing moiety, such as a short alkyl chain, in place of a complementary nucleotide (Figure 3). A model abasic DNA lesion

("Ab") known to destabilize DNA duplexes was also investigated (Figure 3) (Matray *et al.*, 1998). PNA analogues of abasic groups have been demonstrated by Grossman *et al.* (2008) to avoid product inhibition and improve selectivity in chemical ligation systems using PNA (Dose *et al.*, 2006; Ficht *et al.*, 2005). In the present invention, the abasic group results in a template that is missing a base but still contains the canonical phosphate-sugar backbone.

[00039] Achieving sufficient turnover requires that the product duplex be less stable than the nicked duplex (Figure 1). However, because of multivalency, the product duplex is invariably more stable, even with the destabilizing modification (Mammen *et al.*, 1998). A more reasonable goal is to introduce a modification that renders the product and nicked duplexes closer in stability, so that a temperature can be found where both can form but remain labile. To determine whether the duplex stabilities of the destabilizing templates were optimal, their thermal dissociation behavior was monitored. The temperature at which half the duplex has dissociated is the melting temperature (Tm), providing a way to compare duplex stabilities.

[00040] Figures 4A and B show the thermal dissociation curves for the nicked duplexes (template + two fragments) and the product duplexes (template + product), respectively. The 18-base sequence and the position of the nicked site and destabilizing group are provided in Figure 4C. To illustrate the extent of destabilization, these results were compared with the behavior of a perfectly complementary system (Figures 4A and B, black solid traces, where D = thymidine). For all of the destabilizing templates, the decrease in Tm between the nicked and corresponding product duplexes was 18-22 °C. In contrast, the Tm difference was 27 °C for the natural DNA system (Figure 4D). The smaller ΔTm for the destabilized templates suggested that a temperature might be found where hybridization of the nicked duplex (Figure 1, step A) and dissociation of the product duplex (Figure 1, step C) were both possible.

[00041] In ligation experiments, the template was mixed with the complementary reactive strands (containing a 3'-OH and 5'-phosphate at the ligation site) and 1 unit of T4 DNA ligase for each 15-μL reaction. One unit is the amount of enzyme needed to catalyze the conversion of 1 nmol of 32P-labeled pyrophosphate into ATP in 20 min at 37 °C. The 5' end of one of the fragments was labelled with fluorescein to follow ligation via fluorescent

imaging after separation by denaturing polyacrylamide gel electrophoresis. The gel image in Figure 5A illustrates the ligation mixtures with one equivalent of template after 20 hours of reaction time, where a product band was evident for all of the destabilizing templates. This result indicates that T4 DNA ligase tolerates unnatural modifications to the DNA template near the site of ligation. Ligation with all of the destabilizing templates was hampered when the ligation site was opposite the 3' end rather than the 5' end of the destabilizing group.

[00042] To determine whether these destabilizing templates could turn over in the reaction, the ligation reaction was monitored with substoichiometric amounts of template. At 20 °C, when 0.01 equivalents of complementary template (T) were used, only 1.3% of the fluorescent fragment strand was ligated, indicating that the dissociation of the product duplex was unfavorable. In contrast, using the same amount of an abasic template (Ab) led to 3.2% of the ligated product. From the ratio of [product]/[template] the turnover number (TON) was calculated. The amount of turnover for the perfect template (T) was between 1 to 2 for all reaction temperatures using 0.01 equivalents of template (Figure 5B). In contrast, the turnover was greater than two for several of the destabilizing templates. The only inactive templates that yielded little or no ligated product under these substoichiometric conditions were the xylyl template (Xy) and a template containing a deletion of the thymidine (del, data not shown). With the cis-butenyl template, turnovers were between 0.9 and 1.7, which indicated that this rigid linkage did not promote catalytic behavior.

[00043] For all of the active destabilizing templates, the highest TON was observed at 28 °C, well above the melting temperature of their corresponding nicked duplexes (Figure 5B). The highest TON observed at this enzyme concentration was 5.0 for the abasic template (Ab). Without being bound by theory, the decrease in nicked duplex stability at 28 °C may be compensated for by the faster rate of ligation or dissociation of the product duplex. However, at temperatures higher than 28 °C, the decrease in TON suggests that the formation of the nicked duplex becomes unfavorable. At all temperatures, no background ligation was observed in the absence of template (Figure 5A). The enzyme requirement that the DNA be double-stranded eliminated any background reaction illustrating a major advantage to this method. These results also suggest that employing a catalyst which favors double-stranded DNA might be a way to avoid non-templated background in chemical ligations (Ye *et al.*, 2000).

[00044] To determine how the destabilizing template influenced the rate of ligation, the yield versus time was measured in ligations using one equivalent of template. Within ten minutes ligation is complete when natural DNA template (T) is used (Figure 5C). The rate of ligation for perfect template should first be measured to verify the enzyme activity. If the enzyme is less active due to improper storage or handling, no turnover is observed. Most of the destabilizing templates are only a little slower with the Ab and cB template requiring 20 minutes, and the other templates requiring less than 40 minutes. Without being bound by theory, the leveling off of the signal for the ethyl and butyl templates may be attributed to subtle differences in the extinction coefficients used to calculate the DNA concentration. The leveling off of the xylyl suggests that most of the xylyl template is in a conformation that does not allow for hybridization or ligation. Comparing these results with those of chemical ligation methods which demonstrate slower rates of ligation indicates that faster ligation methods lead to greater turnover (Abe *et al.*, 2004; Sando *et al.*, 2004; Dose *et al.*, 2006; Ficht *et al.*, 2005).

[00045] The typical ligase concentration for ligating nicked duplexes was 1 unit enzyme per equivalent of fluorescent fragment strand (1.4 μM, 15 μL). To determine whether increasing enzyme concentration would increase the amount of turnover, ligation reactions using concentrated enzyme (5 units per reaction) were performed with the same amount of template (0.01 equivalents). At higher enzyme concentration, the DNA template (T) still exhibited a TON close to one. In contrast, the Bu and Ab templates generated 18 product strands per template (Figure 5D), which is 5-fold higher than the maximum turnover number of 3.5 previously reported by Grossman *et al.* (2008) using similar probe (1.2 μM) and template (12 nM) concentrations (Dose *et al.*, 2006).

[00046] Embodiments of the present invention may be applied in smart, DNA-based systems; for example, an environmental stimulus can be used to release destabilizing template causing the amplification of a DNA material, for example, DNA-ligated gold nanoparticle aggregates (Claridge *et al.*, 2008). In a method for native DNA detection, another complementary ligation cycle must be included. A cross-catalytic replication strategy may be initiated by a native DNA strand representing a target sequence (Figure 6). The destabilizing template is formed in situ from destabilizing probes by a ligation reaction templated by the

natural DNA target (Figure 6, steps A and B). As a result of ligation, the same product duplex is formed as in the previous cycle. Consequently, the product:template duplex is destabilized, leading to the release of the original target and a newly formed destabilizing template (Figure 6, step C). This destabilizing template can now generate a copy of the original target template, which goes on to catalyze the formation of more destabilizing templates (Figure 6, steps D-E). As the product of each cycle is a template for the other, significant amplification of the original target will ensue.

[00047] To experimentally verify that cross catalysis occurs, the fragment strands listed in Figure 6 were combined, including a fluorescent modified fragment corresponding to the bottom cycle (T_F, fluorescein-modified thymine). A target DNA sequence was introduced which, although active in the top cycle, should have no effect on the fluorescein-labeled fragment; thus, the formation of any fluorescent ligated product would signify cross catalysis. T4 DNA ligase ligates a destabilizing probe terminated with a 5'-phosphate abasic (Ab) group (Verly *et al.*, 1983). Target-initiated cross catalysis occurred for the Ab-substituted system (Figure 7). Self replication was observed in this two cycle system, and the exhibited cross-catalytic TON was greater than that for the single catalytic cycle (TON 14 vs 4.5; CC vs SC, respectively). Decreasing the number of equivalents of template (CC*) further increased the number of cross-catalytic turnovers, indicating that dissociation is favored as the fragment:template ratio becomes larger (Abe *et al.*, 2004; Dose *et al.*, 2006). The highest cross-catalytic turnover of 32 corresponds to the target undergoing on average 32 cycles of self replication (Figure 7, CC*).

[00048] For the cross-catalytic reactions using 1 unit of ligase and the 5' phosphate abasic destabilizing probe, a small level of background was observed (0.5% and 3.7% product at 20 and 24 °C, respectively, with no template present). The more concentrated enzyme (5 units per 15 μL reaction), capable of ligating blunt ends, displayed very high background under identical reaction conditions. In contrast, the 5'-phosphate thymidine fragment exhibited a large amount of background (25% ligation product at 20 °C). Without restriction to a theory, it is believed the reason for the high background in the natural system is based on the probe design, which leads to a one-base overhang of thymidine on one fragment and deoxyadenosine on the other. Although this overhang exists in the Ab-modified system, replacing the thymidine with the abasic group prevents hydrogen bonding with the adenine,

thus minimizing nonspecific joining of the fragments. Consequently, very little background ligation is observed.

[00049] In one embodiment, destabilizing probes are made complementary to the target sequence (Figure 8). Upon mixing the probes and the target, the probes hybridize to the target. T4 DNA ligase ligates the two probes together to form a destabilizing template. This template is generated in situ and is not initially present in the ligation mixture. As a result of the destabilizing group, the target and destabilizing template dissociate, releasing both strands to template more reactions. While the released target continues to template the formation of the destabilizing template, each new destabilizing template that forms and is released hybridizes to another set of probes that are present and causes them to ligate. This newly formed strand is identical to the initial target sequence. Every cross-catalytic cycle thus yields a new target, which can then participate in making more destabilizing template and consequently more target. Such cross-catalytic cycles are essential for achieving exponential amplification of the target sequence, similar to PCR. Eventually some probes become ligated in the absence of the target. This background reaction is observed but it requires longer reaction times than target-initiated reactions.

[00050] By subtracting the yield of a control reaction lacking initial target from a target-initiated replication, target DNA can be detected from the difference in target formed (Measured Target Formed, Figure 9A; ΔTarget Formed, Figure 9B). Multiple sequences may thus be detected, indicating that this destabilization approach is very general. Moreover, the present invention enables detection of single mismatches and genomic DNA from cell lysate, and achieves fM detection limits. Use of a high concentration of T4 DNA ligase leads to very rapid amplification. Consequently, thousands of turnovers within hours may be observed, compared to an earlier report of 30 replication cycles in 20 hours.

[00051] In one embodiment, the invention may be used to detect target sequences specific to pathogens such as anthrax, hepatitis B, and *E. coli*. Probes have also been synthesized that detect for the mutation on codon 526 of the *rpoB* gene that leads to multi-drug resistance in *M. tuberculosis* (Arnold, *CIM*, 2004). These probes can be ligated.

[00052] In one embodiment, the invention enables detection of single-point

mutations with a high degree of discrimination (Figure 10). Since the method is sensitive to the rate of each step in the cycle, the presence of a mutation at the ligation site (G instead of T) slows down ligation, which has a major affect on the amplification rate. Amplification is thus only slightly higher than the background reaction when a mismatch is present at the ligation site. Consequently, at specific points of the amplification process, difference ratios are seen between the mismatch and perfect target samples greater than 100, which is a very high signal ratio for mismatch detection.

[00053] The method of the present invention occurs isothermally owing to the presence of one or more destabilizing groups in the destabilizing probes. Adding mismatches to the probes can lead to enhanced destabilization and quicker amplification. Additionally, using combinations of abasic groups and mismatches, the temperature of the amplification process has been tuned from 13 °C to 30 °C. As long as the mismatches are added away from the ligation site, they promote faster amplification.

[00054] In one embodiment, the amplification method may be optimized by varying the probe concentration and by employing probes containing one abasic and one mismatch (G, not at the ligation site), which has a limit of detection as low as 14 pM of target. Sensitivity of the assay may be improved with a method of serial ligation. In one embodiment, the target sequence is first combined with low probe concentrations (140 nM) and enzyme (1 μL high concentration per 15 μL reaction). At these low probe concentrations, the background reaction is very slow. After 2 hours, an aliquot of this mixture is removed and added to more concentrated probes (1.4 μM) and enzyme (1 μL high concentration per 15 μL reaction). This serial ligation method improves sensitivity, detecting as low as 140 fM DNA (Figure 11).

[00055] The probes form duplexes that have single base pair overhangs, which the enzyme is able to ligate quite efficiently. In order to reduce this reaction, in one embodiment, a system has been developed in which these duplexes have blunt ends, which has led to a reduction in the rate of background ligation. Additionally, T4 DNA ligase can still join blunt ends although this reaction is slow. *E. coli* ligase, an enzyme inefficient at ligating blunt ends, may eliminate the background and allow detection of even lower target concentrations.

[00056] *E. coli* has been detected using probes that are complementary to the *E coli*

DNA ligase gene. This sequence can be detected on plasmid DNA containing approximately 6000 base pairs. To detect the double-stranded genomic DNA, the genomic DNA is first heated in a solution containing the probes, to 95 °C and then allowed it to cool for 20 minutes before adding the enzyme. Additionally, *E. coli* target was directly detected from cell lysate. Several colonies of *E. coli* were dispersed in 50 μL water. The solution was then heated to 95 °C to lyse the cells and inactivate any native enzymes. An aliquot of the lysate was then diluted 10-fold and added it to the probes (14 μM). The solution was then heated to 95°C to dissociate the genomic DNA and the enzyme (5 units) was added after the solution had cooled for 20 minutes. The presence of *E. coli* was easily detected after 3 hours. To confirm that the components of the cell lysate were not influencing the replication rate as they were not present in the reference control, the same experiment was performed using the wrong probes (Hepatitis B probes) in the *E. coli* cell lysate. The amount of replication was indistinguishable from the control lacking any genomic target, which proved that replication was target-initiated for the *E. coli* experiments.

[00057] Polyacrylamide gel electrophoresis has been used to monitor the formation of the target strand during the reaction using a fluorescent label on one of the corresponding probe strands. In one embodiment, the amplification of the target sequence may be detected using real-time or chip-based methods of detecting the product. In one approach, one probe is modified with a fluorescent donor (FAM) and another probe with a fluorescent acceptor (TAMRA or Cy5). Upon ligating the probes to form a target sequence, Förster resonant energy transfer (FRET) is observed, which provides a real-time method for monitoring amplification of the target. The FRET method may be useful with standard plate readers. In another embodiment, one of the probe strands is immobilized to a surface and the other probe strand is modified with a gold nanoparticle. After templated-ligation of the two probes occurs, the gold nanoparticle is covalently attached to the surface, which can be observed by silver plating through the catalytic reduction of silver (I) onto the gold nanoparticles. Surface-based detection can be monitored using a flatbed scanner to observe the silver plating.

[00058] The applicants have demonstrated that DNA templates for enzymatic ligation reactions can turn over in the ligation cycle by introducing a destabilizing modification into the template strand. The turnover numbers are higher than isothermal chemical ligation strategies at similar strand lengths, concentrations, and ratios (Grossman *et*

al., 2008; Silverman *et al.*, 2006). Further, the present invention adds another cycle that leads to a self-replicating DNA system using destabilization to overcome product inhibition. The success of the abasic probes in both cycles is especially promising as this phosphoramidite is commercially available, allowing simple access to templates capable of turnover. The invention not only enables advances in nanotechnology in the replication of DNA materials, but also is useful in isothermal target amplification for the broad field of DNA diagnostics. With simple destabilizing groups and rapid ligation methods, turnover and target initiated self-replication are made possible.

[00059] Exemplary embodiments of the present invention are described in the following Examples, which are set forth to aid in the understanding of the invention, and should not be construed to limit in any way the scope of the invention as defined in the claims which follow thereafter.

[00060] **Example 1 – Preparation of DNA Strands**

[00061] DNA was synthesized on an ABI 380 solid-phase synthesizer using Glen Research reagents. Strands were purified by Glen-Pak DNA Purification cartridges (cat. 60-5200-01) according to the DMT-On protocol. Standard nucleotide phosphoramidite and the following were used: Chemical Phosphorylation Reagent II (cat. 10-1901-90), Fluorescein-dT Phosphoramidite (cat. 10-1056-95), and dSpacer CE Phosphoramidite (cat 10-1914-90) for the abasic (*Ab*) template and fragments. All other destabilizing templates were prepared from the corresponding protected diols with solid-phase synthesis. OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html) was used to determine the extinction coefficients where the destabilizing templates' absorptivity was assumed to be Ac, with ***D*** = a deletion.

Table 1. DNA Strands

	Strands
Ac	3'-AACAATTTA-**D**-AACTATTC-5'; **D** = T or destabilizing group
A	3'-GAATAGTT-A-TAAATTGTT-5'
C	3'-GAATAGTTA-5'
C_P	3'-GAATAGTT$A_{Phosphate}$-5'
B	3'-TAAATTGTT-5'
B_F	3'-TAAATTGTT$_{Fluorescein}$-5'
Cc	3'-AACTATTC-5'
Bc_P	3'-AACAATTTAT$_{Phosphate}$-5'
$BcAb_P$	3'-AACAATTTA$(Ab)_{Phosphate}$-5'

[00062] Example 2 – **Thermal Dissociation Experiments**

[00063] 1.3 nmol of each DNA sequence (Ac and A for the product duplex experiments and Ac, B, and C for the nicked duplex experiments) was combined in PBS buffer (1.0 mL, 10 mM $MgCl_2$, 20 mM pBS, pH 7.0) and allowed to hybridize for about 15 min. While stirring at 100 rpm, absorbance readings at 260 nm were taken from 10 to 60° C at 1° C intervals, with 1 min hold time.

[00064] Example 3 – **Ligation Experiments**

[00065] Strand amounts were for single cycle reactions were: B_F, 1 equiv; C_P, 2 equiv; and 1 or 0.01 equiv of template (Ac); and for cross cycle reactions were: B_F, 1 equiv; C_P, 2 equiv; A, 0.01 or 0.001 equiv; Cc, 2 equiv; and either strand Bc_P or $BcAb_P$, 2 equiv. In a typical ligation, where 1 equiv = 21 pmol, the appropriate amounts of DNA fragments and template were first combined in water in a 400-μL mini-centrifuge tube to reach a final volume of 10 μL and incubated at the desired reaction temperature. While several of these DNA solutions incubated, in a separate mini-centrifuge tube, T4 DNA ligase (8 μL) at lower concentration (1 Unit/μL, Invitrogen cat. 15224-017) or higher concentration (5 Units/μL, Invitrogen cat. 46300-018) was mixed with ligation buffer (24 μL, 5 x concentrated) and water (8 μL). A portion of this ligase mixture (5 μL) was immediately added to each of the DNA solutions (final [DNA] = 1.4 μM for each equivalent). The reactions were then placed in a covered thermal incubator for 20 hours unless otherwise noted. To stop ligation, $EDTA_{(aq)}$ (1 μL, 0.5 M) was added for every unit of enzyme present. For the kinetic experiments, aliquots (3 μL) were removed from the bulk ligation mixture at various reaction times and placed in a separate microcentrifuge tube containing $EDTA_{(aq)}$ (1 μL, 0.5 M). Samples were

stored at 4° C until analyzed by 15% denaturing PAGE.

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[00066] The following references are indicative of the level of skill of those skilled in the art to which this invention pertains.

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WHAT IS CLAIMED IS:

1. A method for amplifying a target nucleic acid sequence comprising the steps of:

(a) providing target probes, having a first end and a second end, that are complementary to an original target nucleic acid sequence segment wherein at least one target probe comprises at least one destabilizing moiety that is located at or near the first end that is to be ligated of the target probe;

(b) hybridizing the target probes to the original target nucleic acid sequence segment and ligating the target probes together to form a first product duplex comprising the original target nucleic acid sequence segment and a newly generated destabilizing template

wherein said at least one destabilizing moiety that was located at or near the first end of the target probe in step (a) is located near a middle of the destabilizing template;

(c) dissociating the first product duplex spontaneously as a result of a destabilizing effect of the destabilizing moiety to release the original target nucleic acid sequence segment and the destabilizing template;

(d) providing template probes complementary to the destabilizing template that when ligated together form a product nucleic acid sequence;

(e) hybridizing the template probes to the destabilizing template and ligating the template probes together to form a second product duplex comprising a destabilizing template and a newly generated product nucleic acid sequence;

(f) dissociating the second product duplex spontaneously as a result of a destabilizing effect of the destabilizing moiety to produce the destabilizing template and the product nucleic acid sequence; and

(g) repeating steps (a) to (f) to produce multiple copies of the destabilizing template and multiple copies of the product nucleic acid sequence,

wherein the destabilizing moiety is selected from the group consisting of: an abasic nucleotide, a 1',2'-dideoxyribose-5'- phosphate, butyl, cis-butenyl, and ethyl group;

wherein the method for amplifying a target nucleic acid sequence is conducted in a liquid; and

wherein the method is an isothermal method and the temperature of the liquid is maintained within a temperature range of about 5° C.

2. The method of claim 1, wherein the product nucleic acid sequence has a composition that is at least 90% the same as original target nucleic acid sequence segment.

3. The method of claim 1, wherein the product nucleic acid sequence has a base quantity of between about 13 and 24 bases.

4. The method of claim 1, wherein steps a-g occur concurrently in a single liquid vessel.

5. The method of claim 1, wherein the target nucleic acid sequence is a DNA sequence.

6. The method of claim 1, wherein the target nucleic acid sequence is a RNA sequence.

7. The method of claim 1, wherein the target probes comprise a DNA sequence.

8. The method of claim 1, wherein the template probes comprise a DNA sequence.

9. The method of claim 1, wherein the target probes comprise a DNA sequence and the template probes comprise of a DNA sequence.

10. The method of claim 1, wherein the original target nucleic acid sequence segment in step (a) of claim 1 is a DNA target sequence formed by the method comprising the steps of:

i) providing a RNA sequence;
ii) providing DNA probes complementary to the RNA sequence; and
iii) reacting the RNA sequence with at least two DNA probes comprising the step of hybridizing, ligating and dissociating

to produce the DNA target sequence segment, which can then undergo steps (a)-(g).

11. A method for amplifying a target nucleic acid sequence

comprising the steps of:

(a) providing target probes that are complementary to an original target nucleic acid sequence segment wherein at least one target probe comprises at least one destabilizing moiety that is located at or near the first end that is to be ligated of the target probe;

(b) hybridizing the target probes to the original target nucleic acid sequence segment and ligating the target probes together to form a first product duplex comprising the original target nucleic acid sequence segment and a newly generated destabilizing template;

(c) dissociating the first product duplex spontaneously as a result of a destabilizing effect of the destabilizing moiety to release the original target nucleic acid sequence segment and the destabilizing template;

(d) providing template probes complementary to the destabilizing template that when ligated together form a product nucleic acid sequence;

(e) hybridizing the template probes to the destabilizing template and ligating the template probes together to form a second product duplex comprising a destabilizing template and a newly generated product nucleic acid sequence;

(f) dissociating the second product duplex spontaneously as a result of a destabilizing effect of the destabilizing moiety to produce the destabilizing template and the product nucleic acid sequence; and

(g) repeating steps (a) to (f) to produce multiple copies of the destabilizing template and multiple copies of the product nucleic acid sequence,

wherein the destabilizing moiety comprises an 1',2'-dideoxyribose-5'-phosphate;

wherein the method for amplifying a target nucleic acid sequence is conducted in a liquid; and

wherein the method is an isothermal method and the temperature of the liquid is maintained within a temperature range of about 5° C.

12. The method of claim 1, wherein at least one of the probes comprise one or more mismatches not at a ligation site.

13. The method of claim 1, comprising the further step of detecting the target sequence or the destabilizing template.

14. The method of claim 13, wherein the step of detecting comprises the discrimination between a perfect target sequence and a sequence with a single base pair mismatch.

15. The method of claim 13, wherein the detection step comprises the steps of separately labeling the probes with a fluorescent donor and a fluorescent acceptor, and detecting Forster resonant energy transfer after ligation.

16. The method of claim 13, wherein the step of detecting comprises the steps of:

immobilizing a probe to a glass surface while another probe in solution is labeled with a gold nanoparticle,

covalently binding said gold nanoparticle to the surface following ligation of the two probes, and

detecting the gold nanoparticle by catalytic silver reduction plating.

17. The method of claim 1, wherein the target probes and template probes each have a concentration within a concentration range of about 140 nM to about 1.4 μM.

18. The method of claim 1, comprising a first ligation stage and a second ligation stage, wherein the first ligation stage comprises steps (a)-(f) of claim 1 at a first target probe and a first template probe concentrations and the second ligation stage immediately follows the first ligation stage and comprises steps (a)-(f) of claim 1 at a second target probe and a second

template probe concentrations that are at least two times higher than the first target probe and the first template probe concentrations.

19. The method of claim 18, wherein the second target or second template probe concentration is about 10 times higher than the first target or first template probe concentration respectively.

20. The method of claim 1, wherein the liquid is kept within a temperature ranging between about 13° C to about 30° C throughout the method.

21. The method of claim 1, wherein the liquid comprises an enzyme that ligates the template probes and/or target probes.

22. The method of claim 21, wherein the enzyme comprises a T4 DNA Ligase.

23. The method of claim 21, wherein the enzyme has a concentration range from about 1 to about 5 Weiss units.

24. The method of claim 1, wherein the multiple copies of a product nucleic acid sequence consists of the same original target nucleic acid sequence segment.

25. A method for amplifying a target nucleic acid sequence comprising the steps of:

(a) providing target probes, having a first and a second end, that are complementary to an original target nucleic acid sequence segment wherein at least one target probe comprises at least one destabilizing moiety that is located at or near the first end of the target probe;

(b) hybridizing the target probes to the original target nucleic acid sequence segment and ligating the target probes together to form a first product duplex comprising the original target nucleic acid sequence segment and a newly generated destabilizing template; wherein said at least one destabilizing moiety that was located at or near the first end of the target probe in step (a) is located near the middle of the destabilizing template;

(c) dissociating the first product duplex spontaneously as a result of a destabilizing effect of the destabilizing moiety to release the original target nucleic acid sequence segment and the destabilizing template;

(d) providing template probes complementary to the destabilizing template that when ligated together form a product nucleic acid sequence;

(e) hybridizing the template probes to the destabilizing template and ligating the template probes together to form a second product duplex comprising a destabilizing template and a newly generated product nucleic acid sequence;

(f) dissociating the second product duplex spontaneously as a result of a destabilizing effect of the destabilizing moiety to produce the destabilizing template and the product nucleic

acid sequence; and

(g) repeating steps (a) to (f) to produce multiple copies of the destabilizing template and multiple copies of the product nucleic acid sequence,

wherein the method for amplifying a target nucleic acid sequence is conducted in a single liquid vessel, wherein the liquid is kept within a temperature between about 13°C to about 30° C throughout the method, wherein the product nucleic acid sequence has a base quantity of between about 13 and 24 bases, wherein the target probe consists of a DNA sequence and the template probes consists of a DNA sequence, and wherein the destabilizing moiety is selected from the group consisting of: an abasic nucleotide, a 1',2'-dideoxyribose-5'-phosphate, butyl, cis-butenyl, and ethyl group; and

wherein said method is an isothermal method and the temperature of the liquid is maintained with a temperature range of about 5° C.

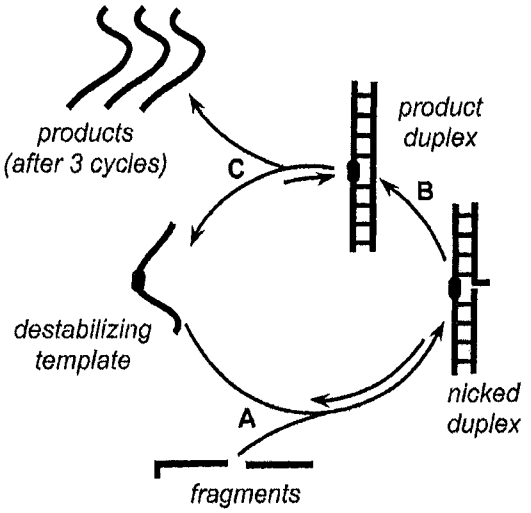

products
(after 3 cycles)
C
product
duplex
B
destabilizing
template
nicked
duplex
A
fragments

FIG. 1

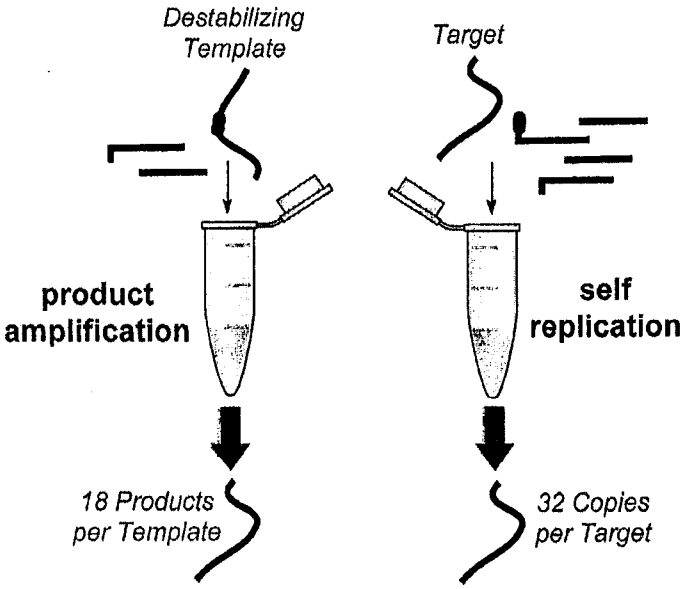

Destabilizing
Template
Target
product
amplification
self
replication
18 Products
per Template
32 Copies
per Target

FIG. 2

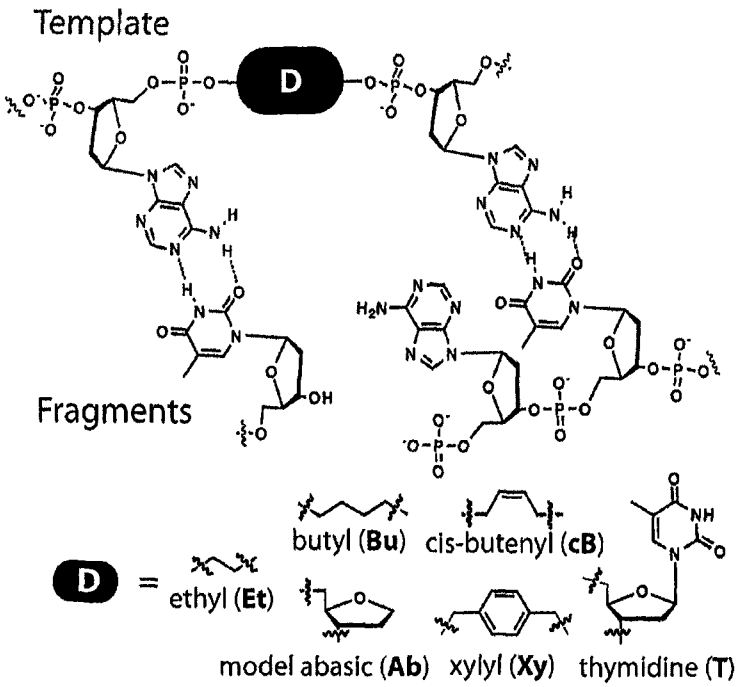
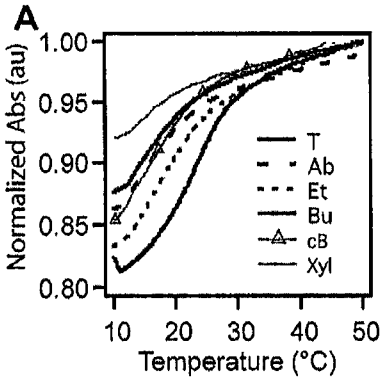
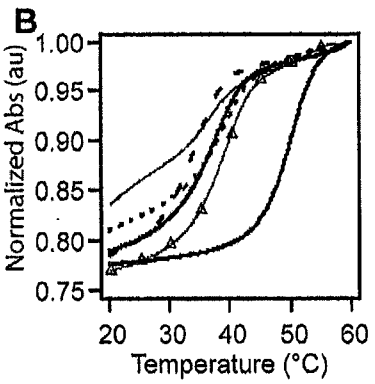

Template
D
Fragments
D = ethyl (Et)
butyl (Bu)
cis-butenyl (cB)
model abasic (Ab)
xylyl (Xy)
thymidine (T)

FIG. 3





C

template

5'-CTTATCAA-DATTTAACAA-3'

product

3'-GAATAGTTATAAATTGTT-5'

fragments

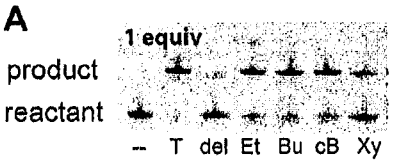
3'-GAATAGTTA-5'

3'-TAAATTGTT-5'

D

Template (D)	Nicked Tm (°C)	Product Tm (°C)
Complement (T)	22.2	49.3
Abasic (Ab)	16.3	34.4
Ethyl (Et)	17.2	38.7
Butyl (Bu)	17.2	37.0
cis-Butenyl (cB)	17.1	37.8
Xylyl (Xy)	16.9	37.3

FIG. 4

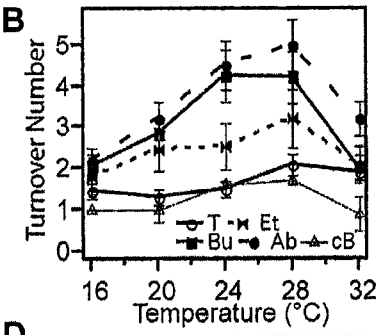


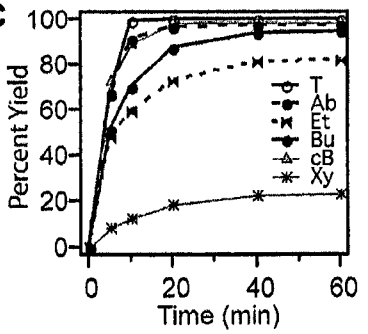
product strand

3'-GAATAGTTATAAATTGTT$_F$-5'

reactant strand

3'-TAAATTGTT$_F$-5'





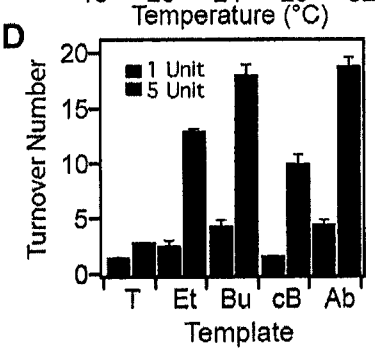


FIG. 5

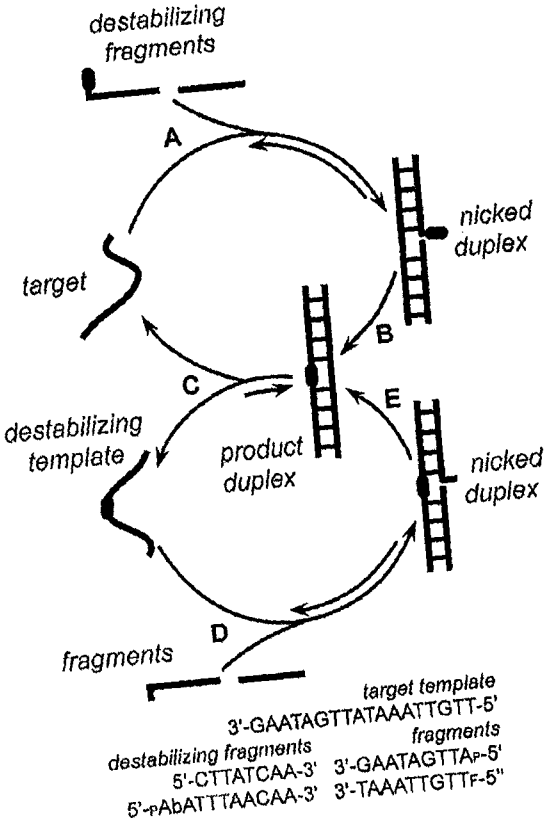

destabilizing fragments
A
nicked duplex
target
B
C
E
destabilizing template
product duplex
nicked duplex
D
fragments
target template
3'-GAATAGTTATAAATTGTT-5'
fragments
destabilizing fragments
5'-CTTATCAA-3'
3'-GAATAGTTAP-5'
5'-pAbATTTAACAA-3'
3'-TAAATTGTTF-5''

FIG. 6

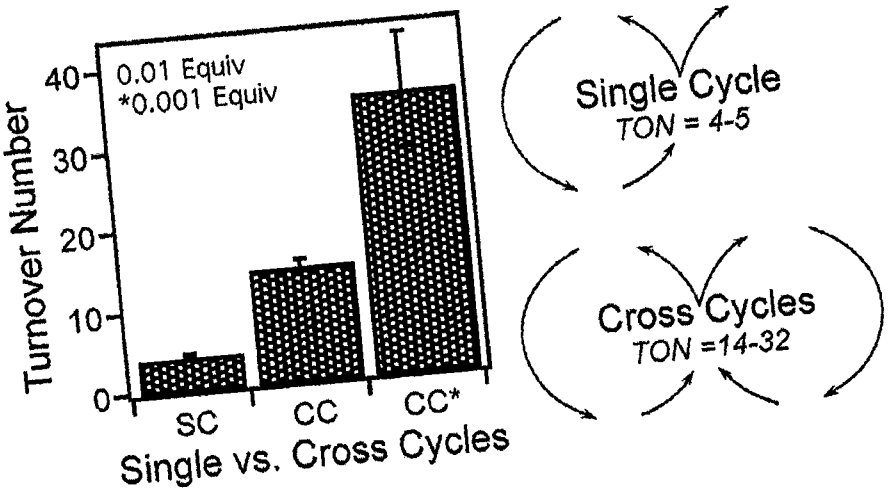
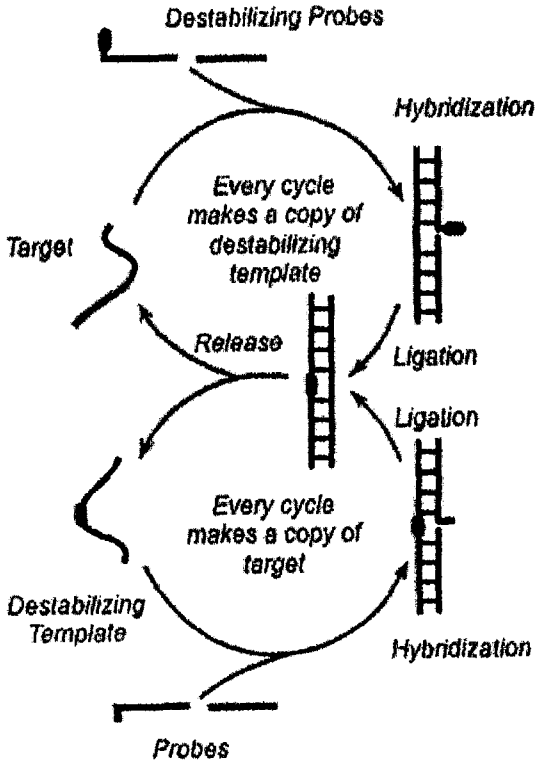

0.01 Equiv
*0.001 Equiv
Turnover Number
40
30
20
10
0
SC
CC
CC*
Single vs. Cross Cycles
Single Cycle
TON = 4-5
Cross Cycles
TON =14-32

FIG. 7



Abasic Destabilizing Group

FIG. 8

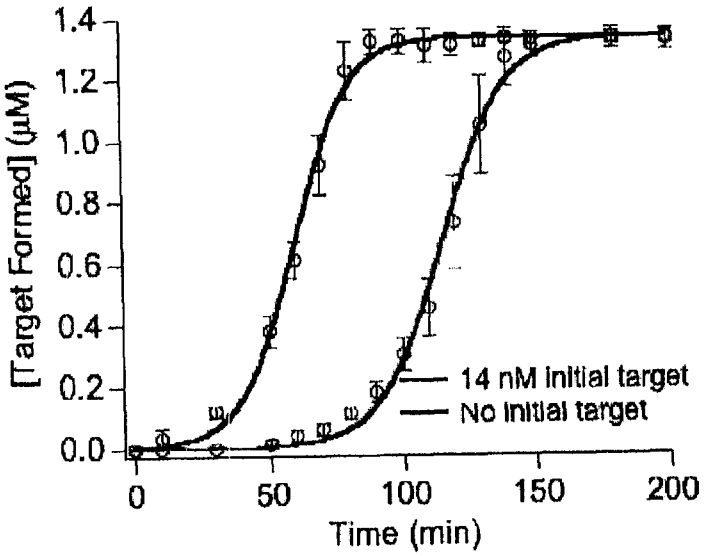

[Target Formed] (μM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
Time (min)
14 nM initial target
No initial target

FIG. 9A

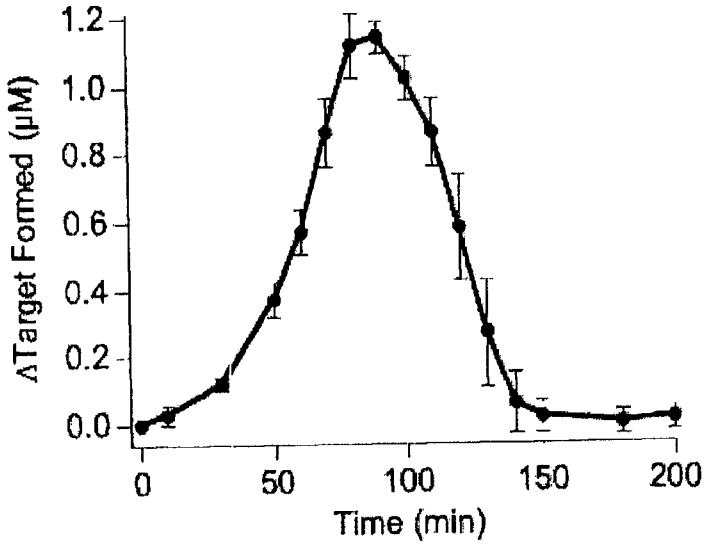
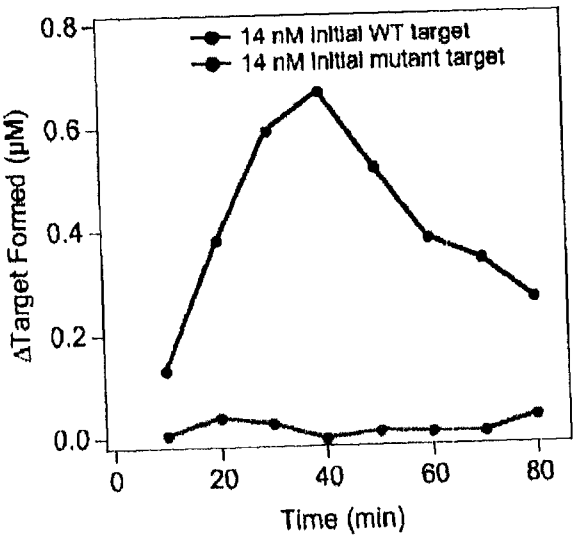

ΔTarget Formed (μM)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
Time (min)

FIG. 9B



Target Sequences :

Wild-Type 5′-TTGTTA AATATTGATAAG-3′

Mutant 5′-TTGTTA AATA**G**TGATAAG-3′

Destabilizing Probes:

3′-AACAATTTA(ab)-5′ 3′-AACTATTC-5′

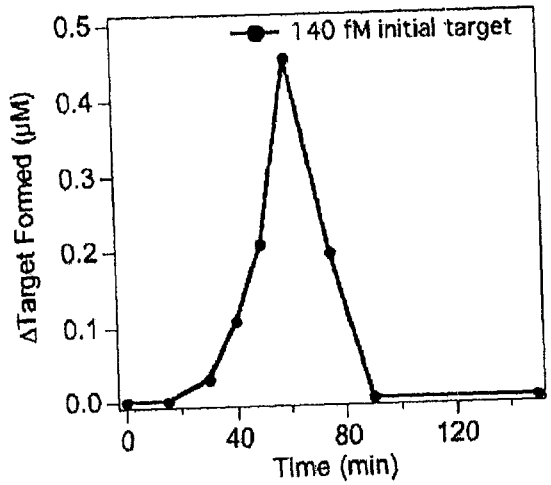
Probes:

5′-T_FTGTTA AAT-3′ 5′-ATTGATAAG-3′

T_F= fluorescent label;

(ab) = abasic destabilizing group

FIG. 10



Target Sequence :

Wild-Type 5′-TTGTTA AATATTGATAAG-3′

Destabilizing Probes:

3′-AACAATTTA(ab)-5′ 3′-AAC**G**ATTC-5′

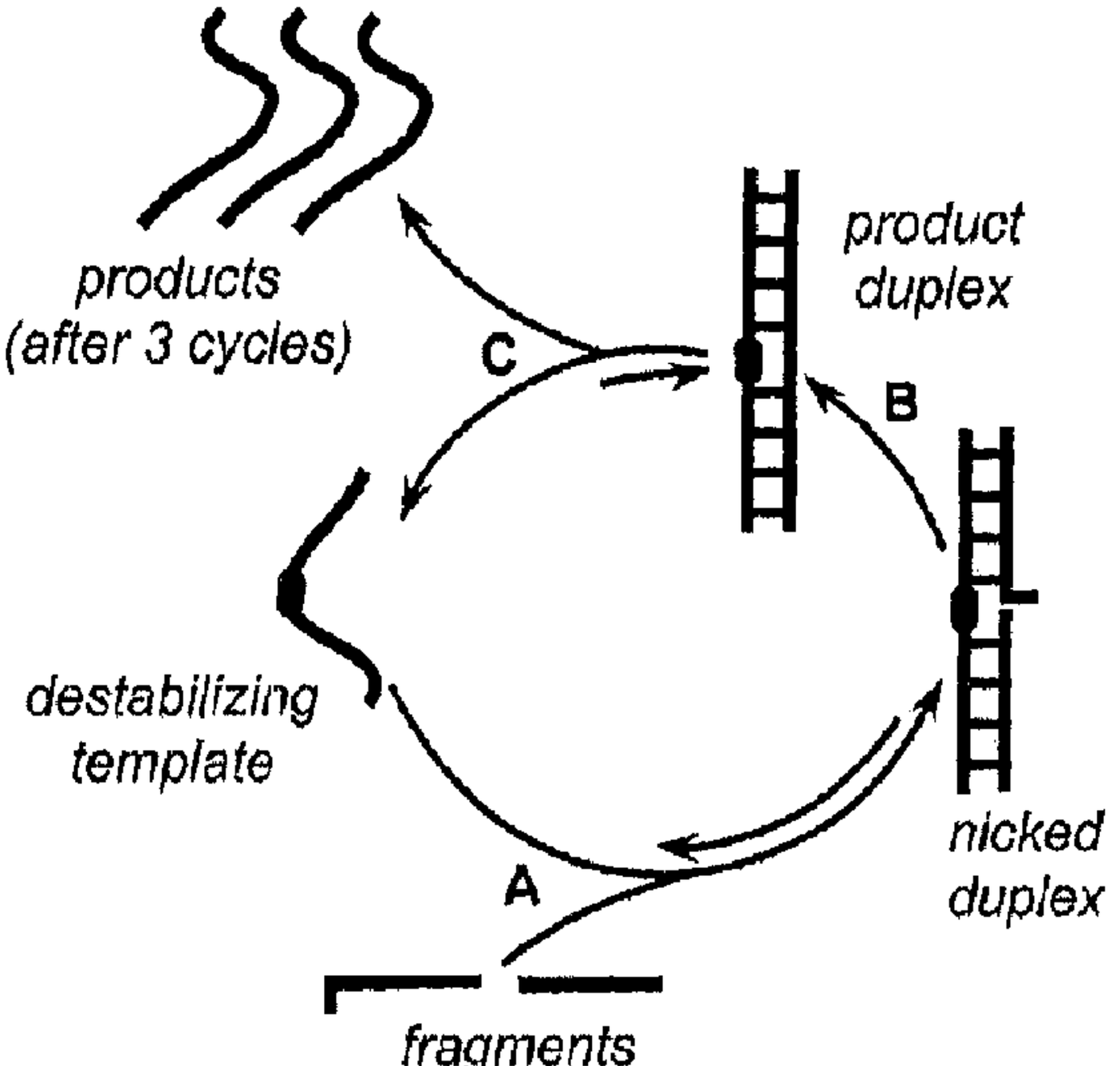
Probes:

5′-T_FTGTTA AAT-3′ 5′-ATTGATAAG-3′

T_F= fluorescent label;

(ab) = abasic destabilizing group

FIG. 11


products
(after 3 cycles)
C
product
duplex
B
destabilizing
template
nicked
duplex
A
fragments