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Isothermal Detection of DNA by Beacon-Assisted Detection Amplification**

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Nucleic acids are routinely used as biomarkers to help diagnose pathogenic infections and genetic disorders. Specific nucleic acids indicating the presence of a disease are often found in only trace amounts in a complex biological extract, so new technologies to detect a unique DNA sequence amongst the millions of nucleotides that constitute a genome are constantly in demand. Analysis of DNA is currently performed by amplifying trace amounts of a specific sequence to levels that are detectable. Several DNA amplification techniques have been developed which can be broadly divided into those that require thermal cycling and those that proceed at constant temperature (isothermal).

The polymerase chain reaction (PCR) is the most widely used thermal cycling technique for DNA amplification.[1] The reaction proceeds exponentially so trace amounts of DNA can be amplified to detectable levels. However, thermal cycling imposes instrumental constraints that limit this technique to a laboratory setting, and the PCR product typically requires characterization at the end of the reaction to determine the specificity of amplification.

Isothermal amplification of DNA has emerged as an alternative amplification technique that often employs a strand displacement polymerase to continuously replicate one strand of a DNA duplex.[2–8] The reaction proceeds at constant temperature, so the time required for DNA amplification is less than that required for thermal cycling techniques. Reactions can be performed without specialized instrumentation and have the potential for "on the spot" testing of DNA. However, the isothermal nature of these reactions can result in non-specific priming and the production of unwanted byproducts.[9] While strategies to make isothermal amplification more specific have been developed,[10–14] advances in the accuracy, sensitivity, speed and simplicity of DNA detection would be beneficial in the laboratory and for "on the spot" testing of DNA.

A new isothermal reaction to simultaneously amplify and detect DNA is reported herein. The procedure designated beacon assisted detection amplification (BAD AMP) is an integrated "biological circuit" designed to detect, amplify and measure a specific DNA sequence in a cellular extract. The circuit is composed of two molecular switches that operate in series. The first switch is designed to be activated by a specific nucleotide sequence. The second switch is designed to initiate DNA amplification and signal transduction.

Detection of a specific DNA sequence is achieved using a molecular beacon. A single-stranded (ss) "DNA trigger" binds to a complementary sequence in the molecular beacon causing it to "switch" conformations and emit a fluorescent "activation" signal (Figure 1 A). As a result of activation, ssDNA at the 3′ end of the beacon is presented to an engaging primer which initiates DNA polymerization on the beacon. The polymerase will also displace the DNA trigger during synthesis of the duplex DNA.[15,16] As a result, the beacon is maintained in an "activated" conformation while the DNA trigger is free to bind to another beacon and initiate a new cycle of triggering, priming and displacement. With each reaction cycle the DNA trigger is regenerated, another beacon is "activated" and a duplex beacon is formed.

The second switch in the biological circuit is designed to initiate exponential amplification of the DNA trigger and signal transduction following detection. The 3′ region of the molecular beacon contains a DNA endonuclease recognition sequence designed to traverse the stem and loop region of the molecular beacon (Figure 1 C). In the absence of a DNA trigger the beacon is "inactive" and the recognition sequence is an unsuitable substrate for the DNA endonuclease. Following "activation" of switch 1, a duplex beacon is produced and the DNA recognition sequence becomes a suitable substrate for the endonuclease nicking of the DNA duplex. This enzymatic "nicking" of the duplex constitutes the second switch in the biological circuit which is designed to initiate amplification of the DNA trigger.

A key component to achieving DNA amplification and signal transduction involves generating multiple copies of the DNA trigger to enable the "activation" of multiple beacons in a single reaction cycle. The amplification reaction depicted in Figure 1 B outlines such a cycle in which the duplex molecular beacon is a substrate for the DNA nicking endonuclease. The 3′ end of nicked DNA primes a subsequent cycle of displacement, nicking and polymerization. During each synthesis cycle a DNA trigger is synthesized which can activate subsequent molecular beacons to initiate additional polymerization reactions. Once initiated, the nicking, polymerization and displacement reactions cycle continuously to produce the ssDNA trigger that "activates" the molecular beacon in a cyclic chain reaction, resulting in signal transduction which is evident by an increase in fluorescent signal.

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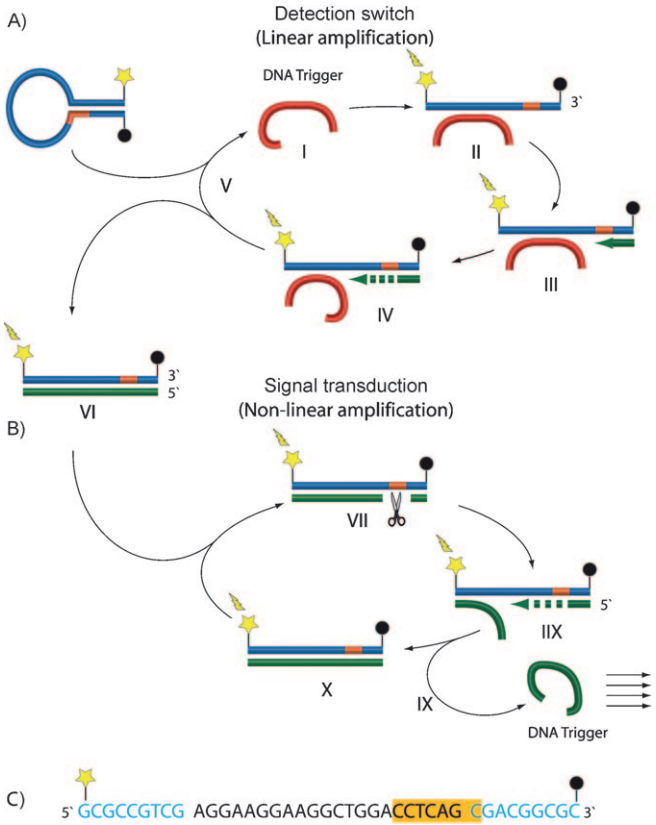

Figure 1. A) Outline of the BAD AMP detection switch: The 16-base DNA trigger (I) has an ambient melting temperature (T_m 46 °C) and can form a stable duplex with the loop region of the molecular beacon at 40 °C. Hybridization displaces the fluorophore from the quencher, resulting in the production of a fluorescence signal (II). This exposes the 3′ stem of the beacon, allowing a short primer (T_m 30 °C) to transiently hybridize (III). The 3′ end of the primer is extended by *Bst* large fragment DNA polymerase, which also displaces the DNA trigger (IV) enabling it to initiate another reaction cycle (V). Polymerization produces a double-stranded (ds) DNA duplex (VI) that maintains the fluorescence signal (T_m 78 °C). The progress of the reaction can be measured in real-time by monitoring the fluorescence intensity. B) Outline of the amplification and transduction switch: DNA detection is performed as outlined in (A) to produce a dsDNA duplex (VI). Exponential amplification is achieved by introducing *NbBvCI* endonuclease into the reaction buffer to "nick" the dsDNA duplex (VII). The 3′ end of the nicked DNA is extended by *Bst* large fragment DNA polymerase which displaces a single copy of the DNA trigger (IIX). The displaced DNA trigger (IX) is free to hybridize to a subsequent beacon and initiate another cycle of BAD AMP. DNA polymerization regenerates the dsDNA duplex (X) to maintain the fluorescence signal and initiate another cycle of nicking, polymerization and displacement. C) The DNA sequence of the molecular beacon (T_m 88 °C) containing 6-FAM (5′) and DABCYL (3′) on the stem (blue), a loop (black) and a *Nb.BbvCI* restriction site (yellow highlight).

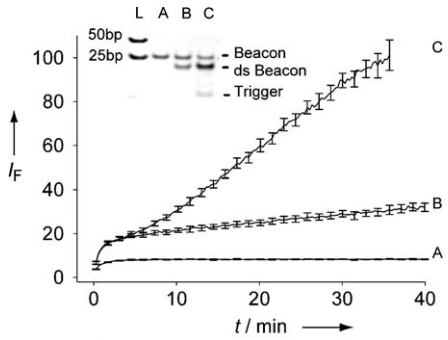

Figure 2. Replicate BAD AMP reactions were prepared, and each reaction was initiated with 1.76 pmol of the DNA trigger. A) The reaction containing only *NbBvC1* endonuclease. B) The amplification reaction containing only *Bst* large fragment DNA polymerase. C) The amplification reaction containing both *Bst* large fragment DNA polymerase and *NbBvC1* endonuclease. The progress of each reaction was monitored by measuring the fluorescence intensity. Values represent the fluorescence mean ± standard deviation of quadruplicate reactions at the indicated time. Inset: Polyacrylamide gel electrophoresis of the above samples. (L: 25 bp DNA ladder, A, B and C are as outlined above).

The products produced by numerous cycles of BAD AMP in an experimental system are depicted in Figure 2. The progress of the reaction was visualized using gel electrophoresis which revealed the presence of the unreacted beacon and the proposed duplex molecular beacon (Figure 2 inset). The exponential behavior of the reaction was characterized by the excessive amount of DNA trigger produced during the reaction. The progress of the reaction is best characterized by monitoring the fluorescence emitted by the product. Within seconds of initiating the reaction there was a large increase in the fluorescence signal (Figure 2). Thereafter, the amount of fluorescence emission increased from approximately 2 units per minute during the early stages to 3 units per minute at the height of the reaction. It is clear that both switches in the biological circuit are dependent on the polymerase since no DNA amplification was detectable when it was omitted from the reaction. Furthermore, signal amplification and transduction are dependent on the nicking endonuclease, since the rate of amplification was substantially lower when it was omitted from the reaction.

Within seconds the signal produced by BAD AMP was 10-fold higher than that of the unamplified molecular beacon (Figure 2). The amount of product doubled every 20–30 seconds but production slowed down during the latter stages of the reaction. The reaction yield was found to increase with the primer length (Supporting Information) which was attributed to the formation of a more stable primer/DNA duplex. The reaction yield also increased as the concentration of the primer or the molecular beacon were increased (Supporting Information). This was attributed to a higher incidence of collisions between the reactants, which resulted in an increase in the rate of the reaction.

A time-course of BAD AMP reactions initiated with different amounts of the DNA trigger is shown in Figure 3. Within the first few seconds there was a noticeable increase in fluorescence signal emitted from reactions that contained a high concentration of the trigger. When the reaction was initiated with 17.6 pmol of DNA trigger there was a 32-fold increase in fluorescence signal within 30 s. This increased beyond 100-fold in 7.5 min. The fluorescent signal emitted from each reaction decreased with the amount of DNA trigger, demonstrating that BAD AMP can be used to measure changes in DNA levels. When the concentration of the target decreased below 88 fmol no detectable increase in

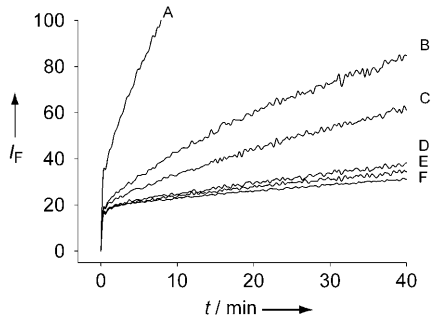

Figure 3. Replicate BAD AMP reactions containing the molecular beacon, *Bst* large fragment DNA polymerase and *NbBvCl* endonuclease were prepared and initiated with a different amount of the DNA trigger (A: 1.76×10^{-11} mol, B: 1.76×10^{-12} mol, C: 8.80×10^{-13} mol, D: 1.76×10^{-13} mol, E: 8.80×10^{-14} mol, F: no DNA trigger). The progress of each reaction was monitored by measuring the fluorescence intensity.

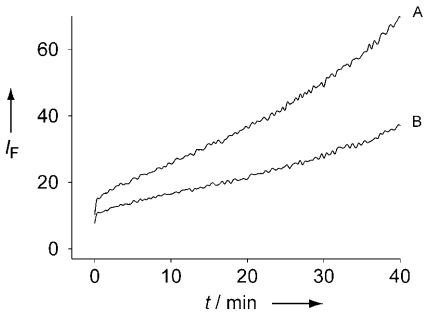

Figure 5. Replicate reactions containing the molecular beacon, *Bst* large fragment DNA polymerase and *NbBvCl* endonuclease were prepared and initiated with 234 ng of DNA extracted from either MCF-7 cells (A) or lambda phage (B). The progress of each reaction was monitored by measuring the fluorescence intensity.

fluorescence was observed within the timeframe of the reaction. In its current form, this represents the detection limit of BAD AMP and corresponds to 5×10^{10} copies of DNA. This detection limit was reproducible in replicate reactions that were standardized by including a fixed amount of fluorescent dye.

The ability to specifically detect a unique DNA sequence in a complex nucleic acid extract was evaluated. 176 fmol of the DNA trigger added to a nucleic acid extract was detectable using BAD AMP. As the amount of DNA trigger added to the extract was increased, the fluorescence signal increased as expected (Figure 4). The level of a specific DNA

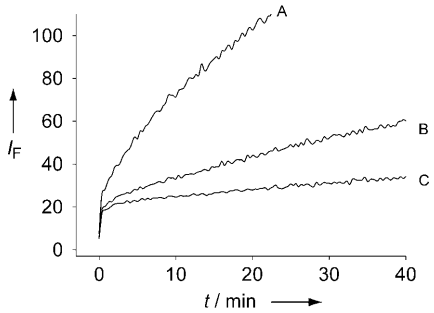

Figure 4. Replicate BAD AMP reactions containing 1.8 μg of heterogeneous RNA, the molecular beacon, *Bst* large fragment DNA polymerase and *NbBvCl* endonuclease were prepared and initiated with a different amount of the DNA trigger (A: 1.76×10^{-11} mol, B: 1.76×10^{-12} mol, C: 1.76×10^{-13} mol). The progress of each reaction was monitored by measuring the fluorescence intensity.

sequence (β-actin) in DNA extracted directly from human cells (MCF-7) was also evaluated using BAD AMP. Within 2 min there was a noticeable increase in the fluorescence signal emitted from the reaction (Figure 5). The fluorescence increased 7.5-fold over 40 min in response to the β-actin "triggering" sequence. The reaction was dependent on the presence of β-actin DNA as extracts of lambda-DNA that lacked the DNA trigger sequence produced a significantly reduced fluorescent signal.

We have developed a new signal amplification and detection strategy for rapid quantification of DNA and have demonstrated its application in DNA derived from human cells. This was achieved by engineering a simple and flexible biological circuit composed of two molecular switches designed to initiate a cascade of events to detect and amplify a specific DNA sequence. This procedure has the potential to greatly simplify the analysis of nucleic acids because amplification is rapid and trace amounts of DNA can be detected and quantified in a single reaction in real-time.

Experimental Section

The molecular beacon (Signa-Proligo) was diluted to 20 μM in 1 mM $MgCl_2$ and 20 mM Tris-HCl pH 8. The BAD AMP primer: GCCGTCGC and DNA trigger (5′CTCTTCCAGCCTTCCTTCC-TAA3′) (Geneworks) were diluted to 80 μM.

BAD AMP reactions contained 5 mM NaCl, 1 mM tris-HCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol pH 7.9, 1.25 mM dNTPs, 2.5 μg BSA, 250 nM molecular beacon, 2 U *Bst* large fragment DNA polymerase, 2.5 U *Nb.BbvCI* nicking endonuclease (New England Biolabs), 2 μM primer and the DNA trigger in 0.1% Triton X100. Reactions were assembled at 4°C and initiated by adding the primer and the DNA trigger then incubating the reaction at 40°C. Fluorescence measurements were made in a real-time PCR machine (Corbett Rotogene) at 17 s intervals using an excitation wavelength of 470 nm and a detection wavelength of 510 nm.

DNA was extracted from MCF-7 cells using a DNeasy kit (Qiagen, Venlo, Netherlands). Lambda-DNA was purchased (Fermentas International Inc). DNA from both sources was precipitated by adding 3 M sodium acetate (pH 5.2) and ethanol. The concentration of purified DNA was determined by measuring its optical density at 260 nm.

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