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Helicase-dependent isothermal DNA amplification

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Abstract

Polymerase chain reaction is the most widely used method for in vitro DNA amplification. However, it requires thermocycling to separate two DNA strands. In vivo, DNA is replicated by DNA polymerases with various accessory proteins, including a DNA helicase that acts to separate duplex DNA. We have devised a new in vitro isothermal DNA amplification method by mimicking this in vivo mechanism. Helicase-dependent amplification (HDA) utilizes a DNA helicase to generate single-stranded templates for primer hybridization and subsequent primer extension by a DNA polymerase. HDA does not require thermocycling. In addition, it offers several advantages over other isothermal DNA amplification methods by having a simple reaction scheme and being a true isothermal reaction that can be performed at one temperature for the entire process. These properties offer a great potential for the development of simple portable DNA diagnostic devices to be used in the field and at the point-of-care.

Keywords: DNA amplification; isothermal; helicase; DNA polymerase; UvrD

Introduction

The polymerase chain reaction (PCR) revolutionized our capabilities to do biological research, and it has been widely used in biomedical research and disease diagnostics. Hand-held diagnostic devices, which can be used to detect pathogens in the field and at point-of-care, are demanded currently. However, the need for power-hungry thermocycling limits PCR application in such a situation. Several isothermal target amplification methods have been developed. Strand-displacement amplification (SDA) combines the ability of a restriction endonuclease to nick the unmodified strand of its target DNA and the action of an exonuclease-deficient DNA polymerase to extend the 3′ end at the nick and displace the downstream DNA strand. Transcription-mediated amplification (TMA) uses an RNA polymerase to make RNA from a promoter engineered in the primer region, a reverse transcriptase to produce complementary DNA from the RNA templates and RNase H to remove the RNA from cDNA. In rolling circle amplification (RCA), a DNA polymerase extends a primer on a circular template, generating tandemly linked copies of the complementary sequence of the template. However, these isothermal methods also have their limitations: most have complicated reaction schemes and are incapable of amplifying DNA targets of sufficient length to be useful for many research and diagnostic applications.

In living organisms, a DNA helicase is used to separate two complementary DNA strands during DNA replication. We have devised a new isothermal DNA amplification technology, helicase-dependent amplification (HDA), by mimicking nature. HDA uses a DNA helicase to separate double-stranded DNA (dsDNA) and generate single-stranded templates for primer hybridization and subsequent extension. As the DNA helicase unwinds dsDNA enzymatically, the initial heat denaturation and subsequent thermocycling steps required by PCR can all be omitted. Thus, HDA provides a simple DNA amplification scheme: one temperature from the beginning to the end of the reaction. In this study, we present the Escherichia coli UvrD-based HDA system, which can achieve over a million-fold amplification.

Results

HDA design

In this system, strands of duplex DNA are separated by a DNA helicase and coated by single-stranded DNA (ssDNA)-binding proteins (SSBs). Two sequence-specific primers hybridize to each border of the target DNA. DNA polymerases extend the primers annealed to the templates to produce dsDNA. The two newly synthesized

dsDNA products are then used as substrates by DNA helicases, entering the next round of the reaction. Thus, a simultaneous chain reaction proceeds resulting in exponential amplification of the selected target sequence. E. coli UvrD helicase was chosen as the DNA helicase for our first HDA system because it can unwind blunt-ended DNA fragments. The SSB in the HDA reaction is either bacteriophage T4 gene 32 protein or RB49 gene 32 protein.

Amplification of a target sequence from plasmid DNA

Two M13/pUC19 universal primers were used in an HDA reaction to amplify selectively a 110 base pair (bp) target sequence from a derivative of pUC19 plasmid. After a 1 hr incubation at 37° C, a 110-bp amplification product was observed on a 2% agarose gel. Sequencing results confirmed that it matched the target DNA sequence. To determine the essential elements, each key component was omitted from the reaction. In the absence of UvrD helicase, no amplification was observed, confirming that helicase is required. In the absence of accessory protein MutL, no amplification product was observed, suggesting that UvrD helicase-mediated amplification requires MutL. MutL stimulates UvrD helicase activity more than tenfold by loading it onto the DNA substrate. In the absence of T4 gene 32 protein, no amplification product was observed, indicating that SSB is required, probably to prevent reassociation of the complementary ssDNA templates. In the absence of ATP, no amplification product was detected, indicating that the helicase cofactor is essential. Target sequences up to 400 bp can be efficiently amplified from plasmid DNA.

Amplification of target sequences from genomic DNA

The E. coli UvrD-based HDA system was used to amplify a 123-bp fragment from the oral pathogen Treponema denticola. The amount of template was varied from 10^7 to 10^3 copies of the T. denticola genome. With 10^3 copies of initial target, about 10 ng of products were generated, corresponding to 10^10 molecules of the 123-bp fragment. Thus, the HDA system is capable of achieving over ten million-fold amplification. The negative control showed no trace of amplified products, proving the specificity and reliability of HDA. The E. coli UvrD-based HDA system can also amplify target sequences from genomic DNAs isolated from Helicobacter pylori, E. coli, Neisseria gonorrhoeae, Brugia malayi and human cells.

One temperature HDA

We tested whether the entire HDA reaction could be carried out at one temperature without prior heat denaturation. The yield of the one-step HDA amplification was about 40-60% of the two-step HDA reaction, but enough product is generated to be detected. This demonstrates that HDA is able to amplify a target sequence from bacterial genomic DNA at one temperature for the entire process.

Amplification from T. denticola cells

A specific product was obtained when using 10^7 to 10^4 cells as template. As the initial cell number was lowered, non-target-specific products (primer-dimers) were observed; nevertheless, the negative control allows us to distinguish target-specific from non-target-specific products. The current HDA system can work on crude samples, such as whole bacterial cells, with only a tenfold loss of sensitivity compared with purified genomic DNA.

Detection of B. malayi DNA in blood

To test the possibility of using HDA on real samples, a pathogen's DNA sequence was amplified in the presence of human blood. A 99-bp fragment of the HhaI repeat of the filarial parasite B. malayi was chosen as target. Decreasing amounts of B. malayi genomic DNA were added to human blood samples. A specific product was detected for samples containing as low as 5 pg of B. malayi DNA, corresponding to 500 copies of the genome. These results demonstrate the feasibility of using HDA to detect a pathogen in a real sample.

Real-time HDA

We developed a real-time detection system using a LUX primer specific to the earIR homologue gene in T. denticola. After 35 min, product accumulation generated a typical sigmoid curve. A semilogarithmic plot revealed an initial first-order reaction with a doubling time of 3 min. Curves from two identical reactions were very similar, suggesting good reproducibility. In the negative control, the fluorescent

signal remained below the threshold line.

Discussion

We report a new isothermal DNA amplification technique, named HDA. It has a significant advantage over PCR in that it eliminates the need for an expensive and power-hungry thermocycler. HDA also offers several advantages over existing isothermal DNA amplification methods. First, it has a simple reaction scheme, in which a target sequence can be amplified by two flanking primers, similar to PCR. In contrast, SDA uses four primers and modified deoxynucleotides, and TMA needs three different enzymatic steps. Second, HDA is a true isothermal DNA amplification method; the entire reaction can be performed at one temperature. In contrast, other isothermal methods such as SDA still need an initial heat denaturation step. Third, the current UvrD system can achieve over a million-fold amplification, and a pathogen genomic DNA can be detected in a human blood sample, demonstrating that HDA can be performed on crude samples and has potential as a diagnostic tool.

E. coli UvrD helicase, a repair helicase, was chosen as our model system, but its speed (20 bp/s) and processivity (less than 100 bp per binding) are limited. This may explain why the current UvrD HDA system is inefficient at amplifying long target sequences. The performance may be improved by testing different helicases. In a recent experiment, we were able to amplify a 2.5-kb target from plasmid DNA using a processive replicative helicase, T7 gene 4 protein, which unwinds DNA at 300 bp/s with high processivity.

Optimization of current HDA systems involves identifying rate-limiting steps. One rate-limiting step could be the coordination between the helicase and the DNA polymerase. A DNA polymerase that can move with the DNA helicase in a coordinated way would be ideal; such coordination is found at the in vivo replication fork where DNA polymerase III interacts with the DnaB helicase. Another rate-limiting step could be the interaction between SSB and DNA. The essential role of SSB is probably to prevent reassociation of the separated DNA strands; no amplification is observed in the absence of SSB.

Future experiments will be directed towards improving the efficiency of HDA by testing different helicases/polymerases and optimizing the concentration of each key component. The simplicity and true isothermal nature of the HDA platform offer great potential for the development of hand-held DNA diagnostic devices for point-of-care or field use.

Methods (summary)

UvrD helicase and MutL were cloned and purified from E. coli as C-terminal fusions with a self-cleavable affinity tag (Impact system, NEB). Gene 32 protein was cloned from RB49 genomic DNA. HDA reactions used two buffers: buffer A (350 mM Tris-acetate pH 7.5, 100 mM DTT) and buffer B (10 mM Tris-acetate pH 7.5, 1 mg/ml BSA, 100 mM magnesium acetate). Reaction component A (template, primers, dNTPs) was heated 2–10 min at 95° C and 1–4 min at 37° C; component B (ATP, exo-Klenow, UvrD, MutL, gp32) was added, and the reaction continued 1–3 h at 37° C. For real-time HDA, fluorescent LUX primers were used and signals measured at 5 min intervals on an iCycler.

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