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Loop-mediated isothermal amplification of DNA

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Abstract

We have developed a novel method, termed loop-mediated isothermal amplification (LAMP), that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. An inner primer containing sequences of the sense and anti-sense strands of the target DNA initiates LAMP. The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem-loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long. The cycling reaction continues with accumulation of 10^9 copies of target in less than an hour. The final products are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity.

Introduction

Nucleic acid amplification is one of the most valuable tools in virtually all life science fields. In addition to the widely used PCR-based detection, several amplification methods have been invented: nucleic acid sequence-based amplification (NASBA), self-sustained sequence replication (3SR) and strand displacement amplification (SDA). Each of these methods has its own innovation to re-initiate new rounds of DNA synthesis. PCR uses heat denaturation; 3SR and NASBA eliminate heat denaturation by using transcription and reverse transcription; SDA eliminates heat denaturation by using restriction enzyme digestion and strand displacement DNA synthesis with modified nucleotides.

These methods can amplify target nucleic acids to a similar magnitude, all with a detection limit of less than 10 copies and within an hour or so, but still have shortcomings. They require either a precision instrument for amplification or an elaborate method for detection due to poor specificity of target sequence selection. The requirement for a high precision thermal cycler in PCR prevents this powerful method from being widely used, such as in private clinics as a routine diagnostic tool. NASBA and 3SR are compromised in specificity, mainly due to the necessity of using a relatively low temperature of 40° C. SDA largely overcomes these shortcomings by using four primers and isothermal conditions, but still has weak points: increased backgrounds due to digestion of irrelevant DNA and the necessity to use costly modified nucleotides.

We have recently developed a novel method that can amplify a few copies of DNA to 10^9 in less than an hour under isothermal conditions and with greater specificity. We describe the mechanism, sensitivity and specificity of this amplification method, termed loop-mediated isothermal amplification (LAMP).

The LAMP method

This method relies on auto-cycling strand displacement DNA synthesis performed by a DNA polymerase with high strand displacement activity and a set of two specially designed inner and two outer primers. In the

initial steps, all four primers are used; later, only the inner primers are used. The forward inner primer (FIP) and backward inner primer (BIP) each contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in later stages. The sequences (typically 23–24 nt) inside both ends of the target region are designated F2c and B2. Two inner sequences (23–24 nt) 40 nt from the ends of F2c and B2 are designated F1c and B1, and two sequences (17–21 nt) outside the ends are designated F3c and B3. FIP contains F1c, a TTTT spacer and the sequence (F2) complementary to F2c. BIP contains B1c, a TTTT spacer and B2. The two outer primers consist of B3 and F3. A DNA sample containing the target and the four primers is heat denatured and rapidly cooled on ice; the LAMP reaction is then initiated by addition of Bst DNA polymerase large fragment and carried out at 65° C for 1 h.

Inner primer FIP hybridizes to F2c and initiates complementary strand synthesis. Outer primer F3 slowly hybridizes to F3c and initiates strand displacement DNA synthesis, releasing a FIP-linked complementary strand that forms a looped out structure at one end. This single-stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement, leading to production of a dumb-bell form DNA, which is quickly converted to a stem-loop DNA by self-primed DNA synthesis. This stem-loop DNA then serves as the starting material for LAMP cycling.

To initiate LAMP cycling, FIP hybridizes to the loop in the stem-loop DNA and primes strand displacement synthesis, generating one gapped stem-loop DNA with an additional inverted copy of the target in the stem and a loop formed at the opposite end. Subsequent self-primed strand displacement synthesis yields one complementary structure of the original stem-loop DNA and one gap-repaired stem-loop DNA with a stem elongated to twice as long. Both products then serve as template for a BIP-primed strand displacement reaction in subsequent cycles. Thus, in LAMP the target sequence is amplified 3-fold every half cycle. The final products are a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. The use of four primers (recognition of six distinct sequences) in the initial steps and two primers (recognition of four distinct sequences) during the subsequent steps ensures high specificity for target amplification.

LAMP amplification of M13 DNA as a model

We chose M13mp18 DNA as a model target and prepared four primers meeting the LAMP requirements. The reaction was carried out at 65° C for 1 h and products separated by agarose gel electrophoresis and identified by restriction enzyme digestion and Southern blot hybridization. The LAMP reaction produced many bands of different sizes from ~300 bp to the loading well. To confirm the structure, the amplified products were digested with restriction endonucleases; the sizes of the fragments generated were in good agreement with the predicted sizes. Southern blot hybridization with three different probes perfectly agreed with the conclusion that the amplified DNA originated from target M13 DNA. The structures were also confirmed by cloning and sequencing.

Optimized conditions for LAMP

The sequences and sizes of the primers were chosen so that their melting temperatures (Tm) fell within certain ranges. The Tm values of F2 and B2 were chosen to fall between 60 and 65° C, the optimal temperature for Bst polymerase. The Tm values of F1c and B1c were set slightly higher so that a looped out structure formed immediately after release of the single-stranded DNA. The Tm values of the outer primers (F3 and B3) were set lower to ensure that synthesis occurred earlier from the inner primers. The outer primers were used at 1/4–1/10 the concentration of the inner primers. A loop of 40 bases or longer between F2c (B2c) and F1c (B1c) gave the best results. The best results were obtained with target DNA of 130–200 bp; DNA of more than 500 bp amplified poorly, so the target size should be less than 300 bp. The best amplification was obtained with Bst polymerase or BcaBEST DNA polymerase. The presence of 0.5–1.5 M betaine (N,N,N-trimethylglycine) or L-proline, which reduce base stacking, stimulated the rate and increased target selectivity with a significant reduction in amplification of irrelevant sequences.

Sensitivity of LAMP

LAMP is highly sensitive and able to detect DNA at as few as six copies in the reaction mixture. In a 45 min reaction six copies of the HBV target were amplified to a detectable level. In the absence of one of the outer primers no significant amplification occurred with 60 copies of HBV target, indicating a strict requirement for recognition of six distinct sequences. LAMP seems less prone to the presence of irrelevant DNA than PCR: the presence of 100 ng of human genomic DNA did not significantly adversely affect amplification efficiency. Single PCR failed to amplify six copies of HBV under the same conditions, though nested PCR overcame this problem.

LAMP for an RNA target

LAMP is also applicable to RNA upon use of reverse transcriptase (RTase) together with DNA polymerase. This method (reverse transcription-coupled LAMP, RT-LAMP) easily detected prostate-specific antigen (PSA) mRNA in one PSA-expressing LNCaP cell mixed with 1,000,000 PSA-negative K562 cells.

Advantages of LAMP

(i) LAMP amplifies DNA with high efficiency under isothermal conditions without significant influence of co-present non-target DNA; its detection limit is a few copies, comparable to PCR. (ii) The products are a mixture of stem-loop DNAs and cauliflower-like structures, which enable simple, easy and selective detection. (iii) LAMP is highly specific, attributable to recognition of the target by six independent sequences initially and four during later stages. (iv) LAMP is simple and easy to perform once appropriate primers are prepared, requiring only four primers, a DNA polymerase and a regular laboratory water bath or heat block. (v) By combination with reverse transcription, LAMP can amplify RNA sequences with high efficiency.

Materials and Methods (summary)

LAMP was carried out in a total 25 l reaction containing 0.8 M each FIP and BIP, 0.2 M each F3 and B3, 400 M each dNTP, 1 M betaine, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 4 mM MgSO4, 0.1% Triton X-100 and target DNA. The mixture was heated at 95° C for 5 min, chilled on ice, 8 U Bst DNA polymerase large fragment was added, followed by incubation at 65° C for 1 h and heating at 80° C for 10 min. Products were analyzed by 2% agarose gel electrophoresis with SYBR Green I staining and Southern blotting.

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