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Accelerated reaction by loop-mediated isothermal amplification using loop primers

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Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions using a set of four specially designed primers and a DNA polymerase with strand displacement activity. We have developed a method that accelerates the LAMP reaction by using additional primers, termed loop primers. Loop primers hybridize to the stem-loops, except for the loops that are hybridized by the inner primers, and prime strand displacement DNA synthesis. Although both inner and loop primers react via the loops, they do so by different mechanisms. The LAMP method presented here uses loop primers to achieve reaction times of less than half that of the original LAMP method. Since the total time of analysis including detection is less than 1 h, this new method should facilitate genetic analysis, including genetic diagnosis in the clinical laboratory.

KEYWORDS: loop-mediated isothermal amplification, LAMP, loop primer, DNA amplification, HBV, strand displacement.

INTRODUCTION

Genetic analysis is preferable in testing a patient for infectious diseases because it can yield detailed genetic information about the virulence and antibiotic resistance of a particular microbial. However, the results of genetic analysis take more than 1 h to obtain. Nucleic amplification is one of the most important tools for many investigators, including molecular biologists. In particular, in application-oriented fields such as clinical medicine, genetic diagnosis is used for monitoring infectious diseases, genetic disorders and genetic traits. Hitherto established target amplification methods include polymerase chain reaction (PCR),[1,2] nucleic acid sequence-based amplification,[3] self-sustained sequence replication,[4] strand displacement amplification[5,6] and loop-mediated isothermal amplification (LAMP).[7,8] PCR, the most extensive of these applied techniques, promotes the next round of DNA synthesis by heat denaturation of double-stranded DNA products. On the other hand, there are signal amplification methods such as branched DNA (bDNA) amplification, Invader, and rolling circle amplification (RCA), the latter of which is also known as a target amplification method[9–11] (Table 1).

One of the advantages of LAMP is its ability to directly amplify specific sequences of DNA under isothermal conditions.[7] Most importantly, this method does not require a denatured DNA template.[8] When combined with reverse transcription, this method can also amplify RNA sequences with high efficiency. Furthermore, single-stranded DNA can be isolated from LAMP products.[12] The LAMP reaction requires a DNA polymerase with strand displacement activity and a set of four specially designed primers, termed

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Table 1. Properties of various nucleic acid amplification technologies

Property	LAMP	PCR	SDA	LCR	NASBA	RCA	bDNA	Invader
Target amplification	○	○	○	○	○	○		
Signal amplification						○	○	○
Target (DNA or RNA)	both	DNA	both	DNA	RNA	both	both	both
Isotherm	○		○		○	○	○	○
Number of enzymes	1	1	2	1	3	2	0	1
Sensitivity (copies)	>10	>10	500	100	100	1	500	6000
Multiplexing amp.	○	○	○		○			

inner and outer primers, which improve specificity. As a first step, a stem-loop DNA structure, in which the sequences of both DNA ends are derived from the inner primer, is constructed as starting material. Subsequently, one inner primer hybridizes to the loop on the product in the LAMP cycle and initiates strand displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long. The final products are stem-loop DNAs with several inverted repeats of the target DNA and cauliflower-like structures with multiple loops, amplifying the amount to 10^9 copies of the target. These reactions occur in less than an hour. However, for genomic diagnosis at the hospital bedside, it is necessary to obtain test result faster than that achieved by the previous method.[7,8]

We have developed a method that accelerates the LAMP reaction by using loop primers. Loop primers hybridize to the stem-loops, except for the loops that are hybridized by the inner primer, and prime strand displacement DNA synthesis. In this report we describe the reaction efficiency and sensitivity of the loop-primer method and its deduced mechanism.

MATERIALS AND METHODS

DNA oligonucleotides

The FIP for lambda DNA consisted of the complementary sequence of F1 (20 nt) and F2 (26 nt): 5′-CAGCCAGCCGCAGCACGTTCGCTCATAGGAGATATGGTAGAGCCGC. The BIP for lambda DNA consisted of B1 (26 nt) and the complementary sequence of B2 (25 nt): 5′-GAGAGAATTTGTACCACCTCCCACCGGGCACATAGCAGTCCTAGGGACAGT. The outer primers were 5′-GGCTTGGCTCTGCTAACACGTT (F3) and 5′-GGACGTTTGTAATGTCCGCTCC (B3). The loop primers were 5′-CTGCATACGACGTGTCT (loop F) and 5′-ACCATCTATGACTGTACGCC (loop B).

The primers used for amplification of the HBs region of the hepatitis B virus (HBV) DNA were as follows. The inner and outer primers for HBV were 5′-GATAAAACGCCGCAGACACATCCTTCCAACCTCTTGTCCTCCAA (FIP), 5′-CCTGCTGCTATGCCTCATCTTCTTTGACAAACGGGCAACATACCTT (BIP), 5′-CAAAATTCGCAGTCCCCAAC (F3) and 5′-GGTGGTTGATGTTCCTGGA (B3), according to Notomi *et al.*[7] The loop primers were 5′-CAGCGATAGCCAGGACAAA (loop F) and 5′-GTTGGTTCTTCTGGACTACC (loop B).

Template DNA

Commercially available lambda DNA (Roche) was used as a material. HBV-positive sera were confirmed by the DNA probe 'Chugai'-HBV kit (Chugai Diagnostics Science). Preparation of genomic DNA was performed using the EXTRAGEN kit (Tosoh) according to the manufacturer's protocol.

LAMP reaction

LAMP was performed in a total 25-μl reaction mixture containing 0·8 μM each of FIP and BIP, 0·2 μM each of the outer primers, 0·4 μM each of loop primers F and B, 1·6 mM dNTPs, 1 M betaine (Sigma), 20 mM Tris-HCl (pH 8·8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 0·1% Triton X-100, 8 units of the *Bst* DNA polymerase large fragment (New England Biolabs), 0·25 μg/ml ethidium bromide, and the specified amounts of double-stranded target DNA.[8] A total of 10^5 copies of lambda DNA were used for LAMP reaction. The mixture was incubated at 60°C for HBV DNA or 65°C for lambda DNA for 1 h and analyzed in real time using the ABI PRISM 7700 sequence detection system (Perkin Elmer Biosystems).[13] This system measures the increase in the fluorescence intensity

of the ethidium bromide that is intercalated into the amplified DNA using the ROX fluorescence channel. No internal control was used.

RESULTS

Construction of the loop primers

The LAMP method can amplify a few copies of DNA within 1 h under isothermal conditions and requires a DNA polymerase and a set of four specially designed primers that recognize total of six distinct sequences on target DNA.[7]

To establish a faster LAMP reaction, addition of two primers, termed loop primers, to the reaction was required, and sequence of the loop primers in the target DNA region was resolved. The position of the loop primer is the region between F2 and F1 (or B1 and B2) in the direction of F1 to F2 (or B1 to B2) (Fig. 1).

Amplification of lambda DNA

A pair of inner primers and a pair of loop primers were used to amplify from the lambda DNA, and the DNA products were analyzed in real time using the ABI PRISM 7700. The analyzed data indicated that the signal increase was detected at 14 min and 44 min with and without the loop primers, respectively, advocating that the use of loop primers accelerated the reaction (Fig. 2A). When the LAMP reaction was performed using primers that consisted of complementary sequences of the loop primers, the signal was detected slightly faster than without the loop primers (Fig. 2A). When this product was digested with a restriction enzyme that recognizes the insert region between F1 and B1, the expected products were obtained (data not shown). Hence, the target-specific amplification was achieved using the loop primers. These results demonstrated that loop primers accelerate the LAMP reaction under isothermal conditions.

Sensitivity of LAMP using loop primers

Using the loop primers, significant amplification occurred from 10^3 copies of lambda DNA (Fig. 2B). In the absence of loop primers, signals were dispersed from 52–92 min in 10^3 copies, and it was thus proved that significant DNA amplification required more than 10^4 copies (Fig. 2C and data not shown). This finding suggests that LAMP reaction with the loop primers provides higher sensitivity.

Amplification of HBV DNA as a model

Six copies of the HBV DNA subcloned in the presence of 100 ng human genomic DNA were amplified to a detectable level by LAMP.[7] We performed the LAMP reaction using HBV DNA extracted from HBV-positive serum of which the initial copy number was unknown (Fig. 2D). When the loop primers were used, the signals were detected in 11 min by ABI PRISM 7700. These results indicate that LAMP using loop primers is able to determine

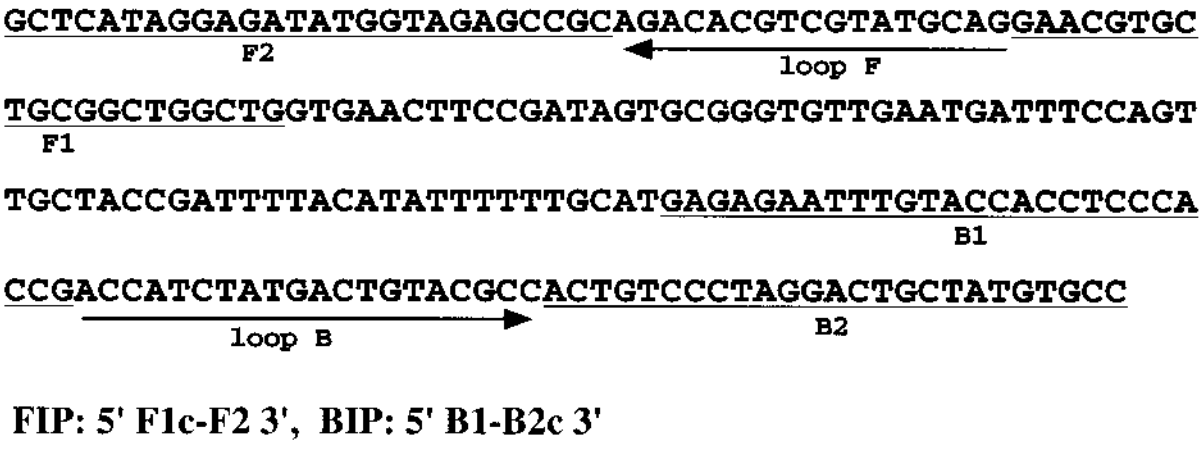

Fig. 1. Nucleotide sequence of lambda DNA used to design the inner and loop primers. Recognition sequences of the inner primer are underlined. Arrows show the DNA sequences used for the loop-primer design. Construction of the inner primers, FIP and BIP, is also shown. F1c and B2c indicate the complementary sequences of F1 and B2, respectively.

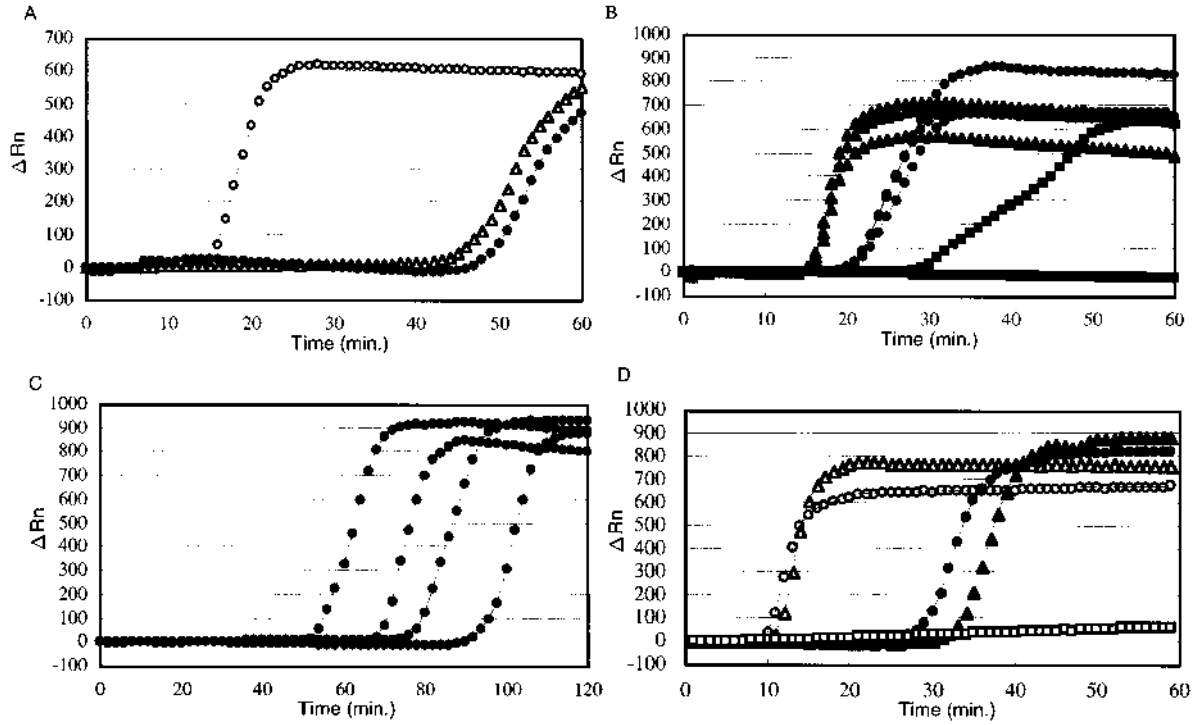

Fig. 2. Analysis of LAMP amplification. (A) Detection of the LAMP amplification signals. A total of 10^5 copies of lambda DNA were used for the LAMP reaction. Open and closed circles show the LAMP reactions with and without the loop primers, respectively. The open triangle shows the LAMP reaction using the complementary sequences of the loop primers. (B) Sensitivity of LAMP using the loop primer: 10^2 (square), 10^3 (circle), and 10^5 (triangle) copies of lambda DNA were used for these LAMP reactions. Each sample was performed in triplicate. In 10^2 samples, one of three was amplified in 1 h. (C) 10^3 copies of DNA were used for the LAMP reaction without the loop primer. Quadruplicate measurement was performed. (D) Detection of DNA derived from HBV. Two sera were used, independently, for LAMP amplification. These two samples were amplified with PCR using a pair of outer primers (data not shown). The open circle and triangle, as the independent samples, indicate reactions with the loop primers. The closed circle and triangle indicate the reactions without the loop primers. The square shows the reaction with the loop primers using HBV non-infective DNA.

The signal reached a plateau after a few minutes, presumably because the free ethidium bromide was depleted by binding to amplified DNA. We confirmed that the amount of DNA was increased after the signal reached a plateau (data not shown). ΔRn is the normalized emission at 615 nm.

rapidly and easily whether or not a patient is infected with various viruses, including HBV.

DISCUSSION

The LAMP reaction was accelerated by the addition of two primers, termed loop primers, that hybridized to the stem-loops except for the loops that had been hybridized by the inner primers. The mechanism and expected reaction steps are illustrated in Figure 3. To accelerate the LAMP reaction, the loop primers act from the elongation steps of the original LAMP (Fig. 3A). The loop primer called loop B hybridizes to a one-loop region of the stem-loop DNA in structure 12 before initiating strand displacement DNA synthesis (Fig. 3B). Then, DNA synthesized from this loop primer is displaced by the extension from the 3′ end of the target DNA, generating structure 15, which is not present in the original LAMP. Another stem-loop in structure 12 is hybridized by the inner primer, FIP, and then FIP primes strand displacement DNA synthesis.[7] Structure 10 returns next to structure 12 as the starting material in this figure (Fig. 3B), and structures 8 and 11 are also reused in the LAMP reaction. It is illustrated that all stem-loop DNAs are used by either inner or loop primers. The loop primers hybridize to the stem-loop region not only in structure 12 but also in the other structures that are generated from the LAMP reaction, resulting in drastic amplification. When primers, which hybridize to the loop region to which inner primer hybridizes, were designed, the reaction rate was slightly accelerated (Fig. 2A). Thus, this result demonstrates that these primers are not involved in accelerating the reaction rate, and consequently that

the mechanism using the loop primers is new. In separate experiments, loop primers were successful in reducing the reaction time using other templates, such as genomic DNA (amplified with SRY gene on chromosome Y) and pBluescript II and M13 mp18 vector DNAs (data not shown).

In our study, we used HBV DNA extracted from HBV-positive serum of which the number of initial copies was unknown, and signal detection was found at 11 min. Using the subcloned HBV DNA as a template, 600 and 6000 copies of DNA were detected at 13 and 11 min, respectively (data not

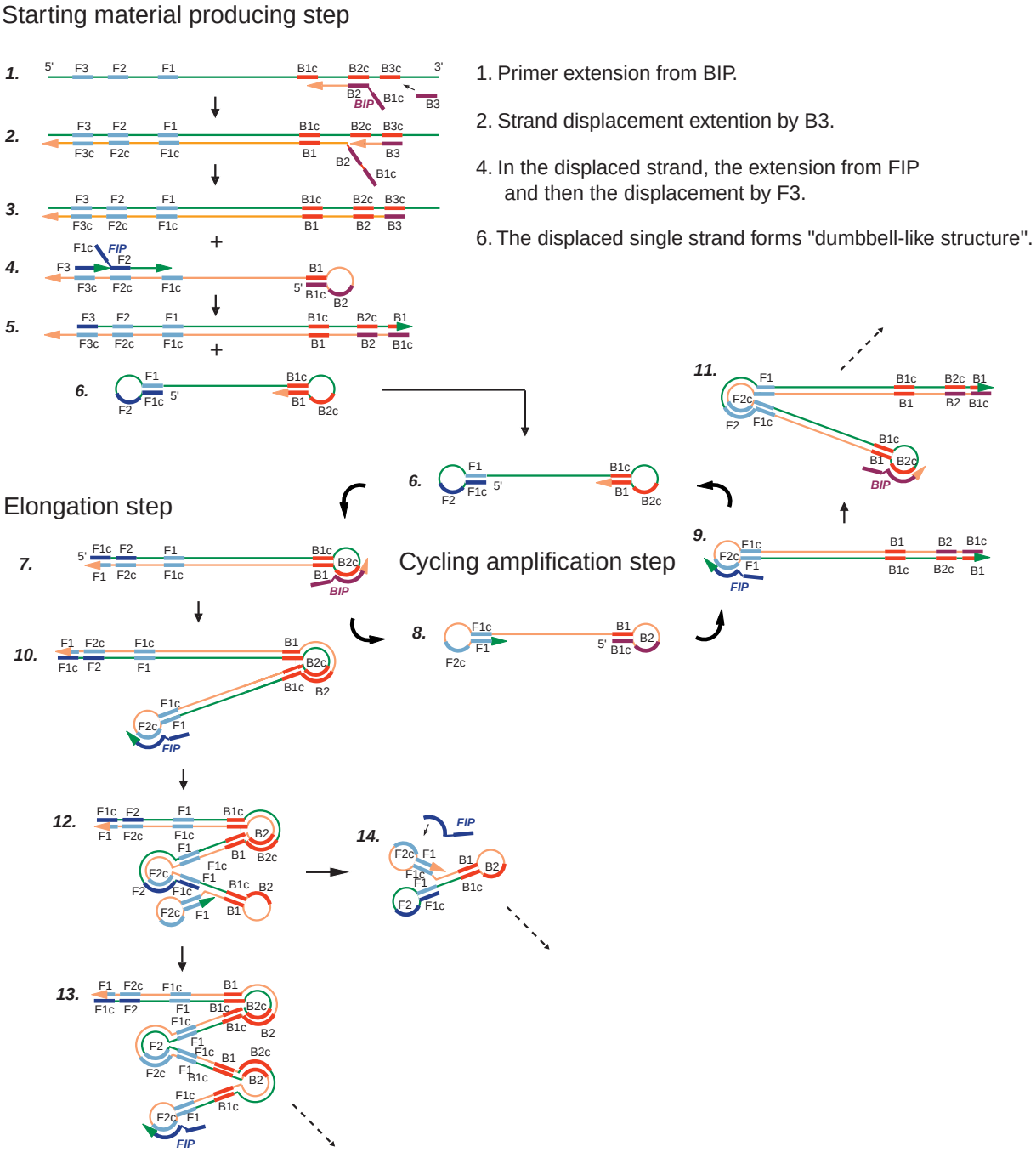

Fig. 3. Schematic representation of the LAMP mechanism using loop primers. (A) Steps in the original LAMP reaction. In the first step, in which starting material is produced, the dumbbell-like DNA form (structure 6) is generated. Then, in cycling amplification step, DNAs of this form are generated continuously. The elongation reactions are started from the sub-products (structures 10 and 11) of the cycling amplification step, generating various sizes of the products. F2c and B2c are hybridized by the inner primers.

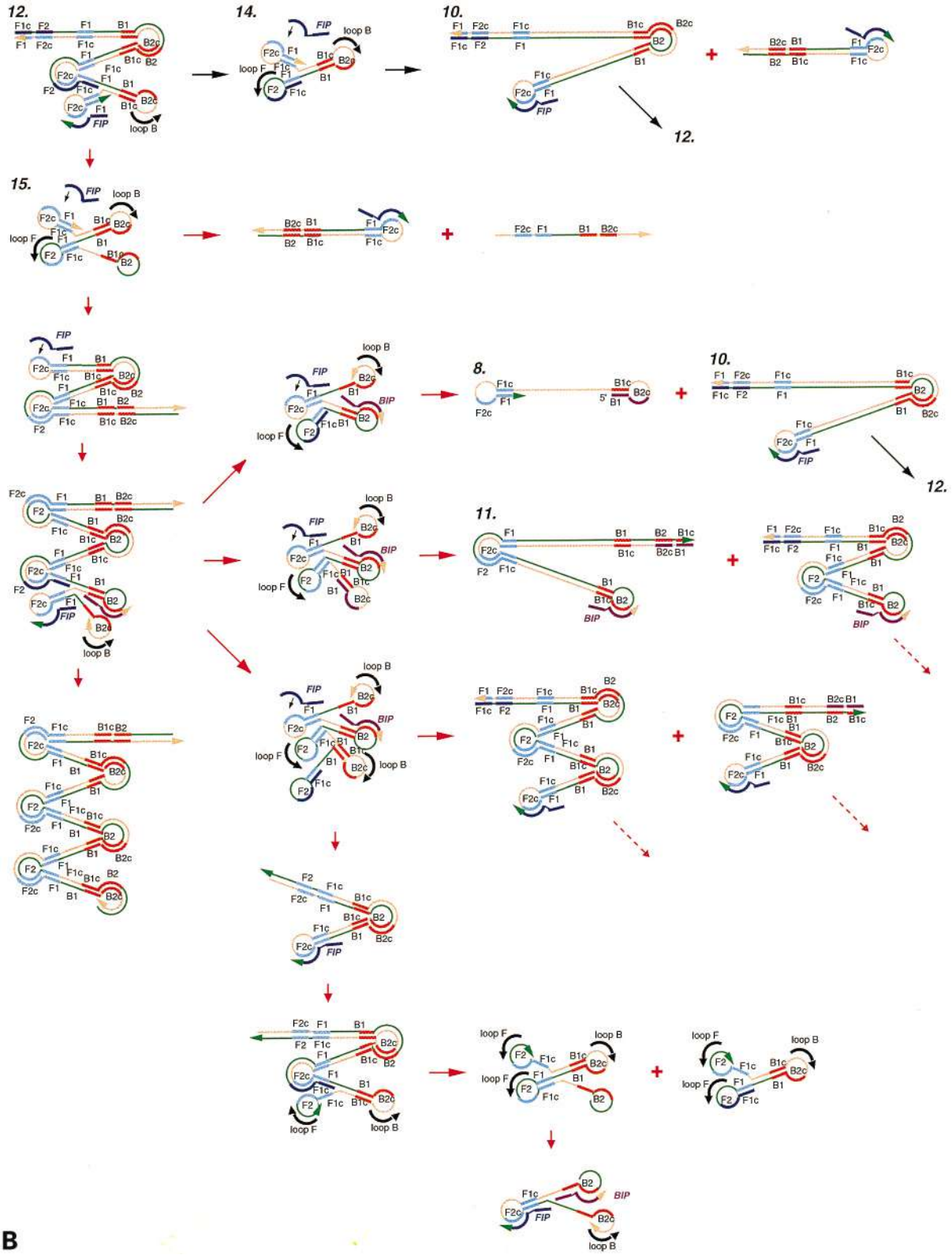

Fig. 3. *Continued*
(B) This figure shows the process that starts from structure 12. Black arrows show the original LAMP reaction pathway. Red arrows show the reaction derived from the loop primers. Red dotted arrows represent continuous reactions. The region between F1 and F2 (or B1c and B2c) is hybridized by the loop primer. The primers that hybridize the 3′ region of the products are not shown because they are irrelevant to the acceleration of amplification.

shown). Comparison of this result with our data suggests there were about 6000 copies of the DNA in the samples. In point-of-care testing (POCT) for hospital and primary care, a recommendation was made that POCT be considered for analytes that have a required reporting turnaround time of less than 30 min.[14] In our study, corresponding to the requirement of genetic POCT, we determined that it is possible to detect in less than 30 min whether or not a patient is infected with an HBV concentration of more than 6000 copies per 4-μl serum.

LAMP is theoretically a method that barely produces non-specific composition products.[7] When appropriate starting material for LAMP cycling was produced, DNA synthesis ensued quickly because the products included sequences that the loop primers recognized. Therefore, it is particularly difficult to produce non-specific products when using loop primers.

This method yielded a large amount of DNA, more than 500 μg/ml. After the reaction, white precipitates, identified as magnesium pyrophosphate, were observed in the reaction mixture.[15] Since the turbidity of the white precipitates in the reaction mixture correlated with the amount of DNA synthesized, this observation implies that it is possible to confirm whether or not the DNA was amplified. The combination of the rapid method using loop primers and detection of the white precipitates is useful for genetic POCT and facilitates genetic analysis, including genetic diagnosis in hospitals and health care facilities.

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