

**Original Research Article****Multi-detection by target mixed loop-mediated  
isothermal amplification****ABSTRACT**

Loop-mediated isothermal amplification (LAMP), a nucleic acid amplification technology, has been characterized as having a short reaction time and as generating large amounts of DNA products under isothermal conditions. We found that LAMP is able to amplify more than one target simultaneously using a mixture of HBV, HCV, and HIV primers, called target mixed LAMP. In this study we also used target mixed LAMP to successfully amplify GAPDH, gamma-actin, TBP, and haptoglobin cDNAs from the pooled cDNA derived from mouse tissues. These findings reveal the usefulness of LAMP for genetic diagnosis including diagnosis of infectious disease, and for conducting gene expression analysis.

*Keywords: LAMP; infectious disease; virus; diagnosis; magnesium pyrophosphate; DNA amplification*

## 1. INTRODUCTION

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method that can instantly amplify DNA or RNA under isothermal conditions [1,2]. The LAMP reaction can be accelerated by using additional primers, termed loop primers, resulting in the reaction times even less than those of the original LAMP [3]. Furthermore, since LAMP generates extremely large amount of DNA products, white precipitate of magnesium pyrophosphate is formed [4]. Its appearance in the reaction mixture can help detect whether the DNA is amplified or not. Since both amplification and detection can progress concurrently, LAMP is thought to be useful for a wide variety of fields, including basic research and clinical medicine. Moreover, single-strand DNA can be isolated from LAMP products; this feature is suitable for genetic analysis such as DNA microarray [5].

In clinical medicine, genetic diagnosis is used to monitor infectious diseases, such as virus and bacterium, and a nucleic acid amplification technology is usually used in these diagnoses. Multiplex nucleic acid amplification is a method by which more than one target sequence can be amplified by including more than one pair of primers in a single reaction, and is known to multiplex PCR and SDA as DNA amplification, and NASBA as RNA amplification [6-8]. Multiplex PCR is used in the diagnosis of infectious diseases [9], but it requires more than one hour of reaction, not to mention expensive machinery.

We have found that LAMP can be amplified with more than one target DNA using more than one pair of primers under isothermal conditions. The target mixed LAMP, including detection of the amplification, is simply achieved in a short time. In this paper, we report on the target mixed LAMP using three targets (HIV, HBV, and HCV) and also report on the amplification of four targets from mouse pooled cDNA.

## 2. MATERIAL AND METHODS

### 2.1 Template DNA

Human immunodeficiency virus-1 (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) plasmid DNAs were purchased from American type culture collection (ATCC numbers 53069, 39629 and 40679, respectively). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), gamma-actin (g-actin), TATA box binding protein (TBP), and haptoglobin cDNAs were cloned into a pBluescript KS (-) vector from mouse 17-day embryo marathon-ready cDNA (Clontech, Palo Alto, CA).

HBV-positive sera confirmed by the measurement of HBs antigen (HBsAg) using Lumispot 'Eiken' HBs-Ag (Eiken, Tokyo, Japan) were used in this experiment. HBV-positive DNA testing by transcription-mediated amplification (TMA) method was entrusted to SRL, Inc. Sera treated with equal volume of 50 mM NaOH and then incubated at 60°C for 10 min were used as templates of LAMP and PCR reaction.

### 2.2 LAMP reaction

LAMP reaction was performed using Loopamp DNA Amplification kit (Eiken, Tokyo, Japan), adding the primers, 0.25 µg/ml ethidium bromide (EtBr) and target DNA. The primers used for LAMP amplification are listed in Table 1. To analyze the target mixed LAMP from cDNA, LAMP products were amplified from mouse pooled embryo cDNA (Marathon-ready cDNA, Clontech). The mixture was incubated at 65°C for one hour and analyzed in real time using the ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, CA) [2].

For target mixed LAMP from HBsAg-positive serum, we used other HBV primers as described previously [1,3]. The reaction mixture containing one  $\mu$ l of the serum was incubated at 62°C for one hour. Primer concentrations were 1200 nM for HBVFIP and HBVBIP, 300 nM for HBVF3 and HBVB3, and 600 nM for loopF and loopB.

## 2.3 PCR reaction

PCR reaction was performed using Expand long template kit (Roche, Mannheim, Germany). HBV DNA was amplified using a primer pair, HBVF3 and HBVB3 used as the outer primer of LAMP, at 94 °C for 3 min, 35 cycles of 94 °C for 30 sec., 62 °C for 30 sec., and 68 °C for 30 sec., having in total time of approximately 1.5 hours.

## 2.4 Dot blot hybridization

The target mixed LAMP was performed using a 5' DIG-labeled loop F primer instead of the non-modified primer. The LAMP products were purified with a PCR purification kit (QIAGEN K.K., Tokyo, Japan). For the dot blot, one  $\mu$ l of the plasmid DNAs (100 ng) were spotted on the Biodyne B nylon membrane (Pall, Port Washington, NY), which was then used in a UV-cross link. The plasmid DNA of the negative control was pBluescript KS (-). The DIG-labeled LAMP products, as probes, were hybridized to the dot blot membrane. Hybridization was performed overnight in PerfectHyb buffer (TOYOBO, Osaka, Japan) at 60°C. Signals were detected using the DIG Detection kit (Roche, Mannheim, Germany) according to the manufacturer's protocol [5].

## 3. RESULTS

### 3.1 Normalization of the primer to the LAMP reaction

We performed the LAMP reaction using target-specific primers at a reaction temperature of 65°C. The concentrations of the primers were determined with the goal of normalizing the reaction time of each sample examined. The groups of samples used in this experiment were HBV, HCV and HIV, and GAPDH, g-actin, haptoglobin and TBP. The primer sequences and decided-upon concentrations are shown in Table 1.

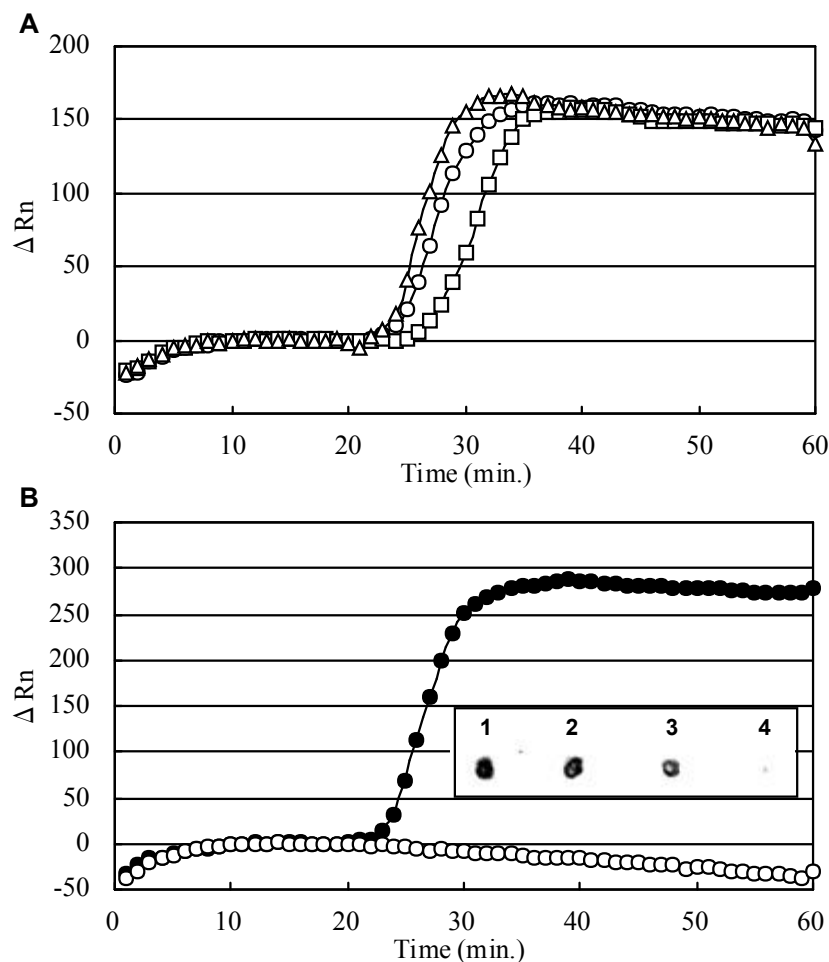
88 **Table 1. List of the primer sequences**

| primer name |        | 5'-primer sequence-3'                        | conc. (nM) |
|-------------|--------|----------------------------------------------|------------|
| HBV         | FIP    | TCAGGGCATATTGACCACAGTTGACTGCCGATTGGTGGAG     | 1300       |
|             | BIP    | CCGAATGCTCCCACTCCTACGGGACTTCAACCCCATCAAGGA   | 1300       |
|             | F3     | GGAGAGATGGGAGTAGGCT                          | 325        |
|             | B3     | TTCGGAGCCCACTCAAAC                           | 325        |
|             | loop F | GTCAACAATTCTCCTCCTGC                         | 650        |
|             | loop B | TGGTTGGCTGCTGGGCAGTGG                        | 650        |
| HCV         | FIP    | CCTTATAGCCCTGAGCTGCATACTCACCTCCATGCTCCACA    | 1200       |
|             | BIP    | TAGTACTCAACCCCTCTGTTGCATCCCATGAGCCTTGGACATG  | 1200       |
|             | F3     | TCTCCACCAGTAGTGCCC                           | 300        |
|             | B3     | CCCGGTCCTGATGTTAGG                           | 300        |
|             | loop F | CTTGGTGCTTTTGCCGCTGCC                        | 600        |
|             | loop B | TGCAACACTGGGCTTTGGTGC                        | 600        |
| HIV         | FIP    | AGCCCTCAGATGCTGCATATAATGGTCTAACCAGAGAGACCCAG | 800        |
|             | BIP    | GGAAAGTCCCCAGCGGAAAGCTGCATCCGGAGTACTTCAAG    | 800        |
|             | F3     | GAGCTCCCAGGCTCAAA                            | 200        |
|             | B3     | GCCTAGCATTTTCATCACGT                         | 200        |
|             | loop F | GCAGCTGCTTTTGCCTGTA                          | 400        |
|             | loop B | TTGTAGCAAGCTCGATGTCAGC                       | 400        |
|             |        |                                              |            |
| GAPDH       | FIP    | TGCATTGCTGACAATCTTGAGTGATGCCCCCATGTTTGTGAT   | 1600       |
|             | BIP    | CTGCACCACCAACTGCTTAGCCCTTCCACAATGCCAAAGT     | 1600       |
|             | F3     | AAACGGGTCATCATCTCCG                          | 400        |
|             | B3     | AGTGATGGCATGGACTGTG                          | 400        |
|             | loop F | GTTGTCATATTTCTCGTGGTTC                       | 800        |
|             | loop B | CTGGCCAAGGTCATCCAT                           | 800        |
| g-actin     | FIP    | TTGCCAATGGTGATCACCTGTGCTGCATCATCTTCCTCCTT    | 1200       |
|             | BIP    | GCACTCTTCCAGCCTTCCTTGGAGTTGAAAGTGGTCTCATGG   | 1200       |
|             | F3     | ATGTTGCCCTGGATTTTGAG                         | 300        |
|             | B3     | GCGGATATCCACATCACACT                         | 300        |
|             | loop F | TCGGGCAGCTCGTAACTCT                          | 600        |
|             | loop B | TGGGCATGGAGTCCTGTG                           | 600        |
| TBP         | FIP    | GCATTTCTTGACGAAGTGCTCTACCGTGAATCTTGGCTGT     | 1600       |
|             | BIP    | TAATCCCAAGCGATTTGCTGCAATCAACGCAGTTGTCCGTG    | 1600       |
|             | F3     | AGAGCTCTGGAATTGTACCG                         | 400        |
|             | B3     | CCTGTGCACACCATTTTTC                          | 400        |
|             | loop F | GCAATGGTCTTTAGGTCAAGT                        | 800        |
|             | loop B | CAGTCATCATGAGAATAAGAGAGC                     | 800        |
| Haptoglobin | FIP    | TTCCTGGTACTTGGTGAGGCGGGGTCAGCCTATCTTGAA      | 800        |
|             | BIP    | GCAGTGCCTTTGCCATTGTCAAAGCTCAGGATCCCA         | 800        |
|             | F3     | TGCCCAGAGAAGAAAACTTG                         | 200        |
|             | B3     | CACACCATACTCAGCGACAG                         | 200        |
|             | loop F | GCCAGCACAGAAGGTGTG                           | 400        |
|             | loop B | GAGGAGGACACCTGGTACGC                         | 400        |

### 3.2 Target mixed LAMP of a mixture of HBV, HCV, and HIV.

When three primer sets for HBV, HCV, and HIV were added to the reaction mixture, LAMP could amplify with each single target and thus each signal was detected at about 24 min (Figure 1A). Next, we tried to amplify with a mixture of the three targets. This result revealed that the reaction rate was the same as when a single target was amplified (Figure 1B). To

95 determine whether all target DNA had been amplified, we performed dot blot hybridization. LAMP products that had been  
 96 amplified with 5' DIG-labeled primers, as probes, were hybridized against each plasmid DNA spotted on the nylon  
 97 membrane, with the result that the probe hybridized with each spotted DNA except for the negative control (Figure 1B,  
 98 inset). These results demonstrated that the amplification of the three targets was coincident in a single tube and that  
 99 target mixed LAMP possesses high specificity against target nucleic acids.  
 100



101

102 **Fig. 1. Target mixed LAMP of HIV, HBV, and HCV.**

103 *A: Real-time monitoring of the LAMP amplification signals. A target mixed LAMP reaction was used for each template. The circle,*  
 104 *triangle, and square show the amplification of HIV, HBV and HCV, respectively. B: The closed circle shows the target mixed LAMP*  
 105 *reaction using three mixed templates. The open circle represents the negative control.  $\Delta Rn$  is the normalized emission at 615 nm. The*  
 106 *inset shows the analysis of dot blot hybridization. The DNA fragment was synthesized from the 5' DIG-labeled primer used as a*  
 107 *hybridization probe. Numbers indicate the position of each plasmid DNA spotted on the nylon membrane. 1: HBV, 2: HCV, 3: HIV, and*  
 108 *4: pBluescript plasmid DNA.*  
 109  
 110

### 111 3.3 Detection of HBV DNA from the infectious sera.

112 We next examined the detection of HBV DNA from the infected sera by target mixed LAMP. In this time, a pair of HBV  
 113 primers which recognize the HBs region was used, because this region was conserved in various subtypes. LAMP  
 114 amplification was occurred two of four sera (Table 2). In sample A, the value of TMA for the positive was 8.1 logarithm of  
 115 the genome equivalents (LGE)/mL and the negative was less than 3.7 LGE/mL which was the lower detection limit [10].

Comparison with TMA, PCR and multiplex LAMP revealed that sample B which could not be detected by TMA and PCR was able to be detected by the target mixed LAMP, so that the reaction efficiency of the LAMP was higher than TMA and PCR. Moreover, the total reaction time of PCR (1.5 hours) was longer than the LAMP (one hour) in this experiment. In addition, using HBV primer only as uniplex LAMP, same result was obtained as that of the target mixed LAMP, suggesting the mixture of the primer does not influence in LAMP reaction (data not shown). These result demonstrated that target mixed LAMP detected the targets from the infectious serum without delay of the reaction time and the efficiency.

**Table 2. HBV DNA amplification by TMA, PCR or target mixed LAMP from HBsAg-positive serum**

|      | sample A | sample B | sample C | sample D |
|------|----------|----------|----------|----------|
| TMA  | +        | -        | -        | -        |
| PCR  | +        | -        | -        | -        |
| LAMP | +        | +        | -        | -        |

### 3.4 Target mixed LAMP from mouse cDNA.

Four cDNAs, GAPDH, g-actin, haptoglobin, and TBP, were independently (using single primer) or multiplexly (using mixed primers) amplified from mouse pooled embryo cDNA (Figure 2). When using a single primer, the amplifications of GAPDH and g-actin were detected at 20 min and TBP and haptoglobin at 25 min (Figure 2A). This result reflects the difference of the expression levels of cDNA, and thus GAPDH and g-actin are more highly expressed than TBP and haptoglobin. These results were concurrent with the results of real time PCR (data not shown).

In target mixed LAMP, the signal detection was 24 min using mixed primers (Figure 2B). The specificity of the products was confirmed by dot blot hybridization, resulting in that all cDNA was amplified (Figure 2B, inset). However, the amplification level of each cDNA was unknown because target mixed LAMP products cannot be shown separately.

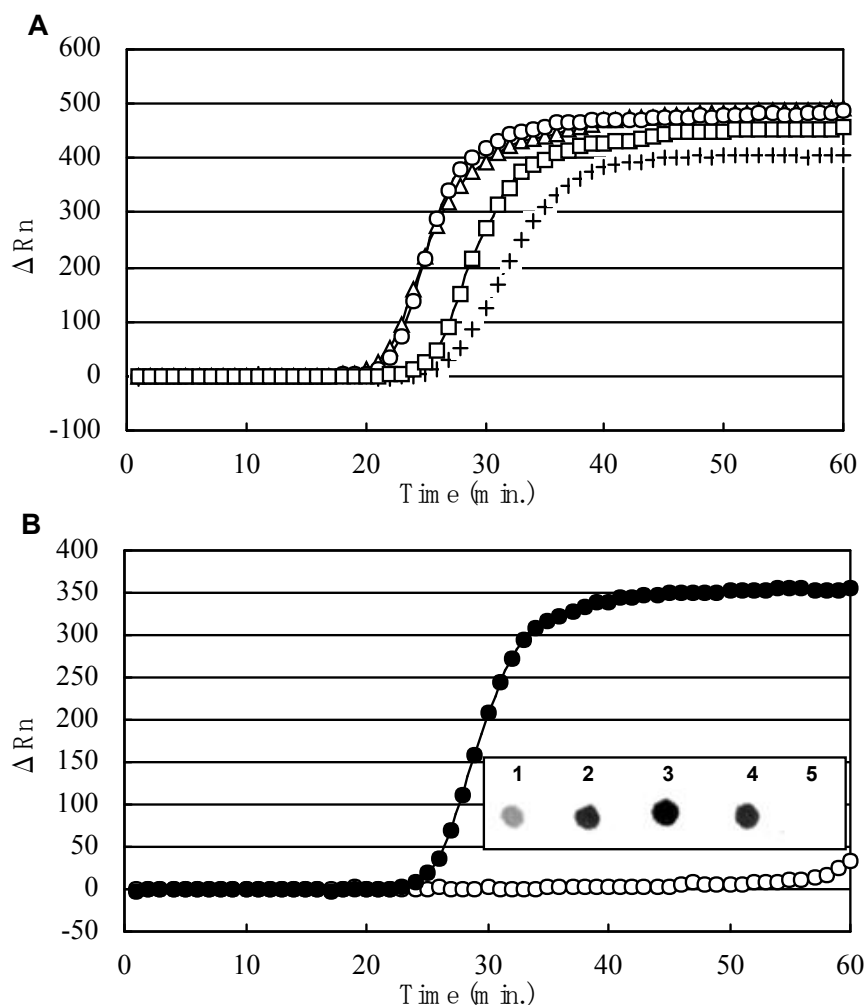


**Fig. 2. Target mixed LAMP of GAPDH, g-actin, haptoglobin, and TBP.**

*A: Real-time monitoring of LAMP amplification signals. LAMP was performed using a single primer from pooled cDNA. The circle, triangle, plus, and square show the amplification of g-actin, GAPDH, TBP, and haptoglobin, respectively. B: Target mixed LAMP reaction. LAMP was performed using mixed primers from pooled cDNA.  $\Delta R_n$  is the normalized emission at 615 nm. The inset shows the analysis of dot blot hybridization. 1: TBP, 2: GAPDH, 3: g-actin, 4: haptoglobin, and 5: pBluescript plasmid DNA.*

#### 4. DISCUSSION

To determine whether a patient is infected with viruses such as HBV, HCV, or HIV, it is not necessary to synchronize with each reaction rate although the primers concentration was prepared in such a way for this experiment. In screening for infection, it is important to detect whether the patients are infected with more than one of HBV, HCV, or HIV. In such a case, it is desirable for each primer to design the conditions by which amplification is performed as soon as possible. We have amplified with HBV DNA prepared from sera [2], but HCV and HIV prepared from sera have not yet amplified. Although RNA must be detected in case of HCV and HIV, the LAMP reaction is able to amplify RNA with the addition of reverse transcriptase [1]. In fact, reverse transcription LAMP was used to detection of RNA virus [11-13]. Our findings suggested that target mixed LAMP can be performed using not only viral DNA but also the RNA prepared from sera. In this study there were two HBsAg-positive sera without DNA amplification. Since the primer used for HBV DNA

amplification from sera by LAMP could detect the level of six copies of the DNA [1], these two sera would contain levels of HBV DNA that were below the detection limit.

To perform target mixed LAMP from cDNA, the first step is to change primer concentration in order to normalize the amplification efficiency. The LAMP reaction is continued until the decay of dNTPs as substrates. If there are divergences in the reaction rates of samples, it is thought that the more highly expressed DNA is dominantly amplified. Therefore, the reaction rates must be synchronized when the expression levels of cDNA is analyzed. In the amplification using each cloned plasmid DNA as a template of which the copy number was known,  $10^5$  molecules of the target DNA could be amplified at the same reaction rate and thus all signals were detected at 22 min (data not shown). This result suggested that GAPDH and g-actin, and TBP and haptoglobin were presented in quantities greater than  $10^5$  molecules and less than  $10^5$  molecules, respectively, in one  $\mu$ l of mouse pooled embryo cDNA.

A number of primer sets seemed to be limited in LAMP reaction, because six primers need to amplify one target and the concentration of the primers becomes too high in a single reaction tube. We consider that at least four test items be measured in blood screening in genetic diagnosis; for example, three test items and an internal control. Walker G.T. *et al.* reported that multiplex SDA could be amplified by a single pair of primers after appending them to the end of target fragments [7]. In addition, we found that eight targets cloned into plasmids with a common priming sequence, could also be amplified by a single pair of amplification primers in target mixed LAMP (data not shown).

The detection of viruses and bacteria has been performed by multiplex PCR in the clinical field [9]. Multiplex PCR does, however, entail expensive machinery and its detection could also require expensive reagents such as Taqman probe [14]. Furthermore it takes some time from amplification until the result is obtained. On the other hand, LAMP is able to achieve a simple amplification in a short time under isothermal conditions and a simple detection using turbidity of magnesium pyrophosphate [4,15]. It is possible to be endpoint detection using a fluorescent metal indicator such as calcein in clinical practice [16].

## 5. CONCLUSION

In this study, we succeeded in amplification with more than one target DNA using more than one pair of primers by LAMP. The target mixed LAMP is thought to be useful for the genetic diagnosis including diagnosis of infectious disease. Especially, we propose the tool for first screening of HBV, HCV, and HIV virus infection.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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