Loop-mediated Isothermal Amplification Reaction Using a Nondenatured Template

To the Editor:

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions *(1)*. The LAMP method requires a set of four specially designed primers and a DNA polymerase with strand displacement activity. The amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops, yielding >500 μg/mL. Although LAMP amplifies DNA under isothermal conditions, the template DNA is heat-denatured. To determine whether LAMP can be performed under isothermal conditions at all steps, we attempted to amplify DNA without using a heat-denatured template.

Hepatitis B virus (HBV) DNA that was obtained from a patient was digested with *Bam*HI and cloned into pBR322 plasmid vector. Plasmid and genomic DNA was prepared using the plasmid Midi reagent set (Qiagen) and EXTRAGEN reagent set (Tosoh), respectively. LAMP amplification of HBV DNA was performed with a pair of primers as described previously *(1)*. The DNA was amplified in a 25-μL reaction mixture containing 1.6 μM each of FIP and BIP, 0.2 μM each of the outer primers, 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 $MgSO_4$, 1.0 mL/L Triton X-100, 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs), and 0.25 mg/L ethidium bromide. Denaturation of plasmid DNA (0.2 ng/mL in Tris-EDTA, pH 7.5) was performed at 95 °C for 5 min followed quickly by placement on ice for 5 min.

Using 0.7% agarose gel electrophoresis, we confirmed that a double-stranded plasmid DNA was prepared by the Plasmid Midi reagent set and that the plasmid was heat-denatured (data not shown). The mixture was incubated at 60 °C for 1 h and analyzed using the ABI PRISM 7700 sequence detection system (Perkin-Elmer Biosystems) *(2)*. This system measures the increase in the fluorescent intensity of ethidium bromide (bound to the amplified DNA) by use of the ROX fluorescence channel. No internal control was used.

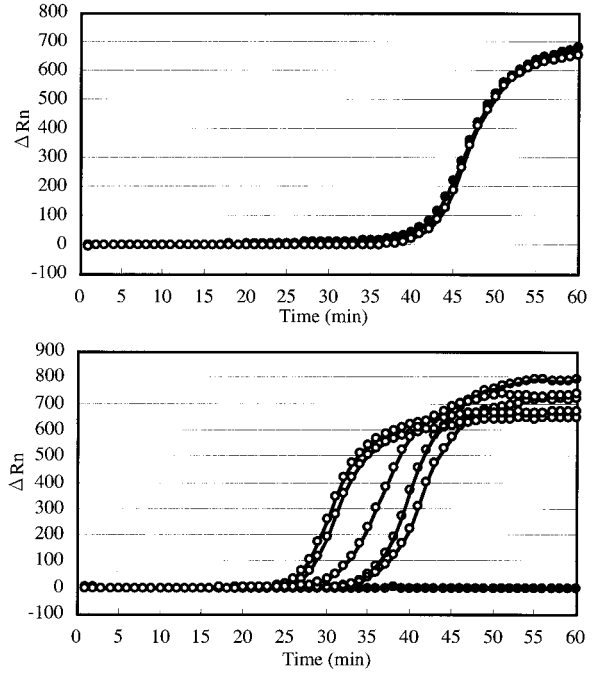

Fig. 1. Detection of HBV DNA by LAMP method.

(*Top panel*), ○ and ● show LAMP reaction using nondenatured and denatured plasmid DNA templates (10^3 molecules), respectively. (*Bottom panel*), ○ and ● show HBV-positive and noninfective DNA, respectively. In each sample, the signal reached a plateau after a few minutes, presumably because free ethidium bromide was depleted by binding to amplified DNA. We confirmed that the amount of DNA increased after the signal reached a plateau (data not shown). Δ*Rn*, normalized emission at 615 nm.

A pair of inner primers was used to amplify denatured and nondenatured double-stranded plasmid DNAs, including the HBV subtype adr sequence *(3)*, and the DNA products were scanned using the ABI PRISM 7700. The scanning data indicated that the signal was detected in both samples after 40 min (Fig. 1, top panel). This result suggests that the LAMP reaction does not require denatured DNA template.

We performed LAMP reactions using genomic DNAs extracted from five HBV DNA-positive serum samples in which the initial copy number was unknown. When we used nondenatured DNA corresponding to 4 μL of serum as template, LAMP amplification was able to detect signals after 25–35 min in five individuals (Fig. 1, bottom panel). This result revealed that the presence of HBV virus can be detected within 1 h from a nondenatured sample.

HBV is not typical because it has double- and single-stranded segments. In separate experiments, however, LAMP was performed successfully without heat denaturation for template DNAs, such as λ DNA, pBluescript II, and M13 mp18 vector DNA, and human genomic DNA (*SRY* gene on chromosome Y), including commercially available material (data not shown). Some of the double-stranded DNA seems to become single-stranded at high temperatures in the presence of high concentrations of betaine, a reagent that facilitates DNA strand separation because it isostabilizes DNA *(4)*. The exact mechanism, however, is unknown.

Because there is no necessity for heat denaturation of the template DNAs, LAMP could be used more easily and rapidly in clinical medicine.

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High-Sensitivity C-Reactive Protein: Product Claims and the Food and Drug Administration

To the Editor:

Several articles in recent issues of *Clinical Chemistry* have made reference to the Dade Behring N *High Sensitivity* CRP (hs-CRP) assay and have indicated that it has been approved by the Food and Drug Administration (FDA) for cardiovascular risk prediction *(1–3)*.

Although the use of hs-CRP assays in the assessment of cardiovascular risk is clearly gaining momentum, the FDA-cleared intended use for these tests is not cardiovascular risk prediction, but rather the quantitative determination of C-reactive protein (CRP) in serum and plasma. Measurements are useful in the detection and evaluation of infection, tissue injury, inflammatory disorders, and associated diseases. The Summary and Explanation section of the FDA-cleared labeling for the Dade Behring hs-CRP assay states that the test may add to the predictive value of other markers used to assess the risk of cardiovascular and peripheral vascular disease *(4)*. I am unaware of any CRP assay being cleared by the FDA for stand-alone use to predict risk of cardiovascular disease.

As a class II in vitro diagnostic medical device, the Dade Behring N *High Sensitivity* CRP assay referenced in these articles is not "approved" by the FDA per se, nor is premarket approval required. Instead, as with most in vitro diagnostic tests, marketing clearance for the N *High Sensitivity* CRP assay was obtained by way of the premarket notification process described in section 510(k) of the Food Drug and Cosmetic Act. This distinction is important to manufacturers inasmuch as FDA regulations state that, "any representation that creates an impression of official approval of a device because of complying with the premarket notification regulations is misleading and constitutes misbranding" *(5)*. To prevent publication of misleading information, I urge the *Clinical Chemistry* editorial staff to exercise specific caution in working with authors to appropriately identify the regulatory status of in vitro diagnostic medical devices.

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Comment on "High-Sensitivity C-Reactive Protein: Product Claims and the Food and Drug Administration"

To the Editor:

Dr. Ayash's letter is helpful in trying to clarify the regulatory terminology pertinent to Food and Drug Administration (FDA) marketing applications. It should be pointed out, however, that it is not, and should not be the responsibility of journal reviewers or editors to police the accuracy of claims statements from a regulatory standpoint. The FDA employs compliance officers who monitor advertised claims by manufacturers to ensure that they are consistent with the approved or cleared labeling for their products. Clinicians and researchers are not subject to the same claims restrictions as manufacturers.

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RE: Europium Nanoparticles and Time-resolved Fluorescence for Ultrasensitive Detection of Prostate-specific Antigen

To the Editor:

We were intrigued to read of the work reported by Harma et al. *(1)*. Their report provides an excellent summary of the potential advantages and disadvantages of implementing an assay that uses small particles containing highly fluorescent rare earth complexes.

In the 1970s, we evaluated the use of reactive latex particles loaded with rare earth chelates. Europium chelates were of particular interest because of their characteristic strong red emission bands and the extended time course of emission following a pulse of excitation light. Although we ultimately chose to pursue assays using different labels, the work was documented in two US patents *(2, 3)*, which may interest readers.