A Fluorescent Confirmation Method for DNA Amplification in PCR through a Fluorescent Pyrophosphate Sensor

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Pyrophosphate (PPi) is involved in several important enzymatic reactions such as the adenylate cyclase catalyzing synthesis of cyclic AMP from ATP, the attachment of a given amino acid to a particular tRNA catalyzed by an aminoacyl-tRNA synthetase in protein synthesis, and the DNA replication catalyzed by a DNA polymerase.[1,2] In addition, patients with calcium PPi dihydrate (CPPD) crystals and chondrocalcinosis have been shown to have high synovial fluid PPi levels.[3] Therefore, the selective and sensitive detection of PPi is a prerequisite for monitoring the above enzyme reactions and for diagnosing the diseases related to the release of certain levels of PPi.

The most common way to confirm the target nucleic acids amplified after PCR (polymerase chain reaction) is by running a gel electrophoresis. Chemical and enzymatic assays have been developed to verify PCR results.[4-7] However, these methods may be too slow and laborious for quick confirmation of DNA amplification in PCR. PPi detection methods based on the formation of visible precipitates with transition metal ions are insufficiently precise.[8] The colorimetric detection for PPi by 1,10-phenanthroline complex with ferrous ion needs incubation at high temperature.[9]

Recently, we developed a fluorescent sensor (**1**·2Zn, Figure 1), which showed a high sensitivity and selectivity for PPi over other anions including nucleotide triphosphates in aqueous solvent.[10] We expected that our sensor can be used as a new fluorescent confirmation method for DNA amplification after PCR, because the sensor can detect a small amount of PPi in the presence of a large excess of ATP.[10]

In this paper, we present a fluorescence method that can determine PPi released during PCR through **1**·2Zn sensor (**1**·2Zn, Figure 1). We expect that the degree of DNA polymerization can be determined by the concentration of PPi released during PCR.

The PPi released from the polymerization reaction bound to the dinuclear zinc complex of the **1**·2Zn sensor (see the Experimental Section for details). Consequently, PPi induced a pronounced red shift of the λ_{max} of **1**·2Zn because the weakening of the bond between the phenolate oxygen and Zn^{2+} induced a more negative charge characteristic on the phenolate oxygen and thereby caused a bathochromic shift of λ_{max} of **1**·2Zn. Therefore, an increased charge characteristic on the phenolate oxygen of **1**·2Zn induced a fluorescent enhancement (Figure 1).

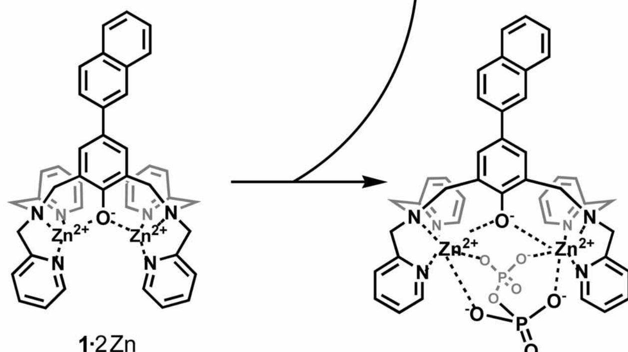

Figure 1. Detection method for DNA amplification in PCR.

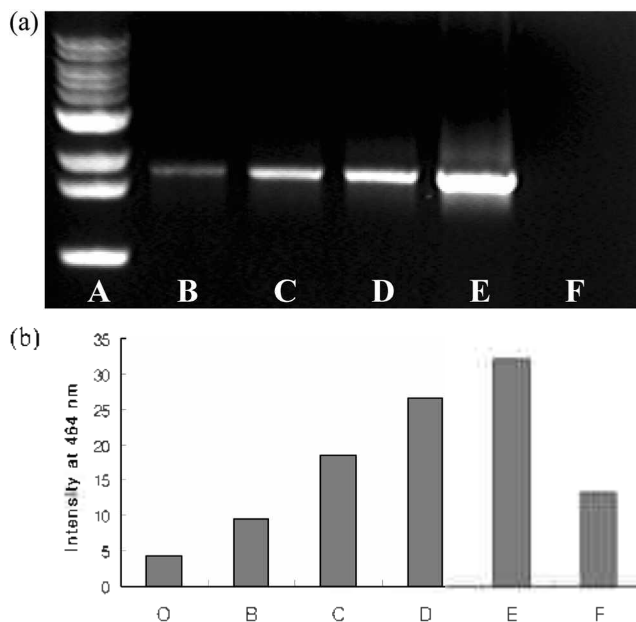

Figure 2. (a) Gel electrophoresis of finished PCR mixtures. (b) Fluorescence intensity of **1**·2Zn sensor (5 μM) at 464 nm upon addition of the finished PCR product mixture in 10 mM HEPES buffer (pH 7.4) at 25 °C: (A) Standard bp ladder, (O) 0 μL (B) 1 μL, (C) 3 μL, (D) 5 μL, (E) 9 μL finished PCR product mixture performed with template DNA and (F) 9 μL finished PCR product mixture performed without template DNA.

Compared with the previous systems, the system we propose here not only enables instant and accurate confirmation but is also an inexpensive way to examine the PCR outcome.

Figure 2 shows the results of gel electrophoresis on the finished PCR mixture (Figure 2a) and the fluorescence intensity at 464 nm for the **1**·2Zn sensor upon the addition of the finished PCR mixture (Figure 2b). Upon the addition of sample E (9 μL of the finished PCR mixture), in which the PCR product was obtained in the presence of template DNA, in an aqueous solution of **1**·2Zn sensor, the fluorescence emission spectrum showed a 7.4-fold enhancement relative to sample O (no PCR mixture). The fluorescence intensity of each DNA band from gel electrophoresis was calculated using fluorescence scanner and compared with that of **1**·2Zn sensor upon the addition of the PCR mixture. In samples B, C, D and E, gel electrophoresis and fluorescence of **1**·2Zn showed the same trend. The signal change of each experiment in B, C, D and E was almost linear, which confirmed our hypothesis that the extent of fluorescence change of sensor **1**·2Zn was not only proportional to the amount of PPi generated from PCR but also DNA amplified.

However, when PCR reaction was performed in the absence of template DNA, as in sample F, the fluorescence was slightly enhanced while the gel electrophoresis showed no significant change. This discordance between the two experimental results arose from the property of the **1**·2Zn sensor, which showed slight fluorescence enhancement in the presence of dNTP. However, this was not considered to be a serious problem since it can easily be overcome through a simple mathematical correction.

This PPi detection method enabled the confirmation of DNA amplification after PCR by the addition of **1**·2Zn to the finished PCR mixture. The PPi released from this polymerization reaction, which bound to the dinuclear zinc complex of the **1**·2Zn sensor, was detected by fluorescence enhancement. This method is expected to enable the simple and quick assessment of the PCR product.

Experimental Section

Template DNA and Primer. *Rattus norvegicus purinergic receptor P2X* was used as the target for PCR and 5'-GAA GGA GTT CCC CTG AGA ATT CG-3' as the primer. The PCR mixture (50 μL) contained template (about 5 ng), primer (2 pM), and 250 μM each of dNTP and buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% v/v Triton X-100). The thermal cycling program with Mastercycler began at 94 °C for 5 min, followed by the addition of 1 unit *Taq* DNA polymerase. The reaction was then run for 40 cycles comprising 1 min denaturation at 94 °C, 1 min annealing at 58 °C, 1 min extension at 72 °C and a final extension at 72 °C for 5 min.

Fluorescence Measurement. The standard procedure for fluorescent confirmation is as follows. To each finished PCR product solution was added 5 μL, 0.3 mM of **1**·2Zn sensor. This solution mixture was diluted to 300 μL with 10 mM HEPES buffer (pH 7.4). No incubation time or temperature change for fluorescence measurement was needed. The fluorescence change was measured at 464 nm and gel electrophoresis was accompanied to compare the existence of amplified DNA bands and corresponding fluorescence changes.

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