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A simple, sensitive, and label-free assay for alkaline phosphatase activity based on target-promoted exponential strand displacement amplification

Chang Yeol Lee [a], Ki Soo Park [b,*], Hyun Gyu Park [a,*]

[a] Department of Chemical and Biomolecular Engineering (BK21 + Program), KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon, 34141, Republic of Korea
[b] Department of Biological Engineering, College of Engineering, Konkuk University, Seoul, 05029, Republic of Korea

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ABSTRACT

We herein describe a simple, sensitive, and label-free strategy for alkaline phosphatase (ALP) activity based on target-promoted exponential strand displacement amplification (eSDA). The developed system utilizes 3′-phosphorylated DNA probes whose phosphate group at the 3′-end blocks the extension by DNA polymerase. Only in the presence of ALP, the phosphate group at the 3′-end is transformed to the hydroxyl group, which enables the extension by DNA polymerase and produces the double-stranded recognition sequence for nicking endonuclease. Consequently, the effective eSDA is executed, generating a large number of duplex products with the high fluorescence signal from SYBR green I. Based on this novel strategy, we determined the ALP activity down to 0.47 U/L with the high selectivity. In addition, the practical applicability of this assaying system was successfully demonstrated by reliably determining the ALP activity in human serum.

1. Introduction

Alkaline phosphatase (ALP) that catalyzes the dephosphorylation of biomolecules including nucleic acids, proteins, and small molecules, plays important roles in phosphate metabolism [1–3]. The abnormal level of ALP is also associated with various diseases such as hepatobiliary diseases, bone diseases, breast and prostatic cancers, and diabetes [4–6]. Due to its biological and clinical significance, numerous methods have been devised to assay the ALP activity, including chemiluminescence [7], electrochemiluminescence [8], surface enhanced Raman scattering [9], electrochemistry [10,11], colorimetry [12,13], and fluorometry [14–19]. Among these, the fluorescent methods have garnered considerable research attention due to their simplicity, reliability, and sensitivity. However, the developed methods cannot be widely utilized in real applications due to the shortcomings such as the expensive modification with fluorophore or quencher, synthesis of nanomaterials or chemicals, long assay time, and low sensitivity (Table S1). Thus, it is necessary to develop a novel and efficient fluorescent strategy for the ALP activity assay.

As a compelling alternative, we herein developed a simple, sensitive, and label-free method for the determination of ALP activity. This approach utilizes specially designed DNA probes whose 3′-ends are phosphorylated, in combination with an exponential strand displacement amplification (eSDA) for the highly sensitive detection. We envisioned that the 3′-phosphorylated DNA probes that are initially resistant to the extension by DNA polymerase, would be activated for DNA polymerase-mediated extension by the ALP-catalyzed dephosphorylation. This would subsequently promote eSDA, producing a large number of duplexes at a constant temperature for a short time through repeated cycles composed of cleavage, extension, and displacement catalyzed by DNA polymerase and nicking endonuclease [20–22]. As a result, the high fluorescence would be generated from double-stranded (ds) DNA specific fluorescent dyes, SYBR green I. With this novel strategy, we successfully determined the ALP activity with the high selectivity and sensitivity and verified the clinical capability by analyzing the ALP activity in human blood serum.

2. Materials and methods

2.1. Materials

All DNA strands used in the study were synthesized from Genotech Co. (Daejeon, South Korea) and purified by PAGE. The

* Corresponding authors.
E-mail addresses: akdong486@konkuk.ac.kr (K.S. Park), hgpark@kaist.ac.kr (H.G. Park).

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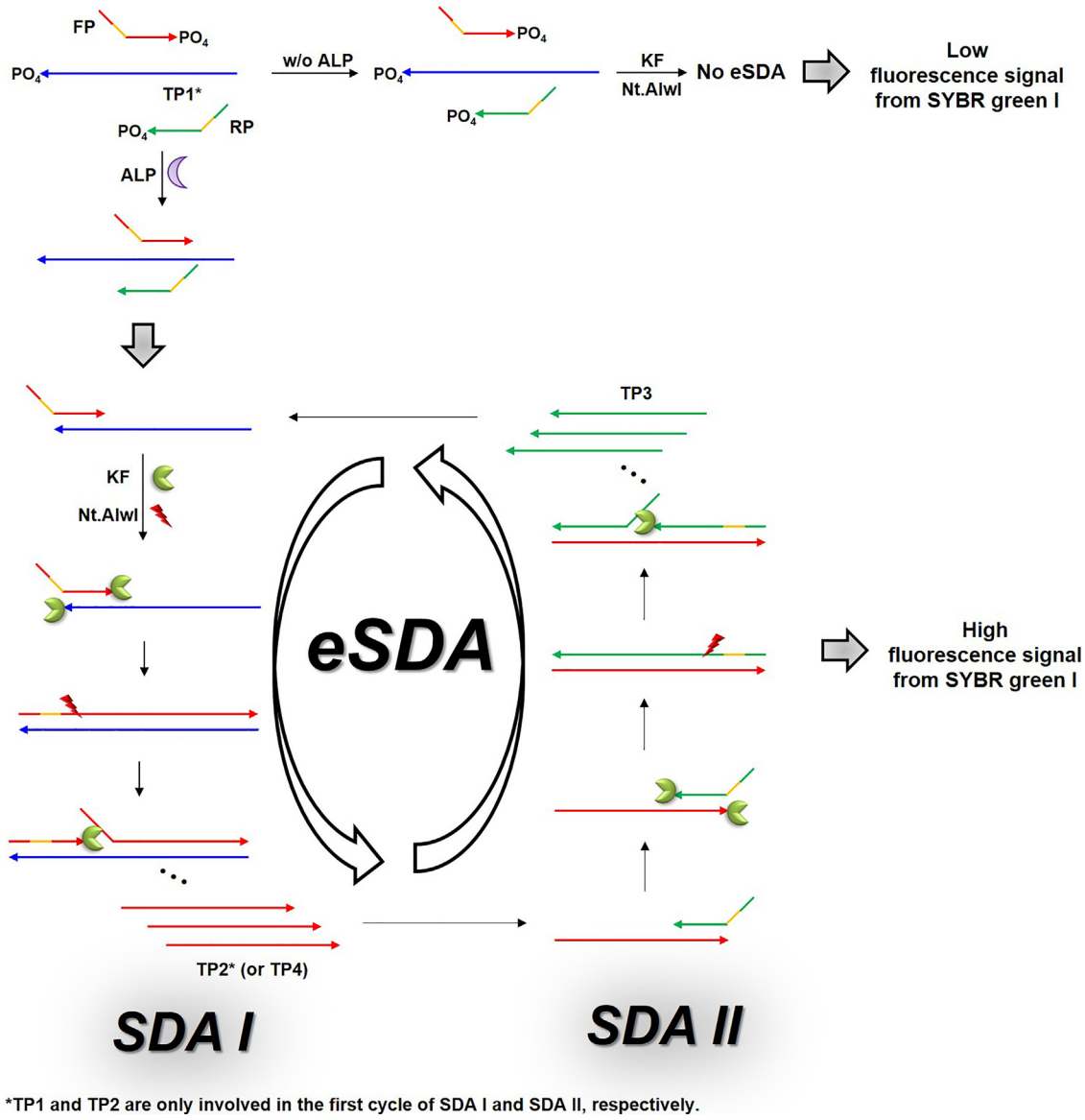

Scheme 1. Schematic illustration of the ALP activity assay based on the target-promoted eSDA. The orange color in FP and RP indicates the single-stranded recognition sequence for nicking endonuclease (Nt.AlwI) and the arrow shows the 3′-end of all DNA probes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sequences of all DNAs are listed in Table S2. Klenow fragment (3′→5′ exo-) (KF), Nt.AlwI, shrimp alkaline phosphatase (ALP), BSA, exonuclease I (Exo I), exonuclease III (Exo III), and uracil DNA glycosylase (UDG) were purchased from New England Biolabs Inc. (Beverly, MA, USA). Lysozyme (Lys) and SYBR green I were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade and used without further purification. Ultrapure DNase/RNase-free distilled water (DW) purchased from Bioneer® (Daejeon, Korea) was used in all experiments.

2.2. Procedure for ALP activity assay

10 μL of DW, 2 μL of FP (2 μM), 2 μL of RP (2 μM), 2 μL of TP1 (2 μM), 2 μL of 10X NEBuffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9 at 1X concentration) and 2 μL of ALP at varying concentrations or other enzymes and protein were mixed to prepare ALP reaction mixtures of 20 μL. The reaction mixtures were then incubated at 37 °C for 1 h, followed by the inactivation of ALP at 65 °C for 5 min. Next, to carry out eSDA, 6 μL of DW, 2 μL of 10X NEBuffer 2, 2 μL of dNTP (2.5 mM), 2 μL of 40X SYBR green I, 4 μL of KF (400 U/mL), and 4 μL of Nt.AlwI (1200 U/mL) were added to the ALP reaction products, making a total volume of 40 μL. The resulting reaction mixtures were then incubated at 37 °C for 15 min, followed by the fluorescence measurement in the range from 510 to 700 nm at an excitation wavelength of 480 nm.

2.3. Gel electrophoresis analysis of ALP activity assay products

The reaction products were resolved on a 16% polyacrylamide gel using 1X TBE as a running buffer at a constant voltage of 120 V for 110 min. After staining with EtBr, a gel image was taken with a UV transilluminator.

2.4. ALP activity assay in human serum (1%)

In order to prevent the interference from human serum proteins, a solution of human serum diluted with NEBuffer 2 was loaded into a centrifugal filter device (MWCO = 100,000 Da, Millipore). The sample was then subjected to the centrifugation at 8000*g*-force for 20 min [23,24]. Finally, ALP was spiked into the filtered human