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Sensitive detection of transcription factors in cell nuclear extracts by using a molecular beacons based amplification strategy

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ABSTRACT

Monitoring transcription factor (TF) levels provides an important assessment of the state of cell populations. Unfortunately, traditional methods for monitoring TF concentration are generally cumbersome and time-consuming. We developed an ultrasensitive one-pot TF detection method that uses target-molecular beacons-dependent amplification (TMDA) fluorescence strategy to circumvent the aforementioned limitations in TF detection. In this assay, we employed a DNA1/DNA2 duplex as the reporting probe and a stem-loop DNA molecular beacon (MB) as the signaling probe. The integration of protein-DNA1/DNA2 duplex and exonuclease III (Exo III) digestion can convert the detection of transcription factors to the detection of reporter oligonucleotides. The subsequent hybridization of the reporter oligonucleotides with the molecular beacons opens the stem-loop structure. The formation of the DNA complex triggers amplification reaction and the recovery of the fluorescence. This assay exhibits high sensitivity with a detection limit of 2.2 pM and a detection range of 3 orders of magnitude, which is superior to most currently used methods for transcription factor detection. More importantly, this method is suitable for the direct detection of TFs in crude nuclear extracts of cancer cells.

1. Introduction

A molecular beacon is a hairpin-shaped single-stranded oligonucleotide which is labeled with a fluorophore at one end and a quencher at the other end. Molecular beacons are a novel class of probes that adopt stem-and-loop structure in solution. The loop portion of molecular beacon is a probe sequence that is complementary to a target sequence. The stem of molecular beacon holds the fluorophore and quencher close to each other, quenching the fluorescence of the fluorophore. In the presence of target nucleic acids, the beacon molecule goes through a conformational change from the hairpin shape to a more rigid, rod-like double helix during the hybridization, and the two arms are forced to move away from each other. As a result, the fluorophore and quencher are separated from each other, restoring the fluorescence. Thus, an increase in the fluorescence intensity of molecular beacons reports directly the presence of target nucleic acid (Duan et al., 2013; Zuo et al., 2010).

In recent years, the applications of molecular beacons can be combined with target amplification technique. The most commonly used target amplification technique is polymerase chain reaction (PCR). By using this strategy, gene mutation and single nucleotide polymorphism (SNP) have been characterized (Marras et al., 1999, 2003; Piatek et al., 1998; Tapp et al., 2000). On the other hand, by synthesizing beacons with different fluorophores, real-time PCR can be conducted in multiplex formats so that multiple genes can be quantified in the same tube (Templeton et al., 2004; Varma-Basil et al., 2004). Besides PCR, other techniques, such as nucleic acids sequence-based amplification (NASBA), have also been used to amplify the targets (Ayele et al., 2004; Leone et al., 1998). These methods shown that molecular beacons hold great potential as excellent probes for nucleic acids. It is interesting to notice that, although molecular beacons were originally developed to detect nucleic acids, more molecular beacons employed amplification method were used in protein detection (Fang et al., 2000; Li et al., 2000). Such molecular beacons-assisted amplification method, with its inherent stability, specificity, and simplicity, has recently emerged as a potential amplification technique for rapid and cost-effective detection of protein. On the other hands, several promising isothermal signal amplification strategies also have been introduced for the protein assay (Hao et al., 2014; Zhang et al., 2014), such as telomeric repeat amplification protocol (TRAP) (Liu et al., 2015) and nicking enzyme signaling amplification (NESA). Recently, several methods devised utilizing the advantages of molecular beacon and the isothermal exponential amplification reaction were proposed (Duan et al.,

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2013). Unfortunately, these methods are limited by their unsatisfactory sensitivity, lengthy assay time, or complicated design.

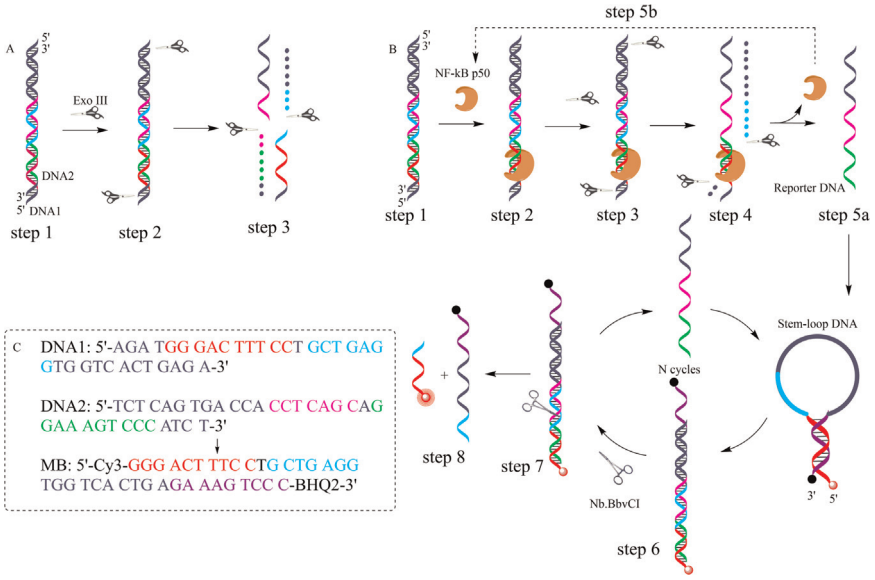
Transcription factors (TFs) are a class of DNA-binding proteins that modulate the flow of genetic information from DNA to RNA (Aboussekhra et al., 1995; Ren et al., 2000). Owing to the pivotal role of TFs in gene expression as well as its close relationship with human diseases, the accurate measurement of transcription factors is of great importance to both medical diagnosis and biological research (Helin, 1998). Recent years have seen the development of a broad class of biosensors for the assay TFs (Bonham et al., 2012; Vallée-Bélisle et al., 2011; Yin et al., 2014; Zhang et al., 2012). Unfortunately, these methods for monitoring TF concentration or binding activity are generally cumbersome and time-consuming. For example, enzyme-linked immunosorbent assay (ELISA) is multistep techniques that require specific antibodies against each new protein target (Renard et al., 2001). Alternative methods have been developed for *in vivo* and *in vitro* microcantilever-based assays (Huber et al., 2006), surface plasmon resonance imaging (Smith et al., 2003), and electrochemical impedance spectroscopy detection (Bogomolova et al., 2009). These newer strategies, however, are have their own drawbacks: the former require extensive modification of the cells and the latter require extensive sample processing due to their difficulty functioning in complex media. Therefore, the development of robust methods for simple, cost-effective, and sensitive detection of DNA-binding proteins, especially the transcription factors, is highly desirable. Alternatively, fluorescence-based methods have been employed for the detection of TFs in homogeneous solution which allows direct measurement of TFs in solution (Xu et al., 2008). In a typical fluorescence assay, the presence of TF leads to the formation of DNA duplexes results in a high fluorescence resonance energy transfer (FRET) signal. However, the protein-DNA binding might induce steric hindrance, leading to a low FRET signal (Bonnet et al., 1999). Therefore, the development of new methods for sensitive and selective detection of transcription factors is highly desirable.

In the present work, we develop an ultrasensitive one-pot transcription factors detection scheme that uses target-molecular beacons-dependent amplification (TMDA) fluorescence assay by integrating protein-DNA interaction, exonuclease III (Exo III) digestion, endonuclease (Nb.BbvCI) and isothermal exponential amplification. In our strategy, the integration of protein-DNA and Exo III digestion can convert the detection of transcription factors to the detection of reporter oligonucleotides. The subsequent hybridization of the reporter oligonucleotides with the molecular beacons (MBs) opens the stem-loop structure. The formation of the DNA complex triggers amplification reaction and the recovery of the fluorescence. More importantly, our method is suitable for the direct detection of TFs in crude cellular extracts of cancer cells.

2. Experimental section

2.1. Materials and chemicals

All oligonucleotides were purchased from Genscript Biotechnology Co., Ltd. (Nanjing, China) and their sequences were listed in Scheme 1C. To obtain the DNA duplexes, the oligonucleotides were mixed at the same molar ratios with the final concentration of 10 μM in the hybridization buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA). The mixture was heated at 95 °C for 5 min and then slowly cooled to 25 °C for about 4 h. The purified recombinant NF-κB p50 (rhNF-κB p50) was purchased from Promega (Madison, WI). Exonuclease III (Exo III) and Nb.BbvCI nicking endonuclease were obtained from New England Biolabs (Ipswich, MA). Other chemicals were of reagent grade and were used without further purification. Solutions were prepared with ultrapure water, which was purified with Milli-Q purification system (Branstead) to a specific resistivity of > 18.2 MΩ cm.



Scheme 1. Schematic diagram showing the principle of TMDA fluorescence assay for the detection of NF-κB p50. (A) In the absence of NF-κB p50, DNA1 and DNA2 were both digested by Exo III at the 3′ terminus (step 2 and step 3). (B) In the presence of NF-κB p50, Exo III digestion cannot proceed past the binding site (step 2 and step 3), and the DNA2 is protected from the digestion and thus part preserved (step 4). The released transcription factor then combined with another DNA1/DNA2 duplex (step 5) to protect the digestion of reporter DNA (part of DNA2). Then the reporter DNA hybridized with the molecular beacons and opened the stem-loop structure (step 6), which broke the FRET between the fluorophore (Cy3) and the quencher (BHQ2), and recovered the fluorescence (step 7). The formation of the DNA complex triggers the selective enzymatic cleavage of the DNA2-DNA3 duplex by Nb.BbvCI, resulting in the release of the reporter DNA and the recovery of the fluorescence (step 8). The released reporter DNA then hybridizes with another molecular beacon to initiate the cleavage of the molecular beacon, liberating the Cy3-DNA-segment and the release of BHQ2-DNA-segment. (C) DNA oligonucleotides sequence used in this strategy. The colors of the sequences are the same as given in part A and part B of Scheme 1. The arrow marked the cleavage point of Nb.BbvCI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)