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A new method for sensitive detection of microphthalmia-associated transcription factor based on “OFF-state” and “ON-state” equilibrium of a well-designed probe and duplex-specific nuclease signal amplification

Kai Zhang [a,*], Ke Wang [a], Xue Zhu [a], Minhao Xie [a], Xiaolu Zhang [b,*]

[a] Key Laboratory of Nuclear Medicine, Ministry of Health, Jiangsu Key Laboratory of Molecular Nuclear Medicine, Jiangsu Institute of Nuclear Medicine, Wuxi, Jiangsu 214063, China
[b] Department of Neurosurgery, Wuxi People's Hospital affiliated with Nanjing Medical University, Wuxi, Jiangsu 214000, China

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ABSTRACT

Herein, we report a new method which the “stage change” of DNA1 and the cleavage feature upon recycled recognition by DSN was used to detect target microphthalmia-associated transcription factor (MITF) in cell nuclear extracts. In this method, we employed a well-designed DNA1 as a recognition element which can converting in two states, “ON-state” and “OFF-state”. Also, the DNA1 is modified with 2-OMe-RNA on hybridization part in the “OFF-state” to prevent meaningless digestion. By taking advantage of the high amplification efficiency of DSN-aided recycling, high sensitivity of MITF is realized with a detection limit as low as 1.1 pM, which is superior or comparable to that of the reported literature. This method is a fast and easy-to-use one-pot method that was carried out in a tube, while being quantitative and applicable to other proteins in the sample without involving complicated procedures or sophisticated instrumentations. It is very simple and fast, needing only mixing of DNA1, DNA2, MITF and DSN enzyme and incubating within 60 min, which is in the homogeneous solution, and not requiring separation and troublesome procedures. Considering the superior sensitivity and specificity, as well as the multiplex and simple-to-implement features, this method holds great promise of becoming a routine tool for simultaneously quantitative analysis of multiple proteins and supplies valuable information for transcription factor-based early stage cancer diagnosis.

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1. Introduction

Molecular beacon (MB) which is a hairpin-shaped single-stranded oligonucleotide, has rapidly become a mainstay for the detection of target since its rapidly and specifically report the presence of a given nucleic acid sequence in solution (Duan et al., 2013; Zuo et al., 2010). MBs are single-stranded oligonucleotides that possess a stem-and-loop structure with a fluorophore attached to one arm and a quencher to the other and holds the fluorophore and quencher which quenching the fluorescence of the fluorophore into close proximity (Marras et al., 1999, 2003; Piatek et al., 1998; Tapp et al., 2000). The MB loop portion is a probe sequence that is complementary to a target sequence. In the presence of target nucleic acids, the MB 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 (Templeton et al., 2004; Varma-Basil et al., 2004). As a result, the fluorophore and quencher are separated from each other, and thereby producing a large increase in fluorescence. Thus, an increase in the fluorescence intensity reports directly in the presence of target nucleic acid (Duan et al., 2013; Zuo et al., 2010). Because of their advantages of detection-without-separation, high sensitivity, and especially excellent selectivity to differentiate single-base mismatched targets, MBs have been widely used in different areas including RNA and DNA monitoring, biosensing, and real-time gene monitoring.

It is interesting to notice that, although molecular beacons were originally developed to detect nucleic acids, more molecular beacons employed amplification method were used for the detection of protein (Ayele et al., 2004; Duan et al., 2013; Leone et al., 1998). Such molecular beacons-assisted amplification approach, with its inherent stability, specificity, and simplicity, has recently

* Corresponding authors.
E-mail addresses: zhangkai@jsinm.org (K. Zhang), doczhangxiaolu@gmail.com (X. Zhang).

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emerged as a potential amplification technique for rapid and cost-effective detection of protein. These methods shown that molecular beacons hold great potential as excellent probes for sensitivity detection of nucleic acids.

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). Therefore, owing to the pivotal role of TFs in gene expression as well as its close relationship with human diseases, analyzing multiple TFs can provide more valuable information for understanding the mechanism underlying gene expression regulation and the roles of various TFs in the cellular processes (Helin, 1998). For this end, a broad class of methods that can simultaneously analyze multiple TFs have been developed for the assay TFs (Bonham et al., 2012; Vallée-Bélisle et al., 2011; Yin et al., 2014; Zhang et al., 2016; Zhang et al., 2012). Unfortunately, these methods for monitoring TF concentration or binding activity are generally cumbersome and time-consuming. Therefore, the development of new methods for general, cost-effective, and sensitive detection of transcription factors are currently in great demand.

Recently, an elegant one-step and sensitive method for targets detection were reported based on duplex-specific nuclease (DSN) signal amplification (Qiu et al., 2015; Xi et al., 2014; Yin et al., 2012; Zhang et al., 2014). Duplex-specific nuclease, a nuclease isolated from the hepatopancreas of the Kamchatka crab, shows a strong preference for hydrolyzing dsDNA (more than 10 base pairs) and DNA in DNA: RNA heteroduplexes rather than ssDNA or RNA. In which DSN was utilized to recycle the target-assisted cleavage of Taqman probes, leading to significant fluorescence signal amplification.

In the present work, we develop an ultrasensitive transcription factors detection method by integrating protein-DNA interaction, DSN cleavage, the advantage of molecular beacons and isothermal exponential amplification in a one-pot reaction. And we employed microphthalmia-associated transcription factor (MITF), which is a kind of TF, as the model. In our strategy, the combining of the duplex DNA and TF can convert the DNA1 from the “OFF-state” to the “ON-state”, in which the hybridization part of DNA1 is functional (Adornetto et al., 2015). The subsequent hybridization of the oligonucleotides with the molecular beacon opens the stem-loop structure. The formation of the DNA complex triggers amplification reaction and the recovery of the fluorescence which is detectable. More importantly, our method is suitable for the direct detection of TFs in crude cellular extracts of cancer cells.

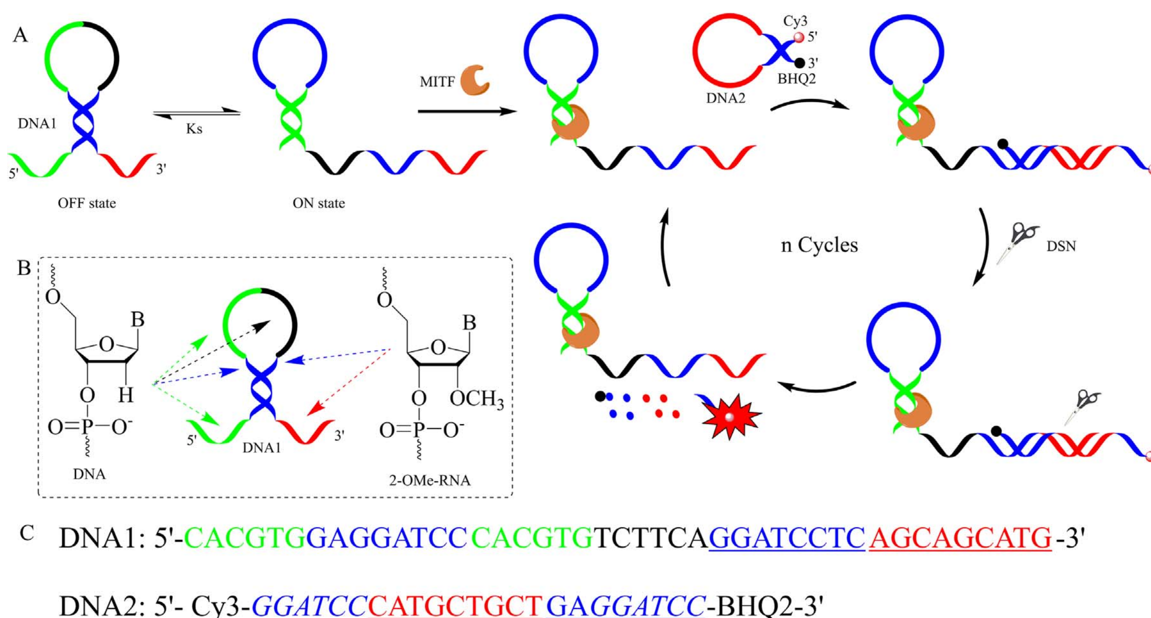
2. Experimental section

2.1. Materials and chemicals

All oligonucleotides were purchased from Genscript Biotechnology Co., Ltd (Nanjing, China) and their sequences were listed in Scheme 1. The purified recombinant Microphthalmia-associated transcription factor (MITF) was purchased from Promega (Madison, WI). Duplex-specific nuclease (DSN) was purchased from Newbornco Co., Ltd. (Shenzhen, China). 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).

2.2. Protein-DNA interaction and DSN cleavage

The MITF (20 μL) with different original concentrations (0, 1, 5, 10, 25, 50, 100, 1000, 2500, 5000 and 10,000 pM) and DNA1 (300 nM, 50 μL) were incubated at room temperature for 30 min in the presence of protein binding buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mg mL^{-1} yeast tRNA, 10% glycerol, 0.25 mM DTT. For the detection of MITF in the nuclear extracts (20 μL), the sample was mixed with the DNA1 (50 μL) and incubated at room temperature for 30 min in the binding buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.25 mM DTT, 2 mM sodium phosphate (pH 7.0), 20 ng μL^{-1} HaeIII-cut *Escherichia coli* (*E. coli*) DNA, and 25 ng μL^{-1} yeast tRNA. For next experiments of the DSN amplification, 0.5 U DSN (Fig. S1) dissolved in 10 × DSN master buffer (13.3 μL, 500 mM Tris-HCl, pH 8.0; 50 mM $MgCl_2$, 10 mM DTT), and 50 μL of DNA2 (with an



Scheme 1.. (A) Schematic diagram showing the principle of DSN-based amplification fluorescence assay for the detection of MITF. (B) The structure of the DNA1: the sequences of DNA or 2-OMe-RNA were marked. (C) The olgonucleotides sequence used in this strategy. The colors of the sequences are the same as given in part A and part B and the underline parts marked the hybridization part of DNA1 and DNA2. (For interpretation of the references to color in this scheme, the reader is referred to the web version of this article.)

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