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A dumbell probe-mediated rolling circle amplification strategy for highly sensitive transcription factor detection

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ABSTRACT

Highly sensitive detection of transcription factors (TF) is essential to proteome and genomics research as well as clinical diagnosis. We describe herein a novel fluorescent-amplified strategy for ultrasensitive, quantitative, and inexpensive detection of TF. The strategy consists of a hairpin DNA probe containing a TF binding sequence for target TF, a dumbbell-shaped probe, a primer DNA probe designed partly complementary to hairpin DNA probe, and a dumbbell probe. In the presence of target TF, the binding of the TF with hairpin DNA probe will prohibit the hybridization of the primer DNA probe with the "stem" and "loop" region of the hairpin DNA probe, then the unhybridized region of the primer DNA will hybridize with dumbbell probe, subsequently promote the ligation reaction and the rolling circle amplification (RCA), finally, the RCA products are quantified via the fluorescent intensity of SYBR Green I (SG). Using TATA-binding protein (TBP) as a model transcription factor, the proposed assay system can specifically detect TBP with a detection limit as low as 40.7 fM, and with a linear range from 100 fM to 1 nM. Moreover, this assay related DNA probe does not involve any modification and the whole assay proceeds in one tube, which makes the assay simple and low cost. It is expected to become a powerful tool for bioanalysis and clinic diagnostic application.

1. Introduction

Transcription factors are proteins that bind to specific DNA sequences, thereby control the transcription of genetic information from DNA to messenger RNA (Latchman, 1997). Transcription factors are essential for the regulation of gene expression and confirmed to be the largest family of human proteins. There are approximately 2600 proteins in the human genome (approximately 10% of genes in the genome) that contain DNA-binding domains, and most of them are presumed to function as transcription factors (Babu et al., 2004).

Transcription factors play critical roles in the regulation of a variety of essential cellular processes, such as cell development, differentiation, and growth (Lee and Young, 2000). Furthermore, the changes in expression level of some transcription factors has been confirmed to closely connect with multiple aspects of oncogenesis (Baldwin, 2001; Libermann and Zerbini, 2006; Wolf et al., 2005). Therefore, quantitative protein detection of transcription factors plays an important part in clinical diagnosis and biomedical research. Many powerful traditional techniques have been developed for transcription factors detection. They include DNase footprinting assay (Galas and Schmitz, 1978), electrophoretic mobility shift assay (EMSA) (Garner and Revzin, 1981), Western blots, and enzyme-linked immunosorbent assay (ELISA) (Burnette, 1981; Engvall and Perlmann, 1971). However, these methods such as DNase footprinting assay, EMSA, Western blots are usually time-consuming and laborious with the involvement of either radioisotopes or fluorescence labels and are not adaptable to assays requiring high throughput (Zhang et al., 2012). Furthermore, they cannot achieve quantitative analysis of protein expression. ELISA is a widely used quantitative detection method for protein and other analytes. While it needs expensive antibodies against each target protein, which elevates the analysis cost. Therefore, it is highly desirable to develop robust methods for simple, cost-effective, and sensitive detection of transcription factors.

Recent years, a broad class of fluorescence-based approaches have emerged, including fluorescence polarization assays, "molecular beacon" based assays, protein–DNA FRET assays (Giannetti et al., 2006; Liu et al., 2012; Lundblad et al., 1996; Moellering et al., 2009). In comparison to conventional methods, fluorescence

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detection is convenient and sensitive. It allows the homogeneous assay of DNA-binding proteins in solution. Nowadays, the rolling circle amplification (RCA) technique has gained considerable attention in nucleic acid and protein determination (Cheng et al., 2010; Ge et al., 2014; He et al., 2014; Ji et al., 2012; Konry et al., 2011; Zhuang et al., 2014). In a typical RCA, a circular template is isothermally amplified by Phi29 DNA polymerase. The DNA amplification proceeds in a linear model, resulting in a long repeated DNA sequences complementary to the circular template. Thus, the amplified fragment which contains thousands of tandem repeats serves as a signal amplifier for the ultrasensitive detection of specific targets.

In this work, we developed a fluorescence-based strategy for the detection of transcription factor by integrating TF–DNA interaction (Zhang et al., 2012), ligation reaction, and dumbbell probe-mediated RCA (D-RCA) strategy (Wang et al., 2008; Zhou et al., 2010). Here, TATA-binding protein (TBP), a transcription factor that binds specifically to a DNA sequence called the TATA box, was used as a target model to demonstrate our strategy. The proposed TF detection system consisted of three components: a hairpin-like TF binding probe (TFBP) with a hanging single DNA sequence and a TF binding sequence integrated at the stem of the hairpin (it was used as target recognition element), an amplification primer, and a dumbbell-probe used for RCA. Introduction of TF protein hindered the hybridization of TFBP with the R-primer because of the formation of TF–TFBP complex, and then the 3-terminal of the R-primer would hybridize with the dumbbell-probe, resulting in the reaction of ligation-RCA. The detection system offers two major advantages over other reported schemes. First, the designed homogeneous assay scheme is simple and suitable for high throughput TF analysis. Second, dumbbell-probe mediated RCA strategy allows us to achieve large dynamic range and femtomolar sensitivity.

2. Experimental

2.1. Reagents and materials

The sequences of the sensing probe were designed by mfold web server (http//mfold.rna.albany.edu) and synthesized by Sango Biotechnology Co., Ltd. (Shanghai, China). They are listed in Table 1. The 5′ end of the dumbbell-probe is phosphorylated.

TBP was purchased from ProteinOne Inc. (Maryland, USA). Phi29 DNA Polymerase, dNTPs, T4 DNA ligase, the universe buffer, and 10 × tango buffer were provided by Thermo Fisher Scientific Inc. (Massachusetts, USA). SYBR Green I was obtained from Invitrogen Inc. (California, USA). All other reagents were of analytical reagents grade. All aqueous solutions were prepared with ultrapure water (≥ 18.3 M, Milli-Q, Millipore).

2.2. Transcription factor–DNA binding

TBP solution (2 μL) at a certain concentration was mixed with 1 μL of 0.5 μM TBP-probe, 5 μL of H_2O, and 2 μL of 5 × TBP binding buffer (100 mM Tris acetate pH 7.5, 20 mM $MgCl_2$, 375 mM potassium glutamate, 25% glycerol, and 0.5 mg/mL BSA). The TBP and TBP-probe binding reaction was allowed to proceed for 5–60 min at room temperature. Then 1 μL of 0.5 μM R-primer was added to the mixture and incubated at 30 °C for 5–50 min, resulting solution I.

2.3. Ligation reaction

For ligation reaction, the prepared solution I was mixed with 20 μL of a reaction mixture containing 10–120 nM dumbell-probe, 0.5 mM ATP, 10 units of T4 DNA ligase, 3 μL of 10 × Tango buffer, and water. The reaction mixture was incubated at 30 °C for 15 min to generate circularized dumbell probes, resulting solution II.

2.4. RCA reaction

For RCA reaction, adding Phi29 DNA polymerase (5 U/μL, 4 μL), dNTPs (10 mM, 1 μL), 0.6 μL of 10 × Tango buffer into solution II to obtain the RCA reaction solution (35.6 μL). The RCA reaction proceeded for 15–180 min to generate RCA products. The resulting mixture was incubated at 65 °C for 10 min to inactivate the polymerase.

2.5. Measurement of fluorescent spectra

The RCA amplification product was mixed with 10 μl 10 × SG dye (Invitrogen) and diluted to final volume of 200 μl with 10 mM PBS (pH 7.3). The fluorescent spectra were measured using a spectrofluorophotometer (F-4600, Hitachi, Japan). The excitation wavelength was 497 nm, and the spectra were recorded between 520 and 610 nm. The fluorescence emission intensity was measured at 530 nm.

3. Results and discussion

3.1. Design of the TF sensor

The strategy is shown in Scheme 1. First, TF binding probe (TFBP) which was designed as a hairpin structure with a hanging single DNA sequence and a TATA box integrated at the stem was dissolved in the buffer. Then the target protein TBP was added and bound to the stem region. The mixture was then incubated with R-primer. Only 5-terminal of R-primer hybridized with the hanging single DNA of the TFBP for the reason of TBP binding on hairpin stem. After incubation, dumbell-probe and ligase, phi29 DNA polymerase was added successively. Ligation and isothermally amplification was carried out in turn. Then RCA products were analyzed via fluorescence from SYBR Green I (SG). On the contrary, in the absence of the target TF, R-primer would form the complementary double-stranded DNA with TFBP, the hybridization of 3-terminal of R-primer with dumbell-probe was thus blocked, and the following ligation and RCA could not proceed, resulting in a low fluorescence signal.

Table 1
Oligonucleotides used in the experiments.

Name	Sequence (5′ to 3′)
Dumbell-probe	p-GCTACACTTCATTCTactctcgtcacgCTTGGACTGAcgtgacgagagtCTTTAGCTTATCAG
TBP-probe	**GTATAAAGA***GCTACACTTCA***TCTTTATAC***AGTCATCACGCTCCGCTCTC*
R-primer	*GAGAGCGGAGCGTGATGACTGTATAAAGATGAAGTGTAGCCTGATAAGCT*

The bold letters indicate the stem sequences of the TBP-probe (it is also the TBP binding site). The italic letters indicate the complementary bases between TBP-probe and R-primer. The underlined letters indicate the complementary bases between dumbell-probe and R-primer. The lowercase letters represent the stem sequences of the dumbell-probe.