Analytical Biochemistry 448 (2014) 105–112

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Analyzing transcription factor activity using near infrared fluorescent bridge polymerase chain reaction

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ARTICLE INFO

Article history:
Received 14 October 2013
Received in revised form 26 November 2013
Accepted 2 December 2013
Available online 9 December 2013

Keywords:
Bridge PCR
Near infrared fluorescence
Transcription factor
NF-κB

ABSTRACT

This study has developed a new method, near infrared fluorescent bridge polymerase chain reaction (NIRF–bPCR), for analyzing transcription factor (TF) activity. This method was first used to detect the activity of purified nuclear factor kappa B (NF-κB) p50. The results demonstrated that this method could quantitatively detect the activity of p50 protein at less than 115 ng (~ 2320 fmol), and the detection limit reached as little as 6.94 ng (~ 140 fmol) of p50 protein. This method was then used to detect TF activity in cell extracts. The results revealed that this method could specifically detect NF-κB activity in HeLa cell nuclear extracts. Finally, this method was used to detect the activities of multiple TFs in a protein sample. The results showed that this method could detect the activities of six TFs—NF-κB, AP-1, TFIID, CREB, NF-E2, and p53—in the TNFα-induced and -uninduced HeLa cell nuclear extracts. Calculation of the fold induction of six TFs revealed that NF-κB, CREB, and AP1 were activated by TNFα induction in HeLa cells, in agreement with the detection results of other methods. Therefore, this study provides a new tool for analyzing TF activity. This study also revealed that NIRF–bPCR may be used as a new method for detecting DNA molecules.

Regulatory transcription factors (TFs)[2] are a group of sequence-specific DNA-binding TFs that control gene transcription via transcription factor binding sites (TFBSs) in genome [1]. For instance, nuclear factor kappa B (NF-κB) is an important regulatory TF that is ubiquitous in various cells [2]. When a cell encounters various stimulators such as tumor necrosis factor α (TNFα) and lipopolysaccharide (LPS), it can translocate into nucleus and bind numerous κB sites in genome to regulate expressions of its many target genes involved in some important biological processes such as immunology and inflammation [3]. For most studies of regulatory TFs, analyzing their DNA-binding activity is inevitable. Therefore, development of techniques for detecting activities of regulatory TFs has attracted much attention. In addition, because regulatory TFs often regulate gene expression in a combination fashion [4], simultaneously analyzing activities of multiple TFs can provide more information for understanding the mechanisms underlying gene expression regulation.

Electrophoretic mobility shift assay (EMSA) is the widely used method for measuring TF activity [5,6]. This method detects the signal of TF-bound DNA in native polyacrylamide gel by using autoradiography [7]. The signal intensity of TF-bound DNA represents TF activity in sample [8,9]. This method has been the "gold standard" tool for TF detection due to its high sensitivity and simple detection process [6]. However, although chemiluminescent EMSA [10] and fluorescent EMSA [11] have been developed to avoid isotopes that have challenged this method, the method is still limited by its low throughput and large sample consumption. In addition, to check the specificity of TF–DNA complex in EMSA, a supershift assay that relies on TF's antibody must be performed.

To improve the throughput of TF detection, another method has been developed [12]. This method was also called DNA–protein interaction enzyme-linked immunosorbent assay (DPI–ELISA) [13] or TF enzyme-linked immunoassay (TF–EIA) [14]. This method detects TF with double-stranded DNA (dsDNA) probe-coupled microplates using an ELISA-like process. This method is proper for detecting a certain TF in multiple samples in a high-throughput format [15]; however, it is not suitable for simultaneously

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[2] *Abbreviations used:* TF, transcription factor; TFBS, transcription factor binding site; NF-κB, nuclear factor kappa B; TNFα, tumor necrosis factor α; EMSA, electrophoretic mobility shift assay; DPI–ELISA, DNA–protein interaction enzyme-linked immunosorbent assay; TF–EIA, TF enzyme-linked immunoassay; dsDNA, double-stranded DNA; bPCR, bridge polymerase chain reaction; NIRF, near infrared fluorescence; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; PBR, protein-binding reaction; RT, room temperature; EB, ethidium bromide; UV, ultraviolet; PBS, phosphate-buffered saline; PBST, PBS and Tween 20; TBP, TATA-binding protein; AP1, activator protein 1 (JUN/FOS); TFIID, TATA box binding protein; CREB, cAMP responsive element binding protein 1; NF-E2, nuclear factor (erythroid-derived) 2; p53, tumor protein p53.

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http://dx.doi.org/10.1016/j.ab.2013.12.003

detecting multiple TFs in a sample due to its dependence on antibodies of TFs. Thus, this method is incapable of analyzing activities of multiple TFs in a small amount of protein sample [16]. In addition, both supershift assay and DPI–ELISA need TF-specific antibody that can bind TF–DNA complex; however, most commercialized TF antibodies are suitable only for Western blot assay, not for binding TF–DNA complex.

To simultaneously analyze activities of multiple TFs in a sample, several new methods have been developed, including TranSignal Protein/DNA Array [17], TF Activation Profiling Plate Array [18], Oligonucleotide Array-Based Transcription Factor Assay (OATFA) [16,19], and Fluorescent Microsphere-Based Multiplexed High-Throughput Assay [20]. These methods analyze multiple TFs in a similar process that consists of incubating DNA probes with sample, isolating protein-bound DNA, and detecting protein-bound DNA with DNA array or beads. These methods allow high-throughput detection of many TFs in a particular protein sample; for example, protein/DNA array was used to detect activities of 108 TFs in two types of cells [21]. These methods, therefore, provide useful tools for studying TFs [22]; however, these methods are still challenged by the autofluorescence of solid supports in visible fluorescence spectrum or variance of enzyme-catalyzed signal development.

This study has developed a new method for detecting TF activity that is based on bridge polymerase chain reaction (bPCR) and near infrared fluorescence (NIRF) techniques. In this method, dsDNA probe was first incubated with protein sample, and the TF-bound DNA was separated from free DNA via native polyacrylamide gel electrophoresis (PAGE). The TF-bound DNA was then recovered from gel and detected with bPCR that contained biotin-labeled dUTP. Finally, the signal was reported by NIRF-labeled streptavidin. This method was fully validated by detecting the DNA-binding activities of purified recombinant NF-κB p50 and NF-κB in HeLa cell nuclear extracts. The method was also enlarged to simultaneously detect activities of multiple TFs in HeLa cells.

Materials and methods

Oligonucleotides used for preparing dsDNA probes and primers

Oligonucleotides were synthesized using the standard phosphoramidate chemistry and were purified by high-performance liquid chromatography (HPLC; Sangon). The oligonucleotides used as dsDNA probes and bPCR primers are shown in Table S1 of the online Supplementary material. To obtain dsDNA, two complementary oligonucleotides were mixed in the same molar ratios at a final concentration of 50 μM in 100 ml of TEN (Tris–EDTA–NaCl) buffer [23]. The mixture was heated for 5 min at 95 °C and cooled slowly to 25 °C.

Cell culture, TNFα stimulation, and nuclear extract preparation

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in 5% CO_2. For TNFα induction, cells were cultivated with serum-free DMEM containing 30 ng/ml TNFα (Sigma) for 30 min. The control cells were simultaneously cultivated with serum-free DMEM. The nuclear extracts were prepared with a Nuclear Extract Kit (Active Motif) according to the manufacturer's instructions. The nuclear extract was qualified with Bradford assay.

Preparation of primer DNA microarray

The amino-modified oligonucleotides (Table S1) used as primers of bPCR were dissolved in sterile water at a concentration of 20 μM and stored at 4 °C. Immediately before spotting, the primer oligonucleotides were diluted with 50% spotting solution (CapitalBio) at a final concentration of 1 μM and two oligonucleotides as a pair of bPCR primers were mixed in the same volume. The mixture was spotted (30 nl/dot) on aldehyde-modified glass slides (CapitalBio) using a spotting robot (AD1500, BioDot). The spotted slides were hydrated at 37 °C for 12 h. The slides were briefly washed with 0.2% sodium dodecyl sulfate (SDS) and sterile water. The slides were soaked in 0.3% (w/v) sodium borohydride solution for 5 min and briefly washed three times with sterile water. The slides were spun dry in a slide centrifuge (Labnet) and kept at 4 °C.

Binding of dsDNA probe with protein sample and isolation of TF-bound DNA

All protein-binding reactions (PBRs) were 10 μl, consisting of DNA-binding buffer [24], 0.1 mg/ml poly(dI-dC) (Amersham, used for nuclear extract), various amounts of purified recombinant NF-κB p50 protein (Promega) or nuclear extract (see figures), and 5 pmol of NC and TF dsDNAs. PBRs were kept at room temperature (RT) for 1 h, followed by mixing with 2 μl of 40% (v/v) glycerol and undergoing native PAGE in 0.5× TBE (Tris–borate–EDTA) at 100 V in a refrigerator for 1 h. Gel was stained with 0.5 μg/ml ethidium bromide (EB; Amersham) for 30 min, and the gel slice with shifted DNA was excised under an ultraviolet (UV) transilluminator. The gel slices were soaked in 20 to 60 μl diffusion buffer [23] overnight at 37 °C. The eluates were directly detected with bPCR.

bPCR and NIRF detection

All bPCRs were 25 μl, consisting of 0.025 U/μl PrimeSTAR HS DNA polymerase (TaKaRa), 1× PrimeSTAR buffer (Mg^{2+} Plus; TaKaRa), 0.2 mM each of deoxynucleotide triphosphates (dNTPs; TaKaRa), 8 μM biotin–dUTP (Fermentas), and various amounts of templates (see figures). PCRs were added in GeneFrames (Thermo Scientific) stuck around primer microarrays on slides, and covered with a coverslip (Thermo Scientific). Slides underwent the following PCR program in a thermocycler: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 15 s, and 72 °C for 45 s, and 72 °C for 5 min. Subsequently, GeneFrames were removed from slides and slides were briefly washed with sterile water. Slides were successively incubated with maleic acid buffer [23] containing 1% (w/v) blocking reagent (Roche) and phosphate-buffered saline (PBS) [23] containing 1:15,000 diluted streptavidin–IRDye 800 (LI-COR Biosciences) for 30 min at RT. Slides were briefly washed with sterile water after each incubation. Finally, slides were spun dry and scanned with an Odyssey Infrared Imaging System (LI-COR Biosciences) at the channel of 800 nm in the following parameters: resolution, 42 μm; preset, membrane; quality, medium; focus offset, 3 mm; intensity, 7.0. NIRF signal was quantified with the Odyssey Infrared Imaging System.

Detection of DNA-binding activity of TF with EMSA

EMSA was performed as described previously [25]. In brief, PBRs built with DNA (biotin-labeled NF-κB dsDNA) underwent native PAGE in 0.5× TBE at 100 V in a refrigerator for 1 h, and DNA was electronically transferred on positively charged nylon membrane (Roche). Membrane was crosslinked with a UV crosslinker (UVP) for 15 min and washed twice with maleic acid buffer–0.3% (v/v) Tween 20. Membrane was successively incubated with maleic acid buffer containing 1% (w/v) blocking reagent (Roche) and PBS