Real-time PCR Assay for Ultrasensitive Quantification of DNA-Binding Proteins

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Background: The specific binding of proteins to DNA is a key step for many cellular activities, such as transcription regulation, DNA replication, recombination, repair, and restriction. The detection of DNA-binding proteins, as well as the identification of specific binding sites, is therefore important to understand gene expression mechanisms and cellular function. We describe an ultrasensitive method for quantification of DNA-binding proteins.

Methods: We combined the common exonuclease III (ExoIII) footprinting assay and real-time PCR for quantification of DNA-binding proteins, for an assay that does not require antibodies against the target proteins. Double-strand DNA probes were designed to monitor the activities of DNA-binding protein. The protein-binding site is at the 5′ end of the forward primer. When a target protein is present, it will specifically bind to the protein-binding site and produce a physical hindrance to ExoIII, which protects the reverse DNA strand from digestion by ExoIII. The remaining single-strand DNA template can be quantitatively detected by real-time PCR. Conversely, in the absence of the target protein, the naked primer regions will be degraded by ExoIII, which then cannot be amplified by real-time PCR.

Results: We detected the binding of 10 different transcription factors in crude cell extracts. The assay quantitatively detected binding at femtomolar concentrations of protein.

Conclusions: This technique is customizable and easy to establish. It has potential applications in research, medical diagnosis, and drug discovery.

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Many proteins with natural, sequence-specific DNA-binding activity are involved in regulating important cellular processes such as cell growth, differentiation, apoptosis, and transformation to cancer (1). Transcription factors, for example, bind to specific promoter sequences and recruit chromatin-modifying complexes and the transcription apparatus to initiate RNA synthesis (2–4). The reprogramming of gene expression that occurs as cells move through the cell cycle, or when cells sense changes in their environment, is effected in part by changes in the DNA-binding status of transcription factors. Distinct DNA-binding proteins are also associated with origins of DNA replication, centromeres, telomeres, and other aspects of genome maintenance (5, 6). Therefore, the quantitative detection of DNA-binding proteins, as well as the identification of specific binding sites, is important to understand gene expression mechanisms and cellular function.

Conventional methods for detecting DNA-binding proteins are electrophoretic mobility shift assay (EMSA) (7), DNA footprinting (8), ELISA (9), and Southwestern blotting (10). They are laborious, time-consuming procedures that typically involve the use of radioisotopes or antibodies against the target proteins, which are not adaptable to high-throughput formats, thus making the procedures unsuitable for more extensive application. In recent years, several techniques have been developed to detect DNA-binding proteins, including DNA microarray (11–15), fluorescence resonance energy transfer (16, 17), and Exo-dye–based assay (18). They are useful tools in studying protein-DNA interaction; however, their lack of

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Nonstandard abbreviations: EMSA, electrophoretic mobility shift assay; IPCR, immuno-PCR; ExoIII, exonuclease III; ssDNA, single-strand DNA; dsDNA, double-strand DNA; TNFα, tumor necrosis factor α; Ct, threshold cycle.
sensitivity makes them difficult to apply in detecting trace amounts of DNA-binding proteins.

PCR and other forms of target amplification have enabled rapid advances in the development of powerful tools for detecting and quantifying DNA targets of interest for research, forensic, and clinical applications (19–21). To extend the scope of PCR to high-sensitivity detection of proteins, the immuno-PCR (IPCR) technique was developed in 1992. IPCR takes advantage of specific antibody–DNA conjugates (22) and has become a well-respected research methodology, having established its general applicability for the sensitive detection of numerous protein antigens, usually providing a 100- to 10,000-fold improvement of the detection limit of conventional ELISA (23). Nam et al. (24) originally developed a high-sensitivity nanoparticle-based assay for protein detection, termed bio-bar-code assay, in which an amplifier nanoparticle is coloaded to the captured target. Because only one of the strands is attached covalently to the nanoparticle, the complementary strand can be released and serves as the surrogate target, which is then detected by hybridization to an array (24). IPCR and nanoparticle-based bio-bar-code approaches have high sensitivity and could be used for the ultrasensitive detection of target proteins. Both, however, involve the use of different antibodies against the target proteins.

Herein, we report a universal real-time PCR assay for the ultrasensitive quantification of DNA-binding proteins that does not require antibodies against the target proteins. The common exonuclease III (ExoIII) footprinting assay is coupled with real-time PCR to monitor the activities of DNA-binding proteins. We used this technique to successfully detect 10 different transcription factors in crude cell extracts. This assay allows ultrasensitive and high-throughput quantification of DNA-binding proteins and can be used extensively in biomedical research.

Materials and Methods

Preparation of Double-strand DNA Target for Binding Assay

The primers and the single-strand DNA (ssDNA) template used in this study, synthesized using accepted phosphoramidate chemistry and purified by HPLC (Invitrogen), are listed in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue4. Briefly, we subjected ssDNA to PCR in the presence of the reverse primer and the forward-tailed primer, which contains different protein-binding sites at the 5′ end (see Table 1 in the online Data Supplement). After PCR amplification and purification, we obtained double-strand DNA (dsDNA) probes with different protein-binding sites for binding reactions.

Preparation of Duplex Competitors for Competition Assay

We synthesized the following oligonucleotides to be used as a cold NF-κB–specific competition probe: 5′-AGT TGA GGG GAC TTT CCC AGG CTT TTT-3′ and 5′-GCC TGG GAA AGT CCC CTC AAC TTT TTT-3′; the underlined sequence represents the NF-κB–binding sites. The oligonucleotides used as a cold NF-κB nonspecific competition probe were 5′-AGT GAT TAC TTT CAC AGG CTT TTT-3′ and 5′-GCC TGT GAA AGT AAT CTC AAC TTT TTT-3′; the underlined sequence represents the nonspecific NF-κB–binding sites. We made competitor probes with 5 protruding bases (T) at each 3′ end for protection from ExoIII digestion. To obtain duplex competitors, we mixed 2 oligonucleotides in the same molar ratios at a final concentration of 50 μmol/L in 100 μL of 10 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, and 1 mmol/L EDTA. The mixture was heated for 5 min at 95°C and cooled slowly to 25°C.

Quantitative Detection of DNA-binding Proteins

This assay involves 3 steps: protein–DNA interaction (protein binding), ExoIII digestion, and real-time PCR amplification and detection. For protein binding, the experiment was performed in binding buffer containing 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 0.5 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 3 mmol/L MgCl₂, 0.05 g/L poly(di-dC) (Roche), 10% vol/vol glycerol, 0.5 g/L bovine serum albumin, and 0.05% Nonidet P40. NF-κB (rhNF-κB p50) was purchased from Promega and preincubated with DNA-binding buffer at 37°C for 10 min. HeLa cell nuclear extract was stimulated with and without tumor necrosis factor α (TNF-α) that was purchased from Takara Active Motif and preincubated in binding buffer containing 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 0.5 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 3 mmol/L MgCl₂, 0.05 g/L poly(di-dC) (Roche), 10% vol/vol glycerol, 2 mmol/L sodium phosphate (pH 7.0), 20 ng/μL HaeIII-cut Escherichia coli DNA (Roche), and 25 fg/L yeast tRNA (Roche) at 37°C for 10 min. For nuclear extracts, interference may arise in this assay due to the endogenous nucleases present. For ExoIII exposure (25). To avoid this problem, we included sodium phosphate and carrier nucleic acids including poly(di-dC), HaeIII-cut E. coli DNA, and yeast tRNA to suppress endogenous nuclease activities in the crude extracts (25, 26).

After incubation, dsDNA probes with different protein-binding sites were added and incubated at the same temperature for 20 min more. At the end of the DNA–protein interaction, a sufficient amount of ExoIII was added and incubated to digest the dsDNA probes at 37°C for 5 min. The reaction was terminated at 70°C for 20 min.

Real-time PCR was run for quantitative detection of DNA-binding proteins by use of a 96-well PCR plate with a final reaction mixture of 25 μL consisting of 600 mmol/L of each primer, 200 mmol/L TaqMan probe (Takara), 200 μmol/L of each deoxyribonucleoside triphosphate, 3.5 mmol/L MgCl₂, and 1× TaqMan Buffer (Takara) under the following conditions: 95°C for 10 min, 40 cycles
at 95 °C for 15 s, and 60 °C for 1 min. The primers and TaqMan probe used in this study are listed in Table 1 in the online Data Supplement.

**DATA ANALYSIS**

The instrument monitors the increase of the normalized fluorescence reporter signal for each cycle. We applied a baseline correction, typically using the first 10 to 15 cycles as background signal. The software calculates the threshold value (Ct), which represents the first PCR cycle at which the reporter signal exceeds the signal of the baseline determined by the user. The threshold signal was determined to be above the background signals for empty wells and was set in the linear signal increment phase according to the data obtained for each experiment. The normalization of the Ct values was achieved as follows. Ct values are inversely proportional to the amount of DNA template and, thus, DNA-binding protein concentration, so we subtracted test sample Ct values from negative control Ct values to generate ΔCt values. The ΔCt values increase with increasing amounts of DNA template and proteins. We analyzed each test in triplicate and calculated mean values of ΔCt to make a calibration curve for quantification analysis of target proteins. We obtained linear regressions of the signals from the enriched samples against the logarithmic concentrations of proteins. We performed statistical analysis and plotting of the data with SigmaPlot 8.0 software.

**Results and Discussion**

**MECHANISM**

Fig. 1 illustrates this assay. Briefly, we prepared dsDNA probes by regular PCR, containing the different protein-binding sites, universal primer regions, and TaqMan probe region. The protein-binding site is at the 5’ end of the forward primer (Fig. 1A). All sequences used in this study are summarized in Table 1 in the online Data Supplement. We incubated DNA-binding proteins with dsDNA probe for a binding reaction. ExoIII was then added to degrade dsDNA. ExoIII is a modifying enzyme, which exhibits the 3’–5’ exodeoxyribonuclease activity specific for dsDNA. It can degrade dsDNA from blunt ends, 5-overhangs, or nicks. In this assay, if a target protein is present, it will specifically bind to the protein-binding site and produce a physical hindrance to ExoIII, protecting the reverse DNA strand from digestion by ExoIII (Fig. 1A). The remaining ssDNA template can be quantitatively detected by use of real-time PCR (Fig. 1B, curve 2). Conversely, in the absence of the target protein (negative control), the naked primer regions will be degraded by ExoIII, which cannot be amplified by real-time PCR (Fig. 1B, curve 3). The positive control is the dsDNA probe without digestion of ExoIII, which shows the original amount of the dsDNA probe (Fig. 1B, curve 1). As clearly shown in Fig. 1B, there is a background signal in the negative control, which probably results from 2 factors. One is incomplete digestion of ExoIII. Alternatively, the ssDNA template could be produced when the dsDNA probe was prepared by regular PCR. ssDNA could not be degraded by ExoIII, which results in a background signal by real-time PCR.

**SPECIFICITY AND GENERAL APPLICABILITY**

To explore more fully the specificity of detection, we performed competition assays: we made 2 duplex DNA competitors, one containing a consensus NF-κB–binding site (GGGACTTTCC) as a specific competitor and the other not containing an NF-κB–binding site as the non-specific competitor. We added the specific and non-specific competitors to the reaction buffer containing NF-κB for a binding reaction and performed ExoIII digestion. The results of competition assays are shown in Fig. 2. The specific competitor decreased the amount of dsDNA template (or increased the Ct value) in the presence of NF-κB (Fig. 2A; Fig. 1 in the online Data Supplement), whereas the non-specific competitor did not affect the amount of dsDNA template or the Ct value (Fig. 2B; Fig. 1 in the online Data Supplement). These results provide strong evidence for the specificity of this assay and also indicate that such competition assays could be used to rapidly assess the relative binding affinity of the protein to different variants of DNA.

To investigate quantitative detection of DNA-binding proteins, we added a series of different concentrations of NF-κB to the same molarity dsDNA probe with an NF-κB–binding site and performed simultaneous ExoIII digestion and real-time PCR. As shown in Fig. 2 in the online Data Supplement, the Ct values decreased when the amount of NF-κB increased from 10 fmol/L to 2 nmol/L (see Fig. 2, inset, in the online Data Supplement), and we observed a detection limit of 10 fmol/L NF-κB (see Fig. 2 in the online Data Supplement). Moreover, linear regression indicated broad dynamic range and high linearity (R² = 0.999; see Fig. 2 in the online Data Supplement), suggesting that, under optimal experimental conditions, this assay can be applied for ultrasensitive quantification of DNA-binding proteins.

**USE WITH NUCLEAR EXTRACTS**

Nuclear extracts contain many DNA-binding proteins that may interfere with the assay. To illustrate applicability to nuclear extracts, we performed the assay shown in Fig. 3 in the online Data Supplement in the presence of the HeLa cell nuclear extract. The specific competitor decreased the amount of dsDNA template (or increased the Ct value) in the presence of NF-κB from nuclear extracts (see Fig. 3, A and C, in the online Data Supplement). In contrast, addition of nonspecific competitor to the same reaction system did not affect the amount of dsDNA template or the Ct value (see Fig. 3, B and C, in the online Data Supplement). Thus nuclear extract–dependent variation of the Ct value was NF-κB specific. Although we added a 100-fold excess of competitor over the amount of DNA probes, a high Ct value was observed (see Fig. 3A in the
For further confirmation, we added an even higher amount of competitor (800 nmol/L) to the reaction. The Ct value obtained indicated that a high degree of amplification was achieved even under these conditions (see Fig. 4 in the online Data Supplement). It appears that although most of the protein binds to the competitor, a substantial fraction is still able to bind to the specific DNA probe. Therefore, the DNA probe could be protected by this specific binding from digestion of ExoIII and be used as the template for further amplification, suggesting that the method has high sensitivity for detecting trace amounts of DNA-binding proteins.

The high amplification might also be caused by nonspecific binding. Therefore, we performed additional experiments to address nonspecific binding of nuclear extracts to the DNA probe lacking the binding site (see Fig. 5

Fig. 1. Real-time PCR for quantification of DNA-binding proteins.
(A), schematic representation of the assay. (B), amplification plots. Curves 1–3 indicate the 2 nmol/L double-strand NF-κB probe without digestion of ExoIII as positive control, the 2 nmol/L double-strand NF-κB probe bound by 10 pmol/L NF-κB and digested by ExoIII, and the 2 nmol/L NF-κB probe with digestion of ExoIII as negative control, respectively.
in the online Data Supplement). The results indicated that the nonspecific binding is very weak, similar to the negative control, thus providing strong evidence that this system is stable and works well to quantitatively evaluate DNA-binding proteins.

To explore quantitative detection of multiple DNA-binding proteins in the nuclear extracts, we selected 10 important transcription factors as target proteins, including SP1, AP2, NFAT, SRE, EGR, YY1, AP1, CREB, OCT1, and NF-κB; detailed information is summarized in Table 1 in the online Data Supplement. The different target proteins in HeLa cell nuclear extract were detected by adding increasing amounts of nuclear extracts to a series of the same molarity dsDNA probes with different protein-binding sites in one 96-well PCR plate. The Ct values decreased with increasing amounts of nuclear extracts (see Fig. 6 in the online Data Supplement). As clearly shown in Fig. 7 in the online Data Supplement, we observed linear regression of signals for the enriched samples against the logarithmic concentration of target proteins, strongly suggesting that this assay could be used for high-throughput quantification of DNA-binding proteins in crude cell extracts.

**Conclusions**

Gene expression is only one of the relevant aspects of cell physiology, another being the misregulation of protein-mediated cellular signaling pathways. The localization and activity of low-abundance oncogenes such as DNA-binding proteins remain difficult to assess with DNA-only approaches. Although proteomic analysis provides useful complementary data, detection is still limited to a fraction of the proteins present in crude cell extracts. Therefore, the development of convenient, specific, sensitive assays for detecting DNA-binding proteins remains an extremely important goal. In EMSA, one of the general DNA-based methods (7), the activity of DNA-binding proteins is evaluated by measuring the intensity of a retarded band produced by DNA-protein complexes in native polycrylamide gel electrophoresis. EMSA can offer a sensitive detection for DNA-binding proteins; however, it is time-consuming and laborious. Moreover, the procedure requires the use of radioactivity and is not adaptable to...
automatic and high-throughput analysis. In ELISA, another conventional DNA-based method, the proteins are measured by capturing DNA-binding proteins with a dsDNA-coupled plate and then detected using ELISA (9). Its sensitivity is limited, however, preventing its utility in detecting trace amounts of DNA-binding proteins.

In this report, we describe a universal real-time PCR coupled with ExoIII protection assay that has broad dynamic range and high linearity for the ultrasensitive quantification of sequence-specific DNA-binding proteins. It does not require antibodies against the target proteins; however, the incomplete digestion of ExoIII has an effect on the detection sensitivity or limit.

We used this assay to successfully detect 10 different transcription factors in the crude cell extracts. Additionally, it could be used to quantitatively evaluate transcription factor NF-κB from the HeLa cell nuclear extract unstimulated and stimulated with TNF-α (Fig. 3), with a ΔCt of 0.87. According to the curvature formula as described in Fig. 2 in the online Data Supplement, we calculated that the concentration of NF-κB increased 7.41 times after the HeLa cell nuclear extracts were stimulated with TNF-α. The results provided strong evidence that this assay has wide potential application in research, medical diagnosis, and drug discovery. Moreover, the assay should be useful for high-throughput measurement of the DNA-binding activity or affinity of proteins. One application could be in screening for drugs targeted to a DNA-binding protein. The technique should also have applications in medical diagnosis, as a means to determine the presence or activity of a DNA-binding protein linked to a particular disease. Our report is proof-of-concept for the assay’s rapid, ultrasensitive, and high-throughput ability to study the DNA-binding activities of cellular extracts and purified and recombinant proteins.

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