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Detection and comparison of protein-DNA interactions using DNA-BIND™ plate and horseradish peroxidase-based colorimetric assay

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Abstract

We describe a procedure for detection and comparison of protein-DNA interactions using DNA-BIND™ plate and horseradish peroxidase (HRP)-based colorimetric assay. Amino-modified oligonucleotide was covalently immobilized on the surface of the DNA-BIND™ plate. After the complementary oligonucleotide was annealed, the plate was incubated with protein to allow sequence-specific DNA-binding. Primary antibody and HRP-labeled secondary antibody were then employed, and colorimetric assay was conducted before the absorbance was read. This method is sensitive, specific and high-throughput, which has been applied not only in the detection of protein-DNA interaction, but also in quantitative comparison of DNA-binding capabilities among wild type and mutant proteins.

Keywords

protein-DNA interaction; DNA-BIND™ plate; HRP-based colorimetric assay

The study of DNA-binding activity of a protein is crucial for elucidating its biological function. The most widely applied technology is perhaps the electrophoretic mobility shift assay (EMSA)¹, whereby proteins are detected by their ability to retard the mobility of a labeled DNA fragment through a nondenaturing gel [1]. EMSA is sensitive but difficult to adapt for high-throughput automatization and is hardly to do quantitative comparison.

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¹*Abbreviations used:* DBD, DNA binding domain; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; HRP, horseradish peroxidase; IRF, interferon regulatory factor; ISRE, interferon-stimulated response element; NOS, N-oxy succinimide; TBS, Tris-buffered saline; TBST, TBS containing 0.5% Tween-20; TMB: 3,3',5,5'-tetramethylbenzidine

Moreover, to gain high sensitivity, EMSA generally depends on radioisotope-labeling which requires safety concerns and handling precautions.

Here, we describe a microwell-based procedure for the detection and comparison of protein-DNA interactions using DNA-BIND™ plate (Costar, Corning, MA) and horseradish peroxidase (HRP)-based colorimetric assay. The principle is illustrated in Fig. 1. This procedure was tested with interferon regulatory factor 7 (IRF-7), which binds to the interferon-stimulated response element (ISRE). The ISRE oligonucleotides (5'-GGT ACC ACA AAC GAA AAA TGG GCC-3', its complementary oligonucleotides 5'-GGC CCA TTT TTC GTT TGT GGT ACC-3' and 5'-NH₂-C12-GGC CCA TTT TTC GTT TGT GGT ACC-3') were synthesized by Invitrogen (Shanghai, China). The AP1 consensus oligonucleotides (5'-CGC TTG ATG AGT CAG CCG GAA-3' and 5'-TTC CGG CTG ACT CAT CAA GCG-3') were purchased from Promega (Madison, WI). Protocol for the detection of protein-DNA interaction using DNA-BIND™ plate and HRP-based colorimetric assay is followed:

1. To the DNA-BIND™ plate, add 100 µl/well of amino-modified C12 linker-armed oligonucleotide in Oligo Binding Buffer (50 mM NaH₂PO₄, pH 8.5, 1 mM EDTA) at a concentration of 25 pmol/well. Incubate overnight at 4°C [2].
2. Remove uncoupled oligonucleotide by washing the plate five times with Tris-buffered Saline (50 mM Tris, pH 7.5, containing 150 mM sodium chloride, TBS).
3. Block the unreacted N-oxysuccinimide ester (NOS) groups by adding 200 µl of 3% BSA in Oligo Binding Buffer. Incubate for 30 min at 37 °C and then decant.
4. Wash the plate five times with 5×SSC (750 mM sodium chloride, 75 mM sodium citrate, pH 7.0).
5. Add 100 µl/well of the complementary oligonucleotide in Hybridization Solution (5×SSC, 0.1% SDS) at a concentration of 25 pmol/well. Incubate for 60 min at 60°C and then decant.
6. Wash the plate with 60°C-preheated 2×SSC containing 0.1% SDS for three times, and with TBS containing 0.5% Tween-20 (TBST) for one more time.
7. Block the plate with TBST containing 5% non-fat milk powder for 60 min at room temperature, and wash three times with TBST.
8. Add 50 µl/well of control protein or partially purified HIS-FLAG tagged wild type or lysine residue mutant IRF-7 [3;4] in DNA Binding Buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM MgCl₂, 5% (v/v) glycerol, 0.5 mg/ml BSA, 100 µg/ml salmon sperm DNA, 0.5 mM phenylmethylsulfonyl fluoride). For competition experiments, 2.5 µg of the specific or non-specific competitor oligonucleotide was added and the salmon sperm DNA was reduced to 2.5 µg/well. Incubate for 60 min at room temperature, and then decant.
9. Wash the plate five times with TBST.
10. Add 100 µl/well 1:5000 diluted anti-FLAG monoclonal antibody (Stratagene, La Jolla, CA) in TBST containing 5% non-fat milk powder. Incubate for 60 min at 37°C.
11. Wash five times with TBST.
12. Add 100 µl/well 1:5000 diluted HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in TBST containing 5% non-fat milk powder, incubate for 60 min at 37°C.

13. Wash five times with TBST, and one more time with TBS.
14. Add 50 μ l/well 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Tiangen, Beijing), incubate for 10 min at room temperature without light exposure.
15. Add 50 μ l/well 2 M sulfuric acid to terminate the reaction.
16. Read the absorbance at 450 nm on a 96-well plate reader (BioTek Instruments, Winooski, VT).

Using this procedure, the ISRE-specific binding of IRF-7 was detected. Very low absorbance (0.058) was read at 450 nm when no DNA-binding protein was added (lane 1, Fig. 2A). Increasing amounts of partially purified IRF-7 resulted in dose-dependent increase of the absorbance at 450 nm (0.066, 0.078, 0.127 and 0.288, lanes 2 to 5, Fig. 2A). Denaturation of IRF-7 by adding 1% SDS in the protein-DNA binding reaction mixture decreased the absorbance to 0.066 (lane 6, Fig. 2A). The absorbance was very low (0.059) without the amino-modified ISRE oligonucleotide. These results demonstrated that the absorbance at 450 nm is dependant on the binding of IRF-7 to the ISRE.

The specificity of IRF-7 binding to ISRE was evaluated by a control protein, a specific competitor and a nonspecific competitor (Fig. 2B). Using the same experimental procedure, the absorbance at 450 nm was as low as 0.056 when a HIS-FLAG tagged control protein was utilized to replace the IRF-7, demonstrating its inability to bind to ISRE (lane 3, Fig. 2B). When the unmodified competitive oligonucleotides were included in the protein-DNA binding reaction mixture, the A450 was decreased, demonstrating that the binding of IRF-7 to the ISRE is specific (lane 4, Fig. 2B). However, when AP1 consensus oligonucleotides were added, the absorbance was not decreased, indicating that binding of IRF-7 to the ISRE was not impaired by nonspecific competitor (lane 5, Fig. 2B). These results demonstrated this method can detect the protein-DNA interaction specifically.

This procedure provided us a simple way to quantitatively compare the DNA binding capabilities among wild type and mutant proteins (Fig. 2C). Various lysine residue mutants of IRF-7 [3;4] were tested for their DNA binding capabilities. The absorbance for mutants K45R (lane 3, Fig. 2C), K61R (lane 5, Fig. 2C), K92R (lane 6, Fig. 2C) and K120R (lane 7, Fig. 2C) were all very low, compared with the wild type IRF-7 (0.541, lane 2, Fig. 2C), indicating that mutations at these lysine residues located in the DNA binding domain (DBD) prevented IRF-7 from binding to the ISRE. However, the absorbance for mutant K50R (lane 4, Fig. 2C) was decreased to 0.294, suggesting that mutant K50R retained partial ISRE binding capability. Western blot analyses revealed that the variation of binding abilities was not due to different amount of wild type or mutated IRF-7 protein (lower panel of Fig. 2C). This result is in agreement with the crystal structure of IRF-7 DBD [5] and our previous findings that lysine residue mutants at K45, K61, K92 and K120 abolished or weakened significantly the ability of IRF-7 to activate IFN promoters while mutant K50R retained partial activity [4].

In this study, we described a simple, quantitative, and high-throughput method for the detection and comparison of protein-DNA interactions. The procedure is originated from the enzyme-linked immunosorbent assay (ELISA) [6], except that the protein is not absorbed to the multiwell plate directly, but through protein-DNA interaction with covalent immobilized oligonucleotides. For ISRE-specific DNA binding of IRF-7 [3], the sensitivity of this procedure was almost as good as EMSA. The specificity of this method was also proved to be good since the HIS-FLAG tagged control protein could not bind to the ISRE, and the specific competitor decreased the ISRE binding of IRF-7 while the nonspecific competitor did not. This method is also stable and reproducible. At the immobilization step, the amine-modified oligonucleotide is covalently coupled to the surface of the multiwell plate through

a nucleophilic substitution reaction [2], which can not be washed off the plate and should be more stable than the linking through hydrogen bonds, for instance, between streptavidin and biotin [7]. In addition, using HRP-based colorization assay should be more general and economical than using alkaline phosphatase-conjugated secondary antibody in similar systems [8]. The procedure described here can be applied in comparisons of DNA-binding abilities of proteins and screening for specific protein binding sites of DNA.

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Fig. 1. Schematic representation of microwell-based procedure for the detection and comparison of protein-DNA interactions using DNA-BIND™ plate and HRP -based colorimetric assay.

Fig. 2.

Detection of the ISRE-specific binding of IRF-7 using DNA-BIND™ plate and HRP-based colorimetric assay. (A) Detection of the ISRE-binding of IRF-7, using no protein (lane 1) or increasing amounts (from 6 ng to 150 ng) of partially purified IRF-7 (lanes 2 to 5). Lane 6 is the same as lane 5 except that 1% SDS was added in the binding reaction mixture. Results are mean values from three independent experiments, and the standard deviations are shown. (B) Detection of the ISRE-specific DNA binding of IRF-7. No protein (lane 1), partially purified IRF-7 (lanes 2, 4 and 5) or control protein that does not bind ISRE (lane 3) were utilized. 2.5 µg competitor (lane 4) or AP1 consensus oligonucleotides (lane 5) were added in the reaction mixture. (C) Quantitative comparison of ISRE-binding abilities of wild type and mutant IRF-7. No protein (lane 1) or wild type (lane 2), K45R (lane 3), K50R (lane 4), K61R (lane 5), K92R (lane 6) or K120R (lane 7) of IRF-7 were utilized to detect the ISRE-binding. Western blot result is shown in the lower panel conducted with anti-FLAG monoclonal antibody.