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Endonuclease-based Method for Detecting the Sequence Specific DNA Binding Protein on Double-stranded DNA Microarray

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Abstract: The double-stranded DNA (dsDNA) probe contains two different protein binding sites. One is for DNA- binding proteins to be detected and the other is for a DNA restriction enzyme. The two sites were arranged together with no base interval. The working principle of the capturing dsDNA probe is described as follows: the capturing probe can be cut with the DNA restriction enzyme (such as EcoR I) to cause a sticky terminal, if the probe is not bound with a target protein, and the sticky terminal can be extended and labeled with Cy3-dUTP by DNA polymerase. When the probe is bound with a target protein, the probe is not capable to be cut by the restriction enzyme because of space obstruction. The amount of the target DNA binding proteins can be measured according to the variations of fluorescent signals of the corresponding probes.

Keywords: Double stranded DNA microarray, DNA binding protein, label-free detection.

Sequence-specific DNA-binding proteins are a large group of proteins which play an important role in regulating cellular processes. Many methods have been developed to detect the activation ability of proteins to their DNA consensus, such as gel-shift assays[1-2] and DNase I foot-printing assays[3]. Generally, the conventional methods are laborious, time-consuming，incapable of high-parallel analysis，and even involved in the use of radioisotopes. Recently, a molecular beacon assay was created for DNA-binding proteins detection, which provided a free-labeled approach for the detection of DNA-binding proteins[4]. Microarray, characterized with its high-parallel capacity of detecting molecular target as DNA and protein in samples, has provided a platform for high-throughput detecting and analyzing the interaction between biological molecules, and

Table 1 Oligonucleatides used for fabricating dsDNA microarray

Name	Sequence	Length (nt)
Oligo I	5'…. NH_2-TTTTTTGTTGCATTTCCGGGTTTGGCAAGCTTTTAA GCTT… 3'	40
Oligo II	5'. . . NH_2-TTTTTTGTGGGGACTTTCCGAATTCGCGATCGC GAATTC …3'	40
Oligo III	5'… NH_2- TTTTTTGTG ATTTCCGG GAATTC GCG AT CGC GAATTC …3'	38

The blocks stand for the sequence which could be hybridized as a hairpin structure and digested by EcoR I. The gray parts on the sequences stand for the DNA consensus of the NF-*k*B p50p50.

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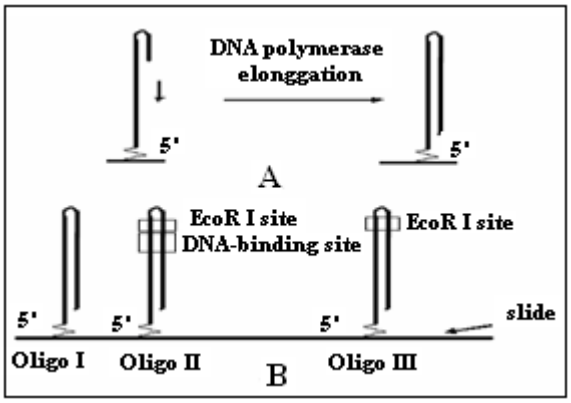
Figure 1 The method of the dsDNA microarray fabrication



A: After the probe spotted on glass surface by 5'end, the 3' end of DNA sequence was annealed to form a short double-stranded hairpin structure, and then ssDNA was enzymatically extended to become a double-stranded DNA probes. **B:** Three kinds of Oligos used to fabricate dsDNA microarrays. Oligo I without any protein binding site was spotted as a negative control. Oligo III only contained an EcoR I site and spotted as a positive control. For the reason of space obstruction, once the NF-*k*B proteins bind to their sites, the EcoR I molecules will not be able to combine with their corresponding DNA sites at the same duplex DNA probes and no fluorescence detected after DNA polymerase extension with Cy3-dUTP. On the other hand, the probes not bound with NF-*k*B proteins would be digested by EcoR I, and the stick terminals will be detected after DNA polymerase extension with Cy3-dUTP.

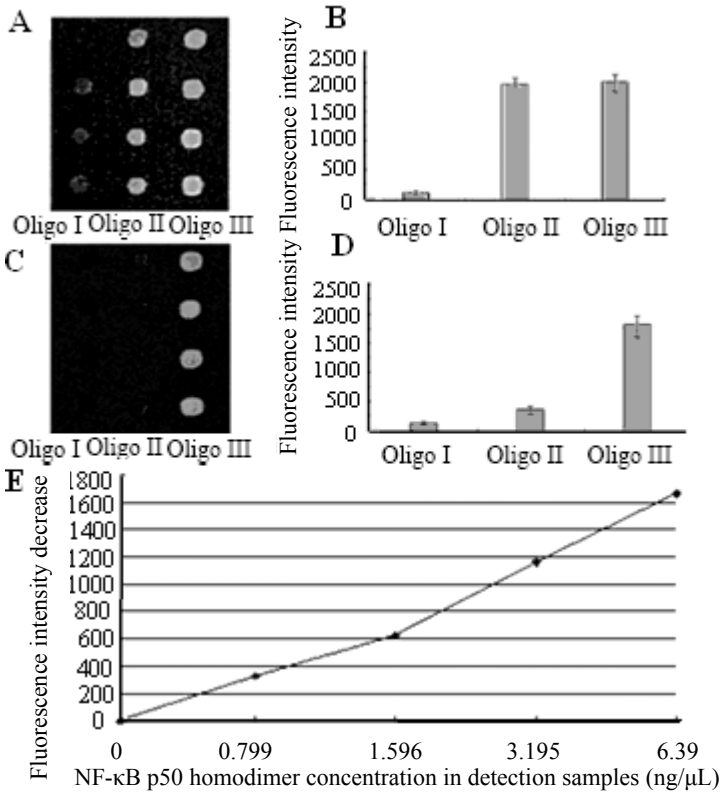
now began to be used in analyzing DNA-binding proteins[5-6]. However, the above dsDNA microarray technology is still inconvenient and easy to introduce interference due to its requirement of labeling the target proteins previously. Here we report a new kind of capturing dsDNA probe system to perform NF-*k*B p50p50 label-free detection, in which the EcoR I recognition site arranged closely to the NF-*k*B binding site.

Fabrication of dsDNA microarray was described elsewhere[6], which combine micro-spotting technology and DNA enzymatic synthesis. In this study, we designed and chemically synthesized three kinds of dsDNA probes (see **Figure1** and **Table 1**) from Shengyou Inc. (Shanghai, China). The 10-base consensus of protein NF-*k*B p50 homodimer was designed as 5'…GGGACTTTCC…3'. Oligo II was designed with a NF-*k*B binding site and an EcoR I site at the same duplex DNA probe.

All of the protocol of the dsDNA microarray preparation and protein binding were described elsewhere[6]. Oligo I, Oligo II and Oligo III were dissolved in sodium carbonate buffer (0.1 mol/L, pH 9.0) at the concentration of 80 μmol/L and spotted by using a microarrayer on glutaraldehyde-derived glass slides and elongated by Klenow DNA polymerase to create dsDNA microarrays. After the fabrication of the dsDNA microarrays, two slides were incubated with 20 μL DNA-binding buffer at RT for 1 h. In the two different 20 μL binding buffers, one did not contain any NF-*k*B protein, the other with 6.39 ng/μL NF-*k*B (p50). After incubation, the slides were washed and then digested by EcoR I reaction system at 37℃ for 2 h. The sticky end of duplex probes on the microarray was extended by Klenow DNA polymerase elongation system with

Cy3-dUTP. All the slides were scanned by using ScanMicroarray® Lite (Packard Biochip Technologies) in the Cy3 channel at 85% laser power, 80% PMT gain, 5 μm resolution.

Figure 2 The images and fluorescence intensity variations of dsDNA microarrays extended with DNA polymerase with Cy3-dUTP



Oligo I (N-control) could not be digested by EcoR I and not be inserted with Cy3-dUTP during the DNA polymerase elongation. **A and B:** Oligo II and Oligo III (P-control) all contained EcoR I site and produce high fluorescence intensity when only treated with EcoR I and extended with Cy3-dUTP. **C and D:** Oligo II which contained EcoR I site and NF-*k*B binding site presented lower fluorescence intensity than Oligo III (P-control) after NF-*k*B proteins binding and EcoR I digestion, subsequently extension with Cy3-dUTP. **E:** The relationship between different NF-*k*B p50 homodimer concentrations in detection samples and fluorescence intensity decreases.

Table 2 Fluorescence intensity variations of dsDNA probes caused by NF-*k*B binding

Fluorescence intensity	No NF-*k*B binding	NF-kB binding
Oligo I	127. 50±23. 78	125. 50±12. 67
Oligo II	2045. 23±45. 56	410. 67±68. 45
Oligo III	2123. 67±75. 33	2075. 35±66. 67
Fluorescence decrease (Oligo III-OigoII)	29. 38	1664. 68
NF-kB concentration(ng/μL)	0	6. 39

The images and fluorescence intensity variations of dsDNA probes caused by NF-*k*B binding were shown in **Figure 2** and **Table 2**, respectively. In **Figure 2**, the Oligo I (as negative control) in microarray with and without NF-*k*B binding all presented very low fluorescence intensity (127.50±23.78 and 125.50±12.67), because of without any EcoR I recognition site. On the other hand, the fluorescence intensity of Oligo III (as positive control) in **Figure 2A** (2123.67±75.33) was approximated to Oligo III in **Figure 2C** (2075.35±66.67). The fluorescence intensity of Oligo II in **Figure 2A** (2045.23±45.56) was extremely higher than Oligo II in **Figure 2C** (410.67±68.45). It implied that the NF-*k*B binding had interfered in the efficiency of EcoR I digestion for the space obstruction. It also can be found in **Figure 2A** that the fluorescence intensity of Oligo II (2045.23±45.56) was close to the intensity of Oligo III (2123.67±75.33). Our experiment results in **Figure 2E** showed that different concentrations of NF-*k*B protein in detection samples binding had caused different fluorescence decreases. It demonstrated that it would be a possible way to detect or analyze sequence specific DNA-binding proteins by measuring the digestion efficiency of EcoR I through the fluorescence decrease of the immobilized probes, and would not be needed to label proteins.

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