Fluorometric assay of DNA binding proteins using class IIS endonuclease

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ABSTRACT
Fluorometric DNA-protein binding assay was developed with class IIS restriction enzyme and FITC'-labeled DNA fixed on magnetic beads. We used this assay with AP1 and FokI as a case study to quantify DNA-protein binding. Fluorescent images of the treated beads showed that the transcriptional factor bound to its DNA site and inhibited the restricted digestion effectively. The affinity of AP1 to its site was estimated as a Kd value, which was approximately 10nM.

(*FITC: Fluorescein-4-isothiocyanate)

INTRODUCTION
Various studies have been conducted on the characterization of transcriptional factors and their regulation sites. (1) Recent advances in combinatorial methods have resulted in improvements in our ability to determine the consensus binding sequences. (2) In this assay, we took a novel approach toward detection of a DNA-protein interaction. The assay utilizes class IIS restriction endonucleases and the competitive inhibition that results from DNA-protein binding. The endonucleases are an unusual class of enzymes that recognize a specific DNA sequence and cleave DNA non-specifically a short distance away from that sequence. Therefore, if a protein binds next to the DNA sequence specific for the endonuclease, the DNA-protein complex will prevent the endonuclease from digesting its restriction site. If there is no DNA-binding complex present then the enzyme will cleave the DNA.

In this study we utilized this assay in conjunction with fluorometric imaging of the remaining FITC-labeled DNA on magnetic beads. We then quantified the amount of DNA-protein binding by measuring the fluorescence emitted from the digested FITC-labeled DNA fragments in solution.

RESULTS AND DISCUSSION
Fluorescence imaging of AP1 binding
We used the transcriptional factor AP1 and the class IIS restriction enzyme FokI to demonstrate this method with custom designed DNA fragments biotinylated at their 5'-end. The DNA fragments were constructed

Figure 1. Fluorescence images of magnetic beads coated with FITC-labeled DNAs. Fluorescence from beads represented the amount of DNAs (A), and almost all DNAs were digested by FokI treatment (B). In the presence of AP1, DNAs were partially digested and some DNAs still remained on the bead (C). When applying the same treatment to another, random DNA sequence in place of the AP1 sequence (N13_template), AP1 did not bind and no decrease was observed in FokI enzyme activity (D).
in the following order: FokI recognition sequence, consensus sequence for AP1 specific binding and FokI restriction site (AP1_template). After extending complementary strands of the AP1_template from FITC-labeled primers with T7 DNA polymerase, all DNA were fixed on magnetic beads. The beads were then incubated with AP1 for 10 min at room temperature, and digested by FokI for 30 min with vigorous shaking at 37°C. These beads were washed and recovered with a magnetic particle concentrator and imaged by fluorescence microscopy. Fluorescence images of DNA-protein binding in Figure 1 show the steric hindrance effect of specific AP1 binding to the DNA fixed on the beads. Fluorescence intensity from each particle indicated the amount of remaining DNA after FokI digestion. These results suggested qualitatively that the DNA binding protein inhibited class IIS restriction enzyme activity when its specific binding site was encoded between the recognition and restriction sites.

Characterization and quantification of AP1 binding
Comparing the particles in Figure 1, the intensity of the fluorescence was found to vary in each experiment. These differences were partly because the beads could not be excited evenly under the microscope because some were always out of focus. Furthermore, some individual particles were aggregated into larger particles. To overcome these technical problems for quantification of DNA-protein binding, we measured the total fluorescence of the DNA fragments following digestion and release from the beads into the supernatant. The dissociation constant for AP1 binding to the AP1 site was measured to demonstrate the utility of the technique. The concentration dependence of AP1 to its specific binding site was determined by the difference between total binding of AP1 to the AP1_template and non-specific binding of AP1 to a N13_template. Applying 0.02mg/ml poly d (I-C) and 0.01% TritonX-100 during the assay kept AP1 DNA complexes stable at low concentrations of AP1. (Data not shown) The measured affinity of the AP1-AP1 DNA site complex (Kd) was approximately 10 nM estimated from the slope of scatchard plot. Comparing the AP1 binding affinities measured by other conventional methods (1) (Kd=34±4nM), the Kd value was appropriate considering different experimental procedures and conditions. In this study, we demonstrated fluorometric applications for the detection of DNA-protein interactions. Introduction of these applications into the various combinatorial approaches will help advance our understanding of transcriptional regulation mechanisms.

ACKNOWLEDGEMENT
This work was supported by a grant-in-aid for Genome Informatics Technology from New Energy and Industrial Technology Development Organization (NEDO), Japan.

REFERENCES