

Development of Heterogeneous and Homogeneous Platforms

for Rapid Analysis of DNA-Protein Interactions

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Abstract

Two plate-based assay platforms have been developed for rapid analysis of DNA-protein interactions. The first is a heterogeneous, ELISA-based platform in which a biotinylated dsDNA molecule is bound to a streptavidin coated plate. Cell lysate, purified protein, or nuclear extract is incubated in the wells. The DNA-protein complex is then detected with target specific antibody conjugate and substrate. Experimental results, based on several model systems, show it to be a versatile tool for screening known and unknown consensus protein binding sequences and for analysis of multi-component protein complex assembly. This platform is non-radioactive, fast, sensitive, and suitable for analysis of multiple DNA fragments and proteins in a high throughput fashion. The second, homogeneous, assay platform utilizes fluorescence polarization to screen known consensus sequence and target protein. Consensus sequence is labeled with a fluorophore and incubated with the target protein, either purified or in cell lysate. After a 30 minute incubation, the signal is ready to be detected on a plate reader. Data from several model systems demonstrate that the assay is highly sensitive and can detect single base mutations within the binding region. The method is adaptable to 96 and 384-well platforms, making it an ideal tool for research and high throughput drug screening applications.

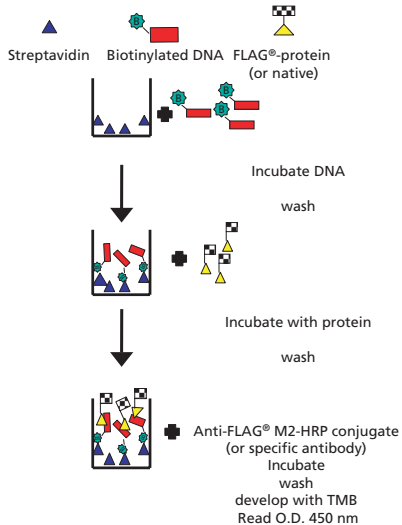
Introduction

Assays that identify and analyze DNA-protein interactions are useful tools for studying cell functions such as gene expression, DNA repair and DNA packaging. These assays can also be useful for determining the affinity, abundance, binding constants and binding specificity of DNA-binding proteins. Screening for unknown consensus sequences can also be achieved. Standard methods for studying these interactions include the use of mobility shift assays. However, these assays can be time and labor intensive and often employ the use of radioactive isotopes. The plate-based assay formats described here are versatile, sensitive, non-radioactive, and fast.

The heterogeneous assay platform utilizes oligonucleotides or PCR fragments that are biotinylated and captured onto a streptavidin coated plate. Cell lysate containing native or affinity tagged proteins is incubated in the well, washed, and DNA-bound protein is detected using a protein specific or epitope-specific antibody. The homogeneous assay platform utilizes oligonucleotides or PCR fragments labeled with fluorescein that are added to the wells of a 96-well plate. Cell lysate or purified protein is then added to the wells. The DNA-protein interaction is detected by measuring the difference in rotational motion in solution when the fluorescein is excited by polarized light (fluorescence polarization).

Results

Overview of Heterogeneous Assay Platform using FLAG™-tagged Proteins



Model Systems

- p65 protein/β-Interferon promoter (β-If)**
 5' Biotin - GCC CTG GGA AAT TCC TTG AA 3'
 3' CGG GAC CCT TTA AGG AAC TT 5'
 β-If-Mutant
 5' Biotin - GCC CTG GAA TCC TCG TTG AA 3'
 3' CGG GAC CTT AGG TCA AAC TT 5'
 β-If (unlabeled competitor)
 5' GCC CTG GGA AAT TCC TTG AA 3'
 3' CGG GAC CCT TTA AGG AAC TT 5'
- p65/Human Ig-K promoter (Ig-K)**
 5' Biotin - GCC CTG GGG ATT TCC TTG AA 3'
 3' CGG GAC CCC TAA AGG AAC TT 5'
 Ig-K-Mutant
 5' Biotin - GCC CTG ATT GTC GGC TTG AA 3'
 3' CGG GAC TAA CAG CCG AAC TT 3'
- P65/α-antitrypsin promoter (α-AT)**
 5' Biotin - AGT TGC AGG AGT TGG AGG GGC TTT CCC CAT AGG CAC CCT GGC 3'
 3' TCA ACG TCC TCA ACC TCC CCG AAA GGG GTA TCC GTG GGA CCG 5'
 α-AT-Mutant
 5' Biotin - AGT TGC AGG AGT TGG ATT TGG CCG GCC CAT AGG CAC CCT GGC 3'
 3' TCA ACG TCC TCA ACC TAA ACC CCG CCG GTA TCC GTG GGA CCG 3'
- P65/Human E-Selectin promoter (ES)**
 5' Biotin - AGC TTA GAG GGG ATT TCC GAG AGG A 3'
 3' TCG AAT CTC CCC TAA AGG CTC TCC T 5'
 ES-Mutant
 5' Biotin - AGC TTA GAG GTT ATC CGG GAG AGC A 3'
 3' TCG AAT CTC CAA TAG CCG CAG TCC T 5'

Figure 1. Oligonucleotide Design for Four Model Systems. Forward and reverse oligonucleotides were synthesized (Sigma-Genosys, Woodlands, TX) for the 1) β-Interferon promoter, 2) Human Ig-K promoter, 3) α-antitrypsin promoter, and 4) Human E-Selectin promoter. Regions highlighted in blue denote the binding domain for p65 protein. Mutant sequences were moderately rearranged. Annealing reactions were performed by heating at 95 °C for 5 minutes, 75 °C for 30 minutes, 60 °C for 60 minutes, and allowed to cool to room temperature. Reactions were checked for completion by 20% TBE PAGE.

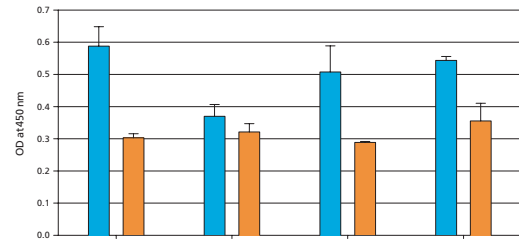


Figure 2. Analysis of DNA-Protein Interaction with Four Model Systems. 200 μl containing 0.5 μM annealed oligonucleotides were coated onto the streptavidin plate, 10 μl p65 transfected cos-7 lysate were incubated in the coated wells containing 180 μl Binding Buffer (10 mM Tris-acetate pH 7.4, 10 mM magnesium acetate, 0.1 mM DTT, 5% DMSO, 0.2 mM EDTA) for 30 minutes at 37 °C with gentle shaking. The plate was washed 4x in TBS and incubated with anti-FLAG M2-HRP conjugate (1:2000) for 1 hour at room temperature with gentle shaking. The plate was washed 4x with TBS and then developed with TMB. Differences in binding affinities between model interactions can be detected.

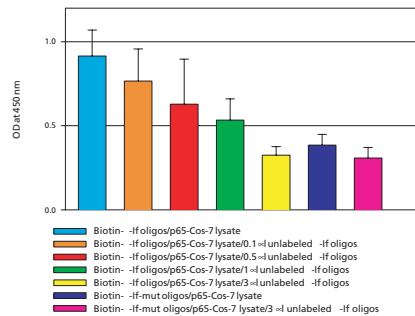


Figure 3. Disruption of p65-β-If Binding Domain Interaction Using Unlabeled Competitor. The p65-β-If interaction was successfully competed off by using increasing concentrations of unlabeled β-If oligonucleotides. The data demonstrates the specific binding of p65 protein to β-If consensus sequence.

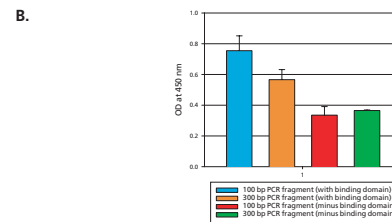
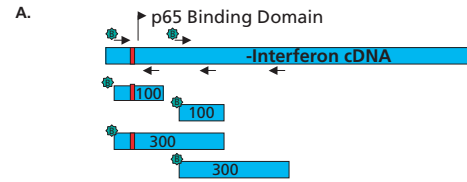
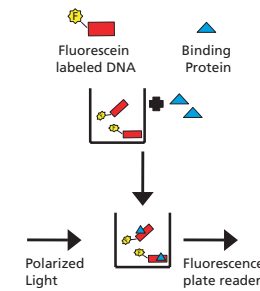


Figure 4. p65-β-If Interaction Using PCR Generated Fragments With or Without p65 Binding Domain. A) 5' biotinylated and 3' primers were designed to amplify varying regions of β-Interferon cDNA. Two PCR fragments (100 bp and 300 bp) contained the p65 binding domain and two fragments (100 bp and 300 bp) were outside the binding domain. B) Interaction between the PCR fragments and p65 transfected lysate shows increased signal from only the fragments containing the p65 binding domain.



Overview of Homogeneous Assay Platform

- Fluorescein-β-Interferon**
 5'F - GCC CTG GGA AAT TCC TTG AA 3'
 3' CGG GAC CCT TTA AGG AAC TT 5'
 β-If (unlabeled competitor)
 5' GCC CTG GGA AAT TCC TTG AA 3'
 3' CGG GAC CCT TTA AGG AAC TT 5'
 Fluorescein-β-If-mutant (negative control)
 5'F - GCC CTG GAA TCC AGT TTG AA 3'
 3' CGG GAC CTT AGG TCA AAC TT 5'
- Fluorescein-c-Fos**
 5'F - CGC TTG ATG ACT CAG CGC TTG A 3'
 3' CGG AAC TAC TGA GTC GGC AAC G 5'
- Fluorescein-c-Jun**
 5'F - CGC TTG ATG ACT CAG CCG GAA 3'
 3' GCG AAC TAC TGA GTC GGC CTT 5'
 Fluorescein-c-Jun-mutant (negative control)
 5'F - CGC TTG ATG ACT TGG CCG GAA 3'
 3' GCG AAC TAC TGA ACC GGC CTT 5'
- Fluorescein-c-Fos-1 bp mutant**
 5'F - CGC TTG ATG CCT CAG CGC TTG A 3'
 3' GCG AAC TAC TGA GTC GGC AAC G 5'
Fluorescein-c-Fos-2 bp mutant
 5'F - CGC TTG ATG ACT TGG CGC TTG A 3'
 3' GCG AAC TAC TGA ACC GCG AAC G 5'
- c-Fos (unlabeled competitor)**
 5' - CGC TTG ATG ACT CAG CGC TTG A 3'
 3' - GCG AAC TAC TGA GTC GGC AAC G 5'

Figure 5. Oligonucleotide Design for Fluorescence Polarization Assays. Forward and reverse oligonucleotides were synthesized for the 1) β-Interferon promoter, 2) c-Fos and 3) c-Jun transcription factor binding domains. 4) One and two base pair mutants for c-Fos were designed by random drawing. Regions highlighted in blue denote binding domain. Red text denotes a mutated sequence. Annealing reactions were performed (Fig. 1).

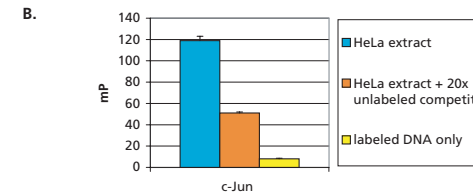
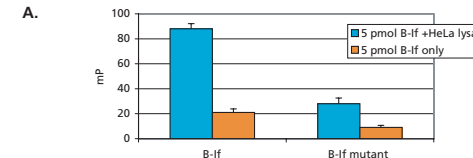


Figure 6. Detection of Endogenous Protein-DNA Interactions Using Fluorescence Polarization (FP). A) Binding of p65 from HeLa lysate to fluorescein labeled β-If/β-If-Mutant oligonucleotides was detected by FP after 30 minutes incubation at room temperature in a black 96-well plate. ΔmP was measured on the Tecan Ultra plate reader (Zurich, Swi). B) 1 μl TNF stimulated HeLa nuclear extract was added to wells containing 5 pmol c-Jun fluorescein labeled oligonucleotides. Binding was competed off by adding 20x unlabeled DNA. Reactions incubated for 15 minutes at room temperature. ΔmP was measured on the Tecan Ultra plate reader.

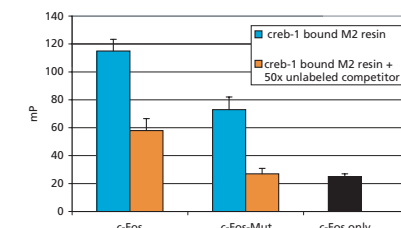


Figure 7. Combined Immunoprecipitation with FP Detection. Creb-1 human transcription factor was cloned into Director-Ready pFLAG-CMV2 and transfected into cos-7 cells. Expressed FLAG-creb-1 was captured from lysate onto anti-FLAG-M2 agarose beads; 2 μl captured creb-1 was incubated with 5 pmol fluorescein labeled c-Fos/c-Fos-Mutant oligonucleotides in a black 96-well plate for 30 minutes at room temperature with gentle shaking. Binding was competed off by the addition of 50x unlabeled DNA.

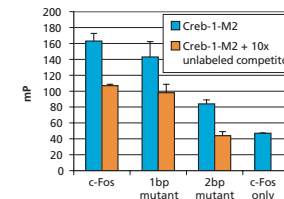


Figure 8. FP Analysis of Binding Affinities for Consensus Sequence Mutants. The sensitivity of the assay was demonstrated by designing a set of fluorescein labeled c-Fos oligonucleotides that contain mutations within the binding domain. (Fig. 5(4)). One and two base pair mutants were chosen by random drawing. Oligonucleotides were added to a black 96-well plate. FLAG-creb-1 was captured from transfected lysate by anti-FLAG-M2 agarose beads. 4 μl was added to the wells and incubated for 30 minutes at room temperature with gentle shaking. ΔmP was measured on the Tecan Ultra plate reader.

Conclusions

- The heterogeneous, ELISA-based assay platform provides rapid analysis of DNA-protein interactions. It is non-radioactive, fast, and sensitive. Assays can be completed in as little as 3 hours.
- The heterogeneous, ELISA-based assay platform is suitable for the analysis of multiple binding domains and proteins in a high-throughput fashion. The platform has been demonstrated using several model systems.
- Both platforms are versatile tools for the screening of known and unknown consensus protein binding sequences.
- Advantages over traditional EMSA include simpler optimization and determination of numerous variables.
- The homogeneous assay platform utilizes fluorescence polarization to screen known consensus sequence and target protein. The platform is highly sensitive, detecting single base pair mutations within the binding region.
- The homogeneous assay platform is adaptable to high-throughput applications, making it an ideal tool for drug screening.

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References

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Related Sigma Products

Product Name	Product Number
Director Ready pFLAG-CMV2 Expression Vector	RDC-L3
SigmaScreen Streptavidin coated 96-well plate, clear	M 5432
Anti-FLAG-M2-HRP conjugate	A 8592
TMB liquid substrate system for ELISA	T 0440
96-well, flat bottom plate, black	M 9685
Anti-FLAG-M2-Agarose	A 2220

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