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Protein-induced fluorescence enhancement detects protein-nucleic acid interactions in microplates

Fulneček J., Valuchová S., Petrov A.P., Tripsianes K., Říha K.
Research group Molecular Biology of Plants, Central European Institute of Technology, Masaryk University, Brno, Czech Republic

- Detect structure and sequence specificities of protein-DNA interactions
- Well-scanning function of BMG LABTECH's multi-mode reader used to increase measurement surface and stability

Introduction

Various biological processes depend on the interaction of proteins with nucleic acids. These comprise packaging of DNA, transcriptional regulation, DNA replication and DNA repair. The binding of proteins to nucleic acids are hitherto detected by electrophoretic mobility shift assays (EMSAs), fluorescence polarization (FP) assays or chromatin immunoprecipitations (ChIP). However, these methods are either laborious (EMSA, ChIP) or limited to specific targets (EMSA, FP, ChIP). A novel fluorescence assay detects interactions of nucleic acids with proteins to identify binding, sequence and structure specificities or dissociation constants. The microplate assay is a quick and cost-effective alternative and can be read on a fluorescence reader with the capability to scan wells such as the FLUOstar® Omega.
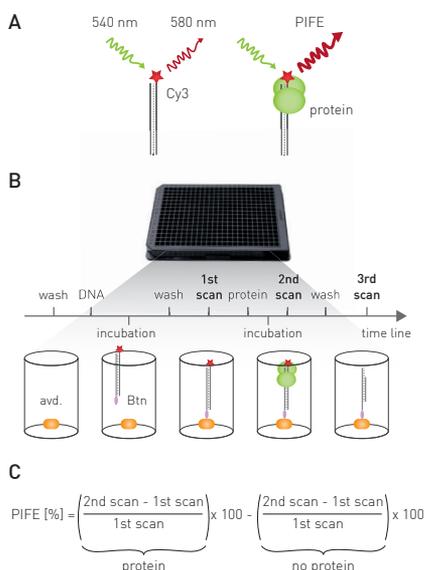
Assay Principle

Fig. 1: Detecting protein-nucleic acid binding by mwPIFE. (A) Fluorescence is enhanced when a protein binds in the physical vicinity of Cy3. (B) Experimental workflow of mwPIFE. Neutravidin is coated on a microwell plate to bind biotinylated oligonucleotide. The fluorescence of a bound oligonucleotide is measured with the FLUOstar Omega. After incubation with protein a second scan is performed. An optional washing step is followed by a third scan. (C) Formula used to calculate PIFE. Figure adapted from Valuchova *et al.*

Cyanine dyes appear as non-fluorigenic cis-isomers and as fluorescent trans-isomers dependent on their local environment[1]. The microwell protein-induced fluorescence enhancement (mwPIFE) assay exploits this phenomenon as it uses Cy3-bound oligomers immobi-lized onto the bottom of a microplate. Protein-binding in proximity to Cy-3 sterically hinders formation of the non-fluorescent cisisomer leading to increased fluorescence (Fig. 1A). Workflow and calculation of PIFE value are shown in Figure 1B and C.

Materials & Methods

- Black 96 well microplate (Greiner, FluotracTM 600, high binding, #655077) coated with 100 µl NeutrAvidin (Pierce, binding capacity ~15pmol D-biotin/well) and blocked with SuperBlock blocking buffer (ThermoScientificic, #15117)
- Proteins*: BamHI (ThermoScientific), XPF/ERCC1 (expressed in Escherichia coli strain BL21 (DE3)), KU70/KU80 (expressed in HI5 insect cells)
- Cy3-labeled oligonucleotides (IDT)*
- FLUOstar Omega microplate reader (BMG LABTECH)

The Cy3-labeled oligonucleotides (100 µl, 25nM) were incubated on the plate for 2 h to bind and sub-sequently washed three times to remove residual nucleic acids. A first scan determined baseline fluorescence, a second scan detected binding of the protein to the oligonucleotide after incubation with 100 µl of the protein solution.

Instrument settings

	FLUOstar® Omega	
Optic settings	Fluorescence Intensity, well scan mode, top optic	
	Excitation: Ex540-10	
	Emission: Em580-10	
	Gain: 2800	
General settings	Settling time: 0.1 s	
	Number of flashes per scan point: 10	
Scan settings	Matrix	
	Scan points: 10 x 10	
	Diameter: 6 mm	

Results & Discussion

Using protein-induced fluorescence enhancement in microplates, the binding of the endonuclease BamHI to its recognition site was verified by a PIFE-value of 30 % (Fig. 2B). Addition of non-labelled oligonucleotides containing the recognition site, but sequence changes

outside the recognition motif decreased the PIFE value down to 5-15 %, depending on concentration and sequence. This indicates lower BamHI-binding to the Cy3-labelled oligo due to competitive binding to unlabeled oligos. Non-labeled probes bearing mutations inside the recognition site gave a PIFE-value of >30 %, representing unperturbed binding of BamHI to the wild-type recognition site.
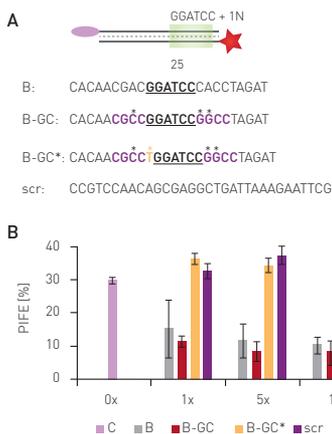
A

GGATCC + 1N

25

B: CACAACGAC**GGATCC**CACCTAGAT

B-GC: CACAA**CGCCGGATCCGGCC**TAGAT

B-GC*: CACAA**CGCCT**GGATCC**GGCC**TAGAT

scr: CCGTCCAACAGCGAGGCTGATTAAAGAATTCG

B

Fig. 2: Sequence specificity of BamHI binding. (A) Sequences of Cy3-labelled DNA probe and competitors (B) dsDNA competitors were added in 1–10x excess over labeled and immobilized DNA probe. Error bars indicate standard deviations from three wells. Data published in Valuchova *et al.*

Having shown that mwPIFE detects sequence preferences of protein-DNA binding, preferential binding of proteins to specific DNA structures was investigated. The XPF/ERCC1 heterodimer incises damaged DNA for repair and specifically binds structures of damaged DNA. A truncated XPF/ERCC1 heterodimer retaining the DNA-binding site was used to determine structure specific interaction. The dimer minimally bound ssDNA or short, blunt ended DNA if competing with the probe displayed in Figure 3B. Reduction of the PIFE-value due to binding of the competing DNA-structure was observed for 10 nt 3'overhang, a hairpin and a splayed DNA-structures (Fig. 3).

A

35/25   HP   Y   Y/10   10ds   10ss

Fig. 3a: Structure specific binding of XPF/ERCC1. Structure of competitor oligonucleotides. Number indicate lenghts of oligonucleotides.

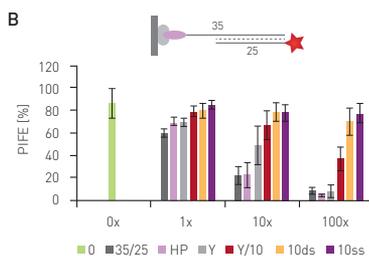*For further information and when publishing data using the mwPIFE please refer to Valuchova *et al.*[3]

B

35

25

Fig. 3b: Structure specific binding of XPF/ERCC1. Competitors were added to DNA-probe-protein complex in 1x, 10x, and 100x excess and PIFE values were calculated. Data published in Valuchova *et al.*

The KU70/KU80 heterodimer is responsible for binding broken dsDNA ends, thereby protecting them from degradation and keeping both ends in proximity to allow for ligation. Increasing the concentration of KU-heterodimer resulted in higher PIFE-values and allowed for the determination of the equilibrium constant KD (Fig. 4).
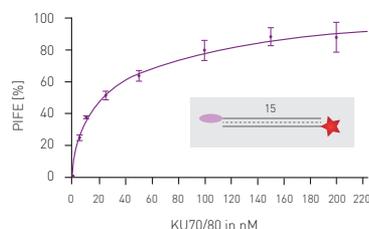
15

Fig. 4: Analysis of Ku-DNA interaction by mwPIFE. The indicated probe was titrated with KU dimer. The binding curve was reconstituted from 3 independent replicas with standard deviations indicated. The calculated Kd value was 10 ± 5 nM. Data published in Valuchova *et al.*

Conclusion

The mwPIFE detects interaction of nucleic acids with proteins and reports on sequence and structure specificities as well as on binding constants. The change in fluorescence intensity needs to be captured on a huge surface of the well to provide stable measurements. This has been enabled by the well-scan function of the FLUOstar Omega combined with its high sensitivity. The simple and cost-effective assay broadens the application to detect structure-specific bindings. Its plate format accelerates the determination of protein-nucleic acid interactions.*

References

1. Hwang H. (2014) *Chem Soc Rev.* **43**(4): 1221–1229
2. Valuchova S. (2016) *Sci Rep.* **23**;6:39653.

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