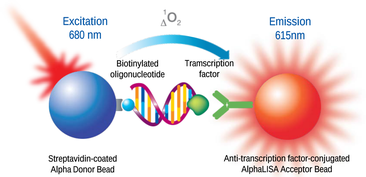
A Fast and Simple, Non-Radioactive Assay to Replace EMSA in the Monitoring of DNA-Protein Interactions

Julie Bédard[1], Mathieu Larivière[2], Daniel Sinnett[2,3], Gregory Cosentino[1] & Nathalie Rouleau[1]

1 Introduction

The interaction of proteins with DNA is central to the control of many cellular processes including DNA replication, recombination and repair, transcription, and viral assembly. The benchmark technique used to study protein:DNA interactions is the electrophoretic mobility shift assay (EMSA). However, standard EMSA suffers from being a radioactive assay and is suitable only for low-throughput applications due to the requirement for a gel-based separation step to identify bound probes. This work describes development of a higher throughput non-radiometric assay to monitor the presence of specific DNA-binding proteins in nuclear extracts using a bead-based luminescent oxygen channeling immunoassay. As proof of concept, we used Hep G2 nuclear extracts to demonstrate binding of Sp1 and HNF1 transcription factors to tagged oligonucleotides containing required cognate response elements. Using as little as 1 µg of nuclear protein extract per well, with 10-30 nM of oligonucleotide, we achieved a specific signal to background ratio of 50.4 and 2.6 for Sp1 and HNF1, respectively. The HNF1 assay window could be increased more than two-fold (S/B of 6.5) by increasing the amount of lysate. Using the Sp1-specific assay, we measured a two-log difference in potency between the untagged wild-type oligonucleotide and corresponding mutated probe in a competition format. These results demonstrate that this novel non-radioactive DNA-binding assay could represent a powerful alternative to EMSA when higher throughput is desired.

2 Assay Configuration



Detection of binding between DNA and transcription factor (TF) is done using Streptavidin-coated Donor beads that capture biotinylated DNA sequence while the TF is recognized by a specific anti-TF antibody which is captured by Protein A- or Protein G- conjugated AlphaLISA® Acceptor beads. Following protein - DNA binding, beads are brought into close proximity to produce an AlphaLISA signal.

3 Materials

- Assay Buffer: 25 mM Hepes, 200 mM NaCl, 0.1% Tween-20 (pH7.4)
- Hep G2 nuclear extracts were prepared according to standard protocols[2] for Sp1 experiments or sourced commercially for HNF1 experiments (Active Motif cat. no. 36011)
- Biotinylated and untagged oligonucleotides were synthesized and annealed to generate dsDNA oligonucleotides per standard protocols
- Anti-Sp1 Ab (sc-17824X) and anti-HNF1 Ab (sc-6547X) were from Santa Cruz
- Protein A- (cat. no. AL101) and Protein G- (cat. no. AL102) conjugated-AlphaLISA® Acceptor beads were from PerkinElmer, Inc.
- Streptavidin (SA)-Donor beads (cat. no. 6760002) were from PerkinElmer, Inc.
- White opaque 384-well Optiplates (cat. no. 6007299) were from PerkinElmer, Inc.
- TopSeal-A (cat. no. 6005185) were from PerkinElmer, Inc.
- Incubator set at 23ºC
- EnVision® reader was from PerkinElmer, Inc.

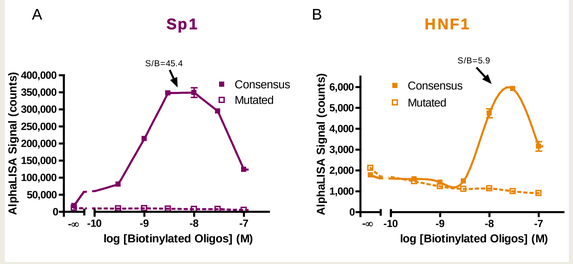
4 Method

- Add 5 µL Assay Buffer (Standard Assay buffer was used for HNF1 experiments. For Sp1 studies, the standard buffer was supplemented with 3 µM $ZnSO_4$)
- Add 5 µL Hep G2 Nuclear Extracts
- Add 5 µL biotinylated Oligos
- Incubate for 30 (Sp1) or 60 (HNF1) minutes at RT
- Add 5 µL of a pre-mix composed of the Ab with Protein A AlphaLISA® beads (20 µg/mL) for Sp1 or the Ab with Protein G AlphaLISA® beads (10 µg/mL) for HNF1
- Incubate for 30 (Sp1) or 60 (HNF1) minutes at RT
- Add 5 µL SA-Donor beads (20 µg/mL for Sp1 or 40 µg/mL for HNF1)
- Incubate for 60 minutes at 23ºC in the dark
- Read plate using an Alpha reader

5 Oligonucleotide Sequences

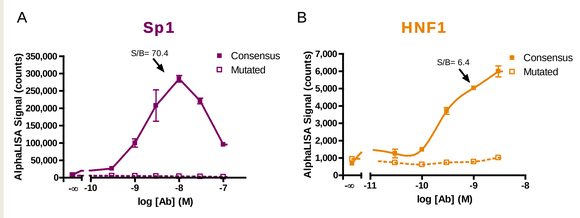
Oligonucleotide	Sequence (5'-3')
Sp1 Consensus	ATTCGATCGGGGCGGGGCGAG
Biotin-Sp1 Consensus	Biotin-TEG-ATTCGATCGGGGCGGGGCGAG
Sp1 Mutated	ATTCGATCGGTTCGGGGCGAG
Biotin-Sp1 Mutated	Biotin-TEG-ATTCGATCGGTTCGGGGCGAG
HNF1 Consensus	TATTATGGTGAGCTAATAAGTTGCAAGTCCCT
Biotin-HNF1 Consensus	Biotin-TEG-TATTATGGTGAGCTAATAAGTTGCAAGTCCCT
HNF1 Mutated	TATTATGGTGAGCCAATAAGTTGCAAGTCCCT
Biotin-HNF1 Mutated	Biotin-TEG-TATTATGGTGAGCCAATAAGTTGCAAGTCCCT
Non-Specific Competitor	GATCGAACTGACCGCTTGCGGCCCGT

6 Biotinylated Oligonucleotide Titrations



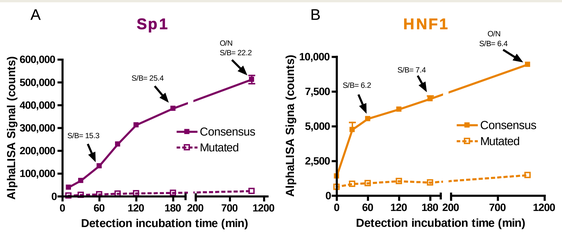
To determine its optimal concentration, an oligonucleotide titration curve was performed using fixed lysate concentration; (A) Sp1 [1 µg/well] and (B) HNF1 [2 µg/well]. Maximum response was observed using (A) 10 nM and (B) 30 nM of oligonucleotides, with signal to background ratio (S/B) value of 45.4 and 5.9, respectively.

7 Antibody Titrations



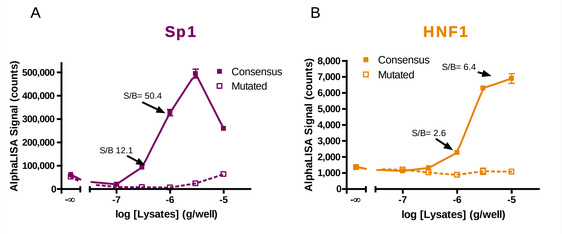
An antibody titration experiment was next performed using optimal oligonucleotide concentration; (A) Sp1 [1 µg nuclear extract/well] and (B) HNF1 [2 µg nuclear extract/well]. The greatest assay windows were obtained with 10 and 1 nM of antibody for (A) Sp1 and (B) HNF1 detections, respectively.

8 Detection Time Courses



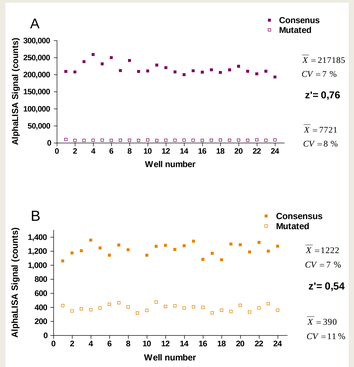
A time course experiment was carried out on the detection step using optimized assay conditions; (A) Sp1 [1 µg nuclear extract/well] and (B) HNF1 [2 µg nuclear extract/well]. A final incubation of 60 minutes was chosen for further experiment assessments even though S/B value could be improved for Sp1 detection by incubating the reaction for a longer period of time.

9 Assay Sensitivity



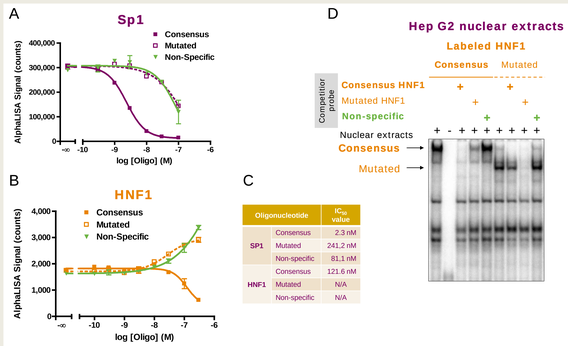
A lysate titration experiment was then conducted for both (A) Sp1 and (B) HNF1 detections using optimal assay conditions. As low as 1 µg/well of non-stimulated Hep G2 nuclear extracts permit the generation of S/B values of (A) 50.4 and (B) 2.6. However, wider assay window (6.4) for HNF1 assay could be generated by increasing the amount of lysate per well to 10 µg.

10 Assay Variability



Assay robustness was then demonstrated by generating Z' value of (A) 0.76 and (B) 0.54 for Sp1 and HNF1 detections, respectively. The assay was performed using optimal assay conditions in the presence of (A) 1 µg/well and (B) 5 µg/well of Hep G2 nuclear extracts.

11 Assay Specificity



The assay specificity was demonstrated for (A) Sp1 and (B) HNF1. Untagged wild-type and non-specific DNA sequences were used as competitor for both assays. (C) A two-log difference in potency was observed between the consensus and non-specific sequences for Sp1 with IC_{50} values of 2.3 and 241 nM, respectively. For HNF1, no competitions occurred with non-specific oligonucleotides and a IC_{50} value of 122 nM was generated with the specific DNA sequence. Results generated from the (D) EMSA did not allow to demonstrate clearly the specificity of the HNF1 assay since partial competition was observed between mutated and consensus DNA sequences compared to a complete competition with the specific probe.

12 Summary

- We have successfully demonstrated the application of a homogenous AlphaLISA® assay to replace EMSA in the monitoring of DNA-protein interaction from nuclear extract samples.

- This novel AlphaLISA detection assay represents a major improvement over EMSA
  - Non-Radiometric Assay
  - Uses less DNA binding element
  - Easier and faster to execute
  - Greater throughput
  - No need for running a gel
  - Higher sensitivity

- The robustness of both assays was demonstrated by generating Z' value greater than 0.54.

- Competition assays have:
  1. Confirmed the specificity of the detection.
  2. Demonstrated the advantage of using Alpha by using less binding element and at least two-fold less nuclear extracts than required to study interactions between TF and their respective DNA sequence with methods commonly used.

References: [1] Vuori et al. *FEBS J.* (2009) ; [2] Schreiber et al. *Nucleic Acids Res.*. **17**, 6419 (1989)

[1]PerkinElmer, Inc., 940 Winter Street, Waltham, MA USA (800) 762-4000 or (+1) 203 925-4602 www.perkinelmer.com
[2] Centre de Recherche, CHU Sainte-Justine, Montréal, Qc, Canada
[3]Département de Pédiatrie, Université de Montréal, Montréal, Qc, Canada