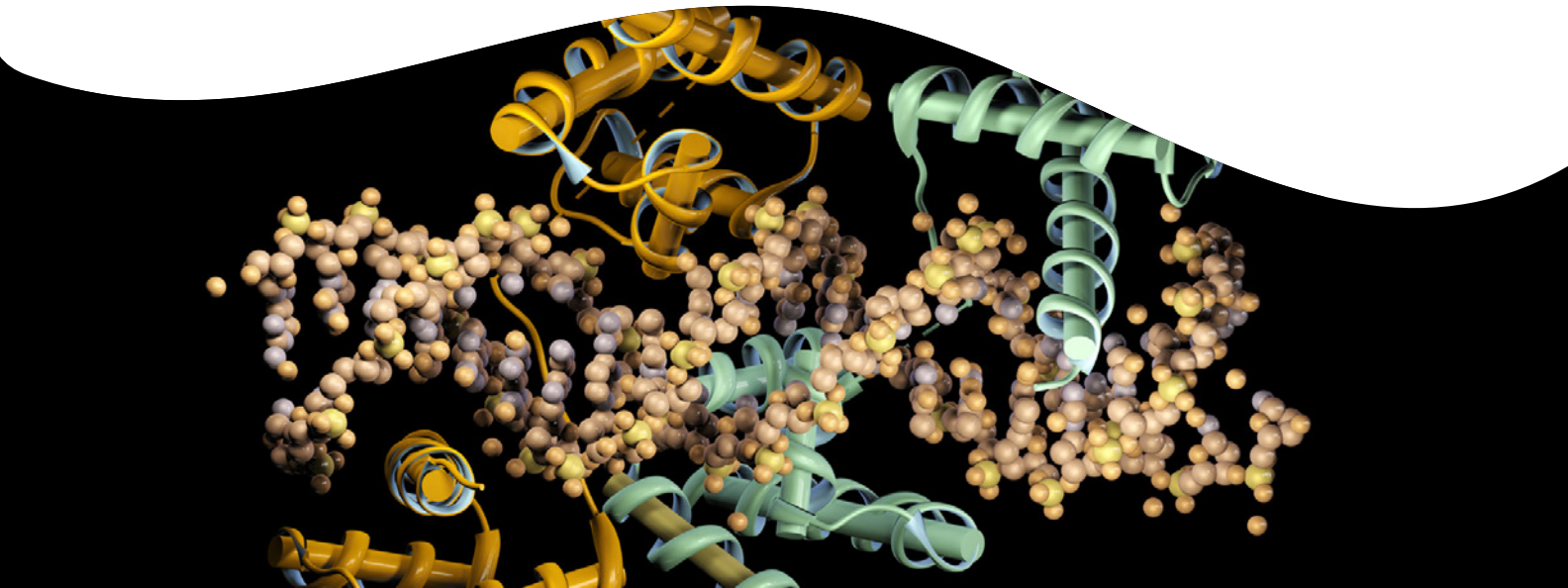
APPLICATION NOTE

A fast and simple chemiluminescent assay for monitoring of DNA-protein interactions.

Introduction

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 that is suitable only for low-throughput applications due to the requirement for a gel-based separation step to identify bound probes. AlphaLISA® bead-based chemiluminescent technology provides a fast and simple non-radioactive assay with a simpler workflow that is highly sensitive (requiring less nuclear extract compared to EMSA), highly reproducible, and amenable to higher throughput. We describe development of an AlphaLISA immunoassay to monitor the presence of specific DNA-binding proteins in nuclear extracts (see Figure 1). As proof of concept, we used HepG2 nuclear extracts to demonstrate binding of Sp1 and HNF1 transcription factors to tagged oligonucleotides containing required cognate response elements. These results demonstrate that the AlphaLISA DNA-binding assay provides a powerful alternative to EMSA.



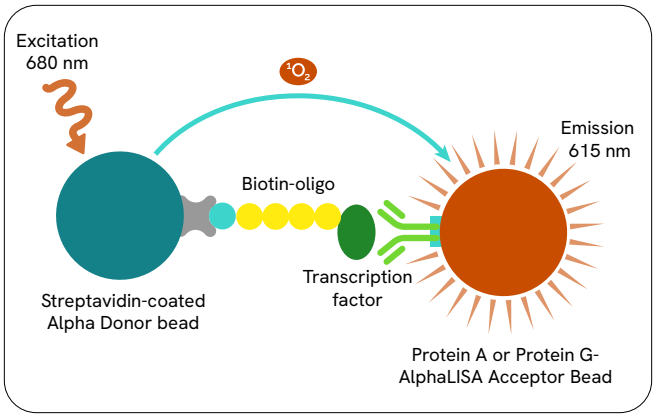


Figure 1: Detection of binding between DNA and transcription factor using AlphaLISA chemiluminescent technology. In this assay, a DNA sequence is biotinylated and captured by streptavidin-coated Donor beads while a specific antibody to the transcription factor is captured by Protein A or Protein G AlphaLISA Acceptor beads. When the beads are brought in proximity, the presence of the nuclear-extracted transcription factors interacting with the biotinylated DNA triggers transfer of energy to the Acceptor bead, resulting in the emission of light.

Materials and methods

Reagents

- Assay Buffer: 25 mM Hepes, 200 mM NaCl, 0.1% Tween-20 (pH 7.4)
- HepG2 nuclear extracts were prepared according to standard protocol for Sp1 experiments, or sourced commercially for HNF1 experiments (Active Motif #36011)
- Anti-Sp1 antibody (Santa Cruz #sc-17824X)
- Anti-HNF1 antibody (Santa Cruz #sc-6547X)
- Protein A AlphaLISA Acceptor beads (Revvity #AL101)
- Protein G AlphaLISA Acceptor beads (Revvity #AL102)
- Streptavidin (SA)-Donor beads (Revvity #6760002)
- White opaque 384-well Optiplates (Revvity #6007299)
- TopSeal-A™ (Revvity #6050185)
- Incubator set at 23 °C
- EnVision® multimode plate reader (Revvity)

Oligonucleotide sequences

Biotinylated and untagged oligonucleotides were synthesized and annealed to generate double-stranded DNA oligonucleotides per standard protocols.

Table 1: DNA sequences for oligonucleotides.

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

Nuclear extraction protocol

The quality of nuclear extract had significant impact on the assay performance and also on assay reproducibility. The following is our recommended protocol:

1. Add 400 µL of ice-cold Buffer 1 to the cell pellet
2. Incubate on ice for 15 minutes
3. Add 25 µL 10% Nonidet NP-40
4. Vortex 10 seconds
5. Centrifuge at 13,000 rpm for 30 seconds
6. Aspirate the supernatant
7. Add 50 µL of ice-cold Buffer 2 to the cell pellet
8. Mix vigorously for 15 minutes at 4 °C
9. Transfer supernatant into pre-chilled microcentrifuge tubes
10. Aliquot and store at -80 °C

Buffer 1: 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 µg/mL Aprotinin and 1X protease inhibitor cocktail tablet

Buffer 2: 20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM DTT, 1 mM PMSF, 10 µg/mL Aprotinin and 1X protease inhibitor cocktail tablet

Assay protocol

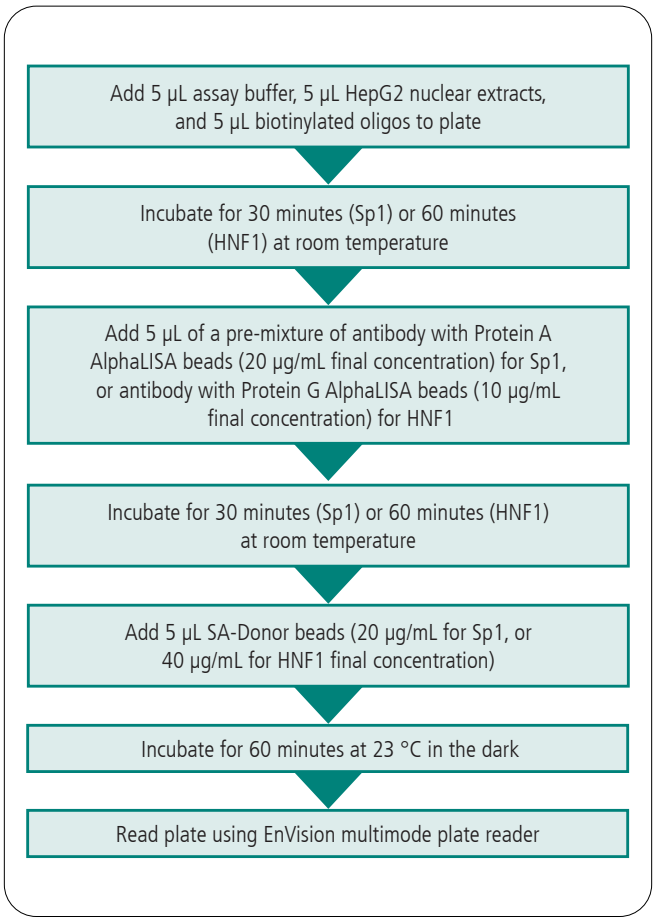


Figure 2: Workflow for AlphaLISA DNA-protein binding assay.

Instrumentation

The AlphaLISA assays were measured using a Revvity EnVision multimode plate reader (Figure 3). The EnVision system offers fast, sensitive Alpha detection technology, in addition to fluorescence, luminescence, absorbance, fluorescence polarization, and time-resolved fluorescence measurement. Incorporation of unique temperature controls within the system ensures accurate, reproducible results for Alpha and AlphaPlex™ assays.

Figure 3: EnVision multimode plate reader.

Assay development

Biotinylated oligonucleotide titration

To determine optimal DNA oligonucleotide concentration, a titration curve was performed using a fixed concentration of lysate. Mutated oligonucleotide was used as a negative control. Results are shown in Figure 4. Increasing concentrations of biotinylated consensus oligonucleotide resulted in increased signal until a hook point was reached, indicating saturation. Maximum response was observed using 10 nM oligonucleotides for Sp1 and 30 nM of oligonucleotides for HNF1, with signal-to-background ratio values of 45.4 and 5.9, respectively.

Antibody titration

An antibody titration experiment was performed using optimized oligonucleotide concentrations from the previous experiment. Mutated oligonucleotide was used as a negative control. Results are shown in Figure 5. For the Sp1 assay, signal increased with increasing antibody concentration until the hook point was reached, indicating saturation. For the HNF1 assay, increasing concentrations of antibody resulted in increasing signal for the range of antibody concentrations tested. The largest assay windows were obtained using 10 nM antibody for Sp1 detection and 1 nM antibody for HNF1 detection.

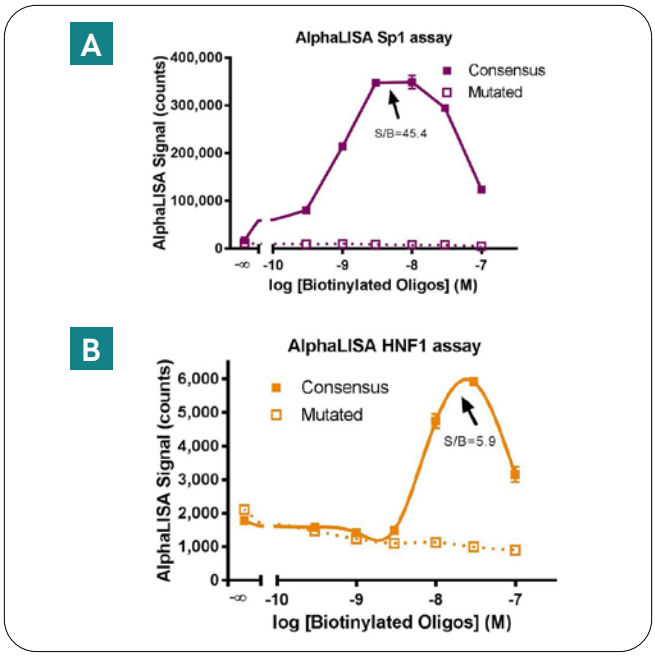


Figure 4: Oligonucleotide titration. A) Sp1 (1 µg nuclear extract/well) and B) HNF1 (2 µg nuclear extract/well). In each assay, 10 nM antibody, 20 µg/mL Protein A or Protein G AlphaLISA Acceptor beads, and 20 µg/mL Streptavidin Donor beads was used (final concentrations in well).

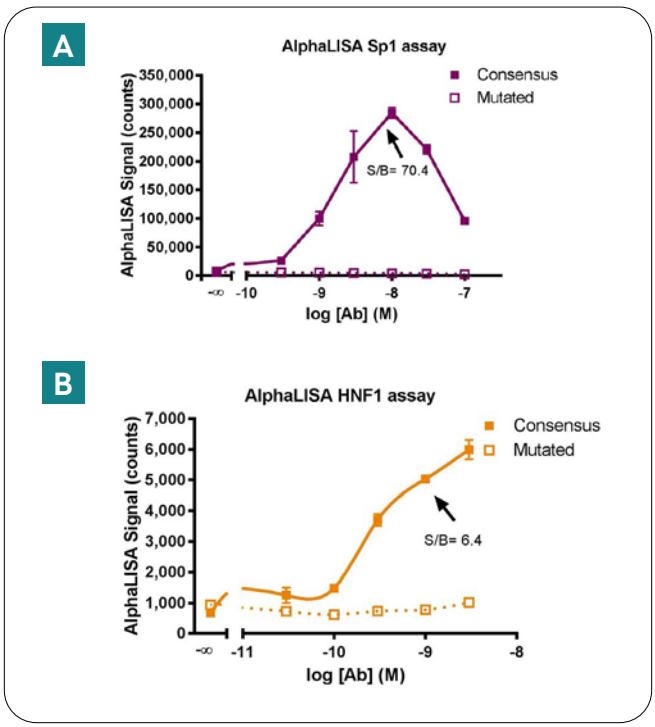


Figure 5: Antibody titration. A) Sp1 assay and B) HNF1 assay. The Sp1 assay used 1 µg nuclear extract/well, 10 nM oligonucleotides, 20 µg/mL Protein A AlphaLISA Acceptor beads, and 20 µg/mL Streptavidin Donor beads. The HNF1 assay used 2 µg nuclear extract/well, 30 nM oligonucleotides, 20 µg/mL Protein G AlphaLISA Acceptor beads, and 20 µg/mL Streptavidin Donor beads. All concentrations are final concentration in the well.

Detection time-course experiment

A time-course experiment was performed using optimized assay conditions from previous experiments. Mutated oligonucleotides were used as a negative control. Assay plates were measured at various time points, up to overnight incubation. Results are shown in Figure 6. A final incubation of 60 minutes was chosen for each assay.

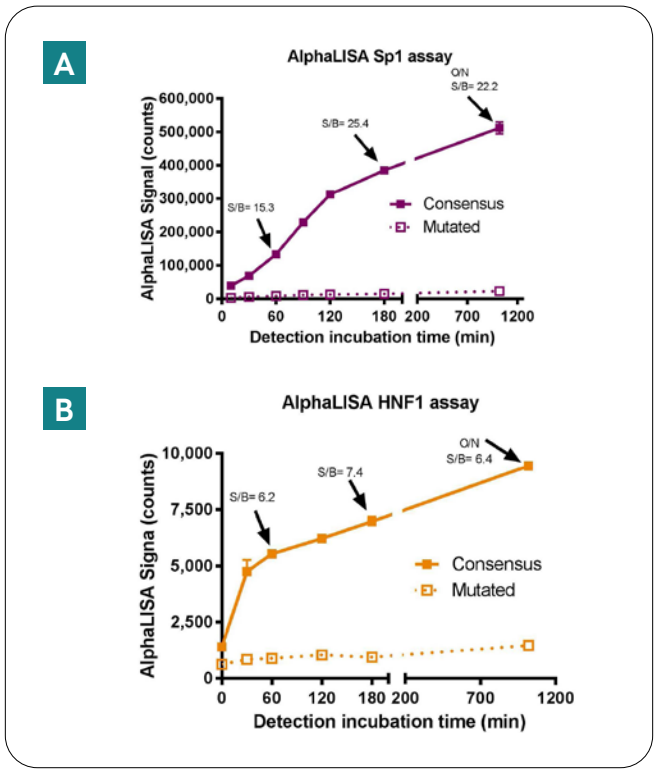


Figure 6: Time-course experiment. A) Sp1 assay and B) HNF1 assay. The Sp1 assay used 1 µg nuclear extract/well, 10 nM oligonucleotides, 10 nM anti-Sp1 antibody, 20 µg/mL Protein A AlphaLISA Acceptor beads and 20 µg/mL Streptavidin Donor beads. The HNF1 assay used 2 µg nuclear extract/well, 30 nM oligonucleotides, 1 nM anti-HNF1 antibody, 20 µg/mL Protein G AlphaLISA Acceptor beads and 20 µg/mL Streptavidin Donor beads. All concentrations are final concentrations in well.

Assay sensitivity

A lysate titration experiment was conducted for both Sp1 and HNF1 detection using optimized assay conditions (Figure 7). As low as 1 µg/well of non-stimulated HepG2 nuclear extracts provided good signal-to-background values (50.4 for the Sp1 assay and 2.6 for the HNF1 assay). A wider assay window (S/B=6.4) could be generated for the HNF1 assay by increasing the amount of extract to 10 µg.

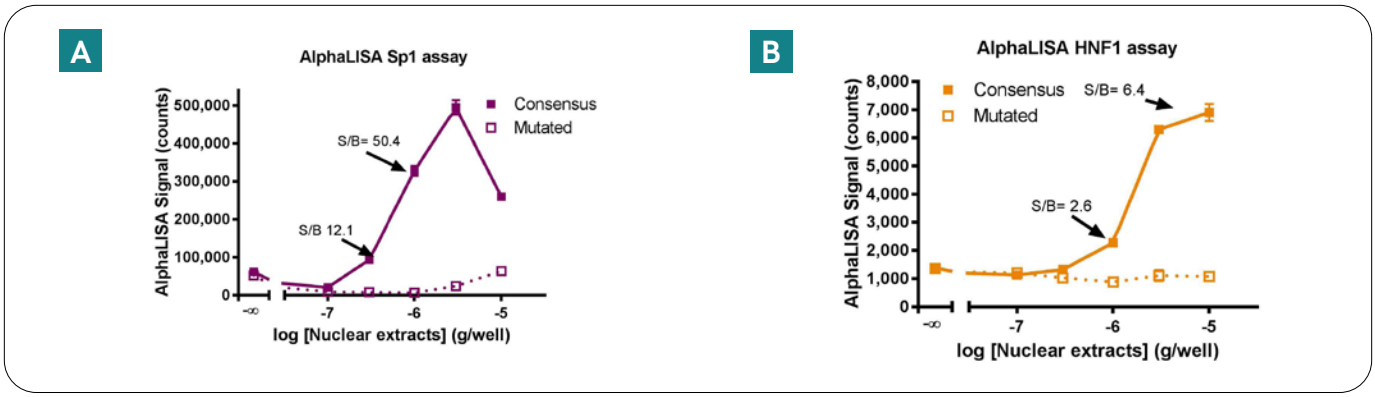


Figure 7: Lysate titration to determine assay sensitivity. A) Sp1 assay using 10 nM oligonucleotides, 10 nM anti-Sp1 antibody, 20 µg/mL Protein A AlphaLISA Acceptor beads and 20 µg/mL Streptavidin Donor beads. B) HNF1 assay using 30 nM oligonucleotides, 1 nM anti-HNF1 antibody, 20 µg/mL Protein G AlphaLISA Acceptor beads and 20 µg/mL Streptavidin Donor beads. Final incubation time was 60 minutes for each assay. All concentrations refer to final concentration in the well.

Assay variability

Assay robustness was demonstrated by generating Z′ values for optimized experiments (Figure 8). Mutated DNA was used to provide a baseline. Z′ values of 0.76 for Sp1 and 0.54 for HNF1 were obtained, indicating good reproducibility.

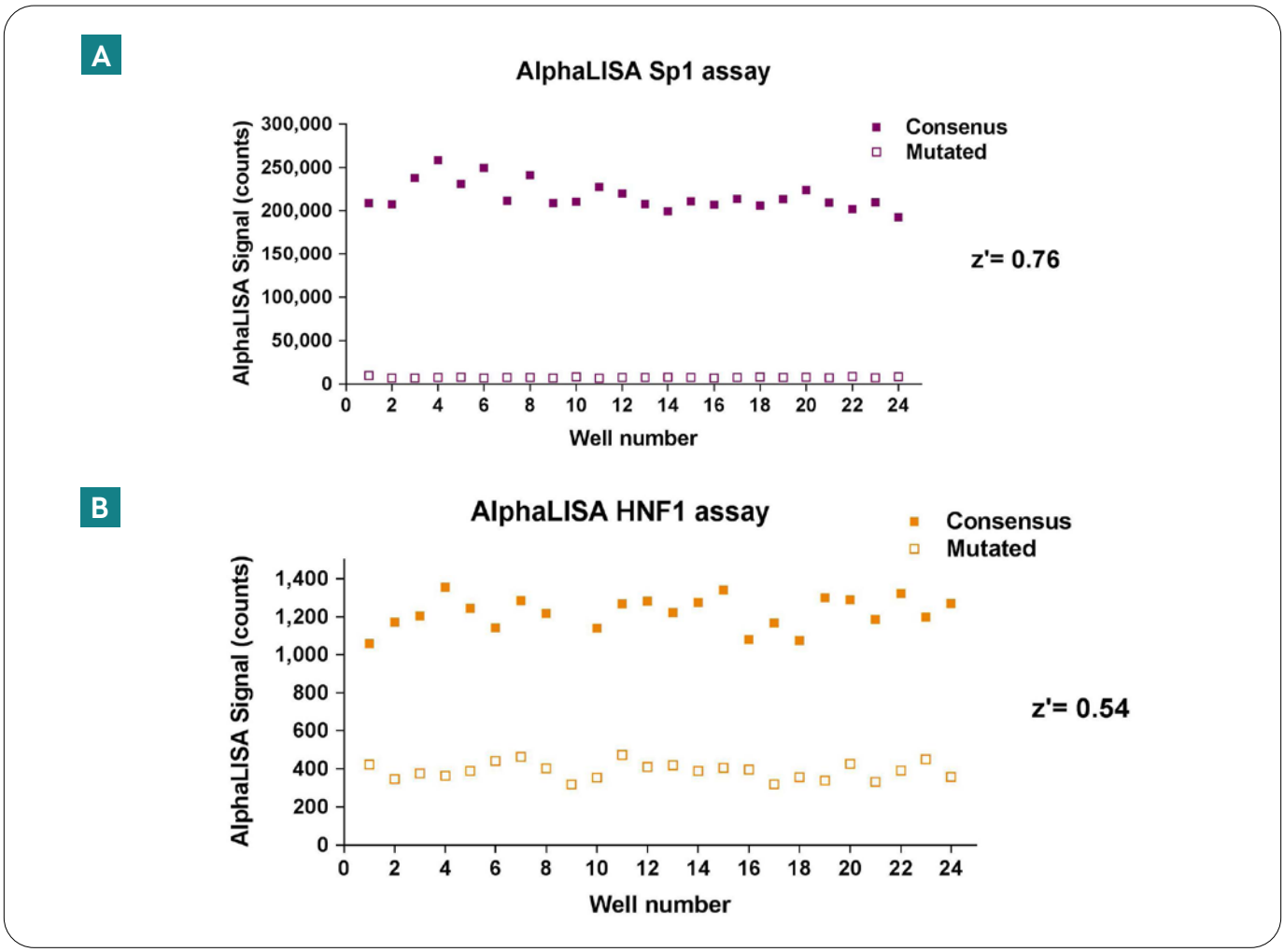
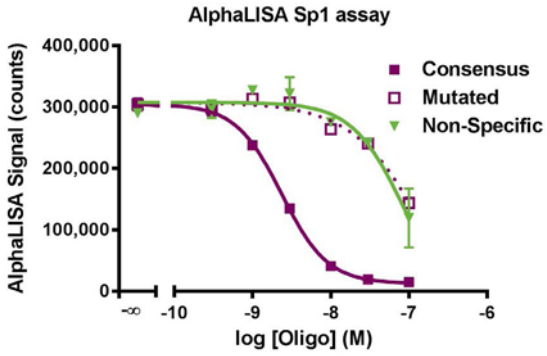


Figure 8: Z′ experiments. A) Sp1 assay. B) HNF1 assay.

Assay specificity

Assay specificity was demonstrated for Sp1 and HNF1 using untagged wild-type and non-specific DNA sequences as competitors for both assays. 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 competition occurred with non-specific oligonucleotides. An IC_{50} value of 122 nM was calculated with the specific DNA sequence. Results generated from the EMSA did not allow us to demonstrate clearly the specificity of the HNF1 assay, since partial competition was observed between mutated and consensus DNA sequences compared to complete competition with the specific probe.

A



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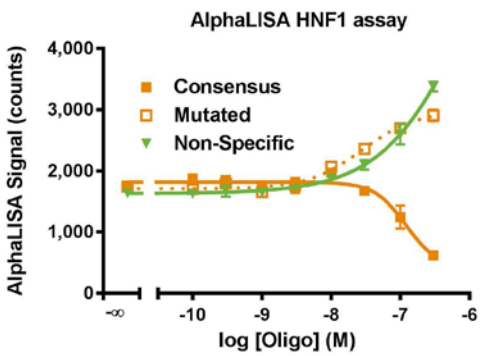

C

Oligonucleotide		IC_{50} value
SP1	Consensus	2.3 nM
	Mutated	241.2 nM
	Non-specific	81.1 nM
HNF1	Consensus	121.6 nM
	Mutated	N/A
	Non-specific	N/A

D

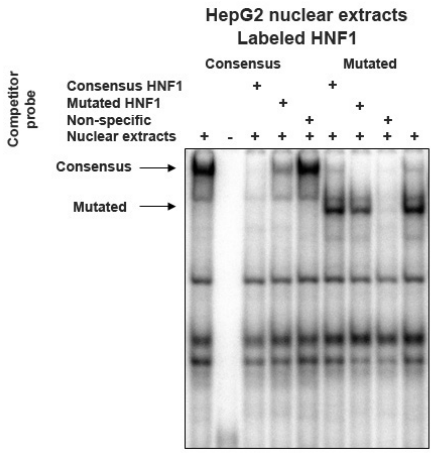

Figure 9: Assay specificity demonstrated for A) Sp1 and B) HNF1. Untagged wild-type and non-specific DNA sequences were used as competitor for both assays. C) A two-log difference in potency was observed between the consensus and non-specific sequences for Sp1 with IC_{50} values of 2.3 and 241 nM, respectively. For HNF1, no competition occurred with non-specific oligonucleotides and an IC_{50} value of 122 nM was generated with the specific DNA sequence. Results generated from the D) EMSA did not enable demonstration of the specificity of the HNF1 assay since partial competition was observed between mutated and consensus DNA sequences compared to complete competition with the specific probe.

Conclusion

We have successfully demonstrated the application of a homogenous, no-wash AlphaLISA assay to replace EMSA in the monitoring of DNA-protein interactions. This AlphaLISA detection assay represents a major improvement over EMSA in providing a non-radioactive alternative with a better workflow that is easier and faster to execute, more sensitive (requiring less binding element and nuclear extract), and amenable to higher-throughput. The robustness of each assay was demonstrated by Z′ value. Specificity of the assays was confirmed using competition experiments.

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