Identification of DNA binding proteins using the NoShift Transcription Factor Assay Kit

Faye Bruggink & Scott Hayes

The NoShift Transcription Factor Assay Kit provides a rapid and sensitive method for the identification of proteins that bind to DNA sequences. The developer of the assay, EMD Biosciences, Inc. (EMD), is a major supplier of premium tools for proteomics and disease pathway analysis known through its global brands Calbiochem, Novabiochem and Novagen. EMD is part of the Life Science and Analytics (LSA) division of Merck KGaA (Darmstadt, Germany) and operates as EMD Biosciences, Inc. in North America and as Merck Biosciences outside North America.

Background

Transcription factors (TFs) help regulate gene expression by binding to DNA, and thus govern essential processes, such as cell division, metabolism, apoptosis and DNA repair. Because they can control regulatory checkpoints that alter gene expression within a cell, TFs are of utmost interest to researchers. Scientists who study protein-DNA interactions frequently rely upon gel shift assays or electrophoretic mobility shift assays (EMSAs), which are based on relative differences in electrophoretic mobility between protein-DNA complexes and uncomplexed DNA under nondenaturing conditions. A 'supershift' assay uses an antibody against the DNA-bound protein to further slow the migration of the complex through the gel and specifically identify the TF. These traditional gel shift assays are tedious and time-consuming, and often require radiolabeled probe or extensive optimization.

As an alternative to EMSAs, EMD has developed the versatile NoShift Transcription Factor Assay system that enables simultaneous analysis of multiple DNA-binding factors in less than 5 h without radioisotopes. With this microassay plate-based approach, researchers can assess sequence-specific binding with cell extracts from different tissues and cell lines that were in different growth stages or were exposed to various drug treatments. The 96-well plate format NoShift Assay system is available in two kit forms (one provides the components for the assay itself, whereas the other also includes components for preparing a nuclear extract) and as separate reagents for different TFs. This technical report compares the performance of the NoShift Assay with that of a conventional gel shift assay and reports the specificity and flexibility of the NoShift Assay with several different TFs and cell lines.

The NoShift Assay system

Each NoShift Transcription Factor Assay Kit comes with one 96-well streptavidin-coated plate (which can be divided into 12

eight-well strips) with sealers, binding reaction buffer, antibody dilution buffer, plate wash buffer, and TMB substrate and provides enough reagents for 100 reactions. The kit is designed for use either

Figure 1 | The NoShift Transcription Factor Assay procedure.

APPLICATION NOTES

Figure 2 | Comparison of the NoShift Assay with traditional gel shift assay. (a) Autoradiograph from a gel shift assay. (b) Data from a NoShift Assay. Both measured AP-1 TF binding activity using nuclear extract from CHO-K1 cells that were either untreated or treated with PMA. In b, the blank contained all assay components except the nuclear extract.

with target-specific NoShift Reagents or with biotin-labeled and unlabeled DNA oligonucleotides provided by the end user that define a protein binding consensus sequence (the capture probes), a primary antibody to the DNA-binding protein, and a horseradish peroxidase (HRP)-conjugated secondary antibody to the primary antibody. The kit reagents include three DNA oligonucleotides (the biotin-labeled and unlabeled wild-type TF consensus binding motif (capture probes), and an unlabeled 'scrambled' sequence of the TF consensus binding motif as a negative control), TF-specific antibody, HRP-conjugated secondary antibody and a positive control nuclear extract. Like traditional gel shift assays, the NoShift Assay can be performed with pure protein, crude extract or recombinant protein, whether from bacteria or insect cells or via translation *in vitro*, although eukaryotic extracts are most commonly used. Because the nucleus contains activated DNA-binding proteins, using nuclear extracts is preferable. For this reason, the version of the NoShift Assay Kit used here includes components (the NucBuster Protein Extraction Kit) that yield a nuclear extract suitable for DNA binding studies in less than 30 min¹.

The NoShift Assay procedure (Fig. 1) is a colorimetric assay in which the TF of interest first binds to a double-stranded, synthetic, biotinylated oligonucleotide containing the protein-binding consensus sequence, and then the TF-DNA complex is captured on a streptavidin-coated plate. The bound TF is then detected through its binding with a specific antibody followed by an appropriate secondary antibody-HRP conjugate and a chromogenic reaction with the 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Thus, the signal intensity (measured as absorbance at 450 nm) reflects the concentration of the bound TF.

NoShift Assay performance: comparison with a conventional gel shift assay

To compare the performance of the NoShift Assay with that of a traditional gel shift assay, we prepared nuclear extracts from untreated CHO-K1 cells and CHO-K1 cells treated with phorbol 12-myristate-13-acetate (PMA), a protein kinase C activator that induces AP-1 TF activity. Using a traditional gel shift assay and the NoShift Assay, we determined AP-1 TF binding activity in both nuclear extracts. The gel shift assay revealed TF-DNA complex formation, evidenced by a change in the mobility of the radiolabeled DNA (Fig. 2a), but determining any difference in band intensity between the treated and untreated extracts is difficult. In contrast, the NoShift Assay clearly provided a relative measurement of the PMA-treated activation (Fig. 2b). As compared to the nuclear extract from untreated cells, the nuclear extract from the PMA-treated cells exhibited nearly a twofold higher signal-to-noise ratio (5:1 versus 2.9:1). This result clearly shows PMA-treated activation of AP-1 and demonstrates the utility of the NoShift Assay for assessing and quantifying the effects of drug treatment on TF activation.

NoShift Assay performance: competitive binding and specificity

Sequence-specific binding of an oligonucleotide with its cognate TF using the NoShift Assay can be demonstrated by competition between a biotinylated and an unlabeled capture probe. Figure 3 demonstrates the highly sequence-specific nature of the assay. Figure 3a shows the results of a competitive analysis of the interaction between the cyclic AMP-responsive element binding (CREB) TF and its biotinylated capture probe in the presence of increasing concentrations of the unlabeled capture probe. The signal-to-noise ratio in the absence of the competing unlabeled capture probe was

Figure 3 | NoShift Assay to detect CREB and Sp1 binding activity. (a) CREB binding activity assays were performed using three capture probes: biotinylated CREB-specific (Sample; competing nonbiotinylated, CREB-specific (S); or competing nonbiotinylated, nonspecific sequence (NS). (b) Sp1 binding activity assays were performed using five capture probes: biotinylated Sp1-specific (Sample); competing nonbiotinylated, Sp1-specific (S); competing nonbiotinylated, nonspecific sequence (NS); or two competing nonbiotinylated probes, each modified to contain a single-point mutation in the Sp1 recognition sequence (mutations 1 and 2). For all assays (performed in triplicate), the blank contained all assay components except the nuclear extract and detection was accomplished with either a CREB- or an Sp1-specific antibody.

Figure 4 | NoShift Assays to detect NF-κB and HIF-1α binding activity. NoShift Assays to detect either NF-κB binding activity (a) or HIF-1α binding activity (b) to its respective capture probe were performed using nuclear extract from HeLa cells that were treated with 200 ng/ml TNF-α or 50 ng/ml TNF-α for 120 min before nuclear extract isolation (for NF-κB binding) or nuclear extract from COS-7 cells that were treated with either 0 μM or 150 μM 2,2-dipyridyl for 17 h before nuclear extract isolation (for HIF-1α binding). Detection was performed with either an NF-κB p65- or an HIF-1α-specific antibody. Each set of reactions was performed in triplicate.

5.3:1, but the signal intensity decreased as the concentration of the unlabeled capture probe increased during complex formation. At an equimolar ratio of unlabeled capture probe to labeled capture probe, the signal intensity decreased to background levels, suggesting that the TF preferentially bound to unlabeled capture probe. In contrast, adding a nonspecific unlabeled capture probe, even at concentrations equimolar with the biotinylated capture probe, did not alter the signal-to-noise ratio, which remained 4.5:1.

We then explored the effect of single-base mutations on binding specificity with Sp1 (Fig. 3b). For this, we designed an unlabeled probe that did not contain the capture sequence and two unlabeled potential competitors each having a single-nucleotide variation in the core recognition sequence (mutation 1 and mutation 2). Neither the nonspecific capture probe nor the two point-mutation variants competed effectively for Sp1 TF binding. The reverse experiment yielded similar information about the specificity of the DNA binding site; double-stranded biotinylated oligonucleotides of each point mutation variant did not produce a signal-to-noise ratio as high as 2:1 (data not shown).

These competition studies demonstrate that the NoShift Assay can effectively differentiate between site-specific binding (to specific TF sequences) and nonspecific binding even when a lower molar ratio of unlabeled capture probe is used than in traditional gel shift assays. These results also indicate the capability of the assay to sensitively discriminate between a TF recognition sequence and a similar sequence that differed by only a single nucleotide.

NoShift Assay performance: detecting activation

The ability to measure differences in TF binding upon activation or repression is a key feature of the NoShift Assays. Figure 4a shows a concentration-dependent effect of tumor necrosis factor-α (TNFα) activation of NF-κB p65, which, in addition to controlling pathways involved in regulating the immune system, affects cellular transformation and oncogenesis. Exposure to TNFα stimulates this TF to bind to its consensus sequence. After treating HeLa cells with 200 ng/ml TNFα for 30 minutes or 50 ng/ml TNFα for 120 minutes, we prepared nuclear extracts, then assessed the relative changes in NF-κB TF binding to its consensus sequence. At low concentrations, TNFα increased NF-κB activity by more than 27-fold, whereas at

higher concentrations, TNFα increased NF-κB activity by more than 50-fold.

Similarly, we measured activation of the hypoxia-induced factor-1α (HIF-1α) in COS-7 cells (Fig. 4b). The HIF-1α TF has been implicated in tumor progression and is stabilized by treatments that inhibit progression through the ubiquitination pathway. Dipyridyl, an iron chelator, prevents HIF-1α ubiquitination. After treating COS-7 cells for 17 h with 2,2-dipyridyl, we prepared nuclear extracts, then determined HIF-1α binding to its consensus sequence. Compared to that in untreated cells, the 17-h dipyridyl treatment of the COS-7 cells increased HIF-1α binding by more than threefold.

Conclusion

The microassay plate-based NoShift Assay offers several advantages over traditional gel shift assays. Most important is the remarkable dual specificity of the assay: the TF binds to the consensus DNA sequence, while the secondary, reporting antibody binds to the complexed TF. Further, the convenient 96-well format of the NoShift Assay permits screening for multiple DNA-binding proteins in the same plate. Thus, the NoShift Assay Kits and convenient target-specific NoShift Reagents offer a fast, sensitive, nonradioactive alternative to traditional gel shift assays.

1. Bruggink, F. & Hayes, S. Identification of DNA binding proteins using the NoShift™ Transcription Factor Assay Kit *inNovations* 15, 9–11 (2002).