Detecting DNA-Binding Protein Activity Using a Membrane-Based Approach

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Abstract

The SAM[2]® Biotin Capture Membrane can be used to study biotinylated molecules based on their affinity for streptavidin. The SAM[2]® Membranes bind linearly and with low non-specific interactions to substrates. For DNA:protein binding studies, they can be used with either biotin-labeled proteins and [^{32}P]-labeled oligos or with biotinylated oligos and [^{35}S]-labeled proteins. This method provides an easy and convenient nongel-based method for detecting the ability of an in vitro-expressed target protein to specifically bind to a DNA oligonucleotide and serves as an alternative to the classic gel mobility shift, electrophoretic mobility shift (EMSA), or gel retardation assays.

The SAM[2]® Biotin Capture Membranes provide an easy and sensitive method for the detection of DNA:protein interactions.

Introduction

The interaction of proteins with nucleic acids has many important functions in regulating cell growth, differentiation and viability. The ability of transcription factors to recognize and bind to consensus binding sites in chromosomal DNA plays a key role in the regulation of mRNA expression. The ability to detect and confirm the interaction of such proteins with various nucleic acid targets provides valuable information about the cell signaling cascades that govern the ability of a cell to divide, migrate, interact with its neighbors, develop and maintain specialized functions, and terminate viability at the appropriate time.

Classically, the ability to detect the interaction of a protein with a nucleic acid target has been investigated using a gel mobility shift assay (1). This assay involves the binding of the target protein (either expressed in vitro or from a cell lysate or nuclear fraction) to a radiolabeled double-stranded DNA oligonucleotide. The complexes are then analyzed using polyacrylamide gel electrophoresis to resolve the unbound oligo from the protein:oligo complex, which exhibits slowed mobility due to its increased mass. The specificity of such complexes are determined by performing competition with unlabeled specific and nonspecific oligos and supershift experiments in which the complex is incubated with an antibody to the DNA-binding protein of interest (1). This method can be quite labor intensive, particularly when simultaneously processing numerous samples.

This paper describes an alternate method for the detection of DNA:protein interactions that does not require gel analysis but instead relies on capture of the labeled DNA:protein complex to a streptavidin-coated membrane (SAM[2]® Biotin Capture Membrane[a]; Cat.# V7861). The method requires that one partner be biotinylated and the other partner be radiolabeled. Thus a biotinylated oligo may be used with a [^{35}S]methionine-labeled protein, or a [^{32}P]-labeled oligo may be used with a biotinylated protein (see Figure 1 for method outline). Labeled proteins are easy to generate using in vitro expression systems such as the TnT® Quick Coupled Transcription/Translation Systems or the TnT® Coupled Wheat Germ Extract Systems. The c-Jun:AP1 complex was used as a model system to confirm the utility of this method. The transcription factor c-Jun can bind as a homodimer to the DNA consensus sequence 5′-TGACTCA-3′, also called an AP1 site (2,3).

In Vitro Expression of DNA Binding Proteins

The c-*jun*-coding region was amplified from HeLa cell total RNA using the Access RT-PCR System, and the resulting RT-PCR[b] product was cloned into the pSP64 poly(A) Vector. This vector contains an SP6 RNA polymerase promoter that allows in vitro expression. This vector also allows the addition of a synthetic poly(A) tail on the transcribed RNA, which enhances protein expression in vitro. The template was then expressed in the TnT® SP6 Quick Coupled Transcription/Translation System (containing rabbit reticulocyte lysate; Cat.# L2080) or the TnT® SP6 Coupled Wheat Germ Extract System (Cat.# L4130) in the presence of [^{35}S]methionine or Transcend™ biotinylated lysine tRNA (Cat.# L5061).

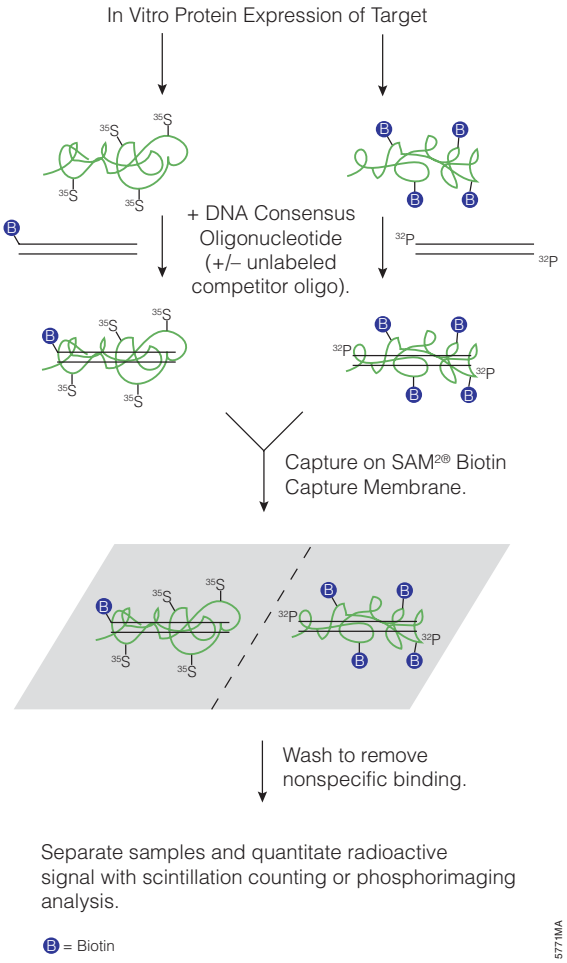

Figure 1. Flow diagram showing the detection of DNA:protein interactions using the SAM2® Biotin Capture Membranes.

Complex Formation Between DNA Binding Protein and DNA Oligonucleotide

Following translation, aliquots of the in vitro-expressed c-Jun protein were incubated with labeled AP1 consensus double-stranded oligonucleotide. The AP1 consensus oligo was 5´ end-labeled with [^{32}P]γ-ATP and T4 Polynucleotide Kinase and purified (as described in Technical Bulletin #TB110). The biotin-labeled AP1 oligo was prepared by annealing a biotinylated top-strand oligo with the complementary unlabeled bottom-strand oligo, and verifying the oligo was double-stranded by polyacrylamide gel analysis. Some reactions were performed in the presence of unlabeled competitor AP1 (specific) or SP1 (nonspecific) oligonucleotide (6pmol/reaction). The gel shift reactions were assembled in a total volume of 15µl and contained 1X Gel Shift Binding Buffer, 1µl labeled AP1 oligo (75,000cpm [^{32}P]-labeled or 2pmol biotin-labeled), and 6–10µl in vitro-expressed c-Jun protein. The assembled reactions were incubated on ice for 20–30 minutes.

Detection of DNA:Protein Interactions using SAM2® Biotin Capture Membrane

Following complex formation, the reactions were spotted onto squares of SAM2® Biotin Capture Membrane, washed three times for 10 minutes each in 1X Gel Shift Binding Buffer without poly(dI-dC)/poly(dI-dC), then each square separated and counted in a scintillation counter.

As seen in Figure 2, capture of the biotinylated-c-Jun/[^{32}P]-AP1 complex onto the SAM2® Membrane could be detected with both the rabbit reticulocyte-expressed c-Jun protein and the wheat germ extract-expressed c-Jun protein. The DNA:protein interaction between c-Jun and the AP1 consensus oligo was specific, as the interaction was competed off by unlabeled AP1 oligo but not by unlabeled SP1 oligo (even at 300-fold molar excess). The background binding of the [^{32}P]-labeled AP1 oligo was very low in the absence of c-*jun* DNA template.

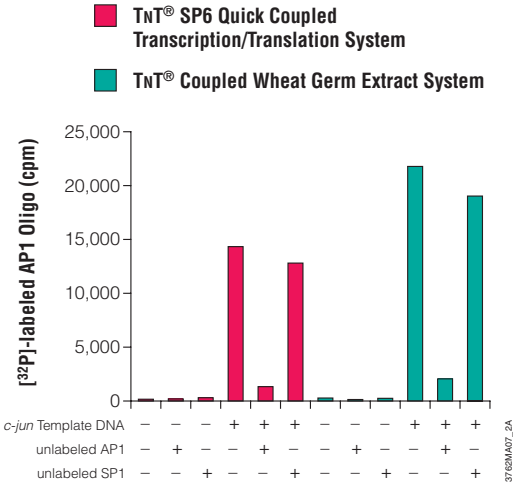

Figure 2. Specificity of binding of in vitro-expressed proteins to [^{32}P]-labeled oligos using the SAM2® Membranes. In vitro translation reactions were performed using the TnT® SP6 Quick Coupled Transcription/Translation System (RRL) or the TnT® Coupled Wheat Germ Extract System (WGE) in the presence and absence of the pSP64 poly(A)-c-*jun* DNA. Competition reactions were performed using an aliquot of each expression reaction (RRL, 6µl, and WGE, 10µl) incubated with [^{32}P]-labeled AP1 Oligos and unlabeled AP1 (specific competitor) or SP1 Oligos (nonspecific competitor). The reactions were spotted onto SAM2® Membranes, and each square was counted in a scintillation counter.

An alternative to using a radiolabeled consensus DNA oligonucleotide is to radiolabel the target protein with [35S]methionine during in vitro expression. Upon incubation of the radiolabeled protein with a biotinylated consensus oligonucleotide, the complex can then bind to, and be detected on, SAM2® Membrane. As seen in Figure 3, specific interaction between the [35S]-c-Jun and the biotinylated AP1 oligo could be detected with c-Jun expressed in the TnT® SP6 Quick Coupled Transcription/Translation System or the TnT® SP6 Coupled Wheat Germ Extract System. Competition with unlabeled AP1 Oligo was seen at a threefold molar excess in both systems. However, using less biotinylated AP1 Oligo for capture would allow more efficient competition by unlabeled oligo.

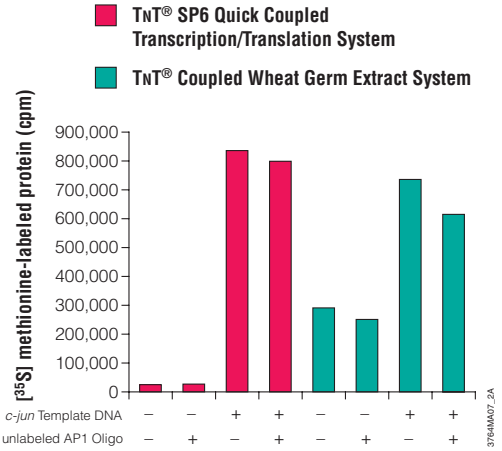

Figure 3. Binding of in vitro-expressed [35S]methionine-labeled proteins to a biotinylated AP1 Oligo and SAM2® Membrane. Duplicate in vitro translation reactions were performed using [35S]methionine and the TnT® SP6 Quick Coupled Transcription/Translation (RRL) or TnT® Coupled Wheat Germ Extract (WGE) Systems in the presence and absence of the pSP64 poly(A)-c-*jun* DNA. An aliquot of each expression reaction (RRL, 6µl, and WGE, 10µl) was incubated with biotinylated AP1 Oligos in the presence or absence of unlabeled AP1 Oligos (6pmol). The reactions were spotted onto SAM2® Membranes and counted in a scintillation counter. The results shown are averages of the duplicates.

Conclusions

The use of the streptavidin-coated SAM2® Biotin Capture Membrane provides an easy and sensitive alternative for the detection of DNA:protein interactions as compared to standard gel mobility shift assays. The target protein of interest can be labeled with [35S]methionine or biotin during in vitro translation and then interact with a biotin- or [32P]-labeled consensus oligonucleotide, respectively. Following complex formation, spotting onto the SAM2® Membrane and washing, the DNA:protein interaction can be detected by simple scintillation counting or phosphorimaging analysis. This method would allow the analysis of many DNA:protein interactions simultaneously and theoretically could be applied to any protein:nucleic acid interaction, including RNA:protein complexes, provided one partner can be radiolabeled and the other partner biotinylated.

References

1. Ausubel, F.M. *et al.* (2000) *Current Protocols in Molecular Biology*, Chapter 12.
2. Fisch, T.M., Prywes, R. and Roeder, R.G. (1987) *Mol. Cell Biol.* **7**, 3490–502.
3. Lee, W., Mitchell, P. and Tjian, R. (1987) *Cell* **49**, 741–52.

Protocols

- *TnT® SP6 Quick Coupled Transcription/Translation System Technical Manual* #TM045, Promega Corporation. www.promega.com/tbs/tm045/tm045.html
- *TnT® Coupled Wheat Germ Extract System Technical Bulletin* #TB165, Promega Corporation. www.promega.com/tbs/tb165/tb165.html
- *Transcend™ Non-Radioactive Translation Detection System Technical Bulletin* #TB182, Promega Corporation. www.promega.com/tbs/tb182/tb182.html
- *SAM2® Biotin Capture Membrane Technical Bulletin* #TB547, Promega Corporation. www.promega.com/tbs/tb547/tb547.html
- *Gel Shift Assay System Technical Bulletin* #TB110, Promega Corporation. www.promega.com/tbs/tb110/tb110.html

Ordering Information

Product	Size	Cat.#
TnT® SP6 Quick Coupled Transcription/Translation System*	40 reactions	L2080
TnT® SP6 Coupled Wheat Germ Extract System*	40 reactions	L4130
Transcend™ tRNA*	30µl	L5061
SAM2® Biotin Capture Membrane*	96 samples	V2861
	7.6 ×10.9cm	V7861
pSP64 Poly(A) Vector	20µg	P1241
AP1 Consensus Oligonucleotide	175pmol	E3201
	35pmol	E3202
SP1 Consensus Oligonucleotide	175pmol	E3231
	35pmol	E3232
Gel Shift Binding 5X Buffer	5 ×200µl	E3581

*For Laboratory Use.

(a) U.S. Pat. Nos. 6,066,462, 6,348,310 and 6,753,157 and other patents and patents pending.

(b) Patents for the foundational PCR process, European Pat. Nos. 201,184 and 200,362, expired on March 28, 2006. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.

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