APPLICATION NOTE

SPA Technologies

The Application of SPA Technology to Study Protein: DNA Interactions

Introduction

Many proteins are able to recognize and subsequently bind DNA. These proteins were first identified by their presence in isolated DNA complexes, by their ability to bind DNA *in vitro*, and by their absolute requirement in many DNA-dependent functions.

This wide range of DNA-binding proteins can affect DNA in many ways and have involvement in DNA structure, packaging, replication, repair, degradation, modification and transport[1]. However, of particular interest is the involvement of DNA-binding proteins in the regulation of transcription. In general, regulation of gene expression is achieved by activating or repressing the transcription of particular genes in specific cell types or in response to specific signals. This control is exerted by a specific class of nuclear proteins, transcription factors, which are either DNA-binding proteins or proteins that interact with and/or modify RNA polymerase II. Some of these proteins are generic factors but many more are specific, associating with particular DNA sequences. It is this specificity that provides challenging therapeutic opportunities.

Techniques used to study protein: DNA interactions

A number of techniques exist for studying the interactions of proteins with DNA.

The principle of Electrophoretic Mobility Shift Assay relies on the fact that nucleoprotein complexes can be resolved from uncomplexed DNA by electrophoresis through non-denaturing polyacrylamide gels[2]. Thus, the mobility shift assay begins to indicate the potential for a gene to be transcribed by providing an assay for the presence of DNA-binding proteins capable of binding to a promoter. Although this is a very simplistic approach compared to the *in vivo* situation it does begin to identify at least the basis of transcriptional control.

Another technique which has been used to study protein-DNA interaction is that of filter binding assays. Here, following the binding reaction the labelled protein-DNA complexes are filtered under vacuum to separate them from free protein and DNA. The filters are then dried and the amount of radioactivity retained determined by liquid scintillation counting.

These conventional techniques have a number of disadvantages in terms of throughput, quantitation, safety and the ability to measure low affinity interactions when compared to the principle of Scintillation Proximity Assay (SPA). The use of SPA to detect and quantitate the interaction of the transcription factor, NF-κB p50 and its DNA consensus sequence has been investigated.

Assay concepts

A number of concepts can be envisaged to study protein-DNA interactions by SPA. The alternatives depend on the location of the radiolabel and the choice of the capture system.

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Radiolabelled DNA-binding protein/biotinylated DNA

Radiolabelled DNA-binding protein is incubated with biotinylated DNA in an appropriate assay buffer, the resulting complexes are captured using streptavidin SPA beads (Figure 1). This concept requires radiolabelling of the DNA-binding protein.

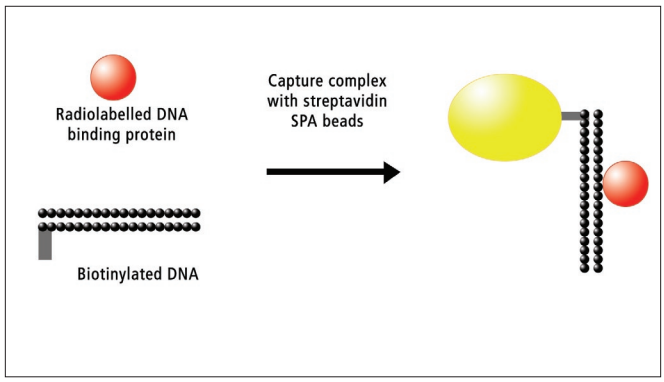

Figure 1. Diagrammatic representation of assay concept using radiolabelled DNA-binding protein/biotinylated DNA

Radiolabelled DNA: DNA-binding protein

Radiolabelled DNA and DNA-binding protein are incubated in an appropriate assay buffer, the resulting complexes are captured using a specific antibody to the DNA-binding protein and protein A SPA beads (Figure 2). If the DNA binding protein is expressed as a fusion protein then clearly an anti-fusion protein antibody could be used for this assay concept. A key consideration is that the antibody used should not interfere with the DNA binding site. Alternatively, biotinylation of the DNA-binding protein and subsequent capture on streptavidin SPA beads could be used

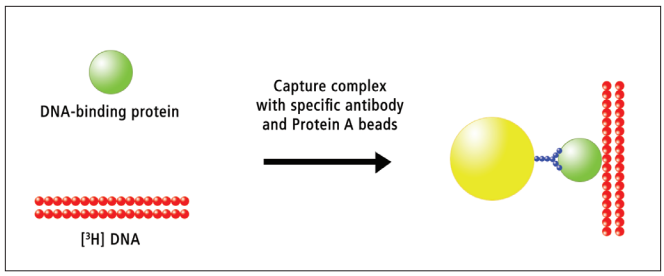

Figure 2. A diagrammatic representation of the assay concept using radiolabelled DNA: DNA-binding protein

Scintillation proximity assay for NF-κB p50/DNA binding

The SPA platform allows dissection of protein complexes and determination of functional sites within the complex. The transcription factor NF-κB is a sequence specific DNA-binding protein. It is constitutively active only in B cells but can be induced in pre-B cells and non-B cells by a number of different factors including T cell mitogens, cytokines, viruses, bacterial lipids and phorbol esters, which cause release of NF-κB from an inhibitory protein, IκB. This allows translocation of NF-κB into the nucleus and subsequent binding to its consensus DNA sequence. NF-κB is a heterotetramer consisting of two 50 Kda (p50) and two 65 Kda (p65) subunits. Both subunits are members of a multigene family, which includes the p49 subunit, also capable of forming heterodimers with p65 and the c-rel protooncogene. The p50 subunits are predominantly responsible for DNA binding while the p65 subunits contain the transcriptional activation function and the binding site for IκB.

NF-kB is involved in transcriptional control of a large number of genes induced during the inflammatory response, for example, TNF ("Tumor Necrosis Factor "), IL-2 and its receptor. In addition, NF-κB may regulate proliferation of HIV-1, cytomegalovirus and adenovirus. Inhibition of NF-κbinding may thus have therapeutic potential in the control of inflammation and viral proliferation[4].

Assay concept for NF-κB p50/DNA binding

A number of the assay concepts outlined above have been experimentally investigated. The most successful format used radiolabelled consensus DNA, NF-κB p50, an antibody to NF-κB p50 and protein A SPA beads to capture the complex (Figure 3). Using an antibody directed to the nuclear localization signal sequence of NF-κB p50, i.e. an antibody which should not inhibit DNA binding by the protein, it has been possible using the above format to generate specific SPA counts.

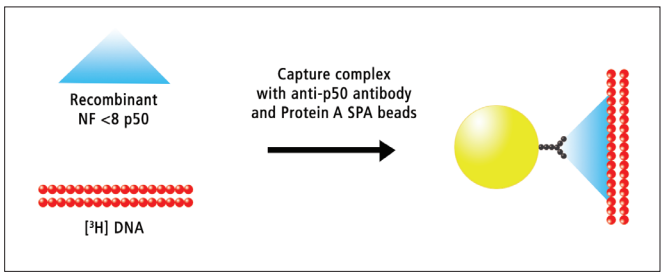

Figure 3. Diagramatic representation of the assay concept for NF-κB p50/ DNA binding

Experimental data

Figure 4 shows a titration of labelled DNA against a fixed amount of NF-κB p50 protein.

The binding correlates with the concentration of [³H]DNA present. A signal:noise ratio of >50:1 has been obtained using this assay format. When the anti-NF-κB antibody is not added to the reaction the non specific binding is low. Also, the addition of a control peptide to which the antibody was raised abolishes binding of the DNA, indicating that the binding event is specific. Furthermore, the binding of radiolabelled DNA can be competed out using unlabelled consensus DNA (Figure 5).

Generally assays were performed as described in Figure 4. However, it has also been shown that it is possible to pre-couple the binding protein to the SPA bead and then probe with the consensus DNA. It is envisaged that this assay would be appropriate for T0 type additions.

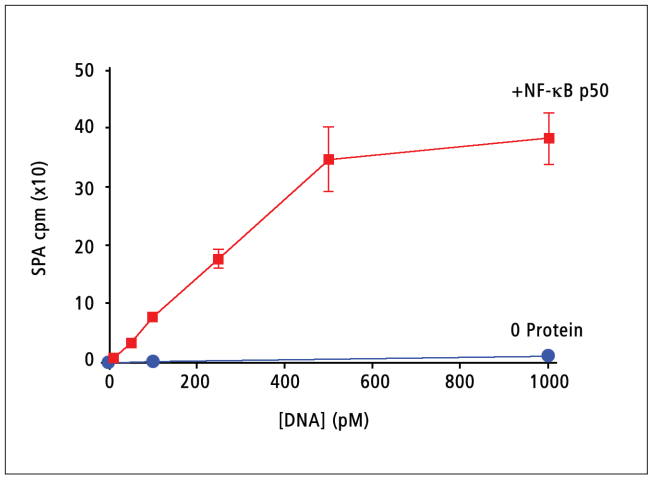

Figure 4. Correlation of DNA concentration with SPA counts. NF-κB p50 (100 ng) was incubated for 30 minutes at room temperature with [^{3}H]consensus DNA (0-5 ng), in assay buffer (10 mM TrisHCl, pH7.5, 0.1M NaCl, 5% glycerol, 1 mM EDTA, 5mM DTT and 1mg/ml BSA) to a volume of 100µl. Anti-NF-κB antibody (0.5 µg) and protein A SPA beads (1 mg) were added. Reactions were incubated for a further 30 minutes at room temperature before counting. Controls were performed by omitting NF-κB p50.

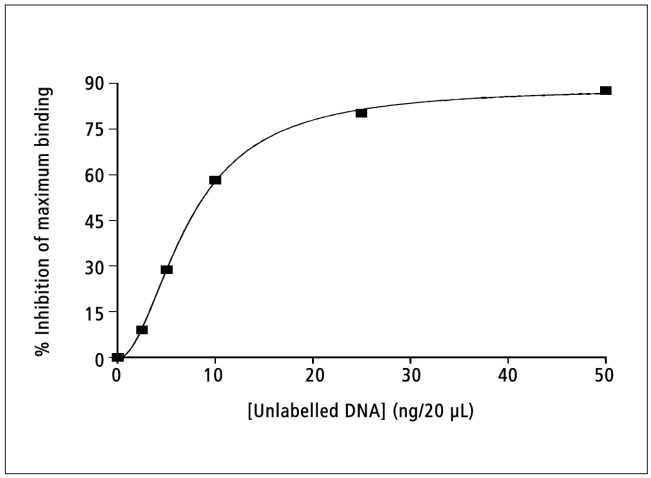

Figure 5. Inhibition of binding by unlabelled DNA. The assay was performed as in Figure 4 but with the inclusion of unlabelled consensus DNA (0-50 ng)

The assay has used both [^{3}H]labelled DNA and [^{33}P]labelled DNA (Figure 6). In the case of [^{33}P]labelled DNA, sedimentation of the reaction mixture either by settling or centrifugation is necessary to generate a signal:noise approaching that of the [^{3}H] based assay. Settling or centrifugation of the SPA beads harvests the energy of the specific binding interaction more efficiently and reduces the non proximity effects of the unconjugated [^{33}P]labelled DNA.

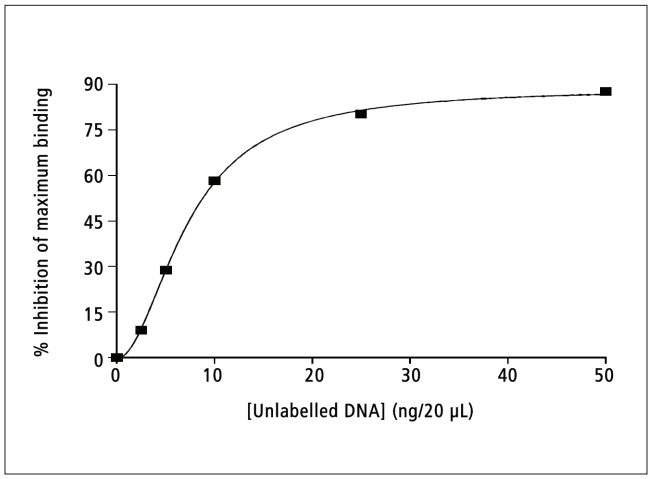

Figure 6. NF-κB p50 binding to a range of [^{3}H] and [^{33}P]DNA concentrations

Assay optimization

As with conventional assays, optimization of the assay conditions is essential to achieve an acceptable signal:noise ratio. The key considerations for the SPA system are outlined below:

1. Capture system – It is important to ensure that sufficient antibody (if required) and SPA bead are present to capture all of the product generated.

2. Radiolabelled DNA – Numerous methods exist for radiolabelling DNA. It is essential to ensure that the specific activity of the labelled DNA is sufficient for the assay. Using Sequenase™ and [^{3}H]TTP (NET52)V), a specific activity of 1-2Ci/:mol was achieved for use in the NF-κB p50/DNA binding assay.

3. Buffer system – The assay buffer should be optimized for pH and reagent concentration. The inclusion of salt eg. sodium chloride or sodium acetate should be considered as this has been shown to have a significant effect on binding. Also, the presence of DTT has been shown to improve binding.

Summary

Using the NF-κB p50/DNA model system, the use of SPA to investigate protein-DNA interaction has been demonstrated. Under optimized assay conditions excellent signal:noise ratios have been obtained with both [^{3}H] and [^{33}P]labelled DNA (>50:1 and >40:1 respectively). By the use of appropriate controls the binding has been shown to be specific. This assay offers significant potential to identify the functional domains with protein complexes and better than existing conventional techniques in terms of throughput, quantitation and safety.

References

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940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
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