A Scintillation Proximity Assay (SPA) for DNA binding by human p53

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INTRODUCTION

Assays to quantitate changes in DNA binding proteins can lead to earlier diagnosis of disease, while the development of drugs to counteract the loss of gene regulation is the likely future of biomedicine (1). The p53 tumor suppressor controls the cell cycle of normal cells (reviewed in 2-4). Mutations in this gene are associated with increased risk in developing metastatic disease, poorer prognosis and decreased sensitivity of cancer cells to chemotherapeutic agents (5-8). Thus functional assays for p53 DNA binding are important for determination of appropriate modes of therapy. Generally DNA binding has been measured by electrophoretic mobility shift assays (EMSAs) or by filter binding (9,10), but neither are amenable to high throughput screens. We therefore developed a scintillation proximity assay (SPA) to measure DNA binding of human p53. (Figure 1).

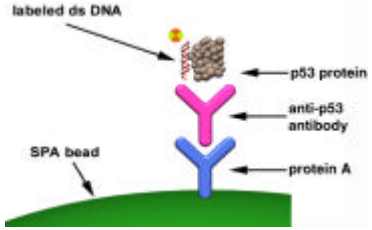

***Figure 1**. Scintillation proximity assay (SPA) for DNA binding by human p53*

METHOD

We prepared a double stranded DNA probe, sequence 5'-$(A)_{19}$TGCCAAGGCTTGCCCGGGCAGGTCTGGCCT/AGG-CCAGACCTGCCCGGGCAAGCCTTGGCA-3' from the cyclin G promoter region that was known to bind p53 in a specific manner (11). This was used to develop the SPA DNA binding assay.

The DNA fragment was labelled using [^{3}H]TTP and terminal transferase to a specific activity of 420Ci/mmol. We prepared human p53 using recombinant baculovirus (kindly provided by K. Okamoto, Columbia University), quantitated the total protein in cell extracts using bicinchoninic acid (Sigma) and the p53 protein specifically using a p53 ELISA kit (Oncogene Research Products, Cambridge, MA). We confirmed DNA binding by p53 using EMSAs, then used those conditions to develop the SPA DNA binding assay. These assay conditions were 0.08µCi (0.19pmoles) [^{3}H] labelled DNA, 100ng anti-p53 monoclonal antibody (pAb421, Oncogene Research Products), 1µg non-specific DNA (double or single stranded polydAdT) with 0.5-100ng p53 in binding buffer (20mM Hepes, pH7.5, 1mM EDTA, 1mM DTT, 10mM ammonium sulfate, 30mM KCl and 0.2% Tween-20) in 20µl. The assay was configured in a standard 96-well white microplate and incubated at room temperature for 5-30 minutes, followed by the addition of 0.5-2mg of PVT protein A SPA beads in 50-100µl binding buffer. The radioactivity bound to the SPA bead was measured using a TopCount™ Microplate Counter (Packard Biosciences, Meriden, CT).

RESULTS

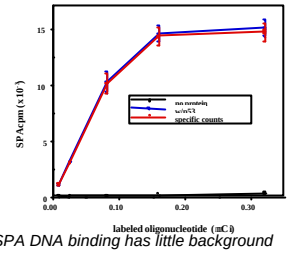

***Figure 2**. SPA DNA binding has little background*

The DNA binding of the recombinant human p53 was first confirmed using EMSAs (data not shown) then monitored using SPA with [^{3}H] labelled DNA. Wells contained 5.8ng p53 or buffer, various amounts of oligonucleotide and 500µg SPA beads. Minimal background was found with this configuration while specific counts varied directly with the amount of label added. (Figure 2, results shown as means, +/-SEM, n=2).

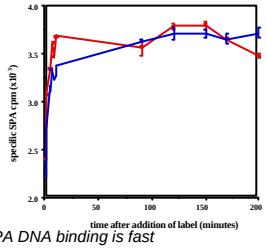

***Figure 3**. SPA DNA binding is fast*

There appeared to be a very fast association of the beads with the p53 DNA complex. Initial changes in SPA signal were complete in 3 minutes (Figure 3). This was followed by a slow 10-20% increase in the counts detected until maximum counts were detected at 2-6 hours. Wells were configured with 6.0ng p53, preincubated with 1mg beads for zero (-o-) or 10 minutes (-x-) before addition of the labelled oligonucleotide and counts determined for 200 minutes. (Results shown as means, +/-SEM, n=2).

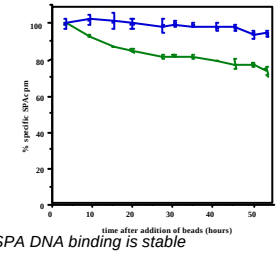

***Figure 4**. SPA DNA binding is stable*

The DNA binding assay was configured with 45ng (-o-) or 15ng p53 (-x-) and 1mg beads and the specific counts determined over 3 days. Approximately 80% of maximum counts were still detectable 50 hours after addition of the label. (Figure 4, results shown as means, +/-SEM, n=2).

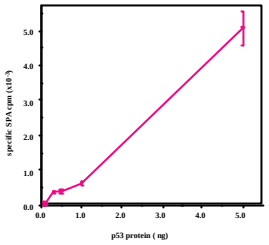

***Figure 5**. SPA DNA binding is sensitive*

Different levels of p53 protein were added to a standard assay and specific counts were determined. Consistently 0.5ng p53 could be detected at twice the background level of counts. (Figure 5, results shown as means, +/-SEM, n=2). This is comparable to the EMSA sensitivity in our hands (not shown).

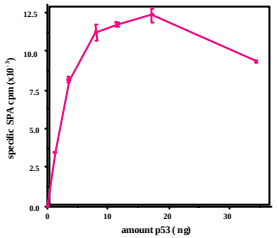

***Figure 6**. SPA DNA binding is saturable*

Increasing amounts of p53 were added using standard assay conditions and specific counts determined. Saturation of the bead under these conditions occurs at approximately 20ng of p53. (Figure 6, results shown as means, +/-SEM, n=2).

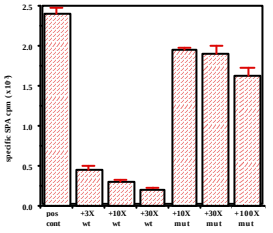

***Figure 7**. SPA DNA binding can be competed*

To confirm the specificity of DNA binding we competed for the counts with different amounts of unlabelled wild-type or mutant oligonucleotide (three base alterations, known not to compete for DNA binding by EMSA [11 and data not shown]). As expected, the wild-type DNA competed efficiently for the SPA counts while the mutant competed very poorly. (Figure 7, results shown as means, +/-SEM, n=2).

DISCUSSION

Using a baculovirus produced human p53, we have developed a DNA binding assay for this protein with SPA technology. We have shown this assay is fast and stable, specific and sensitive and thus offers a clear alternative to the common EMSA method for analyzing DNA binding. Table 1 compares and contrasts these two techniques.

Property	EMSA (gel)	SPA
Time	4.5 + hours	30 minutes
Label	[^{32}P]	[^{3}H]
Quantitation	Extra Steps	Automated
Sensitivity	0.5ng p53	0.5ng p53
Adaptable to HTS?	NO	YES
Miniaturization possible?	NO	YES

***Table 1**. Comparison of SPA and EMSA methods for analyzing p53 DNA binding*

We have found the DNA binding SPA to be faster than EMSA and as it requires more stable radioactive labels, can be more conveniently used over many months. The assays as described were used in a 96-well microplates, but modifications to 384 and 1536 well formats would conserve more reagents, while the EMSA cannot be further reduced.

A future application for this assay will be in analyzing human cell extracts and tissues for levels of p53 DNA binding activity, thus lending itself as a tool for being able to direct appropriate modes of therapy for cancer patients. The format of this SPA is also amenable for high throughput screening, enabling one to identify drugs able to modulate the function of mutant forms of this tumor suppressor (1).

CONCLUSIONS

We have developed a p53 DNA binding SPA that is fast and stable, sensitive and specific. It provides a viable alternative to EMSA assays for monitoring DNA binding particularly for high throughput screening and drug development.

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