APPLICATION NOTE

SPA Technology

Assay Development Guide to Protein:Protein Studies

Introduction

Interactions between proteins are a key feature of many biochemical processes, for example cell signalling. In the absence of any enzymatic activity, measurement of protein:protein interactions has presented problems. Scintillation Proximity Assay (SPA) technology permits the direct measurement of binding of one protein to another. In addition, in cases where the dissociation rate of the interaction is high, SPA provides a means of equilibrium counting in an homogeneous assay format. An SPA system for the measurement of the direct binding of the GTP-ase activating protein, Neurofibromin-1 (NF1) to an oncogenic Ras protein (Leu-61 Ras) has been developed[1]. The assay development and optimization process is used to illustrate the key features in designing a protein-protein interaction SPA.

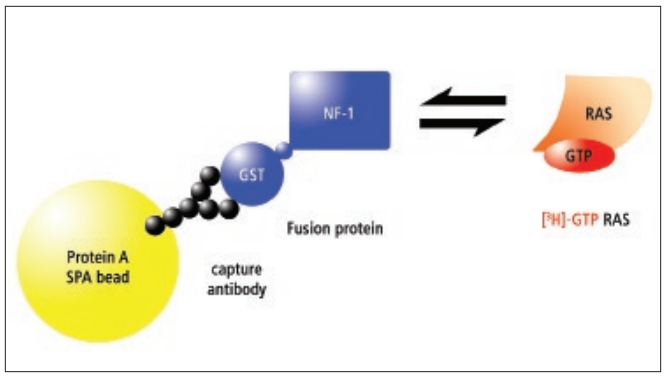

Figure 1. Schematic representation of the NF1-Ras SPA utilizing Protein A SPA beads and anti-GST antibody

Assay Concepts

Antibody Capture Format

There are several alternative assay concepts potentially applicable to a protein-protein interaction assay. The NF1-Ras SPA format exploited the fact that the NF1 protein was expressed as a GST (*Schistosoma japonicum* glutathione-s-transferase) fusion protein, i.e. GST-NF1. The GST-NF1 fusion protein was coupled to a PVT Protein A SPA bead via a commercially available anti-GST antibody.

An obvious alternative bead for this type of format would be one of the anti-species antibody binding beads, e.g. anti-rabbit, anti-mouse or anti-sheep, according to the source of the capture antibody. The specificity, affinity and purity of the antibody will effect the maximum level of signal achievable and choice of bead. It is not possible to predict whether the anti-species antibody binding beads or Protein A beads would perform better. Both should be evaluated empirically as an initial experiment in the development process. This format offers a generic design for the capture of any protein when a suitable antibody exists. An SPA assay utilizing Protein A (RPNQ0019, RPN143) beads and an anti-GST antibody has recently been published for the measurement of the binding of GSTRaf (a serine/threonine kinase) to Ras[2]. However, the antibody capture method does theoretically present opportunity for non-specific inhibition of the antibody/GST fusion protein binding event.

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Streptavidin-biotin format

If one of the proteins can be biotinylated without loss of biological activity, then the use of the streptavidin bead becomes an option. The avidity and stability of the biotin-streptavidin bond can be an advantage. GST-fusion proteins have been biotinylated for other assays without interfering with the functional activity of the protein (Figure 2). This format has been used for an SPA system designed to measure the interaction between Ras and Raf[4]. In this assay, a GSTRaf fusion protein was biotinylated using sulfosuccinimidyl-6-(biotinamido) hexanoate.

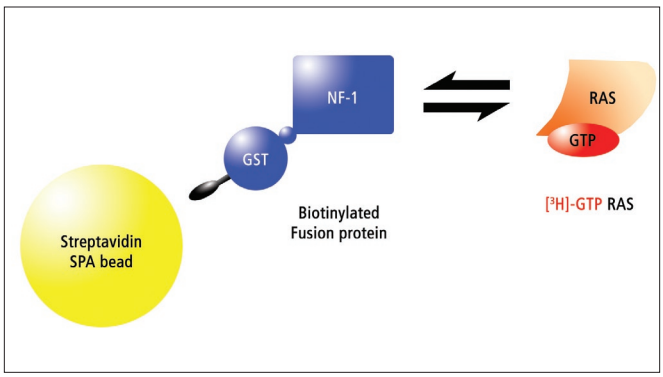

Figure 2. Schematic diagram of potential Streptavidin-biotin capture format for the NF1-Ras SPA

If biotinylation is considered, it is necessary to optimize the ratio of biotinylation reagent to protein. This must be done empirically for the system under study. There is currently no method for the precise determination of the number of biotins that become attached to a protein.

A suggested protocol would be to biotinylate the protein using increasing molar ratios of biotinylation reagent to protein from 1:1 up to 50:1.

Having biotinylated the protein and separated it from the biotinylation reagent, the biotinylated protein could be tested in a simple binding experiment using 1mg of streptavidin bead and a constant amount of the radiolabelled protein. Once an optimal ratio of biotinylation reagent to protein is established, the ratio of bead to biotinylated protein can be determined.

An important point to remember with all the formats described in this section is that the bead or bead-antibody has to be present in excess with respect to the protein to maximize capture by the beads.

Radiolabelling

For detection, radiolabelling one of the proteins is a requirement. In order to achieve a good signal, a chemically pure protein labelled to a high specific activity is desirable. The methods for tritiation and iodination of proteins are described in the course manual. Kahl et al[4] compared both iodine and tritium for labelling Ras directly using conventional labelling methods and for loading Ras with radiolabelled GTPγS. The use of GTPγS permits the use of wild type Ras proteins. For the NF1-Ras SPA, an oncogenic Ras protein (Leu61 Ras) was used. This mutant does not hydrolyze GTP even upon binding of a GTP-ase activating protein. This enabled the Leu61 Ras to be loaded with [^{3}H]GTP using an exchange reaction performed in the presence of a GTP regenerating system (phosphoenol pyruvate and pyruvate kinase) under optimized conditions[1]. GDP and GTP rapidly exchange from the protein into solution and any GDP in solution will be converted into GTP.

Assay Optimization

Factors affecting the signal:noise ratio will be the relative amounts of each reagent present, quality and affinity of the capture antibody and the affinity of the two proteins participating in the interaction.

As has been mentioned earlier, to obtain a maximum signal it is necessary that the capture protein (either biotinylated or coupled to the antibody) be present in excess to the radiolabelled protein and then that bead be present in excess to capture the complex. This section will focus on the optimization process followed for the NF1-Ras SPA.

Antibody

For all assays using an antibody capture format, the purity and affinity of the antibody will greatly influence the amount of antibody (and hence the amount of bead) required for maximal signal. It will be essential that the antibody:protein interaction does not interfere with the protein:protein interaction.

For the NF1-Ras assay, the initial experiment assessed the titre of the anti-GST antibody required. The anti-GST antibody was diluted in assay buffer and a constant volume of each dilution added to each assay well. Maximal binding was achieved using a dilution of 1:20.

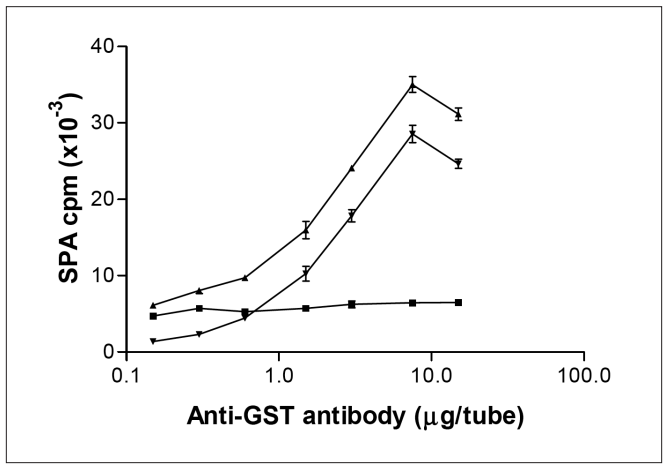

Figure 3. The effect of anti-GST antibody dilution on the binding of HRasL61. GTP to GST-NF1 in the presence of 1 mg Protein A bead in 50 mM Tris.HCl, pH7.5, 2mM dithiothreitol. ▲ = SPA cpm bound in absence of GST-NF1, ▼ = SPA cpm bound in presence of 30pmol/well GST-NF1, ■ = specific SPA cpm bound.

SPA Bead

As shown in Figure 4, four different bead concentrations were tested in the presence of constant amounts of anti-GST antibody, GST-NF1 (Figure 3).

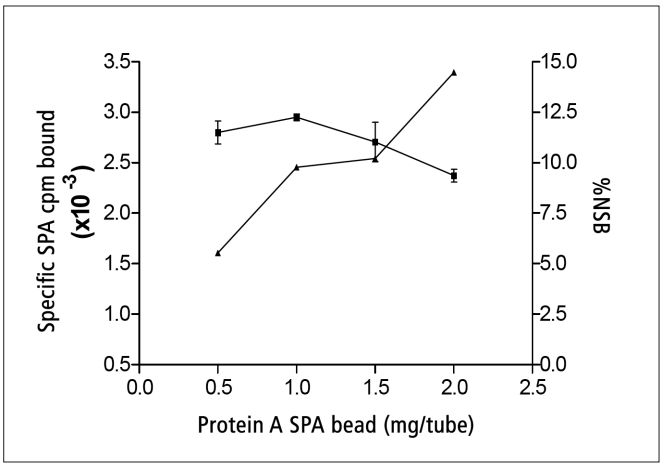

Figure 4. Effect of increasing Protein A bead concentration on the specific SPA cpm bound (■) and percentage non-specific binding (NSB) (▲). Assay conditions: 10 pmol/well H-RasL61.[^{3}H]GTP, 3.75 µg/well GST-NF1 in 50 mM Tris.HCl, pH7.5, 2 mM dithiothreitol.

The maximal signal under the described conditions is achieved with 1mg Protein A bead. However, using 0.5mg Protein A bead, the signal is only slightly reduced but the reduction in the percentage of nonspecific binding is in the region of 50%. An important point demonstrated by this figure is the increase in non-specific binding caused by increasing the concentration of bead.

GST-NF1 fusion protein / Ras [^{3}H]GTP

To optimize the concentration of the GST-NF1 fusion protein, increasing concentrations of GST-NF1 were added to each assay in the presence of 0.5mg Protein A SPA bead and a constant amount of Ras [^{3}H]GTP. The titration of GST-NF1 was performed at four concentrations of Ras [^{3}H]GTP (Figure 5).

The signal increases with the concentration of GST-NF1 until all the available binding sites (i.e. anti-GST antibody bound to the Protein A beads) are saturated. Further increases in the GST-NF1 concentration result in a reduction in signal as a competition is established for anti-GST antibody by GST.NF1 and a complex of GST.NF1 bound to Ras [^{3}H]GTP. Improved signal was also observed by increasing the concentration of Ras [^{3}H]GTP.

Assay buffer and interfering substances

For the NF1-Ras SPA, a simple Tris buffer was used. The presence of dithiothreitol was found to be essential to achieving consistent assay results. It is probable that the dithiothreitol is necessary for the GST fusion protein as a similar requirement for dithiothreitol has been observed with other GST fusion proteins e.g. the SH2 fusion proteins and NF-κB.

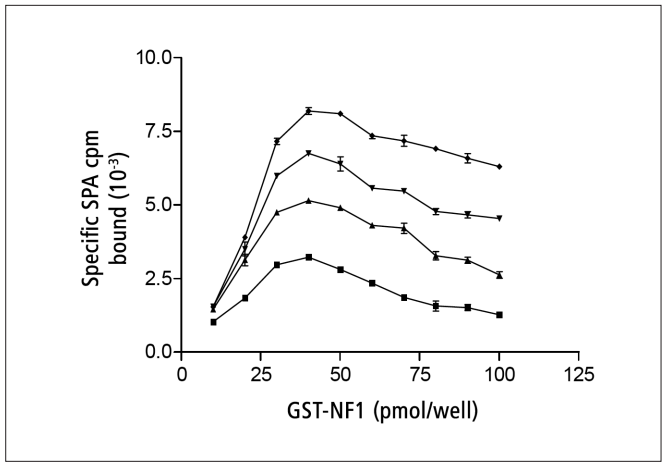

Figure 5. Effect of increasing GST-NF1 and H-RasL61.[^{3}H]GTP concentration on specific SPA cpm bound. Assay conditions: 3.75mg anti- GST antibody (Molecular Probes), 0.5 mg Protein A bead in 50 mM Tris.HCl, pH7.5, 2 mM dithiothreitol. ■ = 10p mol/well HRasL61.[^{3}H]GTP, ▲= 20 pmol/well H-RasL61.[^{3}H]GTP, ▼ = 30pmol/well H-RasL61.[^{3}H]GTP, ◆ = 40 pmol/well H-RasL61.[^{3}H]GTP.

The effect of ionic strength of the buffer was also examined. Both sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) were shown to elicit a significant dose dependent decrease in signal (see figure 6). Increasing ionic strength is reported to dramatically reduce the affinity of interaction between Ras and Neurofibromin[3]. Skinner et al demonstrated the increase in NaCl or $MgCl_2$ concentration effected only the interaction between Ras and NF1 and not the interaction between the anti-GST antibody and the GST fusion protein by the use of a GST-Ras [^{3}H]GTP fusion protein[1].

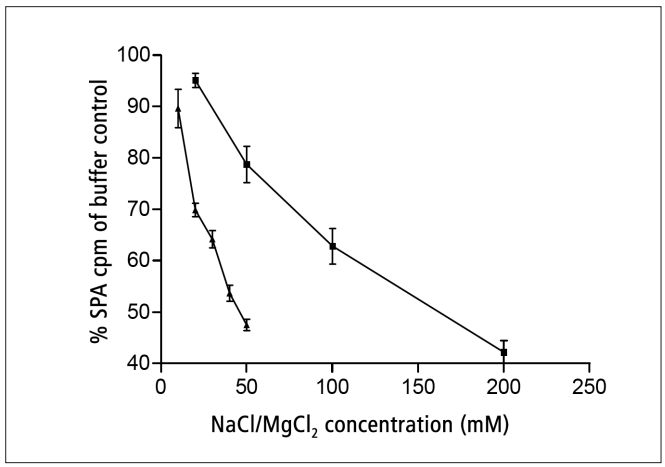

Figure 6. Effect of ionic strength on the capture of Ras.[^{3}H]GTP. ■ = NaCl, ▲=MgCl2. Assay conditions as for figure 5 using 10pmol HRasL61.[^{3}H]GTP and 40 pmole GST-NF1.

Agent	Concentration	% of control binding
DMSO	0.25%	100
	1.0%	96
	2.5%	92
	10%	97
Ethanol	1%	87
	10%	80
Methanol	1%	99
	10%	99
Acetone	1%	93
	10%	65
HECAMEG	10%	98
SDS	10%	0
Tween 20	10%	88
CHAPS	10%	28
Glycerol	10%	72
Zwittergent	10%	90
Deoxycholate	10%	23
Triton X-100	10%	108
Polylysine	10%	96
K^+(Cl)	10 mM	113
Ca^{2+}(Cl)	10 mM	25
Mg^{2+} (Cl)	10 mM	93
SO_4^{2-} (Na)	10 mM	88
PO_4^{3-} (Na)	10 mM	65
CO_2^{3-} (Na)	10 mM	62

Table 1. Effect of potential interfering substances on the NF1-Ras SPA assay

Other potential interfering substances, e.g. detergents and organic solvents were also examined. (Table 1). As expected, acetone significantly reduces the signal due to its effect on the integrity of the bead. Some detergents (SDS, CHAPS and deoxycholate) significantly reduce the signal.

Order of Addition

The NF1 Ras SPA was designed as a T0 addition assay. This means that each reagent was added at the start of the incubation and allowed to equilibrate. This format is convenient for high throughput screening purposes.

As has been briefly mentioned, it is possible that the antibody:protein interaction could inhibit the protein:protein interaction. This would occur if the epitope recognized by the antibody was located close to, or within the binding site of the second radiolabelled protein. To remove any potential interference, a delayed addition assay format can be utilized. In this format, the two proteins will be permitted to equilibrate and bind prior to the addition of the antibody and beads.

Equilibrium Counting

A common feature of all the potential formats discussed here will be the need for establishing equilibrium counting conditions. The signal will vary until all the interactions occurring in the assay attain equilibrium. Due to the homogeneity of the SPA technology, the time course of the assay can easily be followed by repeat counting.

Assay Validation

The specificity of the NF1-Ras interaction was determined using unlabelled GTP, ATP and GDP over a range of concentrations in a SPA competition binding assay (Figure 7). GTP appeared to have an inhibitory effect on the NF1-Ras interaction under normal assay conditions with IC_{50} values of 0.1 to 1.0 mM being observed.

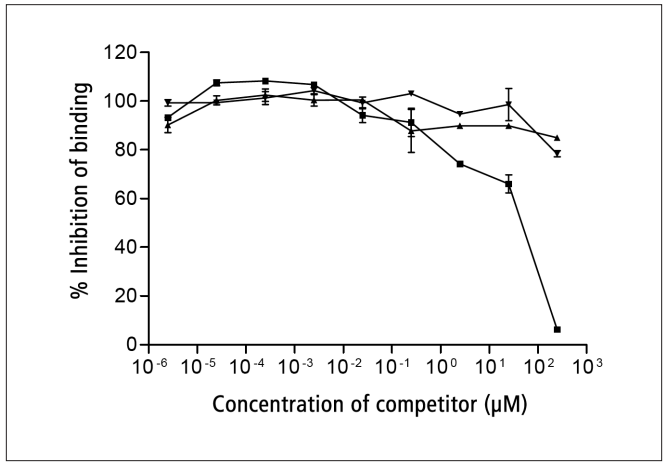

Table 2. Inhibition of H-RasL61.[^{3}H]GTP to GST-NF1 by GTP (■), ATP (▼) and GDP(▲). Assay conditions as for figure 5 using 10 pmol HRasL61.[^{3}H]GTP and 40 pmole GST-NF1

Skinner et al[1] used a neutralizing anti-Ras monoclonal antibody Y13- 259 and a detergent n-dodecyl maltoside (a specific inhibitor of NF1 catalytic activity) to demonstrate specific binding. Both reagents abolished the signal from the NF1-Ras SPA but neither effected the signal from a control SPA in which a [^{3}H] GTP. GST-Ras fusion protein was bound to Protein A beads.

The interaction between Ras and NF1 is known to show a high degree of specificity for the GTP-bound form of Ras. This was demonstrated using both Ras [^{3}H]GTP and Ras [^{3}H]GDP where no binding of Ras [^{3}H]GTP to GST.NF1 was detectable. The NF1-Ras assay has been used to examine the reported inhibitory effect of mitogenic lipids such as arachidonic acid on the GTP-ase activating activity of NF1. Sermon et al[3] showed that Arachidonic acid could abolish the assay signal with 50% inhibition occurring at 5-10 mM arachidonic acid.

The kinetics of the NF1-Ras interaction and also the Ras-Raf interaction have been studied[1,2,3]. The homogeneous nature of the SPA technology permits the kinetic analysis of such interactions despite the very fast dissociation rates (>1min-1). Skinner et al[1] were able to calculate the affinity of the NF1-Ras interaction, giving a Kd of approximately 40 nM.

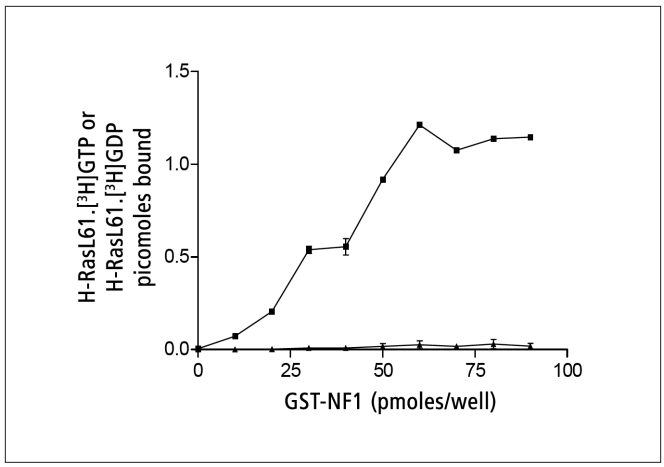

Figure 7. Binding of H-RasL61.[^{3}H]GTP (■) and H-RasL61.[^{3}H]GDP (▼) to GST-NF1.

Typical Assay Conditions

The following assay conditions are those optimized for the NF1-Ras SPA.

- 20 µl 50 mM Tris.HCl, pH7.5, 2 mM dithiothreitol
- 20 µl GST-NF1 (50 pmoles/assay)
- 50 µl Anti-GST antibody (3.75 µg/assay) Molecular Probes
- 10 µl Leu-61 Ras[3H]GTP (10 pmoles/assay)
- 50 µl Protein A SPA bead (0.5 mg/assay)
- 50 µl Sample/buffer
  Total assay volume = 200 µl
  Incubation conditions: One hour at 22°C

Conclusions

The measurement of protein:protein interactions in the absence of enzymatic activity is feasible using SPA technology. The NF1-Ras SPA format demonstrates that interactions of micromolar affinity and fast dissociation rates can be measured using SPA, permitting kinetic analysis and inhibitor screening.

References

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