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Homogeneous Detection and Measurement of Micromolar Affinity Interactions Using AlphaScreen™

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Abstract

Measurement of micromolar affinity interactions is difficult to achieve using common radioactive as well as non-radioactive homogenous screening technologies. The major impediment is the necessity of using concentrations of tracer molecule close to the Kd of the given interaction (at least 10% of that value). Using hundreds of nanomolar of radioactive or fluorescent tracer per well produces assays showing very low signal to background ratios. Furthermore, performing HTS with that amount of radioactive tracer per well would be very costly and also represent potential biohazards. One alternative is to use low specific activity tracer molecules but once again this produces assays with very low signal to background ratios. AlphaScreen eliminates the needs of using high concentrations of tracer to measure weak affinity interaction. Using AlphaScreen reagents it is possible to artificially create conditions reproducing high local concentrations of a given molecule (ex. biotinylated-tracer). For example, we have developed a microformat assay to measure the interaction between a biotinylated peptide derived from the N-terminus of p53 and a GST-HDM2 fusion protein. Competition studies performed with the non-biotinylated p53 peptide generated sigmoidal curves (Hill coefficient = 1) with an IC_{50} of approximately 1 µM. This assay yielded signal to background ratios reaching 500. Other examples recently produced involve the detection of glycosylated binding partners to various lectins such as Wheat Germ Agglutinin (WGA) and Concanavalin A (ConA). Biotinylated-a1-acid glycoprotein binding to WGA was inhibited by 300 µM N-acetyl-glucosamine and 10 nM a1-acid glycoprotein. D-glucose and D-mannose produced competitive inhibition of biotinylated-mannose binding to ConA with IC_{50} values of 3 and 0.6 mM respectively. In all cases, competition curves were sigmoidal and showed Hill coefficient of 1. These results taken together show that AlphaScreen is a very valuable screening platform to efficiently detect and measure low affinity interactions.

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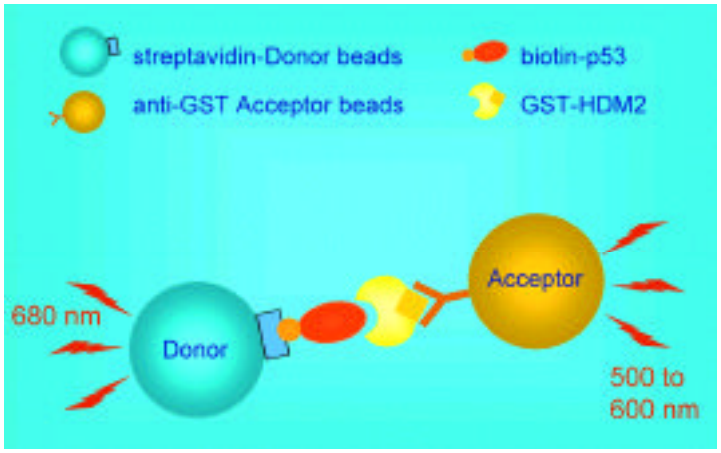
p53 / HDM2

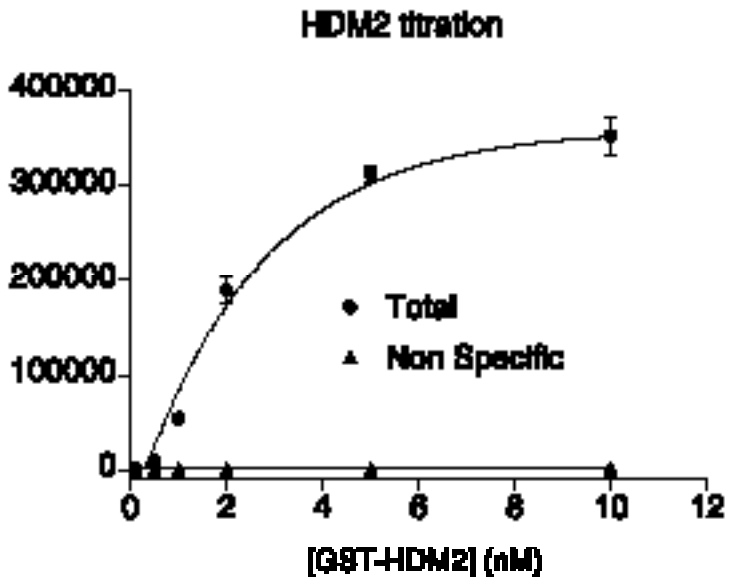


3 Methods

All assays were performed in 10 µl final volume in 384 PerkinElmer ProxiPlate plates (working volume: 15 µl). The standard protocol for the p53 competition assay is the following:

- add 2 µl p53 peptide or buffer
- add 4 µl anti GST-acceptor beads / GST-HDM2 / biotin p53 mix
- incubate 60 minutes at RT
- add 4 µl streptavidin-donor beads
- incubate 60 minutes at RT
- read plate

4 HDM2 Titration



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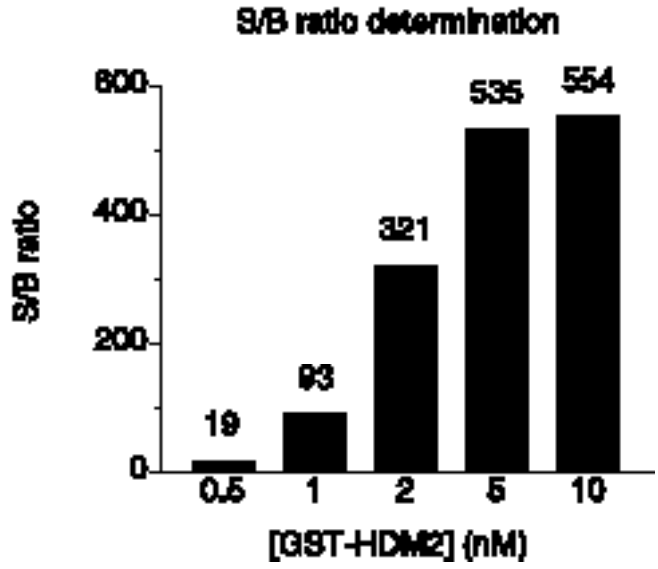
HDM2 Titration S/B Ratio Determination



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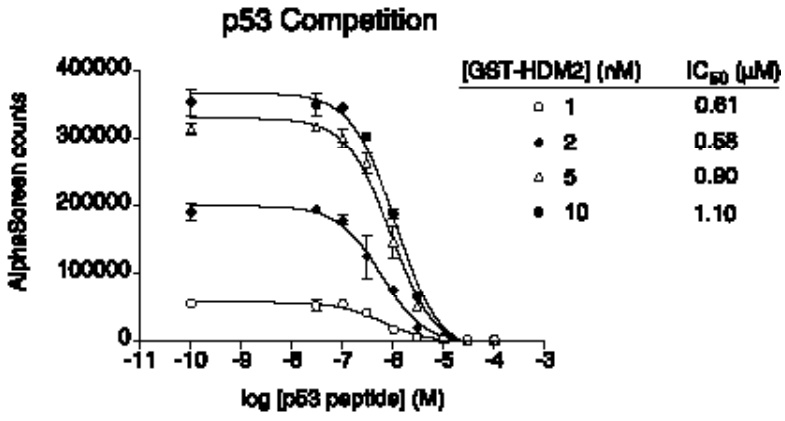
GST-HDM2 Titration p53 Competition



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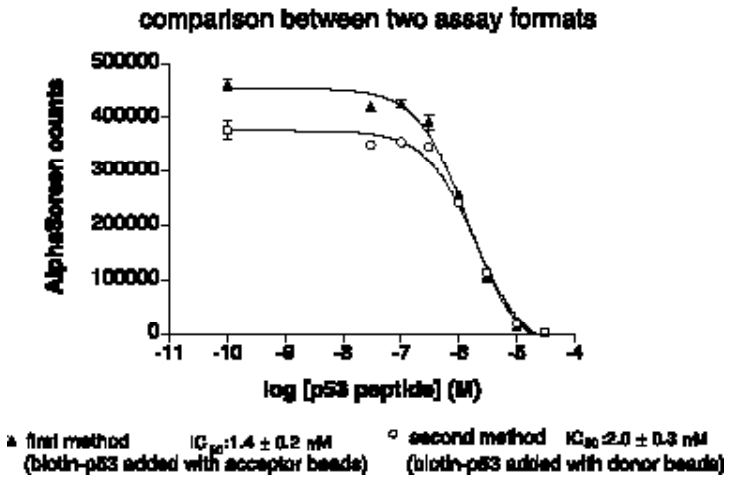
p53 Competition of biotin-p53 Binding to GST-HDM2
Comparison Between Two Assay Formats



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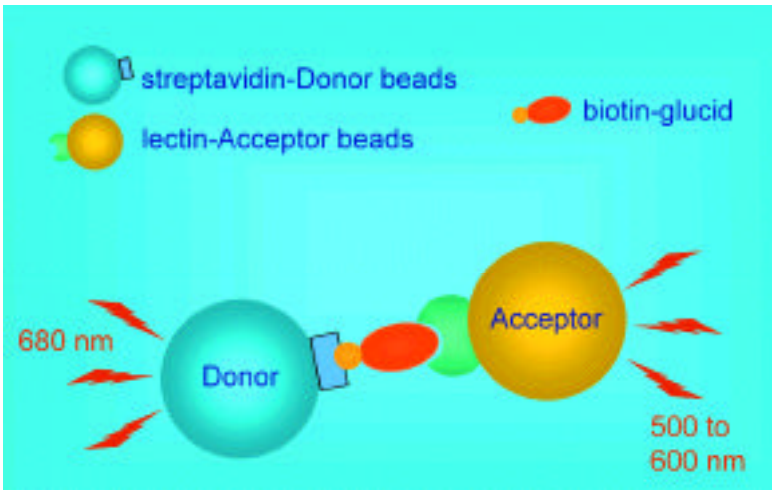
Lectins/Carbohydrates



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Methods

All assays were performed in 25 µl final volume in PerkinElmer 384 well OptiPlates. The standard protocol for carbohydrate competition assay is the following:

- add 10 µl carbohydrate derivative or buffer
- add 5 µl Lectin-Acceptor beads (20 µg/ml final)
- incubate 30 minutes at RT
- add 5 µl biotinylated lectin derivative (tracer)
- add 5 µl streptavidin-Donor beads (20 µg/ml final)
- incubate 60 minutes at RT
- read plate

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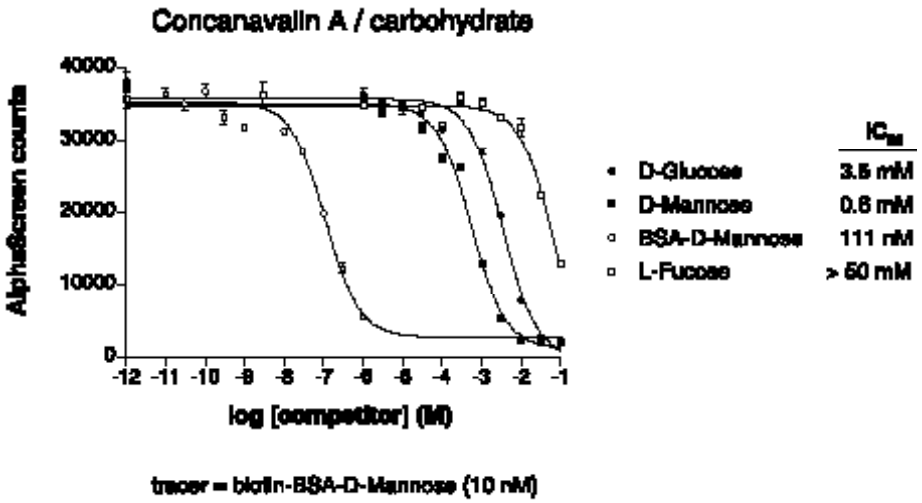
AlphaScreen™ Low Affinity Detection Convanavalin A/Carbohydrate



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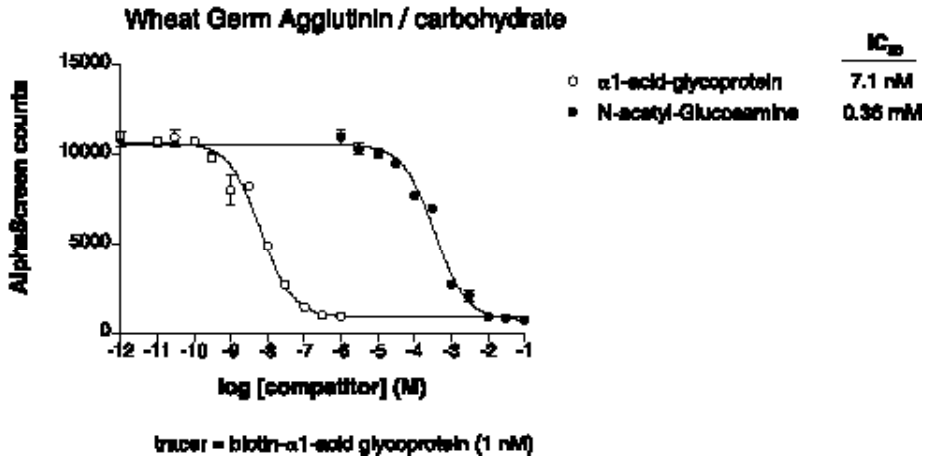
AlphaScreen™ Low Affinity Detection Wheat Germ Agglutinin/Carbohydrate



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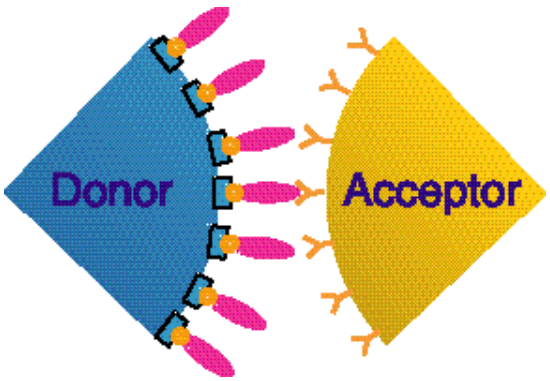
How is it possible to measure µM - mM Affinity Interactions using nM Concentration of biotinylated tracer?



Binding partners captured by their respective beads act in a very limited volume when beads become in close proximity (≈10^{-17} l). nM biotinylated binding partner then reach high local concentration (≈µM)

13 Conclusion

AlphaScreen was used to develop microformat assays to measure the low-affinity interactions involving p53/HDM2 and lectin/carbohydrates. The IC_{50} values obtained, were in agreement with affinity constants already reported by other groups using either isothermal titration calorimetry (p53/HDM2 and lectin/carbohydrates) or SPA (p53/HDM2). At concentration as low as 1 nM of biotinylated tracers, highly robust S/B ratio (close to 100) were obtained. Genuine pharmacodynamic parameters, combined with adequate assay reproducibility and dynamic range, indicate the efficiency of the AlphaScreen technology at measuring low affinity interactions.

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