Assay development (biochemical protein-protein interaction assays)

Before you begin:

- The Donor beads used in Alpha assays are somewhat light sensitive. We recommend working under subdued lighting conditions when working with the beads (less than 100 Lux - the level of light produced on an overcast day). For example, you can turn half of the laboratory lights off and work at a bench away from windows and where the overhead light is not on. Incubate the plate in the dark (for example, placing the covered or sealed plate in a drawer).
- The Alpha signal is temperature-dependent. If you will be performing incubations at 37°C or other temperatures, we recommend that you equilibrate the plate back to room temperature before reading to ensure signal uniformity across the plate.
- We recommend preparing only what you need for the day's experiments. Do not store working dilutions of beads for more than one day.
- Alpha assays require a special reader capable of measuring an Alpha assay. Many standard time-resolved fluorimeters and luminometers cannot read Alpha assays.

1. The first experiment: protein cross-titration

The first experiment is a protein cross-titration. In this experiment, you will be keeping the concentrations of beads constant (20 µg/mL final concentration of each bead) and varying only the concentration of each protein. The plate map for this assay will be designed to test multiple possible combinations of each protein's concentration in a matrix. The assay is performed in singlicate (one well per condition).

Note: This protocol assumes you are performing an assay that uses two proteins that bind directly to the Donor and Acceptor beads, without the use of an antibody. If you also have an antibody in your assay, please refer to Section 2.

A. Preparation of reagents

1. Prepare 200 µL of a 4X working solution (1.2 µM) of Protein X in your assay buffer. If you are not sure what assay buffer to use, try using 1X PBS + 0.5 % BSA. The BSA is important to prevent non-specific interactions.
2. Perform a serial dilution of the 1.2 µM stock in Eppendorf tubes as follows:

Tube	[Final] (M)	[Intermediate] (M) (4X)	Vol of dilution	Assay buffer
1	3×10^{-7}	1.2×10^{-6}	(200 µL of 1.2 µM Protein X)	
2	1×10^{-7}	4×10^{-7}	66 µL of Tube 1	132 µL
3	3×10^{-8}	12×10^{-8}	66 µL of Tube 2	154 µL
4	1×10^{-8}	4×10^{-8}	66 µL of Tube 3	132 µL
5	3×10^{-9}	12×10^{-9}	66 µL of Tube 4	154 µL
6	1×10^{-9}	4×10^{-9}	66 µL of Tube 5	132 µL
7	3×10^{-10}	12×10^{-10}	66 µL of Tube 6	154 µL
8	0	0	300 µL of buffer	

3. Prepare 200 µL of a 4X working solution (1.2 µM) of Protein Y in your assay buffer
4. Perform a serial dilution of the 1.2 µM stock of Protein Y in Eppendorf tubes as follows:

Tube	[Final] (M)	[Intermediate] (M) (4X)	Vol of dilution	Assay Buffer
A	3×10^{-7}	1.2×10^{-6}	(200 µL of 1.2 µM Protein Y)	
B	1×10^{-7}	4×10^{-7}	66 µL of Tube A	132 µL
C	3×10^{-8}	12×10^{-8}	66 µL of Tube B	154 µL
D	1×10^{-8}	4×10^{-8}	66 µL of Tube C	132 µL
E	3×10^{-9}	12×10^{-9}	66 µL of Tube D	154 µL
F	1×10^{-9}	4×10^{-9}	66 µL of Tube E	132 µL
G	3×10^{-10}	12×10^{-10}	66 µL of Tube F	154 µL
H	0	0	300 µL of buffer	

5. (During 1st incubation – refer to protocol below): Prepare a 4X working solution (80 µg/mL) of Acceptor beads:

   16 µL Acceptor beads (5 mg/mL) + 984 µL assay buffer

6. (During 1st incubation – refer to protocol below): Prepare 4x working solution (80 µg/mL) of Donor beads, keeping protected from light:

   16 µL Donor beads (5 mg/mL) + 984 µL assay buffer

B. Assay protocol for a 96-well ½ AreaPlate (Total assay volume of 40 µL)

Refer to the plate map on the next page. You can use a multi-channel repeat pipettor to quickly dispense reagents into the plate.

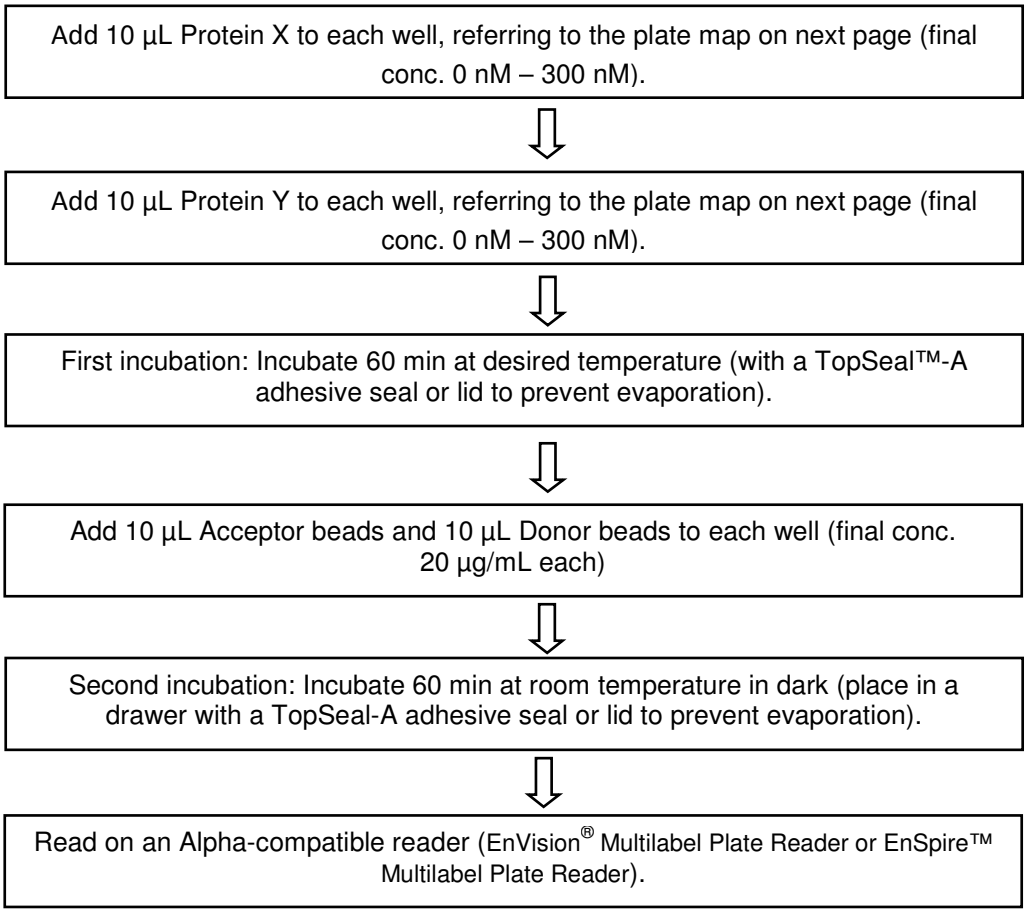

Figure 1. 96-well plate map for cross-titration experiment

- This is a cross-titration matrix. For example, well 5C will contain 3 nM Protein X and 30 nM Protein Y.
- The 0 nM wells are an important control, and should not be omitted. These are your background samples. These wells will include both Donor and Acceptor beads.

	1	2	3	4	5	6	7	8	9	10	11	12
	300 nM Protein X	**100 nM Protein X**	**30 nM Protein X**	**10 nM Protein X**	**3 nM Protein X**	**1 nM Protein X**	**0.3 nM Protein X**	**0 nM Protein X**	**(Empty)**	**(Empty)**	**(Empty**	**(Empty)**
A 300 nM Protein Y												
B 100 nM Protein Y												
C 30 nM Protein Y												
D 10 nM Protein Y												
E 3 nM Protein Y												
F 1 nM Protein Y												
G 0.3 nM Protein Y												
H 0 nM Protein Y												

Notes :

- If your protein-protein interaction is very strong (Kd in pM range), you may want to shift the range of tested concentrations lower.
- If you are using weak affinity beads and your protein-protein interaction is weak (Kd > 100 nM), you may want to include higher concentrations of protein on this plate. For example, when working with Ni-NTA beads or GSH beads, the His-tagged or GST-tagged protein should be titrated up to 1000 nM.
- If one of your proteins can bind more than one bead (for example, a protein that has two or more biotins can potentially bridge two streptavidin-coated Donor beads), you should add that particular associating bead (for example, the streptavidin Donor beads) last to allow other interactions to occur first. Refer to “Order of addition”, Section 3.

In the protocol for the data below, a 3-step assay was performed (biotinylated EGF and EGFR-Fc added first, followed by a 60 minute incubation, Protein A AlphaLISA Acceptor beads added second, followed by a 60 minute incubation, and streptavidin Donor beads added last, with a final 30 minute incubation before reading the plate). The streptavidin Donor beads were added last (after the second incubation) to prevent the multi-biotinylated EGF from bridging two streptavidin Donor beads before other interactions could occur. The data are presented two different ways: as a 3-D bar graph generated using Microsoft Excel, and as a titration curve plotted using GraphPad Prism (Figure 3). A hook point is reached at ~10 nM biotinylated EGF, and ~3 nM EGFR-Fc, after which point the signal begins to decrease.

Sample data:

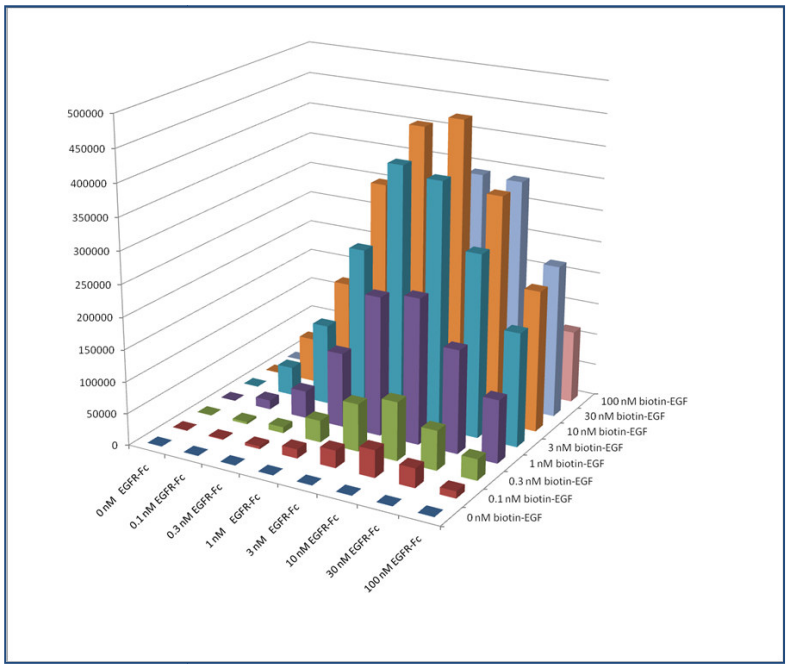

Figure 2. Cross-titration data for EGFR-Fc binding to biotinylated EGF, using streptavidin Donor beads and Protein A AlphaLISA Acceptor beads. The expected Kd for this interaction is ~2.8 nM [Lax, I. *et al.*, 1988).

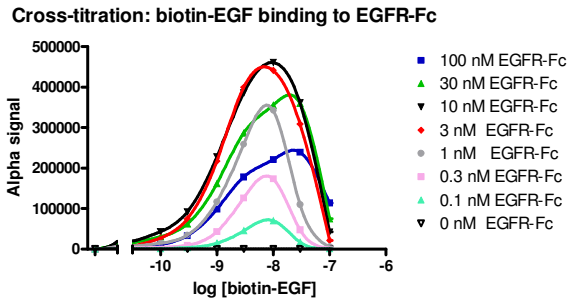

Figure 3. Titration curve in GraphPad Prism of the same data presented in Figure 2.

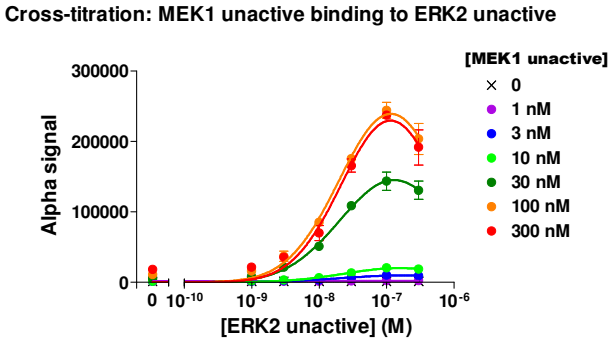

Figure 4. A second example of a protein-protein cross-titration experiment. This interaction is between His-tagged MEK1 and GST-tagged ERK2, with capture and detection using Glutathione Donor beads and Nickel chelate AlphaLISA Acceptor beads. The expected Kd for this interaction is ~ 29 µM [Bardwell, A.J. *et al*., 2001]. A hook point is reached at ~ 100 nM GST-ERK2, and between 100 nM and 300 nM His-MEK1.

2. Cross-titration when you are using antibodies to capture proteins to beads

If you will be using antibodies to capture your proteins to the Donor and/or Acceptor beads, you will also need to choose a concentration of antibody to use in the assay. For most assays, a final antibody concentration of 1 nM, 3 nM, or 10 nM will usually give good results. Depending on the affinity of your antibody for the protein, you may be able to titrate the protein concentration higher than described above (if the antibody has weak affinity for the protein).

Figure 5. Suggested 96-well plate map for cross-titration experiments that involve the use of an antibody to capture Protein X to beads

	1	2	3	4	5	6	7	8	9	10	11	12
	1 µM Protein X	**100 nM Protein X**	**10 nM Protein X**	**1 nM Protein X**	**0.1 nM Protein X**	**0 nM Protein X**	**1 µM Protein X**	**100 nM Protein X**	**10 nM Protein X**	**1 nM Protein X**	**0.1 nM Protein X**	**0 nM Protein X**
A 300 nM Protein Y												
B 100 nM Protein Y												
C 30 nM Protein Y												
D 10 nM Protein Y			(10 nM antibody)						(1 nM antibody)			
E 3 nM Protein Y												
F 1 nM Protein Y												
G 0.3 nM Protein Y												
H 0 nM Protein Y												

3. Order of addition

Order of addition can influence the signal generated to a large extent. The optimal order in which assay components interact should always be determined empirically. Some binding partners may interfere with the association of other binding partners if allowed to interact in the wrong order.

The protocol presented above is a two-step protocol: the two proteins are added first and incubated, and then the two beads are added in a second addition before the final incubation. It is possible to take the protocol above and split this into three steps (adding the beads one at a time). It is also possible to perform a one-step assay, where all of the assay components (proteins, beads) are added to the well at the same time. The effect of these protocol changes will need to be determined experimentally for each assay.

As a note, for proteins or antibodies that are biotinylated at multiple positions, it is generally recommended to add the streptavidin-coated bead last. This is because a protein that is biotinylated at multiple positions could bridge two streptavidin-coated Donor beads before any other interaction can occur, as the streptavidin-biotin interaction is of extremely higher affinity in comparison to other interactions. This would likely make the biotinylated protein unavailable for any further interaction, leading to a false negative result.

Additionally, pre-incubating each protein with its associating bead is usually not recommended, as the bead will slow down the rotation of the protein in solution. This could slow down the kinetics of the protein-protein interaction, requiring longer incubation times. Also, the bead may create steric hindrance, preventing the protein from binding to its partner protein at all. Pre-incubation of a bead with an antibody that will eventually be associating with one of the proteins is sometimes performed.

Examples of different order-of-addition protocols:

Add Protein X and Protein Y ⇩ Incubate ⇩ Add Donor and Acceptor beads ⇩ Incubate ⇩ Read plate	Add Protein X and Protein Y ⇩ Incubate ⇩ Add Acceptor beads ⇩ Incubate ⇩ Add Donor beads ⇩ Incubate ⇩ Read plate	Add Protein X, Protein Y, Donor beads, and Acceptor beads ⇩ Incubate ⇩ Read plate

4. Displacement assay (assay validation)

One way of validating the assay is to demonstrate that the association between Protein X and Protein Y can be disrupted by adding an untagged version of one or the other protein. Increasing concentrations of the untagged version of Protein X or Protein Y can displace the tagged reagent from the assay, disrupting the association between the beads. The signal should decrease with increasing concentrations of the untagged "competitor".

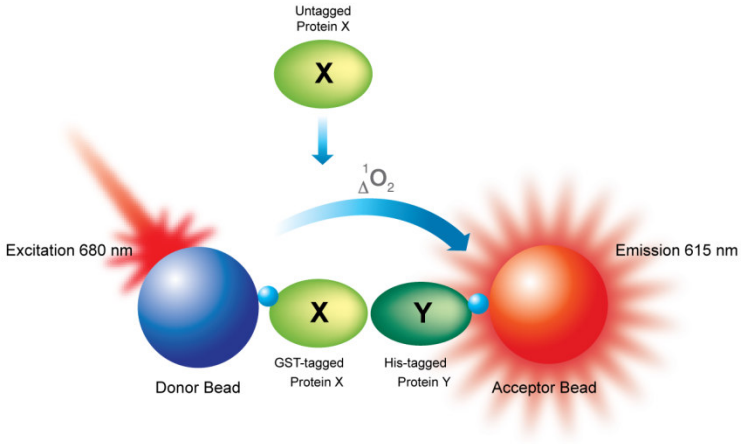

Figure 6. Assay principle for displacement assay. Untagged Protein X competes with GST-tagged Protein X for binding to Protein Y. Because untagged Protein X cannot bind to the Donor bead, a signal cannot be generated from an untagged Protein X/His-tagged Protein Y complex. As a result, signal decreases with increasing concentrations of untagged Protein X.

A. Preparation of reagents

1. Prepare a 5X working solution of tagged Protein X in your assay buffer. Choose a concentration based on data from your cross-titration experiment.
2. Prepare a 5X working solution of tagged Protein Y in your assay buffer. Choose a concentration based on data from your cross-titration experiment.
3. Prepare a 5X working solution of untagged Protein X in your assay buffer (suggested concentration of 5X solution: 100 µM). If you happen to know the expected Kd for your protein-protein interaction, choose a concentration that is above the Kd value if possible.

4. Perform a serial dilution of the untagged Protein X in Eppendorf tubes as follows:

Tube	[Final] (M)	[Intermediate] (M) (5X)	Vol of dilution	Assay Buffer
1	1×10^{-4}	5×10^{-4}	(500 µM untagged Protein X)	
2	3×10^{-5}	1.5×10^{-4}	66 µL of Tube 1	154 µL
3	1×10^{-5}	5×10^{-5}	66 µL of Tube 2	132 µL
4	3×10^{-6}	1.5×10^{-5}	66 µL of Tube 3	154 µL
5	1×10^{-6}	5×10^{-6}	66 µL of Tube 4	132 µL
6	3×10^{-7}	1.5×10^{-6}	66 µL of Tube 5	154 µL
7	1×10^{-7}	5×10^{-7}	66 µL of Tube 6	132 µL
8	3×10^{-8}	1.5×10^{-7}	66 µL of Tube 7	154 µL
9	1×10^{-8}	5×10^{-8}	66 µL of Tube 8	132 µL
10	3×10^{-9}	1.5×10^{-8}	66 µL of Tube 9	154 µL
11	1×10^{-9}	5×10^{-9}	66 µL of Tube 10	132 µL
12	3×10^{-10}	1.5×10^{-9}	66 µL of Tube 11	154 µL
13	0	0	300 µL of buffer	

*It may not be feasible to prepare a 500 µM solution of your untagged protein. Start at the highest concentration possible

* If the Kd for your protein-protein interaction is know, you will want to center the final concentrations of untagged protein around the Kd if possible. Ideally, you want to cover a 3-4 log range.

5. (During 1st incubation): Prepare 5x working solution (100 µg/mL) of Acceptor beads:

   5 µL Acceptor beads (5 mg/mL) + 245 µL assay buffer

6. (During 1st incubation): Prepare a 5X working solution (100 µg/mL) of Donor beads, keeping protected from light:

   5 µL Donor beads (5 mg/mL) + 245 µL assay buffer

B. Displacement protocol for a 96-well ½ AreaPlate (total assay volume of 50 µL)

Refer to the plate map on the next page. You can use a multi-channel repeat pipettor to quickly dispense reagents into the plate.

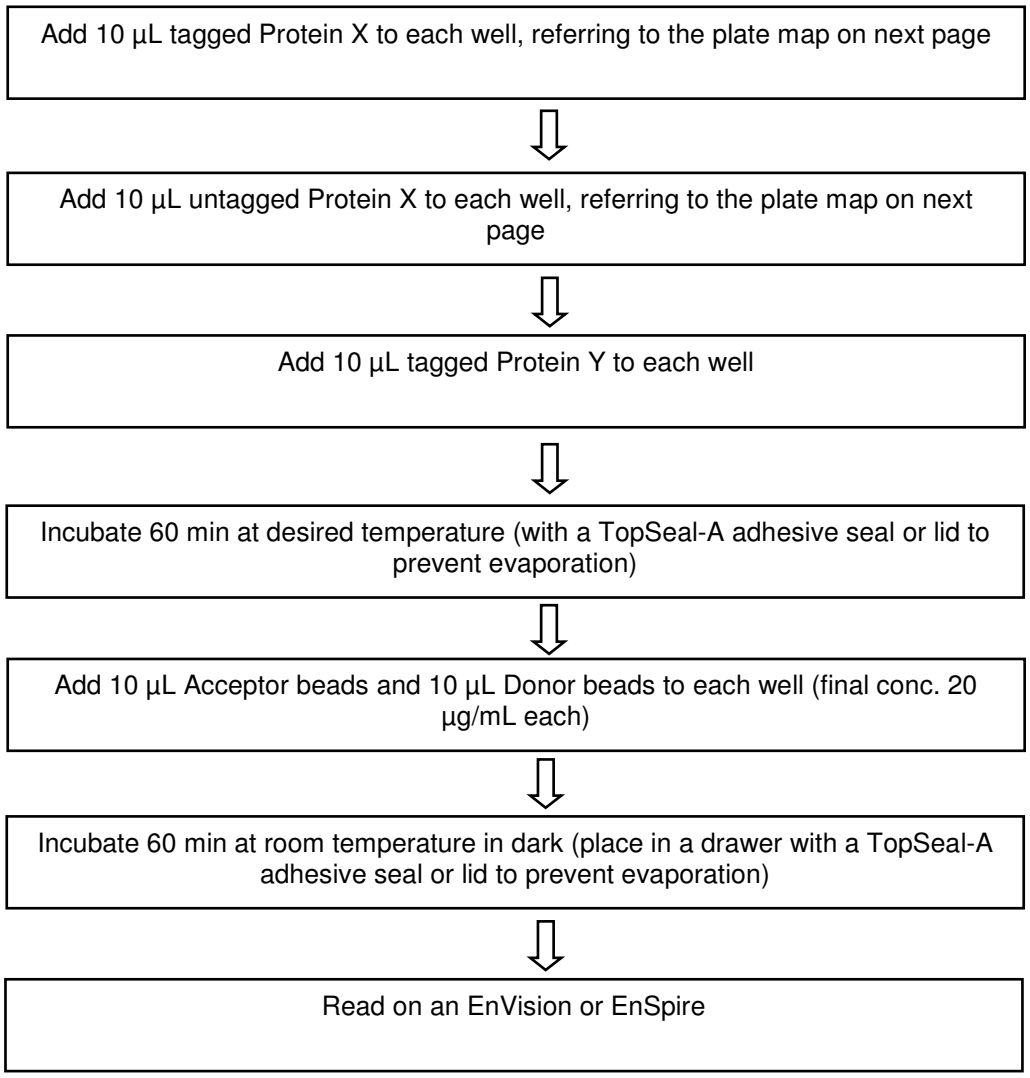

Figure 7. 96-well plate map for displacement experiment

- Samples are set up in triplicate – note that wells D1-F1 are only used to provide more wells so that 0 nM untagged Protein X can be measured. These particular wells should not have 100 µM Protein X.

	1	2	3	4	5	6	7	8	9	10	11	12
	100 µM untagged Protein X	**30 µM untagged Protein X**	**10 µM untagged Protein X**	**3 µM untagged Protein X**	**1 µM untagged Protein X**	**300 nM untagged Protein X**	**100 nM untagged Protein X**	**30 nM untagged Protein X**	**10 nM untagged Protein X**	**3 nM untagged Protein X**	**1 nM untagged Protein X**	**0.3 nM untagged Protein X**
A												
B												
C												
D	(0 nM untagged Protein X)											
E												
F												
G												
H												

Example data:

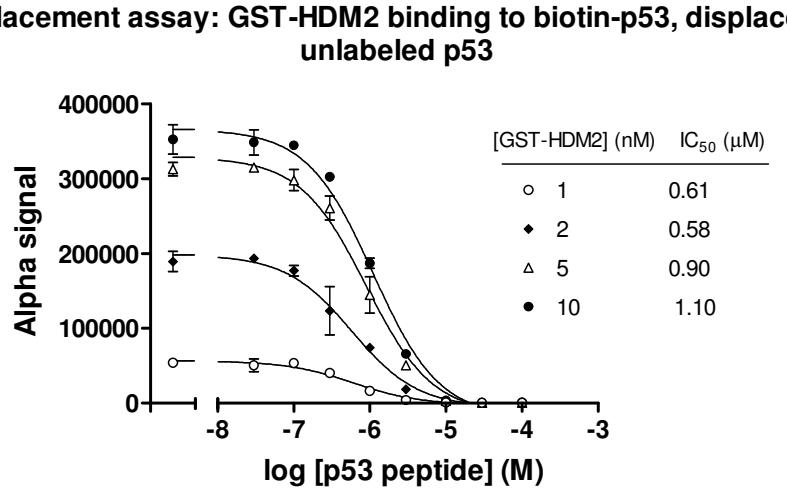

Figure 8. Displacement assay for biotinylated p53/GST-HDM2 interaction, using streptavidin Donor beads and anti-GST AlphaScreen Acceptor beads. Untagged p53 peptide was titrated from 30 nM to 100 µM. Increasing concentrations of untagged p53 competed with biotinylated p53 for binding to GST-HDM2, resulting in a decrease in signal (untagged p53 cannot associate with the streptavidin Donor bead, and therefore cannot generate signal).

References

1. Bardwell, A.J., Flatauer, L.J., Matsukuma, K., Thorner, J. & Bardwell, L. A conserved docking site in MEKs mediates high-affinity binding to MAP kinases and cooperates with a scaffold protein to enhance signal transmission. *J. Biol. Chem* **276**, 10374-10386 (2001).

2. Lax, I. *et al*. Chicken epidermal growth factor (EGF) receptor: cDNA cloning, expression in mouse cells, and differential binding of EGF and transforming growth factor alpha. *Mol. Cell. Biol* **8**, 1970-1978 (1988).