User's guide: Alpha protein:protein interaction assays

Table of contents

1. Introduction

AlphaScreen® and AlphaLISA® are bead-based assay technologies used to study biomolecular interactions in a microplate format. The acronym "Alpha" stands for Amplified Luminescent Proximity Homogeneous Assay. The assay does not require any washing steps. Binding of proteins or other binding partners captured on the beads leads to an energy transfer from one bead to the other, ultimately producing a luminescent signal. Alpha assays require two bead types: Donor beads and Acceptor beads. Each bead type contains a different proprietary mixture of chemicals, which are key elements of the Alpha technology. Donor beads contain a photosensitizer, phthalocyanine, which converts ambient oxygen to an excited and reactive form of O_2, singlet oxygen, upon illumination at 680 nm. Within its 4 µsec half-life, singlet oxygen can diffuse approximately 200 nm in solution. If an Acceptor bead is within that distance, energy is transferred from the singlet oxygen to thioxene derivatives within the Acceptor bead, resulting in light production at 520-620 nm (AlphaScreen) or at 615 nm (AlphaLISA). If the Donor bead is not in proximity of an Acceptor bead, the singlet oxygen falls to ground state and no signal is produced (Figure 1).

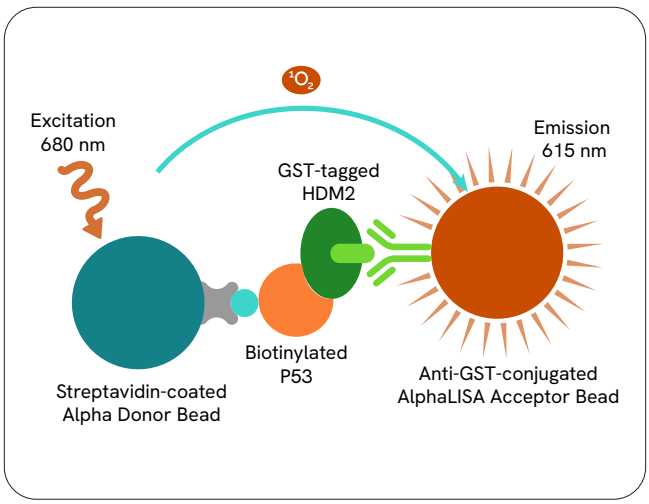


Figure 1: Illustration of an Alpha protein:protein interaction assay, using streptavidin-coated Donor beads, AlphaLISA anti-GST coated Acceptor beads, biotinylated p53, and GST-tagged HDM2.

In an Alpha protein:protein interaction assay, one protein is captured on the Donor beads, and the other protein is captured on the Acceptor beads. When the two proteins interact, the Donor bead is brought into proximity of the Acceptor bead, and excitation of the Donor bead will result in signal generation dependent on the presence of an interaction.

Here are a few advantages that the Alpha technology offers over other technologies in the study of protein:protein interactions:

1. **Distance:** because the singlet oxygen can travel up to 200 nm or more in solution, the distance between the Donor and Acceptor beads can be very large and still generate signal. This allows the measurement of very large proteins and other large complexes. In contrast, traditional FRET (fluorescence resonance energy transfer) requires that the two FRET fluorophores be within ~9 nm of each other for efficient energy transfer. Moreover, in FRET, the energy transfer occurs through nonradiative dipole-dipole coupling, meaning that orientation is also an important parameter. In Alpha assays, the energy transfer between the two beads is chemically-mediated via singlet oxygen.
2. **Broad range of affinities:** Alpha can detect a broad range of affinities with dissociation constants (K_d) ranging from picomolar to low millimolar. Because the assay is homogeneous (no wash steps are required), transient interactions can be measured.
3. **Variety of interactions:** Alpha has been used to study a wide variety of interactions, including protein:protein, protein:peptide, protein:DNA, protein:RNA, protein:carbohydrate, protein:small molecule, receptor:ligand, and nuclear receptor:ligand interactions. Both cell-based and biochemical interactions have been monitored, and applications such as phage display, ELISA, and EMSA (electrophoretic mobility shift assay) have been adapted to Alpha.

4. **Avidity:** because each bead has multiple binding sites, low affinity interactions can be detected using nanomolar concentrations of proteins or other binding partners. This allows you to minimize the amount of proteins required to perform the biomolecular interaction assay.
5. **Ease-of-use:** the assay is homogeneous, meaning that no wash steps are necessary. Many types of pre-coated beads are available to capture biotinylated, FITC-labeled, DIG-labeled, GST-tagged, 6X His-tagged, and other tagged binding partners. Protein A, Protein G, Protein L and anti-species beads are available to capture protein to a bead using an antibody. Unconjugated beads are available for direct conjugation of an antibody or other reagent of choice to a bead. Custom bead conjugation and custom assay development services are also available.

2. Assay design

Please consider the following factors when measuring protein:protein interactions with an Alpha assay.

2.1 Bead choice

2.1.1 AlphaLISA vs. AlphaScreen

Both AlphaLISA and AlphaScreen technologies rely on the use of the Alpha Donor bead. However, this Donor bead can be paired with either an AlphaLISA Acceptor bead or an AlphaScreen Acceptor bead. The main difference between these two Acceptor beads is the final fluorophore used to generate signal. AlphaScreen Acceptor beads use rubrene as the final fluorophore, emitting light between 520 and 620 nm. AlphaLISA Acceptor beads use a Europium chelate as the final fluorophore, emitting light in a narrower peak at 615 nm (Figure 2). This makes the AlphaLISA Acceptor bead less prone to interference from buffer components or other chemicals that absorb light between 520 and 600 nm, though this typically isn't a concern for protein:protein interaction assays (whether biochemical or cell-based).

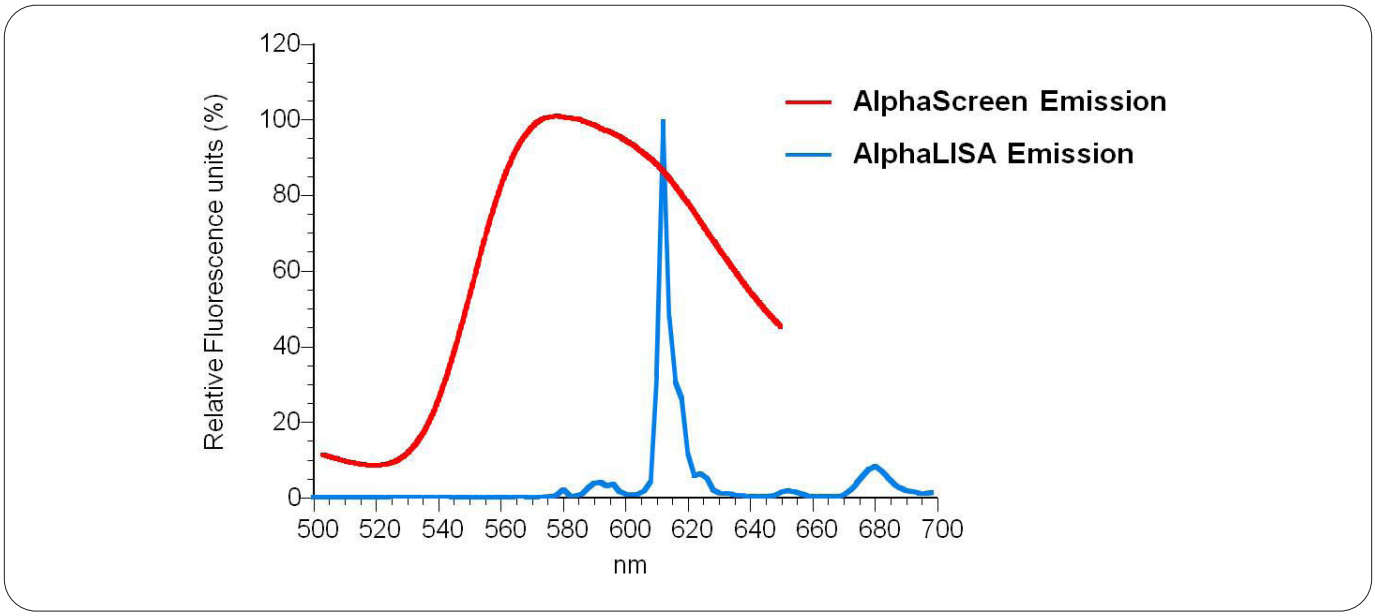


Figure 2: Emission spectra of AlphaScreen and AlphaLISA Acceptor beads.

The decision of whether to use an AlphaLISA bead or AlphaScreen bead will likely depend most on convenience and type of bead coating offered.

- **AlphaLISA beads** are offered as individual toolbox reagents (Donor beads are offered separately from Acceptor beads), allowing the ability to mix-and-match bead options.
- **AlphaScreen beads** are typically packaged as kits, pairing streptavidin Donor beads with AlphaScreen Acceptor beads that bind to a particular tag or label, as indicated in the product name. The AlphaScreen kits also include an appropriate biotinylated "probe" (such as biotinylated 6X His, biotinylated GST tag, etc.) that can be used as a positive control to check that the beads are working correctly.

2.1.2 Bead coating options

A variety of standard Alpha Donor and Acceptor beads are offered as regular catalog items (Table 1). The type of bead coating selected will most likely depend on whether the proteins being studied are available biotinylated, tagged (expressed as GST fusion proteins, 6X His-tagged proteins, etc.) or otherwise labeled (FITC/fluorescein labeled, digoxigenin labeled). If the protein is unlabeled, you could use an antibody directed against the protein to capture the protein onto a bead. It is also possible to directly conjugate a protein, antibody, DNA oligo, lectin, etc. to an uncoated bead. Uncoated beads are coated with a dextran-aldehyde. These aldehyde groups can be easily covalently attached to NH_2 groups in any protein or peptide through reductive amination. The reaction is simple and detailed protocols are available. Other chemistries can also be used.

If you have some flexibility regarding the tagged or labeled state of your proteins, you may want to consider bead capacity and sample type when choosing a bead.

Bead capacity

In a protein:protein interaction, the amount of protein:protein complex formed is dependent on the concentrations of each binding partner within the well. Increasing the concentration of either protein will push the equilibrium to more protein:protein complex.

$$A + B \leftrightarrow AB$$

$$K_d = \frac{[A]\,[B]}{[AB]}$$

Each type of Alpha bead has a characteristic bead capacity (Table 2). This is the point at which the beads are saturated with associating protein, and when additional protein will not be able to associate with the bead. If you have saturated a bead, you may see a "hooking effect". Below the hook point, both Donor and Acceptor beads become progressively saturated by the target molecule, and the signal increases with increasing target concentration. At the hook point, either the Donor or the Acceptor component is saturated with the target molecule and a maximum signal is detected. Above the hook point, an excess of target molecules oversaturates the Donor or the Acceptor beads, which inhibits their association and causes a progressive signal decrease (Figures 3 and 4). The hook effect is a common phenomenon found when using any sandwich-type assay (for example, ELISA assays).

Table 1: Alpha bead products and catalog numbers

Bead coating	Toolbox Alpha Donor beads	Toolbox AlphaScreen Acceptor beads	Toolbox AlphaLISA Acceptor beads	AlphaScreen bead kits (contain streptavidin Donor beads, AlphaScreen Acceptor beads, and a biotinylated probe)
Streptavidin	6760002		AL125	
Strep-Tactin®	AS106		AL136	
Anti-GST			AL110	6760603 (Streptavidin Donor + Anti-GST AlphaScreen Acceptor)
Glutathione	6765300		AL109	
Ni chelate	AS101		AL108	6760619 (Streptavidin Donor + Ni chelate AlphaScreen Acceptor)
Anti-His			AL128	
Anti-c-myc			AL111	6760611 (Streptavidin Donor + anti-c-myc AlphaScreen Acceptor)
Anti-FLAG	AS103		AL112	6760613 (Streptavidin Donor + anti-FLAG AlphaScreen Acceptor)
Anti-DIG			AL113	6760604 (Streptavidin Donor + anti-DIG AlphaScreen Acceptor)
Anti-HA			AL170	6760612 (Streptavidin Donor + anti-HA AlphaScreen Acceptor)
Anti-FITC			AL127	6760605 (Streptavidin Donor + anti-FITC AlphaScreen Acceptor)
Anti-V5			AL129	
Anti-GFP			AL133	
Anti-Maltose Binding Protein (MBP)			AL134	
Protein A	AS102	6760137	AL101	6760617 (Streptavidin Donor + Protein A Acceptor)
Protein G			AL102	
Protein L			AL126	
Anti-rabbit IgG	AS105*		AL104*	6760607 (Streptavidin Donor + anti-rabbit IgG AlphaScreen Acceptor)
Anti-mouse IgG	AS104*		AL105*	6760606 (Streptavidin Donor + anti-mouse IgG AlphaScreen Acceptor)
Anti-mouse IgG			AL164	
Anti-human IgG			AL103*	
Anti-rat IgG			AL106*	
Anti-goat IgG			AL107*	
Anti-sheep IgG			AL132*	
Anti-mouse IgE			AL161	
Anti-mouse IgA			AL163	
Anti-mouse IgM			AL130	
Anti-chicken IgY			AL131	
Unconjugated	6762013	6762003	6772001	
Anti-human IgG1			AL153	
Anti-human IgG2			AL154	
Anti-human IgG3			AL155	
Anti-human IgG4			AL156	
Anti-mouse IgG1			AL157	
Anti-mouse IgG2a			AL158	
Anti-mouse IgG2b			AL159	
Anti-mouse IgG3			AL160	
Anti-mouse IgE			AL161	
Anti-mouse IgM			AL162	
Anti-mouse IgA			AL163	

*Fc-specific antibody

Table 2: Relative capacities of various beads (beads used at 20 µg/mL final concentration in assay).

Bead coating	Used to bind/capture	Theoretical binding capacity[1] (provided for relative comparison)	Molecule and partner bead used to determine theoretical binding capacity
Streptavidin	Biotinylated peptides, proteins, oligos, sugars, small molecules, etc.	30 nM	Biotinylated peptide
Strep-Tactin®	*Strep*-tag® II, One-STrEP-tag, or biotin-tagged targets	100-300 nM	*Strep*-tag® IL-6X His
Anti-GST antibody	GST-fusion proteins and peptides	3 nM	Biotinylated GST with streptavidin bead
Anti-6X His antibody	His-tagged proteins and peptides	100 nM	6X His-GST with glutathione beads
Anti-FLAG antibody	FLAG-tagged proteins and peptides	100 nM	Biotinylated FLAG with streptavidin bead
Anti-maltose binding protein (MBP) antibody	MBP-tagged proteins	1 nM	Biotinylated MBP with streptavidin beads
Anti-HA antibody	Hemagluttinin-tagged proteins and peptides	10 nM	Biotinylated-PEG-HA with streptavidin bead
Anti-c-myc antibody	C-myc-tagged proteins and peptides	100 nM	Biotinylated c-myc with streptavidin bead
Anti-DIG antibody	Digoxigenin labeled proteins, peptides, oligos, etc.	1 nM	Biotinylated digoxigenin with streptavidin bead
Anti-FITC antibody	FITC or fluorescein-labeled proteins, peptides, oligos, sugars, small molecules, etc.	>1 nM	Biotinylated-ERE-FITC with streptavidin bead
Anti-V5 antibody	V5-tagged proteins/targets	3 nM	Biotin-Chromalink V5 (14 aa) with streptavidin beads
Anti-GFP antibody	GFP-tagged (green fluorescent protein-tagged) proteins and peptides	3 nM	Biotinylated GFP with streptavidin beads
Glutathione (GSH)	GST-fusion proteins and peptides	300 nM -1 µM	6X His-tagged GST with nickel chelate bead
Nickel chelate (Ni^{2+})	His-tagged proteins and peptides	300 nM -1 µM	6X His-tagged GST with glutathione bead
Protein A	Antibodies[2]	3 nM (antibody[3])	Biotinylated rabbit IgG with streptavidin bead
Protein G	Antibodies[4]	1 nM (antibody[3])	Biotinylated rabbit IgG with streptavidin bead
Protein L	Antibodies[5]	1 nM (antibody[3])	Biotinylated human IgG (kappa) with streptavidin beads
Anti-human IgG	Fc portion of human IgG antibodies	3 nM (antibody[3])	Biotinylated human IgG with streptavidin bead
Anti-rabbit IgG	Fc portion of rabbit IgG antibodies	1 nM (antibody[3])	Biotinylated rabbit IgG with streptavidin bead
Anti-mouse IgG	Fc portion of mouse IgG antibodies	3 nM (antibody[3])	Biotinylated mouse IgG with streptavidin bead
Anti-mouse IgM	Mouse IgM immunoglobulins	0.3 nM (antibody[3])	Biotin-mouse IgM with streptavidin beads
Anti-rat IgG	Fc portion of rat IgG antibodies	1 nM (antibody[3])	Biotinylated rat IgG with streptavidin bead
Anti-goat IgG	Fc portion of goat IgG antibodies	3 nM (antibody[3])	Biotinylated goat IgG with streptavidin bead
Anti-sheep IgG	Fc portion of sheep IgG antibodies	1 nM (antibody[3])	Biotinylated sheep IgG with streptavidin beads
Anti-chicken IgY	Fc fragment of chicken IgY immunoglobulins	0.3 nM (antibody[3])	Biotinylated chicken IgY with streptavidin beads

[1] The numbers provided are examples given to compare relative bead capacities, and are derived from probe titration experiments, as shown in the QC data on the product tech data sheets (**www.revvity.com**). The actual bead capacity for your bead system should be determined empirically for a given assay. The bead capacities given in this table are influenced by partner bead and the size of the probe used in these assays. The actual bead capacity will be influenced by size of the protein associating with the bead, as well as affinity of the protein (or antibody) for the bead.

[2] Protein A interacts strongly with particular subclasses of antibodies, including human IgG1, IgG2, and IgG4; mouse IgG2A and IgG2B; and rabbit, human and mouse total IgG. Protein A also has weaker affinity for other antibody subclasses.

[3] The bead capacity for these products refers to the capacity for antibody. The amount of protein you will then be able to bind to the antibody on the bead will be dependent on the affinity of your antibody for your protein and the size of your protein, and may range anywhere from 1 pM to 1 µM.

[4] Protein G binds to all subclasses of human IgG and mouse IgG. In addition it binds to rat, goat, sheep, guinea pig, rabbit, cow, pig and horse antibodies.

[5] Protein L binds efficiently total human IgG, IgM, IgA, IgE, IgD, mouse IgG and rat IgG. It binds only poorly mouse IgM and rabbit IgM and it does not bind human IgG (lambda light chain), rabbit, sheep, goat and bovine IgG and rat IgM.

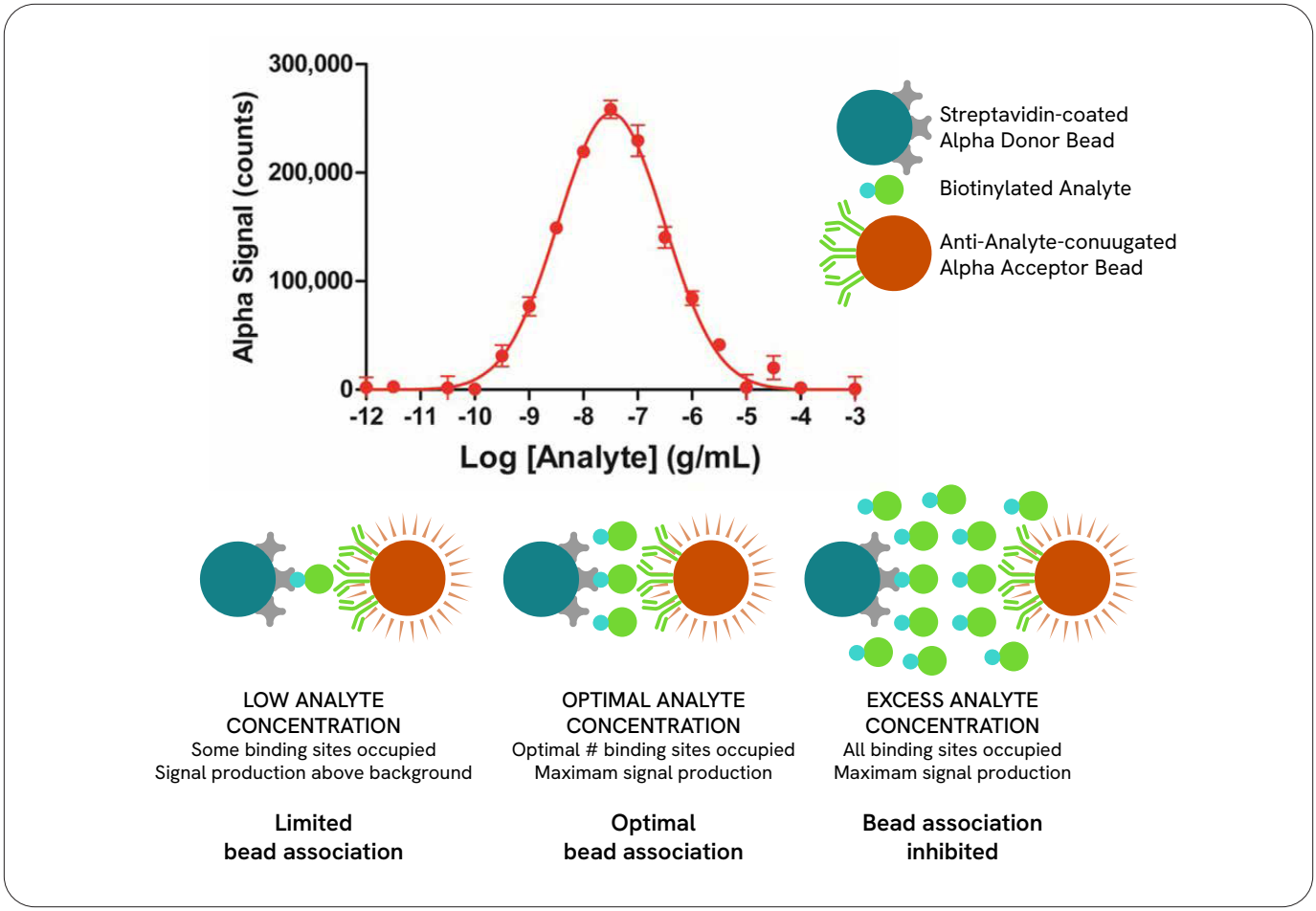


Figure 3: Illustration of the hook effect in a protein detection assay.

Hooking can occur on either the Donor beads or the Acceptor beads. The hooking effect is sometimes masked in Alpha protein:protein interaction assays, resulting in what can look like a traditional saturation curve that reaches a plateau, rather than hooking (Figure 5). In this case, two competing equilibria are occurring: the signal may be decreasing because of the hooking effect on the bead, but the protein:protein interaction may still be increasing because the equilibrium of the protein:protein interaction is being driven to more protein:protein complex with higher concentrations of protein. You will want to choose protein concentrations below the hook point for your assay. In any case, it is useful to have a rough idea of the binding capacity of the various beads, particularly if you are studying a weak (K_d > 100 nM) interaction.

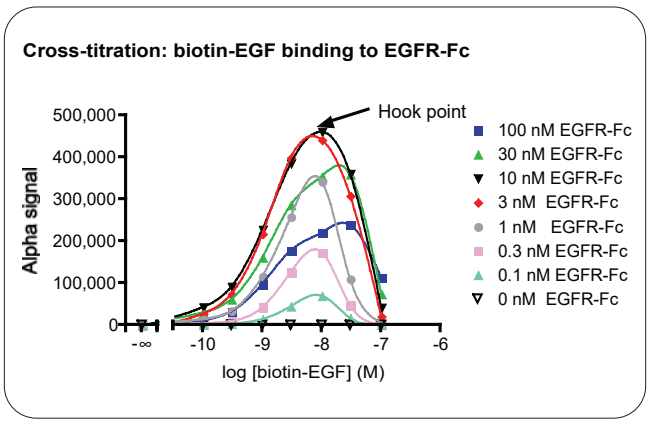


Figure 4: High affinity protein:protein interaction, illustrating the hooking effect. A cross-titration was performed to characterize the interaction between biotinylated- mouse EGF and Fc-fusion human EGFR receptor. Streptavidin Donor beads and AlphaLISA Protein A Acceptor beads were used to capture the proteins and generate the assay signal. The expected K_d for this interaction was ~2.8 nM [Lax, I. et al., 1988]. A hook point is reached at ~10 nM biotinylated EGF, and ~3 nM EGFR-Fc.

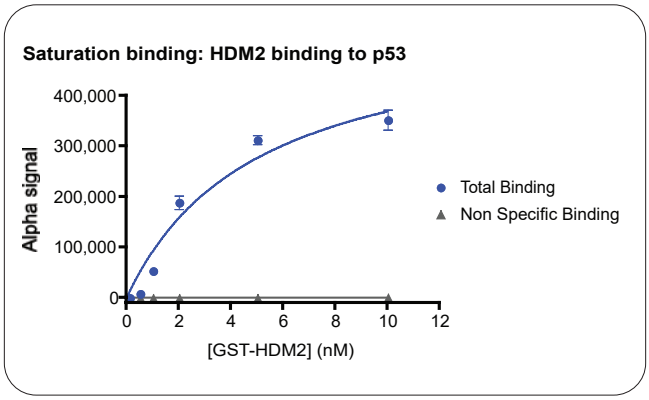


Figure 5: Low affinity protein:protein interaction on low capacity beads, illustrating how the overlapping effects of hooking and increasing protein:protein interaction can resemble a saturation curve. The interaction studied is between biotinylated p53 and GST-tagged hDM2. Streptavidin Donor beads and Anti-GST AlphaScreen Acceptor beads were used to capture the ligands and generate the assay signal. The expected K_d for this interaction was ~ 0.3 µM [Dawson, R. et al., 2003], but deriving a K_d value from the half-maximal signal would give an incorrect value of ~2 nM. See Section 4 for K_d calculation using Alpha assays.

Bead capacities are influenced by:

- Size of the protein associating with the bead: more small peptides will fit on the surface of a bead, compared to large antibodies. For example, a streptavidin-coated bead used at 20 µg/mL usually saturates at around 30 nM biotinylated peptide, but will saturate at around 2-3 nM biotinylated antibody.
- Affinity of the bead for the associating reagent: for a GST-tagged protein, one can choose either anti-GST antibody-coated beads, or glutathione beads. However, the affinity of glutathione for GST is weaker compared to the affinity of anti-GST antibody for GST. This means that one would be able to add higher concentrations of GST-tagged protein to a glutathione bead before saturating/hooking, because for a given concentration of the protein more GST-tagged protein is not associated with the glutathione bead. You may find that the hook point is reached on anti-GST beads at around 20 nM GST-tagged protein, but at around 200 nM GST-tagged protein on glutathione beads. For weak interactions, use of a weaker affinity bead may be desirable (FIgure 6). For strong protein:protein interactions, the protein concentration may not need to be titrated to as high a level, and a stronger signal may be obtained with a higher affinity bead because less protein is dissociated from the bead.

A note about bead capacity: Weak protein:protein interactions (even in the high µM or low mM affinity range) can be studied using Alpha, even though the maximum bead capacities tend to fall in the nM range. The high signal produced by Alpha assays usually means that you can choose nM concentrations of proteins and still get a good signal-to-background for detection of weak interactions. Dissociation constants (K_d) for weak interactions can also be determined by using a competition assay rather than a saturation assay. See Section 4 for information on K_d determinations.

Antibody affinity

If your assay is being designed using an antibody to associate a protein to a bead, you may want to consider the use of weaker affinity antibodies when studying weak interactions (K_d > 100 nM). If you are able to choose between antibodies with weaker or stronger affinities for a given protein, you may find that more target protein can be added before saturating the beads when using a weaker affinity antibody-coated bead. If studying a weaker protein:protein inter-action, it is desirable to use more protein in the assay to push the protein:protein binding equilibrium to more protein:protein complex. By using a lower affinity antibody, more protein can be added to the assay before saturating the bead.

However, if the protein:protein interaction being studied is strong, you will likely generate a higher signal using a higher affinity antibody. In this case, less protein would be dissociated from the antibody at equilibrium.

Sample type

The type of sample may also influence decisions regarding bead selection. For example, glutathione (GSH) beads and nickel chelate (Ni^{2+}) beads may perform well in a biochemical assay but may not work well in a cell-based assay. This is because other components in the cell lysate may have the ability to interact with the GSH and Ni^{2+} beads, causing sample interference. In such cases, it may be preferable to use a bead coated with anti-tag antibody.

Additionally, if you have purified your GST-tagged proteins or His-tagged proteins using an affinity column and will be using a GSH or Ni^{2+} bead in your Alpha assay, you will need to dialyze away any glutathione or imidazole in your purified protein preparation. These components will interfere with the interaction between the tagged protein and the bead.

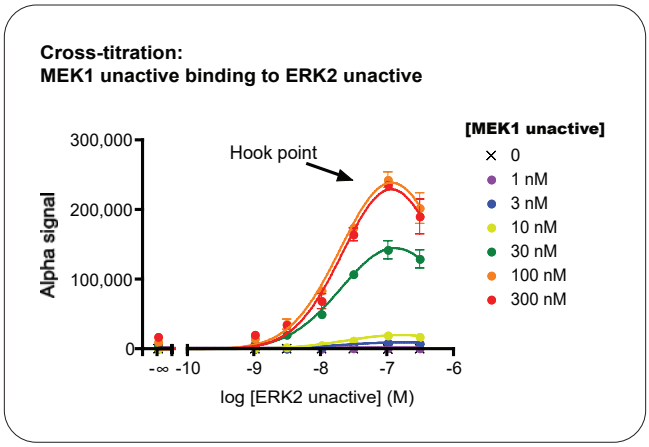


Figure 6: Low affinity interaction on higher capacity beads, illustrating a higher bead capacity as depicted by a higher hooking point. A cross-titration was performed to characterize the interaction between His-tagged MEK1 and GST-tagged ERK2. Glutathione (GSH) Donor beads and nickel chelate AlphaLISA Acceptor beads were used to capture the proteins and generate the assay signal. 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.

2.2 Buffer choice

The information that follows is based on experiments performed using the AlphaScreen TruHits™ beads. This kit contains streptavidin-coated Donor beads and biotinylated AlphaScreen Acceptor beads, which interact in the absence of any binding partners. TruHits beads are commonly used as a control when evaluating potential interference from buffer components or from compounds being tested in the assay. Other bead types may exhibit different sensitivities to various buffer components (for example, a nickel chelate bead is more sensitive to EDTA than a streptavidin bead). Additionally, while some buffer aspects may not affect the Alpha signal itself, they may affect the conformation or interaction of the proteins being studied or the associations of these proteins with the beads.

2.2.1 General concepts

Buffer choice can be very important. Choose pH, buffering capacity and salt concentration that will facilitate the desired interactions between the components of your assay. If metal co-factors are needed for correct conformational integrity of your proteins, it is best to titrate these components appropriately as certain metal ions can interfere with Alpha assays. If excessive non-specific binding is observed, a variety of different detergents may be used such as Tween 20 (0.01–0.1%), Triton X-100 (0.01–0.1%), or CHAPS (0.1% or less). For most Alpha applications, a BSA concentration of 0.1% (w/v) is sufficient to minimize non-specific interactions. Some assays may require slightly higher concentrations of BSA. Try to avoid azide as a preservative as this is a potent scavenger of singlet oxygen and will inhibit the Alpha signal. Proclin (Sigma-Aldrich Cat. No. 48912-U) or Kathon (Sigma-Aldrich Cat. No. 48175-U) is recommended as a preservative and anti-microbial agent.

2.2.2 Buffer components that may interfere

Here are some guidelines for choosing a buffer that will allow for optimal Alpha assay readouts. It is important to note that these guidelines refer to optimal conditions for the Alpha detection reagents themselves, namely the Donor and Acceptor beads, as well as the chemistry that leads to light emission. The proteins you are working with may also have their own restrictions, in terms of assay buffer composition. For more support on optimal assay conditions, please contact our Technical Support group to discuss your specific needs.

Choosing the buffer

Alpha Technology is very tolerant to the types of buffering agents, buffer concentrations and buffer pH used in most assays. The following buffers have been tested in Alpha assays and have been shown to give excellent performance at concentrations from 10 mM to 100 mM:

- Acetate
- Bis-Tris
- Bis-TRIS propane
- CAPS
- Carbonate
- Citrate
- Formate
- HEPES
- MES
- MOPS
- Phosphate
- PIPES
- Tris

Buffer pH

A range of pH (from pH 2.5 to 9) has no influence on the performance of Alpha beads and signal generation. Even pH values up to 10.5 can be accommodated, but a slight loss of performance should be expected. Nevertheless, keep in mind that the assay conditions should also be optimized in terms of their effect on the actual protein:protein interaction being studied.

Buffer salts

Buffers used in biological assays will usually include a variety of salts to generate ionic strength and to satisfy the specific requirements of an assay component such as an enzyme or protein. Alpha assays are highly tolerant of the presence of a wide variety of salts, and can tolerate the following ions up to 300 mM in solution:

- Li^+
- Na^+
- K^+
- Cs^+
- Mg^{2+}
- Ca^{2+}
- NH^{4+}
- F^-
- Cl^-
- Br^-
- I^-
- Borate
- Acetate
- Bicarbonate
- Carbonate
- Phosphate (monobasic and dibasic)
- Sulfate
- Pyrophosphate
- Tartrate

Heavy metals have to be considered carefully. Alpha assays have shown sensitivity to heavy metals in solution, most likely because those ions can react with singlet oxygen to form insoluble oxides. Table 3 describes the effects of commonly used heavy metals on Alpha assay performance:

Table 3: Interference levels in AlphaScreen TruHits™ Assay.

Metal ion	Recommended level	Interference level (IC_{50})
Cobalt (Co^{2+})	0.7 mM or less	3.6 mM
Iron (II) (Fe^{2+})	2 mM or less	0.95 mM
Iron (III) (Fe^{3+})	2 mM or less	9 mM
Manganese (Mn^{2+})	7 mM or less	37 mM
Nickel (Ni^{2+})	0.5 mM or less	2.8 mM
Zinc (Zn^{2+})	0.12 mM or less	2.69 mM

Note: Due to their steep inhibition curves, a concentration of five times (5X) less than the IC_{50} can be considered as having no effect on the assay. The concentrations listed are the final concentrations in the presence of beads. The heavy metal concentration can be higher than the value shown at initial stages of the assay, so long as it is at or below the recommended level after addition of the final assay components.

Detergents and background-reducing proteins

Many assays will require the presence of additives to prevent aggregation, to ensure solubility and stability of components, and to reduce non-specific signal (background). Alpha assays can tolerate the presence of a wide variety of detergents and protein additives, but care must be taken in some cases.

Detergents

The following detergents will have no negative effect on Alpha assays at up to 3% final concentration in solution, unless otherwise indicated:

Brij-35

Dodecane Sulfonate

EMPIGEN

IGEPAL CA630

Guanidinium chloride (up to 100 mM)

Mowiol 4-88

Nonidet P40

NSB195

NSB201

NSB256

Tergiton NP9

Triton X-100

Tween-20

Tween-80

Dodecyltrimethylammonium bromide (DTAB)

Octyl sulfate

Some detergents, when used at high concentration, will have a significant effect on Alpha assays (Table 4). This effect is most likely due to denaturation of proteins present on the surface of the beads. The following table illustrates those detergents and their effects.

Table 4: Interference of detergents in AlphaScreen TruHits Assay.

Detergent	Recommended level†	Interference level (IC_{50})
BHDA*	0.005% or less	0.03%
Benzalkonium chloride	0.005% or less	0.03%
CHAPS	0.02% or less	0.10%
Chenodeoxycholate	0.002% or less	0.02%
Cholate	0.002% or less	0.15%
Decyl sulfate	0.1% or less	0.50%
Deoxycholate	0.003% or less	0.03%
Myristyl sulfobetaine	0.1% or less	0.50%
Pluronic F127	0.1% or less	0.30%
Sodium lauryl sulfate (SDS)	0.02% or less	0.20%

* Benzylhexadecyldimethylammonium chloride

†These recommended concentrations have been obtained by analysis of a dose-response curve using the TruHits kit (Streptavidin Donor beads, Biotinylated AlphaScreen Acceptor beads).

Proteins and polymers

Proteins and other polymers are commonly added to reduce assay background. Alpha assays are tolerant of the following additives at up to 0.5% in solution.

- Bovine serum albumin (BSA), casein, gelatin, heparin, poly-lysine, salmon sperm DNA, Dextran T500 (both from natural and synthetic sources)

However, sources of BSA and casein have been found to vary in quality. These proteins must be of a high purity to avoid the presence of contaminants that could interfere with the assay. One such impurity is biotin, which is often present in casein of low grade. We recommend the following suppliers and catalog numbers for Alpha assays.

- BSA (Sigma Cat. No. A7030)
- Casein 5% solution (Novagen Cat. No. 70955)

Anti-foaming agents

Anti-foaming agents are increasingly used in high-throughput screening assays. These molecules are designed to break the colloidal interactions between molecules of detergents and proteins such as BSA and thereby eliminate the presence of bubbles. Bubble formation can greatly hinder liquid handling and reading. The most common anti-foaming agents are Antifoam A (Cat. No. A5633) and Antifoam 204 (Cat. No. A8311) from Sigma. These agents show no interference with Alpha assays at concentrations up to 0.1%. In one comparison, Antifoam A was shown to be more efficient in reducing bubbles than Antifoam 204.

Cofactors, reducing agents and preservatives

Many assays require the presence of cofactors, reducing agents or preservatives in the assay buffer. Alpha assays are tolerant of a wide variety of these molecules, including the following categories (Table 5).

Table 5: Compatibility with cofactors, reducing agents and preserving agents.

Category	Alpha-compatible additives
Nucleotides, up to 10 mM	All nucleotides derived from adenine, guanine, thymine and uridine, including free nucleotide, mono-, di- and triphosphate and cyclic nucleotides
Reducing agents, up to 10 mM	Dithiothreitol (DTT), 2-mercaptoethanol, tris (2-carboxyethyl) phosphine (TCEP)
Preservatives, up to 0.1%	Kathon™, thimerosal, ProClin® 300

Note: Sodium azide is one of the most popular preservatives used with commercial products. However, it is known as a potent singlet oxygen quencher and thus it will quench Alpha signal strongly (IC_{50} of 0.005%). Sodium azide should be avoided in Alpha assays.

3. Assay development (biochemical protein:protein interaction assays)

The information in this section presents a possible series of experiments to perform to optimize your Alpha protein:protein interaction assay.

Before you begin:

- Alpha assays require a special reader capable of measuring an Alpha assay. Only Revvity multimode detection plate readers have been validated to read Alpha technology. Alpha technology has not been optimized by Revvity for use with other systems.
- We recommend preparing only what you need for the day's experiments. Do not store working dilutions of beads for more than one day.
- 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.

3.1 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 3.2 Cross-titration when you are using antibodies to capture proteins to beads.

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 first 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 first 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 (Figure 7). You can use a multi-channel repeat pipettor to quickly dispense reagents into the plate.

Add 10 µL Protein X to each well, referring to the plate map on next page
(final conc. 0 nM – 300 nM).

↓

Add 10 µL Protein Y to each well, referring to the plate map on next page
(final conc. 0 nM – 300 nM).

↓

First incubation: Incubate 60 min at desired temperature
(with a TopSeal™-A adhesive seal or lid to prevent evaporation).

↓

Add 10 µL Acceptor beads and 10 µL Donor beads to each well
(final conc. 20 µg/mL each).

↓

Second incubation: Incubate 60 min at room temperature in dark
(place in a drawer with a TopSeal-A adhesive seal or lid to prevent evaporation).

↓

Read on an Alpha-compatible reader (EnVision® Multilabel Plate Reader
or EnSpire® Multimode Plate Reader).

- 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												

Figure 7: 96-well plate map for cross-titration experiment

Notes:

- If your protein:protein interaction is very strong (K_d 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 (K_d > 100 nM), you may want to include higher concentrations on this plate.
- 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 3.3.

In the protocol for the following data (Figures 8 and 9), 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 9).

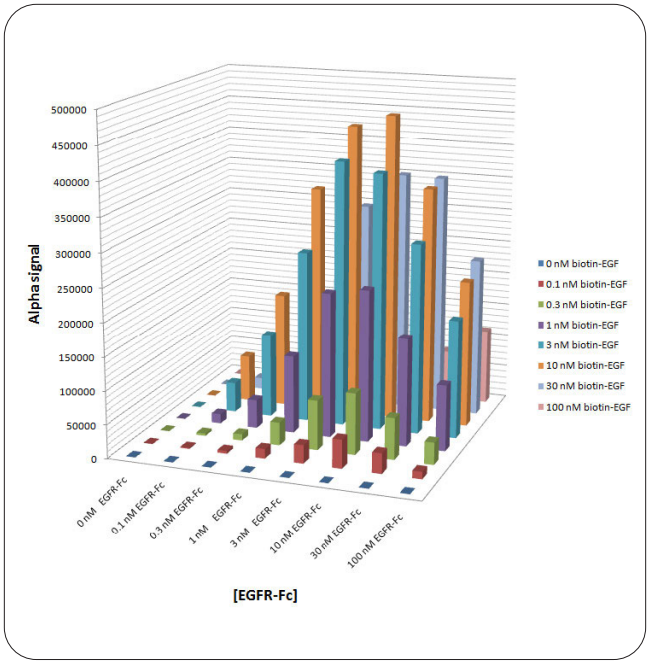


Figure 8: Cross-titration data for EGFR-Fc binding to biotinylated EGF, using streptavidin Donor beads and Protein A AlphaLISA Acceptor beads. The expected K_d for this interaction is ~2.8 nM [Lax, I. et al., 1988).

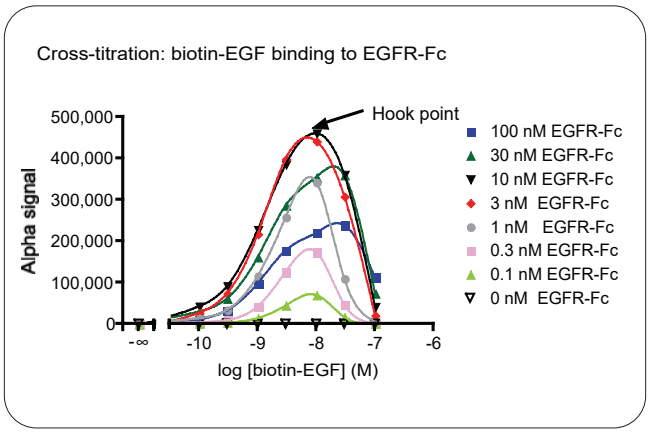


Figure 9: Titration curve in GraphPad Prism of the same data presented in Figure 8.

A hook point is reached at ~10 nM biotinylated EGF, and ~3 nM EGFR-Fc, after which point the signal begins to decrease. Figure 10 shows an example using MEK1 and ERK2. You will most likely want to choose a pair of concentrations that give good S:B while conserving protein. If you will be deriving affinity constants in later experiments, you will need to choose concentrations of proteins that are below the bead capacity – refer to section 4 for more information.

3.2 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. Refer to Figure 11 for a sample plate map. 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).

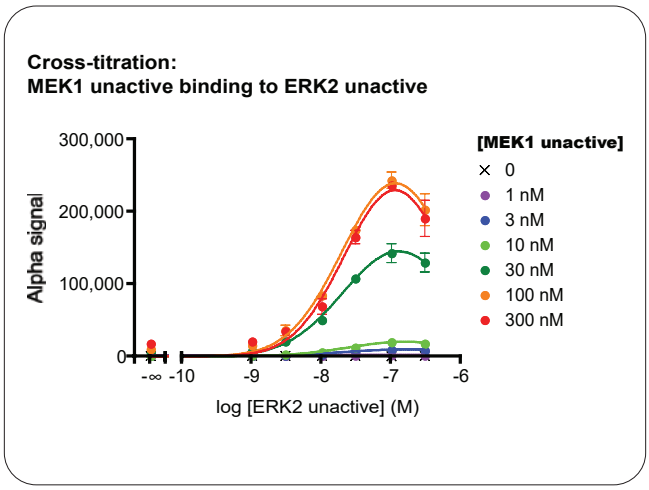


Figure 10: A second example of a protein:protein cross-titration experiment. This interaction is between His-tagged MEK1 and GST-tagged ERK2, with capture and detection using glutathione Donor beads and nickel chelate AlphaLISA Acceptor beads. The expected K_d for this interaction is ~ 29 µM [Bardwell, A.J. et al., 2001]. A hook point is reached at ~ 100 nM GST-ERK2, and between 100 nM and 300 nM His-MEK1.

	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												

Figure 11: Suggested 96-well plate map for cross-titration experiments that involve the use of an antibody to capture Protein X to beads

3.3 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. Keep in mind that some binding partners may interfere with the association of other binding partners if allowed to interact in the wrong order.

The protocol presented in Section 3.1 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 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 (Figure 12). 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 occurs quickly 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.

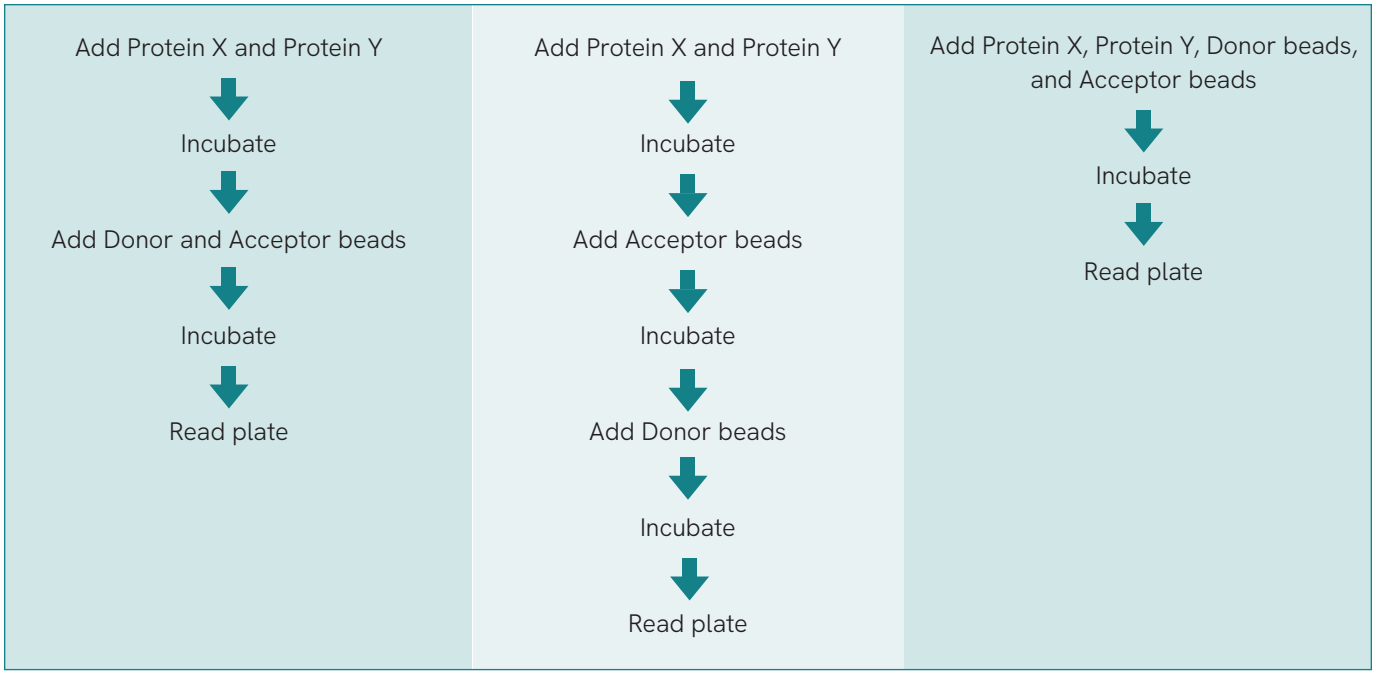


Figure 12: Examples of different order-of-addition protocols

3.4 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" (Figures 13 and 14).

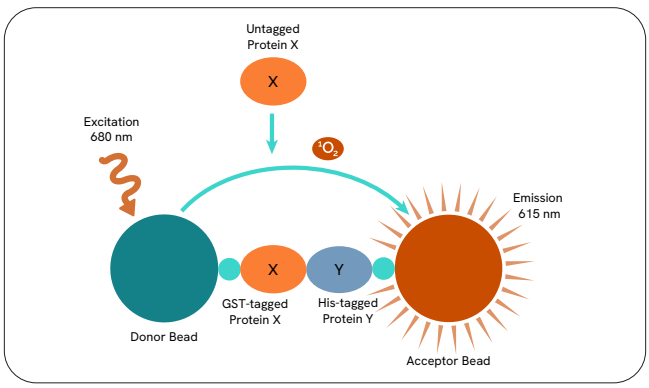


Figure 13: Assay principle for displacement assay. Untagged Protein X competes with GST-tagged Protein X for binding to Protein Y. Because untagged Protein X cannot bind to the Donor bead, a signal cannot be generated from an untagged Protein X/ His-tagged Protein Y complex. As a result, signal decreases with increasing concentrations of untagged Protein X.

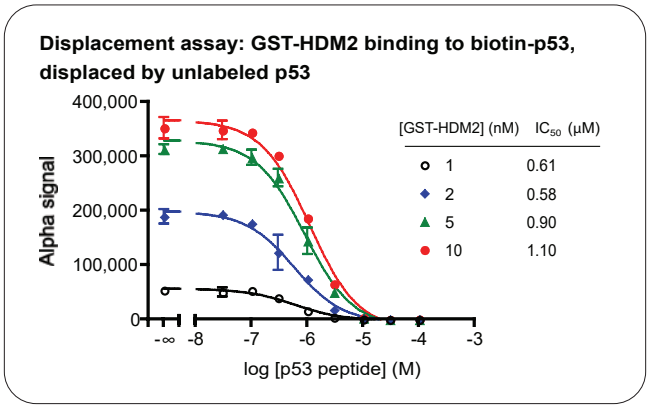


Figure 14: Displacement assay for biotinylated p53/GST-HDM2 interaction, using streptavidin Donor beads and anti-GST AlphaScreen Acceptor beads. Untagged p53 peptide was titrated from 30 nM to 100 µM. Increasing concentrations of untagged p53 competed with biotinylated p53 for binding to GST-HDM2, resulting in a decrease in signal (untagged p53 cannot associate with the streptavidin Donor bead, and therefore cannot generate signal).

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: 500 µM). If you happen to know the expected K_d for your protein:protein interaction, choose a concentration that is above the K_d 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	

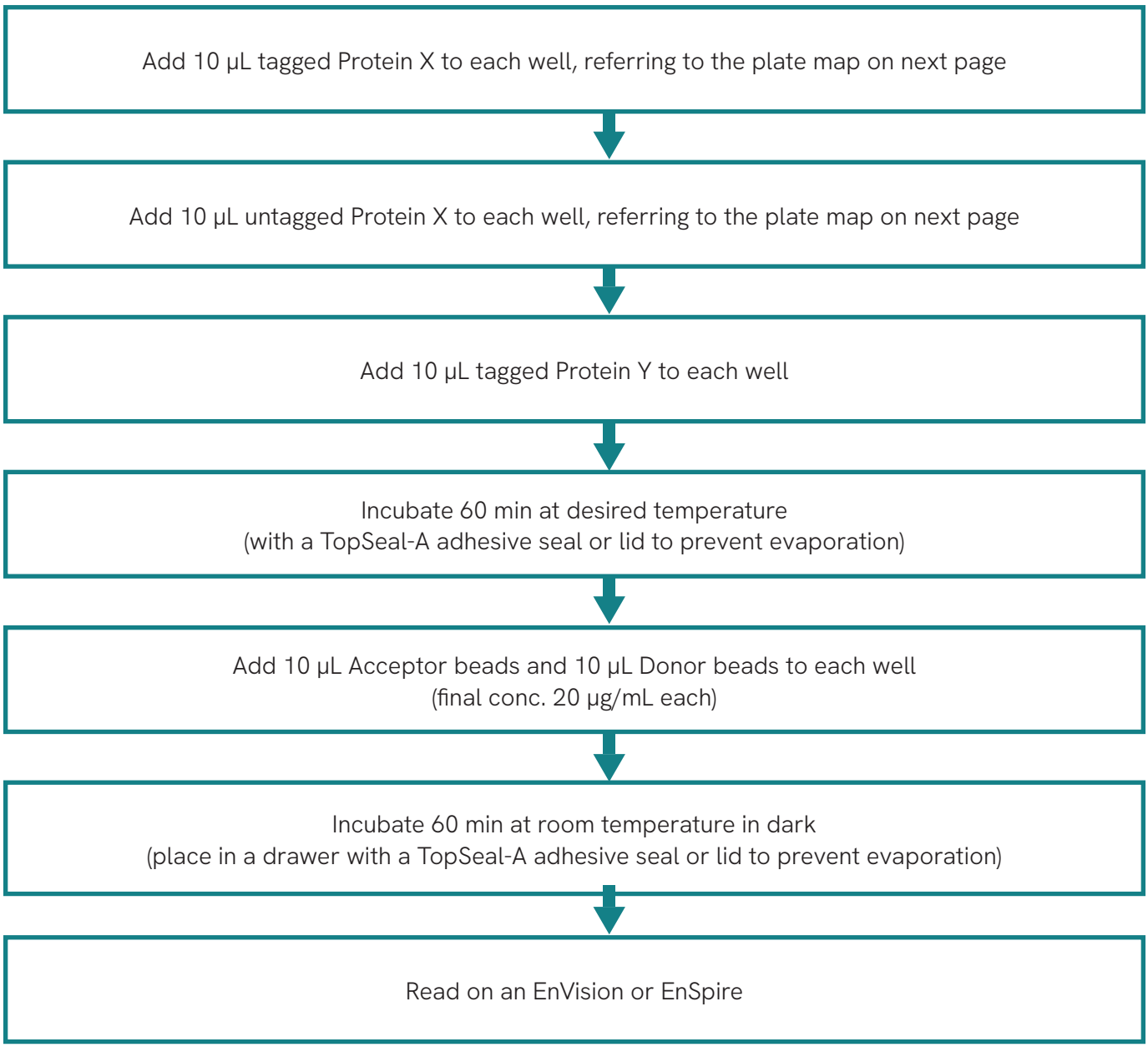
*It may not be feasible to prepare a 500 µM solution of your untagged protein. Start at the highest concentration possible

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

5. (During first incubation): Prepare 5x working solution (100 µg/mL) of Acceptor beads:
   5 µL Acceptor beads (5 mg/mL) + 245 µL assay buffer
6. (During first 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 (Figure 15). You can use a multi-channel repeat pipettor to quickly dispense reagents into the plate.



- 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 untagged 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	1 nM untagged Protein X	3 nM untagged Protein X	0.3 nM untagged Protein X
A												
B												
C												
D	(0 nM untagged Protein X)											
E												
F												
G												
H												

Figure 15: 96-well plate map for displacement experiment

4. Determining K_d in a biochemical assay

The K_d of a protein:protein interaction may be determined with Alpha assays using one of two approaches: saturation curves (section 4.1) or competition assays (section 4.2). Of these, competition assays are applicable to a wider range of assay conditions.

4.1 Saturation curves for determination of K_d

In limited situations, saturation curve assays can be performed to determine the affinity constant (K_d) for the protein:protein interaction in Alpha format. In a saturation assay, one binding protein is titrated across a range of concentrations to generate a saturation curve.

The K_d can be derived from the data as the protein concentration at which half maximal signal is reached. Saturation curves can be used to determine K_d only if the assay meets the following criteria:

1. The K_d for the protein:protein interaction is far enough below the binding capacity of each bead so that all protein concentrations used to derive the K_d (usually up to 5X K_d) fall below the bead binding capacities. (This typically means that the K_d needs to be in the sub-nanomolar range, though some beads have a binding capacity up to ~300 nM – refer to Table 2.)

2. There is an excess of the binding partner that will be varied in comparison to the concentration of the fixed binding partner. For example, if you are varying the concentration of Protein Y in the saturation curve, Protein Y should be in at least 10X excess (molar concentration) compared to Protein X. Otherwise, there is a ligand-depletion (protein:depletion), meaning that the percentage of "bound" protein can be above 10%. In this case, the amount of free ligand (protein) cannot be estimated as being equal to total ligand (protein) for the curve fitting equation.

If the expected K_d is not below the binding capacity of the beads used in the assay, a competition binding assay must be used to determine the K_d for the protein:protein interaction. The reason for this is that the bead system will become saturated when the binding capacity is reached, and two competing equilibria will be occurring: excess protein will be dissociating the interaction between the two beads, decreasing signal (the hooking effect), while the excess protein may simultaneously be driving the interaction between the two proteins, increasing signal. The curve generated will be a composite of these two equilibria, once the binding capacity has been reached. This composite curve may appear to reach saturation, may appear to begin to hook (decrease) slightly, or may continue increasing without reaching saturation (depending on the actual K_d, the concentrations of proteins being tested, and the binding capacities of the beads used in the assay).

Symptoms that a saturation curve cannot be used to accurately predict the K_d:

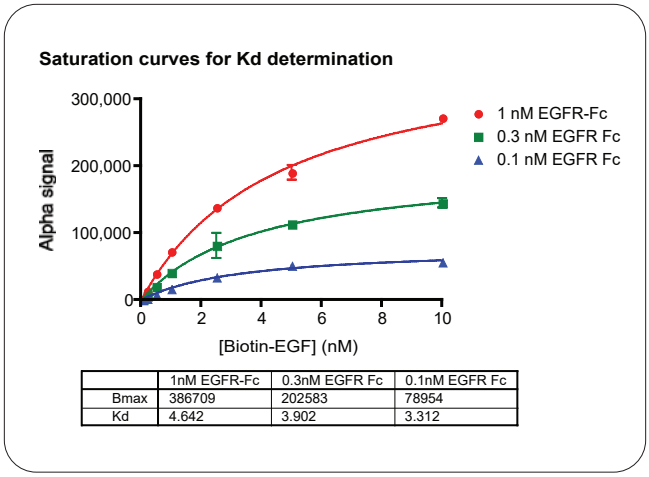
1. Underestimated K_d in Alpha assay. If the Alpha data is giving a K_d that is below what is expected for the protein:protein interaction, it is possible that bead saturation is skewing the data. A competition binding curve should be tested for K_d determination.

2. K_d is calculated to be 5-10 nM when using streptavidin beads. Because the streptavidin-biotin interaction is a relatively tight interaction, the binding capacity of these beads is fairly predictable. If the calculated K_d is between 5 and 10 nM and streptavidin beads are being used, it is likely the binding capacity of the beads has been exceeded. A competition binding curve should be tested for determination of K_d.

Example data:

An excellent example of the use of saturation curves to determine K_d in an Alpha assay format is in a paper by Cassel et al. (2010). In this work, the K_d of the interaction between TAR DNA binding protein 43 (TDP-43) and biotinylated single-stranded TAR DNA and 6 TG repeats was determined to be 0.75 nM and 0.63 nM, respectively. Association and dissociation rates were also calculated and used to derive K_d values. In both cases, the K_d derived was consistent with previous reports using other methods.

In Figure 16, saturation curves were used to determine the K_d for the interaction between biotin-murine EGF and Fc-fusion human EGFR. Streptavidin Donor beads and Protein A AlphaLISA Acceptor beads were used in the assay. The expected K_d is ~2.8 nM as determined by a radioligand binding assay [Lax, I. et al., 1988]. In the protocol for this data, a 3-step assay was performed as follows: 1) biotinylated EGF and EGFR-Fc addition, 60 minute incubation. 2) Protein A AlphaLISA Acceptor bead addition, 60 minute incubation. 3) Streptavidin Donor bead addition, 30 minute incubation followed by 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.



	1nM EGFR-Fc	0.3nM EGFR Fc	0.1nM EGFR Fc
Bmax	386709	202583	78954
Kd	4.642	3.902	3.312

Figure 16: Saturation curve used to determine K_d of the interaction between biotinylated EGF and EGFR-Fc in Alpha format. The expected K_d for this interaction is ~2.8 nM. This approach is generally suitable for low (sub-nanomolar) K_d values.

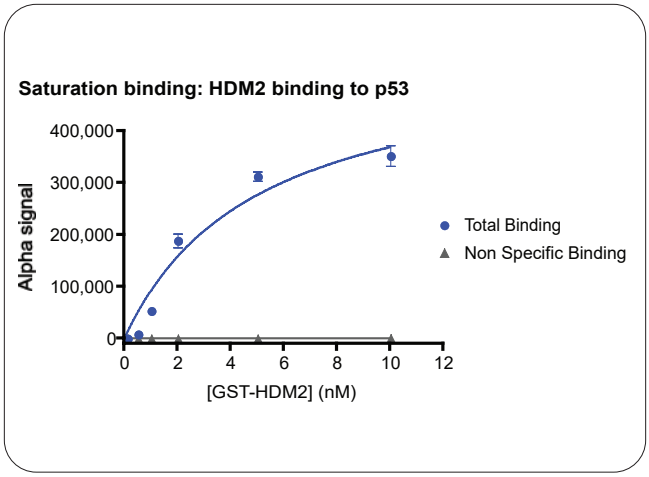


Figure 17: Saturation curve for interaction between GST-HDM2 and biotinylated p53. The expected K_d for this interaction is ~ 0.3 µM. Because the expected K_d is above the estimated binding capacity of the anti-GST-coated Acceptor beads and the streptavidin-coated Donor beads, a saturation curve should not be used to determine K_d.

Example data where K_d *cannot* be determined by a saturation curve:

In Figure 17, the expected K_d of the interaction between HDM2 and p53 peptide is ~ 0.3 µM. This is well above the expected binding capacity of the beads used in the Alpha assay, which is an indication that the saturation method should not be used to determine K_d; instead, a competition binding assay should be used. Based on these data, the K_d would be incorrectly derived to be ~ 2 nM. (10 nM biotinylated-p53 peptide was used in the assay.)

When performing a saturation curve to derive K_d, it is important to choose protein concentrations above, below, and centered around the expected K_d. If possible, at least eight protein concentrations should be chosen, ranging from 10X K_d to 0.1X K_d. The concentration of only one binding partner (protein) should be varied – the other protein should be held at a constant concentration that is below the binding capacity of its associating bead and at least 10X below the highest concentration of the varied protein.

4.2 Competition binding assays for determination of K_d

In many cases, a competition binding assay will need to be used to determine the K_d in Alpha format. This is because many protein:protein interactions have dissociation constants that are above the binding capacity of the beads. In the competitive binding assay, increasing concentrations of the untagged version of Protein X or Protein Y are used to displace the tagged reagent from the assay, disrupting the association between the beads (competition). The K_d value can then be calculated from the IC_{50} value based on a method introduced by Cheng and Prusoff (Figure 18).

The information above is written from the point-of-view of a receptor-radioligand binding assay. In an Alpha protein:protein interaction assay, the "target" becomes one of the tagged proteins being caught to the bead, and the "tracer" becomes the second tagged protein binding to the other bead. If the K_d is significantly higher than the concentration of either

Definitions:

[T] = target
[L*] = tracer
$K_{d\ L^*T}$

Binding
GPCR
radioligand

AlphaScreen
Protein X captured onto Acceptor beads
Protein Y captured onto Donor beads

1) Rule of thumb

$$[T]_{(0.1\ nM)} \overset{10x}{<<} [L^*]_{(1\ nM)} \overset{10X}{<<} [K_d]_{L^*T\ (10\ nM)}$$

2) Cheng & Prusoff

$$K_d = \frac{IC_{50}}{1 + \frac{[L^*]}{K_{d\ L^*T}}}$$

Combining 1 & 2)

$$K_d = \frac{IC_{50}}{1 + \frac{[L^*]}{K_{d\ L^*T}}} \quad \text{(bottom term} \to 0\text{)} \qquad \mathbf{K_d = IC_{50}}$$

Therefore an IC_{50} determination from a carefully crafted competition assay should approximate the K_d

Figure 18: Cheng and Prusoff Equation.

protein, the value for $[L]/K_d$ in the Cheng & Prusoff equation approaches zero. The full equation then becomes $K_d = IC_{50}/(1+0)$, and the K_d approximates the IC_{50} in the competition binding assay.

In order to derive a K_d in this type of assay, a few criteria need to be met:

1. The K_d needs to be at least 10X higher than the concentration of either tagged protein used in the optimized assay. These protein concentrations should be chosen from the first cross-titration experiment (see Section 3.1), keeping in mind that it is not necessary to pick the concentrations that give the highest signal-to-background. As long as the assay window is acceptable, lower concentrations may be chosen.
2. The concentrations of each tagged protein used in the assay need to be below the binding capacity of their respective bead (refer to Table 2 for guidance).
3. The concentration of one labeled protein ("target") should be at least 10X below the concentration of the other labeled protein ("tracer"). If you are using tagged Protein Y, untagged Protein Y, and tagged Protein X in the competition assay, tagged Protein X is the target and tagged Protein Y is the tracer.

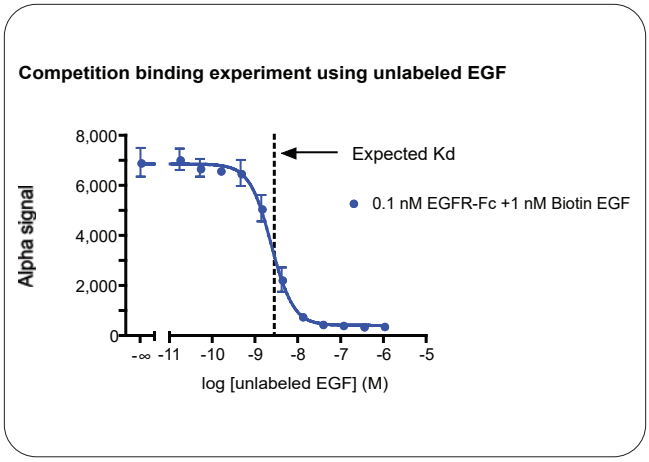


Figure 19: Competition binding assay to determine K_d for a biotinylated EGF/EGFR-Fc protein:protein interaction. Streptavidin Donor beads and Protein A AlphaLISA Acceptor beads were used in this assay. Untagged EGF was titrated from 0.1 pM to 1 µM. The expected K_d is ~2.8 nM as determined by a radioligand binding assay [Lax, I. et al. 1988]. Based on the data, the calculated IC_{50} is 2.4 nM.

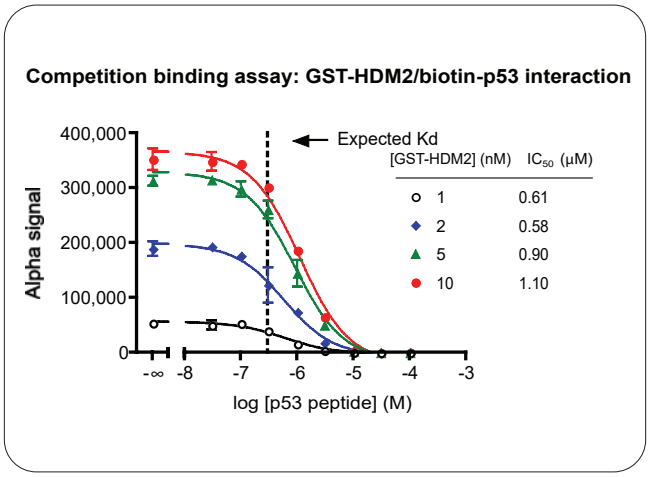


Figure 20: Competition binding assay to determine K_d for a biotinylated p53/GST-HDM2 protein:protein interaction. Streptavidin Donor beads and anti-GST AlphaScreen Acceptor beads were used in this assay. A few concentrations of tagged protein (GST-HDM2) were tested, and the IC_{50} values derived were fairly consistent. Untagged p53 was titrated from 30 nM to 100 µM. Concentration of biotinylated p53 used in the assay was 10 nM. The expected K_d for this interaction is ~ 0.3 µM [Dawson, R. et al., 2003]. Compare to Figure 17, where data derived from a saturation curve erroneously gave a K_d of 2 nM.

Essentially, the same displacement assay used for assay validation (see Section 3.4) will be used. However, it will be important to choose concentrations of the untagged protein that are centered around the expected K_d, in order to derive an accurate IC_{50}. Ideally, a range of concentrations of untagged protein ranging from 0.01X to 100X the K_d would be used. If the expected K_d is not known, it may be necessary to perform two experiments – one using a very broad range of untagged protein concentrations. Then once this data has been evaluated, a second experiment can be set up using a narrower concentration of untagged protein. Additionally, it is advisable to test the assay with two or more different concentrations of tagged proteins (that still adhere to the two criteria above) to see if the IC_{50} changes significantly, depending on the tagged protein concentration. If the IC_{50} does vary dramatically between the two concentrations tested, it is likely the lower concentration is more accurate – the assay may need to be re-run with still lower concentrations of tagged protein. Figures 19 and 20 show two examples where competition binding experiments were used to determine K_d.

Why can a signal be obtained even when working at tagged protein concentrations that are well below the K_d? Because Alpha beads have avidity (multiple binding sites per bead), the microenvironment of the bead artificially increases the concentration of tagged protein in the vicinity of the bead.

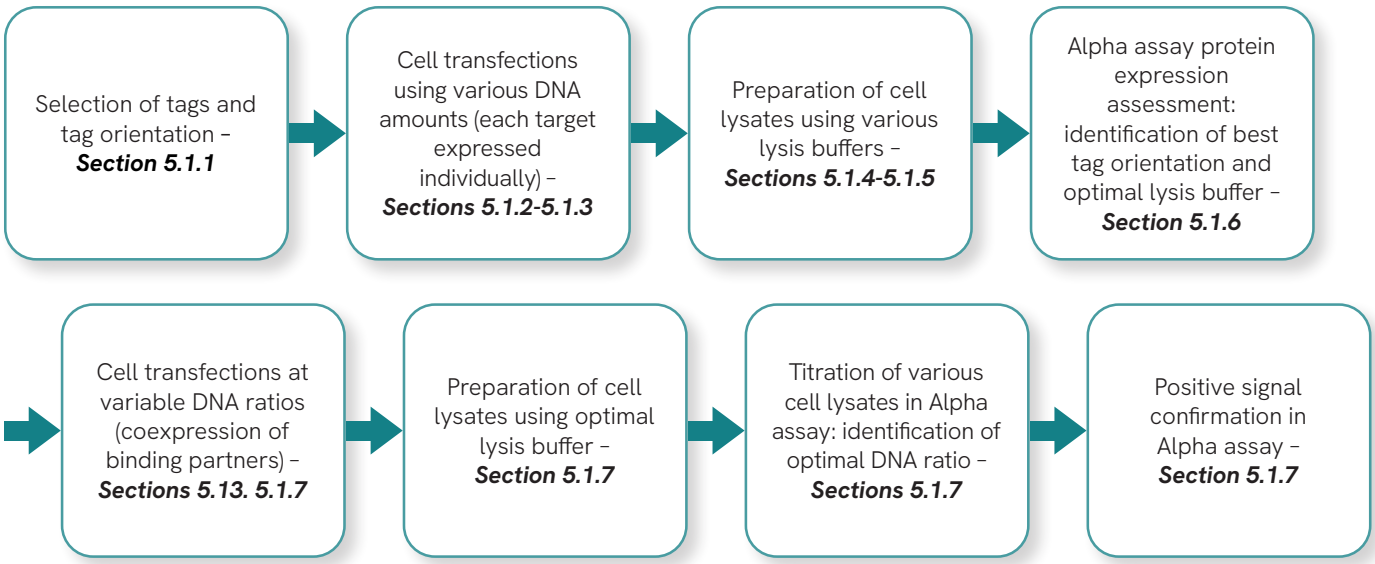
An excellent example of the use of competition binding curves to determine affinity in an Alpha assay format is in a paper by Lazar et al., (2006). The IC_{50} of the interaction between three Fc variant antibodies and Fcgamma receptor was determined to be 380 nM, 60.3 nM, and 7.94 nM, respectively. These IC_{50}s were in close agreement with SPR (surface plasmon resonance)-derived K_d values (252 nM, 30 nM, and 2 nM, respectively).

5. Cell-based assays

While performing biochemical assays is the most straightforward approach to study protein:protein interactions, often the cellular environment can be essential to generate the proper interaction. Cell-based assays to study these interactions using Alpha assays can roughly be divided into three categories:

- Alpha assays involving tagged recombinant proteins, transfected into cells: this approach is the preferred one due to the high protein expression levels achieved in transfected cells, and the availability of good anti-tag antibodies (see Section 5.1).
- Alpha assays involving endogenously expressed proteins: the success of these assays will greatly depend on the endogenous levels of expression of each protein and the availability of specific antibodies directed against the two proteins (see Section 5.2).
- Alpha assays involving non-tagged recombinant proteins, transfected into cells: while this approach relies on antibody detection, it presents an advantage over the second approach due to higher amounts of proteins expressed in transfected cells (see Section 5.3).

5.1 Studying protein:protein interactions using overexpressed tagged proteins



5.1.1 Selection and orientation of protein tags

Documented cases, showing successful use in conjunction with AlphaLISA, include FLAG, GST, His and E-Tag (see Section 5.4). The use of a protein tag can be especially convenient when working with Alpha technology since the expression of each protein can be confirmed before proceeding to the actual interaction assay (see 5.1.6). Revvity offers standalone detection reagents (AlphaLISA Acceptor beads and Alpha Donor beads) or complete AlphaScreen detection kits for these tags (Table 6).

Table 6: Alpha reagents for Tag detection.

Tag	AlphaScreen kits	AlphaLISA Acceptor beads	Alpha Donor beads
anti-GST	6760603	AL110	
Ni chelate	6760619	AL108	AS101
anti-His		AL128	
anti-c-myc	6760611	AL111	
anti-FLAG	6760613	AL112	AS103
anti-DIG	6760604	AL113	
anti-HA	6760612		
anti-FITC	6760605	AL127	
anti-V5		AL129	
anti-GFP		AL133	
anti-Maltose Binding Protein (MBP)		AL134	
***Strep*-Tactin®**		AL136	AS106

Please visit **www.revvity.com** for additional product information and available sizes.

As seen in the products table above, some anti-tag antibodies are available coupled to Donor beads, allowing further assay flexibility for the user.

Note that the glutathione (GSH) detection system is not listed here and is not recommended for cell-based applications, as lysates contain high concentrations of free glutathione.

The orientation of the selected tags on the recombinant proteins (N- or C-terminal) should be in accordance with current knowledge regarding the interaction of interest such that any possible obstruction toward the interacting regions is minimized. When there is no available information on this, the two proteins should be produced with the chosen tag in both the N- and C-terminal regions for a total of four different products. The different tagging combinations can then be transfected in parallel to find the most optimal one(s). Section 5.1.6 will explain how to evaluate and compare the different tag orientations in terms of protein expression.

5.1.2 Generation of expression vector

The protein genes containing the chosen tags should be cloned into appropriate expression vectors.

5.1.3 Transient cell transfection

Many different technologies are available to transfect the expression vectors into cells (Calcium Phosphate, Electroporation, Ballistic Particles, DEAE Dextran, Cationic Matrix, and Lipofection). The lipofection protocol is a relatively simple method that has been used for high throughput screening. FuGENE®6 has been observed to work best in the presence of serum and resulting in little or no toxicity. Below is an example of a transfection protocol for CHO cells using FuGENE®6:

Transfection method for one 100mm Petri dish (0.5 Million CHO cells seeded 16h before):

1. In a 100 mm Petri dish, seed 5 x 10^6 CHO cells 16 hours before transfection. Place in a CO_2 incubator.
2. Add 576 µL of serum-free media to a 2 mL sterile polypropylene tube (no antibiotics or fungicide).
3. Allow the FuGENE®6 tube to equilibrate to room temperature before opening. Vortex well before opening.

4. With a sterile pipet tip, add 9 µL of Fugene6 directly to the serum-free media. **Do not touch the sides of the tube**. Vortex to mix and wait 5 minutes.
5. Add 1-6 µg of DNA to the tube. Wait 15 min.
6. Add the transfection mix to the Petri dish containing the cells and growing media in a drop-wise manner over the entire surface. Swirl to mix properly.
7. 24-48h post-transfection, harvest the cells and prepare the cell lysate.

   We recommend proceeding first with single transfections, in the cell line of your choice, for the two proteins using variable amounts of DNA. This will allow confirmation that each plasmid is producing a functional protein in the cells. Ideally, optimization experiments for DNA amount and post-transfection culture time should be conducted prior to performing double-transfection experiments.

5.1.4 Cell treatment

In certain conditions, treatment of the cells is recommended to achieve an efficient cellular interaction between the two proteins. In some cases, starving the cells may constitute a treatment that will favor the desired protein:protein interaction. In other cases, treating the cells with a known modulator of one of the proteins of interest (agonist, antagonist, inhibitor, etc.) might be an elegant way to generate a control cell lysate. In this example, these would be a ''negative'' (or basal state) lysate and a ''positive'' (stimulated) lysate. It will then be possible to calculate an assay window based on the signals produced with the positive and negative lysates. If no treatments are available, the negative control cell lysate could be a lysate prepared from cells transfected with one protein or the other.

5.1.5 Cell lysis

Lysis buffers

The measurement of protein:protein interactions using Alpha requires the production of cell lysates to release the proteins from their cellular environment. This is a critical step since the lysis per se is a disruptive process involving detergents that could break the protein:protein interaction under investigation. To address that particular need, various non-denaturing formulations of moderate strength have been developed over the years for pull-down and immunoprecipitation assays. The efficiency of lysis buffers to break cells and solubilise proteins will also depend on cell type. We therefore recommend testing several cell lysis buffers. Some that have been successfully used with AlphaLISA are listed below. A protease inhibitor cocktail should be added to all lysis buffers tested. We recommend testing different lysis buffers to select the optimal one.

- 200 mM Tris-HCl pH 7.5, 1% Triton X-100, 50 mM NaCl
  - (used for CHO cells, *see* Waller et al.)
- 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% NP40.
  - (used for BSR T7/5 cells, see Rahman et al. & Mohamed et al.)
- 50 mM Tris-HCl pH 7.5, 125 mM NaCl, 5% glycerol, 0.2% NP40, 1.5 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4.
  - (used for HEK 293T cells, see Lavens et al.)
- 50 mM Tris-HCl pH 7.5, 0.1% CHAPS.
  - (used for CHO cells, *unpublished results from Revvity*)

We also recommend the following commercially available cell lysis buffers:

- **AlphaLISA lysis buffer** (Revvity, Cat. No. AL003) (proprietary formulation)
- **Cell lysis buffer (10x)** (Cell Signaling Technology, Cat. No. 9803)

  (1X Cell lysis buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin)
- **M-PER® Mammalian protein extraction reagent** (Thermo Scientific, Cat. No. 78501) (proprietary formulation)
- **Pierce IP lysis buffer** (Thermo Scientific, Cat. No. 87787) (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol)

Preparation of lysis buffer

1. Dilute the lysis buffer to 1X (if needed) prior to use.
2. Add a standard protease inhibitor cocktail (Sigma Cat. No. P2714 or Roche Cat. No. 05 892 791 001) to the lysis buffers prior to use. Reconstitute according to the manufacturer's instructions. Other cocktails can be used as well.

Cell lysis procedure

1. Treat the cells if needed (Section 5.1.4).
2. Detach the cells if required.
3. Determine the cell density of the culture. Harvest the cells by centrifugation.
4. Discard the supernatant and resuspend the cells in 1X Lysis buffer. The optimal number of cells must be determined experimentally. However, 4 x 10^6 cells/mL are recommended as a starting point. Add 5 µL of 1X Lysis buffer to 20,000 cells.
5. Incubate for about 5-10 minutes at room temperature on a rocker to maximize the lysis.
6. Centrifuge for 5 minutes at high speed (13,000 RPM) in a benchtop centrifuge to clarify the lysate. Discard the pellet.
7. Prepare aliquots and freeze at -80 °C.

5.1.6 Protein expression assessment

Once the bulk cell lysates are prepared, as a preliminary step it is important to evaluate the relative levels of expression of each protein in the cells. We cannot stress enough the importance of gathering solid evidence for expression of the respective proteins before proceeding to the actual detection of the interaction. Although this does not guarantee success in measuring a protein:protein interaction, it is certainly the most important step towards that goal. Examples and recommendations using Alpha assays are given below, but other methods may also be used (see next section).

Tag-based detection with Alpha technology

Typically, the confirmation of protein expression in a cell lysate can be done by performing a competition-type assay set up to detect a tagged positive control peptide included in an AlphaScreen kit (for example, a biotinylated and flagged peptide). To this assay are added increasing amounts of lysate containing the expressed protein bearing the expression tag (see Figure 21). A signal decrease proportional to the amount of cell lysate is expected, as the expressed protein can compete with the positive control. This step should be done for each protein. If necessary, the amount of DNA transfected can be adjusted to achieve the desired expression levels.
Non-transfected cells or cells transfected with an unrelated target should be included as negative controls to rule out any interference coming from non-specific components of the lysates.

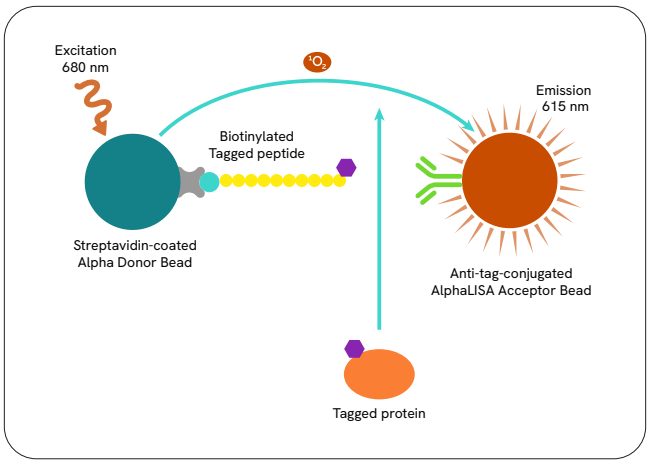


Figure 21: Schematic diagram of an assay used to confirm cellular expression of the tagged protein. The overexpressed tagged protein competes for binding of biotinylated tagged peptide to anti-FLAG conjugated Acceptor beads.

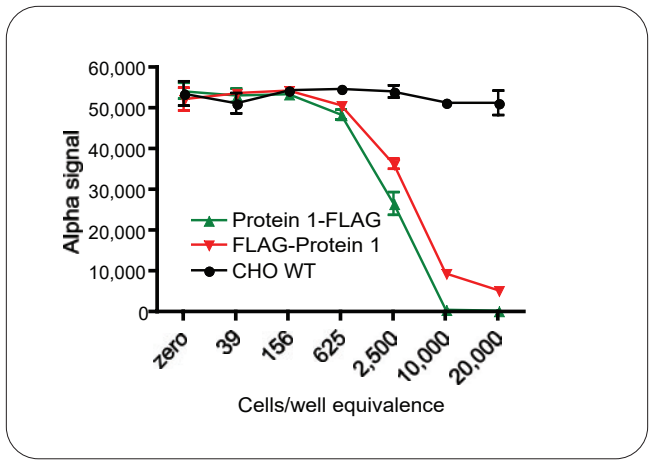


Figure 22: Detection of a FLAG-tagged protein in two transfected lysates using an Alpha assay. The detection of FLAG-tagged Protein 1 (with either an N- or C-terminal tag) was done using an AlphaScreen FLAG detection kit. Serial dilutions of the lysates were tested for their ability to compete with the biotin-FLAG peptide. The amounts of lysate are reported in cells/well equivalence (20,000 corresponding to the undiluted lysates). Untransfected CHO WT lysates were included as a negative control. Here, the Protein 1-FLAG transfect (C-terminal FLAG tag) displayed a slightly higher expression level compared to the FLAG-Protein 1 transfect (N-terminal FLAG tag), indicating that the FLAG tag in the C-terminus may be more optimal.

Figure 22 is an example where the expression of a FLAG-tagged protein was confirmed in transfected CHO cells. The assay was performed in a 384-well OptiPlate™ for a 25 µL final assay volume according to the following procedure.

1. Add 10 µL Anti-FLAG Acceptor Beads (20 µg/mL final) diluted in assay buffer.
2. Add 5 µL CHO lysate dilutions in lysis buffer.
3. Incubate 15 min at room temperature.
4. Add 5 µL of biotin-FLAG peptide (5 nM final) in assay buffer. Incubate 15 min at room temperature.
5. Add 5 µL of streptavidin Donor beads (20 µg/mL final) diluted in assay buffer.
6. Incubate 30 min at room temperature.
7. Read on an Alpha-capable reader (EnVision® Multilabel Plate Reader or EnSpire® Multimode Plate Reader).

In a similar fashion, the expression of Protein 2 tagged with GST could be confirmed using Anti-GST Acceptor beads and the same Anti-GST antibody biotinylated and captured by streptavidin-Donor beads (Figure 23). Note that the Anti-GST antibody is polyclonal, which allows a sandwich capture of the GST protein.

As a result, the signal increases with increasing target protein concentrations. The assay was performed in a 384-well OptiPlate for a 25 µL final assay volume.

1. Add 10 µL Anti-GST Acceptor Beads (20 µg/mL final) diluted in assay buffer.
2. Add 5 µL CHO lysates dilutions in lysis buffer.
3. Incubate 15 min at room temperature.
4. Add 5 µL of biotin-Anti-GST (1 nM final) in assay buffer.
5. Incubate 30 min at room temperature.
6. Add 5 µL of streptavidin Donor beads (20 µg/mL final) diluted in assay buffer.
7. Incubate 15 min at room temperature.
8. Read on an Alpha-compatible reader (EnVision or EnSpire Multimode Plate Readers).

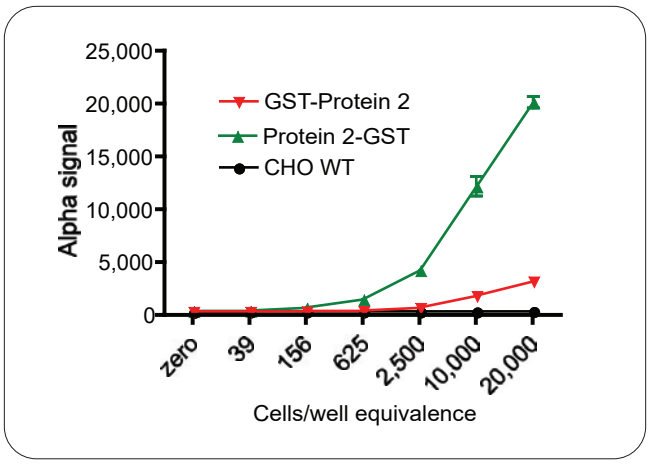


Figure 23: Detection of a GST-tagged protein in two transfected lysates using an Alpha assay. The detection of GST-tagged Protein 2 (with either an N- or C-terminal tag) was done in a sandwich assay involving Anti-GST Acceptor beads and a biotinylated goat Anti-GST antibody. Serial dilutions of lysates were tested in the sandwich assay. The amounts of lysate are reported in cells/well equivalence (20,000 corresponding to the undiluted lysates). Untransfected CHO WT lysates were included as a negative control. Results indicated that expression levels were distinctly higher when the GST tag was located at the C-terminal end of Protein 2.

Additional ways to assess protein expression

The experimental examples given here can help in confirming the expression of the tagged proteins. However, this methodology does not constitute a definitive proof that the cell expresses a fully functional tagged protein. For example, the protein may be truncated or cleaved and the tag could not be attached to the protein. We therefore suggest performing a Western blot detection to verify that the expressed tagged proteins display the expected molecular weights.

If an antibody is available to the tagged protein, one could use an Alpha assay where the tagged protein is detected using both the tag and a specific antibody in a capture assay format. Compared to the tag-based detection strategy described in section 5.1.6, this type of assay can provide more compelling proof for the functional expression of the tagged proteins since the antibody epitope and the tag need to be present on the same protein to generate an Alpha signal.

Based on the results obtained in this section, the best constructs and the optimal lysis buffer should be selected for use in subsequent double-transfection and protein:protein interaction experiments.

5.1.7 Alpha protein:protein interaction assay

When you are ready to perform the double-transfection experiments for the actual detection of an interaction, we suggest that various ratios of DNA be tested in transfections since a 1:1 stoichiometry in DNA amount will not necessarily result in comparable expression levels for the two proteins. Various factors may affect the efficiency of transfection, protein transcription and maturation.

Selection of assay buffer

While there is no universal assay buffer that can be recommended to suit all protein:protein interaction studies, the following two buffers were shown to be successful in some cases:

- PBS + 0.1% BSA (ref. Rahman et al.; Mohamed et al.)
- 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% BSA (Revvity unpublished results)

In addition, test any assay buffers that have been documented in the literature for use with the protein:protein interaction of interest. One general recommendation is to avoid the use of detergents in the assay buffer composition. Some detergent will most likely be introduced to the assay by the sample. (The sample lysis buffer will be diluted 5-fold if 5 µL of sample is added in a 25 µL assay.) Excessively high levels of certain detergents could affect the protein:protein interaction and the Alpha technology as well. Also it is important to verify that the buffer does not contain substances known to interfere with the Alpha technology. The tolerance of Alpha technology to various detergents and other substances is detailed in section 2.2.2.

Confirmation that a positive signal is a valid protein:protein interaction

Once the double transfections have been performed at various DNA ratios and cell lysates have been prepared, the validity of the protein:protein interaction assay should be confirmed. One or more of the following approaches may prove useful in validating that a positive Alpha assay signal from double-transfected cell lysates is indeed due to the desired protein:protein interaction.

- As negative controls, test single-transfected lysates in the protein:protein interaction assay.
- To confirm that the signal observed correlates with the amount of double-transfected cell lysate, perform a titration of the amount of double-transfected lysates added to the assay (Figure 24).
- To confirm that the signal observed is due to the presence of the two specific proteins, when possible, perform a competition experiment in which increasing concentrations of a recombinant form of one of the two proteins is added to the cell lysates (Figure 25).
- To further confirm the specificity of the Alpha assay signal, reference compounds or peptides known to interfere or modulate the protein:protein interaction can be tested for their effect on the assay.

Titration of double-transfected cell lysates in Alpha assays

A cell lysate titration experiment should be performed on each of the double-transfected cell lysates. The assay should be set up to detect the interaction of the two overexpressed proteins under study. Serial dilution of the lysate should correlate with a decreasing signal in the assay. Ideally, a negative control should also be performed using lysates of non-transfected cells or cells transfected with a non-related target cDNA.

Figure 24 shows the results of a serial dilution of lysate from a cell transfected with one ratio of Protein 1-FLAG / Protein 2-GST DNA. The Alpha assay employed a biotinylated anti-GST antibody and anti-FLAG-conjugated Acceptor beads. In this example, the data confirmed that the amount of signal correlated with the amount of lysate added. The assay was performed in a 384-well OptiPlate with a 25 µL final assay volume using the following protocol.

1. Add 10 µL of CHO lysates prepared in lysis buffer.
2. Add 5 µL Anti-FLAG Acceptor beads (20 µg/mL final) diluted in assay buffer.
3. Incubate 30 min at room temperature.
4. Add 5 µL Biotinylated Anti-GST antibody (1 nM final) diluted in assay buffer.
5. Incubate 60 min at room temperature.
6. Add 5 µL streptavidin Donor beads (20 µg/mL final) diluted in assay buffer.
7. Incubate 60 min at room temperature.
8. Read in Alpha-compatible reader (EnVision or EnSpire Multimode Plate Readers)

Competition protocol to validate protein:protein interaction assay

Where a recombinant form of one of the target proteins is available, this can be added to the double-transfected cell lysate in increasing concentrations (Figure 25). A reduction in signal with increasing concentrations of this competing protein suggests that the assay is detecting the desired protein:protein interaction. One essential requirement for such a competition experiment is that the recombinant protein must not contain either of the two tags used in the detection assay (FLAG and GST in this example). The assay shown

here was performed as follows in a 384-well OptiPlate using a 25 µL final assay volume.

1. Add 5 µL Anti-FLAG Acceptor Beads (20 µg/mL final) diluted in assay buffer.
2. Add 5 µL recombinant Protein 2, diluted in assay buffer.
3. Add 5 µL CHO lysates (prepared at 4M cells/mL).
4. Incubate 60 min at room temperature.
5. Add 5 µL of biotin-Anti-GST (1 nM final) diluted in assay buffer.
6. Incubate 60 min at room temperature.
7. Add 5 µL of streptavidin Donor Beads (20 µg/mL final) diluted in assay buffer.
8. Incubate 60 min at room temperature.
9. Read on an Alpha-compatible reader (Envision or EnSpire Multimode Plate Readers).

Once a positive Alpha assay signal is validated, users can refer to the AlphaLISA Assay Development Guide from Revvity for subsequent steps.

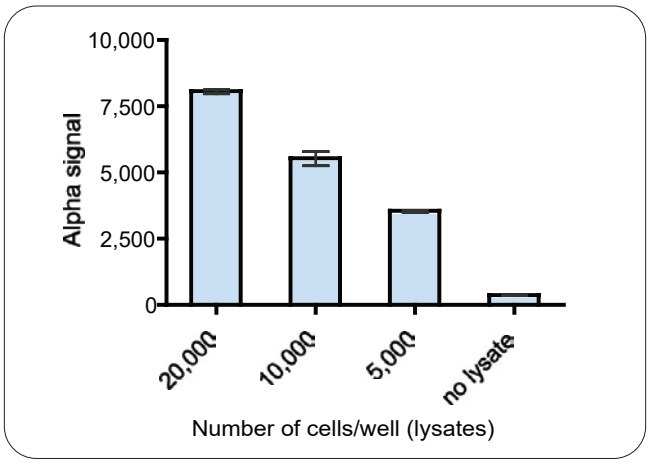


Figure 24: Alpha assay detection of tagged Protein 1:Protein 2 interaction, illustrating that the signal correlates with varying amounts of protein:protein complex. Lysate dilutions prepared from cells transfected with Protein 1-FLAG / Protein 2-GST DNA were tested in an Alpha assay. AlphaScreen Anti-FLAG Acceptor beads and streptavidin Donor beads were used at 20 µg/mL final, and biotinylated anti-GST was used at 0.3 nM final.

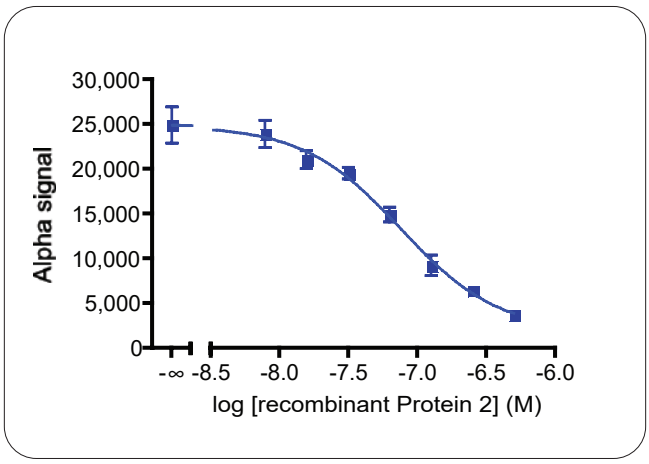
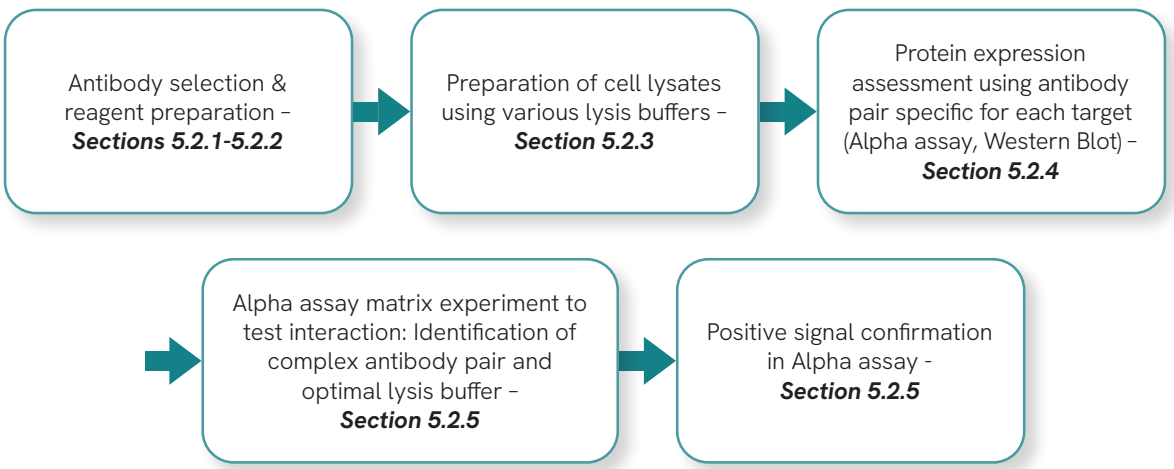


Figure 25: Competition assay performed to validate a protein:protein interaction assay for Protein 1-FLAG and Protein 2-GST. Lysates prepared from CHO cells double-transfected with Protein 1-FLAG and Protein 2-GST DNA were tested in the presence of increasing concentrations of recombinant Protein 2. An IC_{50} value of 73 nM was calculated.

5.2 Studying protein:protein interactions using endogenous proteins



The detection of an interaction between endogenously expressed proteins must rely on the use of specific antibodies, since the proteins will not have expression tags. As for the tagged overexpressed proteins discussed in section 5.1, it is crucial to gather good evidence that the two endogenous proteins to be studied for interaction are indeed expressed in the cellular model chosen.

In the case of endogenous non-tagged proteins, the most popular technique for achieving this is the Western blot. One can also use an antibody-based Alpha assay to detect each protein individually. In this instance, a variety of antibodies should be tested in order to maximize the chance of finding at least one robust pair of antibodies for the assay.

When data from ELISA experiments are available, one should first select the same pair of antibodies to try in an Alpha assay. If this is not successful, other antibody pairs can then be investigated.

5.2.1 Antibody selection

The selection of antibodies should be made based on the available literature for the interaction. The chosen antibodies should bind to an epitope that is as distant as possible from the interacting domain(s) of the proteins to avoid steric hindrance. When the map of the interaction is unknown, we suggest selecting and testing multiple antibodies that target different regions of the protein. Combinations of these antibodies can then be tested in a matrix experiment to find the optimal pair. A practical search engine for commercially available antibodies can be found in the Biocompare® site: www.biocompare.com.

When available, recombinant versions of the studied proteins could prove helpful in selecting antibodies and possibly in confirming the specificity of the putative protein:protein interaction. When testing different antibody pairs in an Alpha assay, the assay can be relatively quickly developed using a recombinant protein as the target. Once the protein:protein assay format has been established, the recombinant protein can be used to show assay specificity by competing with the interaction of the endogenous proteins being studied (See section 5.1.7 for an example of this approach using overexpressed proteins.)

5.2.2 Antibody labeling

All of the selected antibodies should be prepared for use in both of the possible orientations in the Alpha assay. In other words, an aliquot of each antibody should be biotinylated (for capture on streptavidin Donor beads) and a separate aliquot should be directly coupled to Acceptor beads. (Protocols are supplied in the AlphaLISA Assay Development Guide). For each antibody pair, the Alpha assay should be tested in both configurations, i.e. with Antibody 1 Acceptor beads and biotinylated Antibody 2, and with Antibody 2 Acceptor beads and biotinylated Antibody 1.

For some antibodies where the formulation prevents bead coupling or biotinylation, secondary antibodies can be used as an alternative capture method (e.g. AlphaLISA Anti-mouse IgG Acceptor beads) so long as there is no problem of cross-reactivity to the other antibody in the assay. Revvity offers stand-alone detection reagents and complete detection kits along with Donor beads for these antibodies (Table 7).

Table 7: Alpha beads for indirect antibody capture.

Tag	AlphaScreen kits	AlphaScreen Acceptor beads	AlphaLISA Acceptor beads	Alpha Donor beads
Protein A	6760617	6760137	AL101	AS102
Protein G			AL102	
Protein L			AL126	
Anti-human IgG			AL103*	
Anti-rabbit IgG	6760607		AL104*	AS105*
Anti-mouse IgG	6760606		AL105*	AS104*
Anti-rat IgG			AL106*	
Anti-goat IgG			AL107*	
Anti-sheep IgG			AL132*	
Anti-mouse IgM			AL130	
Anti-chicken IgY			AL131	

* Fc specific antibodies

Please visit **www.revvity.com** for additional product information and available sizes.

5.2.3 Cell lysis

Please consult section 5.1.5 for detailed information on cell lysis.

5.2.4 Protein expression assessment

Once the bulk cell lysates are prepared, a preliminary step consists in evaluating the relative level of expression of each protein in the cells. This can be done in an Alpha assay by capturing each individual protein with specific antibodies or by Western blot analysis. Different lysis buffers may be tested at this stage. As for tagged proteins (section 5.1.6), it is important to verify that the assay signal is specific for the targeted protein and is correlated with the amount of lysate added to the assay.

5.2.5 Alpha interaction assay for endogenous proteins

Matrix experiments

The initial search for a positive signal in an Alpha assay demonstrating a protein:protein interaction event will involve exploring two important parameters: lysis buffer and antibody selection. The first experiments should consist in matrix assays where all the combinations of the various lysis buffers and antibody pairs will be tested in parallel (Figure 26).

Please see section 5.1.7 for help in selection of the assay buffer in which the protein:protein interaction will be performed prior to cell lysis.

For each antibody pair tested, we recommend including a negative control for which no lysate is added (lysis buffer only). Alternatively, if the interaction is known to be modulated by a cell treatment, treated versus untreated cell lysates might serve as positive and negative controls. This should help discard any potential cross-reactivity or non-specific signal that could mistakenly be thought to originate from the interaction occurring between proteins.

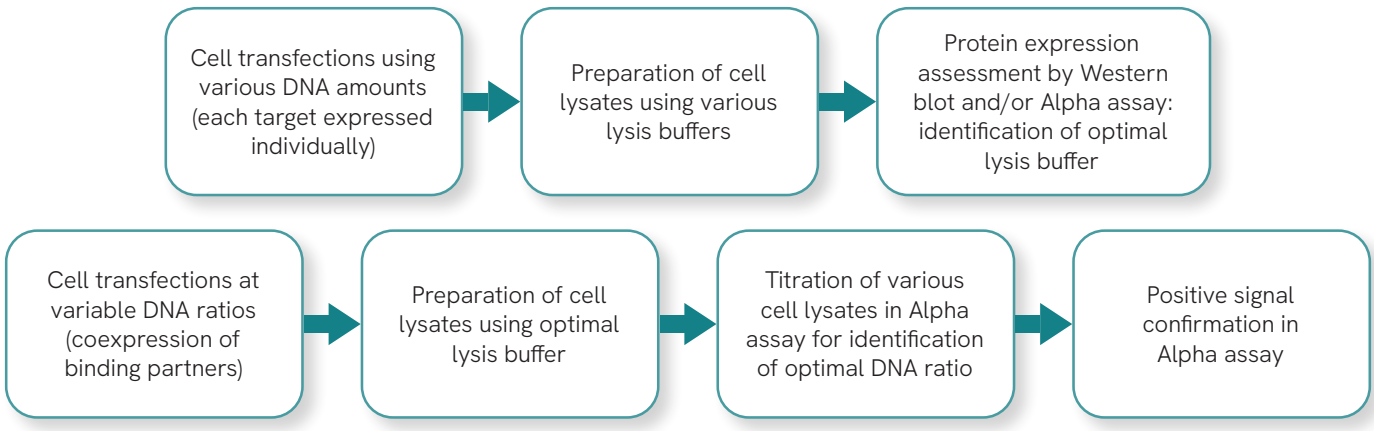
Lysis Buffers		A						B						C						D					
Antibody Pairs		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	A																								
2	B																								
3	C		Cell lysate			Negative Control																			
4	D																								
5	E																								
6	F																								
7	G																								
8	H																								
	I																								
	J																								
	K																								
	L																								
	M																								
	N																								
	O																								
	P																								

Figure 26: Example of a matrix assay for selection of a lysis buffer and antibody pairs. In this 384-well microplate setup, four lysis buffers and eight antibody pairs are tested in triplicate.

Confirmation that a positive signal is a valid protein:protein interaction

Refer to section 5.1.7 for examples of experiments that can confirm the validity of your protein:protein interaction Alpha assay.

5.3 Studying protein:protein interactions on non-tagged recombinant proteins



The approach taken here is a combination of the fundamentals covered in Sections 5.1 and 5.2. It relies on specific antibodies directed against the recombinant proteins, and, for that reason, these assays are more challenging than those involving tagged recombinant proteins. On the positive side, they are in general easier to develop than the assays for endogenous proteins due to the higher expression levels of recombinant proteins.

See sections 5.1.2 through 5.1.5 for information on preparing the cell lysates for analysis. As for other applications, lysates should be prepared and tested from cells expressing each individual protein before working with lysates from cells coexpressing the two binding partners.

When the proteins to be studied do not include any tag, the confirmation of expression and selection of the best lysis buffer can be made using techniques such as Western blotting. It is also possible to create Alpha assays for detecting each protein individually by using two specific antibodies in a sandwich assay format. Performing these validation assays in an Alpha assay format may require some additional time and effort initially, but will ultimately save hands-on time as compared to Western blotting. These benefits can prove especially useful when handling a large number of experimental conditions (e.g. optimization of transfected DNA amounts for each protein).

See Section 5.1.7 for help with setting up the Alpha protein:protein interaction assay.

5.4 Cell-based references

Literature examples of Alpha cell-based assays using recombinant and tagged proteins:

Reference	Tags used	Lysis buffer	Lysis protocol	Assay buffer	Lysate amount
Becker et al., Virology 2008	FLAG and HA	100 mM Tris, pH 8.0, 100 mM NaCl, 0.5% NP-40, 0.2 mM PMSF containing Roche complete protease inhibitor)	Twenty-four hours post-transfection, cells from one well of a 6-well microplate were rinsed with PBS and lysed on ice for 5 minutes. The cellular extracts were then separated following centrifugation for 10 min at 13,000 g at 4 °C.	PBS + 0.1% BSA	2 µL of lysate from one well was diluted in 1:10 in assay buffer
Rahman et al., PLoS Pathogens 2009	GST and His	100 mM Tris pH 8.0, 100 mM NaCl, 0.5% NP-40, containing Roche complete protease inhibitor	Forty-eight hours post-transfection, cells were collected in 500 mL PBS, pelleted and then lysed by suspending in 20 mL lysis buffer. The cellular extracts were then separated following centrifugation for 5 min at 13,000 rpm.	PBS + 0.1% BSA	Cells seeded in 24 well plates. Forty eight hours post transfection, cells were collected in 500 mL PBS, pelleted and then lysed by suspending in 20 µL lysis buffer. 5 µL of this cell extract were mixed to a total assay volume of 25 µL.
Lavens et al., Nucleic Acids Research 2009	Etag and FLAG	50 mM Tris-HCl pH 7.5, 125 mM NaCl, 5% glycerol, 0.2% NP40, 1.5 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4, Complete protease inhibitor without EDTA cocktail. Lysates were cleared by centrifugation.		not disclosed	not disclosed
Mohamed et al., PNAS 2009	His and GST	100 mM Tris pH 8.0, 100 mM NaCl, 0.5% NP-40, containing Roche complete protease inhibitor	Forty eight hours post transfection, cells were collected in 500 mL PBS, pelleted and then lysed by suspending in 20 mL lysis buffer. The cellular extracts were then separated following centrifugation for 5 min at 13,000 rpm.	PBS + 0.1% BSA	Cells seeded in 24 well plates. Forty eight hours post transfection, cells were collected in 500 mL PBS, pelleted and then lysed by suspending in 20 µL lysis buffer. 5 µL of this cell extract were mixed to a total assay volume of 25 µL.
Werden et al., Journal of Virology 2010	His, FLAG and HA	Not disclosed	Not disclosed	PBS + 0.1% BSA	Not mentioned
Waller et al., Journal of Virological Method 2010	FLAG, GST, His and HA (His and HA for transfected cells)	Following a PBS wash: 200 mM Tris-HCl pH 7.5, 1% Triton X-100, 50 mM NaCl	The cell lysates were incubated for 30 min on ice and then centrifuged at 9,357×g in a microcentrifuge for 5 min at 4° C.	100mM HEPES pH-7.5, 1mM EDTA, 5mM DTT, 0.1% CHAPS, 5% glycerol	CHO cells grown in 6 well plate, transfected and grown for 48 h then lysed in 100 µL. 18 µL of this lysates is used per well of Alpha.
Internal data presented in this guidebook	FLAG and GST	Bulk produced at 4M/mL in 50 mM Tris-HCl pH 7.4, 0.1% CHAPS + protease inhibitor cocktail (note: CHAPS was documented as a detergent that preserves the studied interaction).	see Section 5.1.5	50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% BSA	5 µL of 4M/mL lysates in 25 µL total assay volume

Quick start guide to Alpha protein: protein interactions

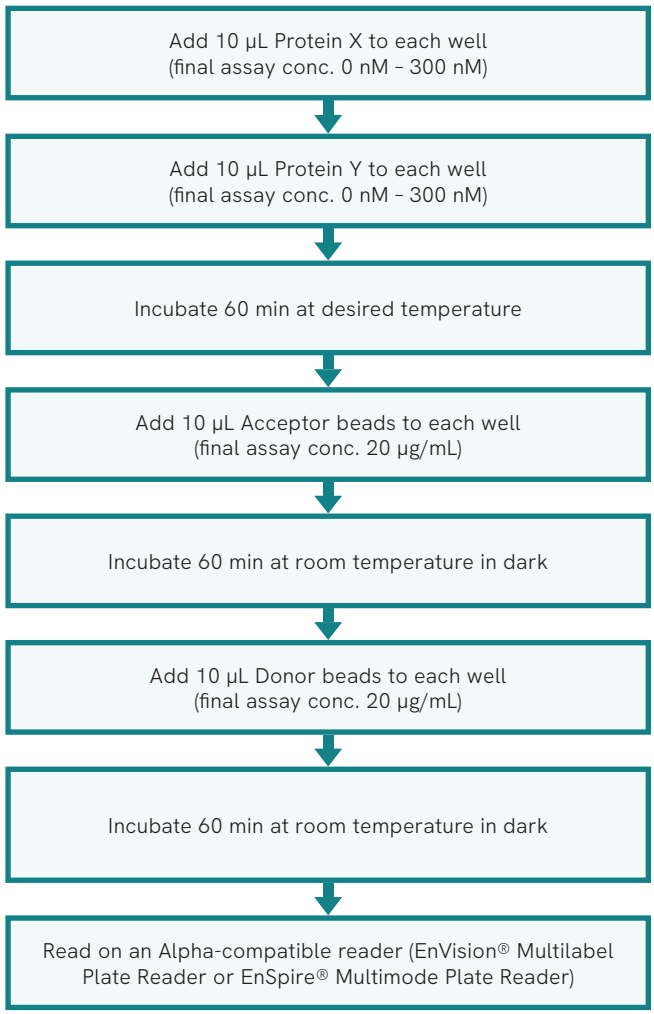
Before you begin:

- Alpha assays require a special reader capable of measuring an Alpha assay. Many standard time-resolved fluorimeters and luminometers cannot read Alpha assays.
- We recommend preparing only what you need for the day's experiments. Do not store working dilutions of beads for more than one day.
- 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). 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.

Assay configuration: A variety of Alpha Donor and Acceptor beads are offered to design your protein:protein interaction assay, including streptavidin coated beads, anti-FITC and anti-DIG coated beads, anti-fusion tag antibody coated beads, nickel chelate and glutathione beads, anti-species antibody-coated beads, Protein A-, Protein G- and Protein L-coated beads. Unconjugated beads are also available, would you need to create your own beads. Different configurations may give different results. Contact Revvity for more advice.

Biochemical assays

In this initial 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 in a 40 µL (final assay volume) reaction. The plate map for this assay (Figure 27) 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).



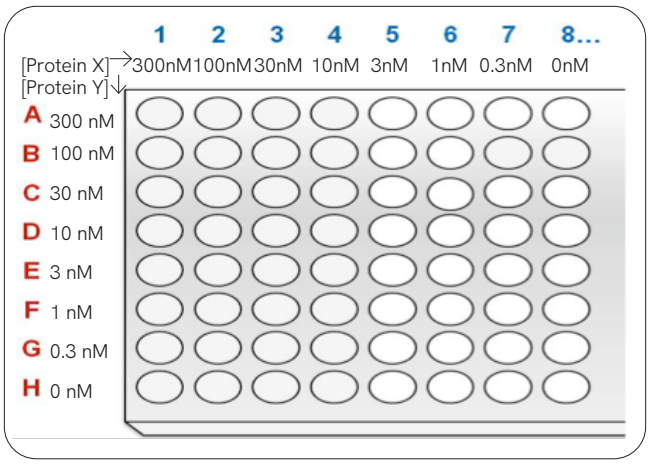


Figure 27: 96-well white ½ AreaPlate map (40 µL final assay volume)

Next optimization steps, if desired:

1. Order-of-addition (adding both beads at same time, or all proteins and beads at same time, etc.)
2. Displacement assay (demonstrating that tagged Protein X or tagged Protein Y can be displaced using untagged protein)
3. Incubation time optimization (up to overnight)

Cell-based Assays

Lysis buffer selection

The measurement of cell-based protein:protein interactions using Alpha requires the production of cell lysates. We recommend testing several cell lysis buffers. A protease inhibitor cocktail (such as Sigma-Aldrich® Cat. No. P2714 or Roche® Cat. No. 05 892 791 001) should be added to all lysis buffers tested. The optimal number of cells must be determined experimentally. For the protocols below, we recommend preparing a lysate equivalent to 2 x 10^6 cells/ mL lysis buffer. Some recommended lysis buffers:

- **AlphaLISA lysis buffer** (Revvity, Cat. No. AL003)
- **Cell lysis buffer (10x)** (Cell Signaling Technology®, Cat. No. 9803)
- **M-PER®** Mammalian protein extraction reagent (Thermo Scientific®, Cat. No. 78501)
- **Pierce IP lysis buffer** (Thermo Scientific®, Cat. No. 87787)

Protein expression assessment

Once the bulk cell lysates are prepared, it is important to evaluate the relative levels of expression of each protein in the cells. For tagged proteins, this can be done with a competition-type assay using the tagged probe included in an appropriate AlphaScreen kit. For endogenous proteins, this can be done using an Alpha assay to capture each individual protein with specific antibodies, or by Western blot.

Alpha cell-based protein:protein interaction protocol *(refer to Figures 28 or 29 for plate map, as applicable)*

1. Add 10 µL of lysates in a white 96-well ½ AreaPlate.
2. Add 15 µL Acceptor beads (20 µg/mL final assay conc.) diluted in assay buffer.
3. Incubate 30 min at room temperature.
4. If applicable: Add 10 µL Biotinylated (Anti-tag) antibody (1 nM final) diluted in assay buffer.
5. Incubate 60 min at room temperature.
6. Add 15 µL streptavidin Donor beads (20 µg/mL final assay conc.) diluted in assay buffer.
7. Incubate 60 min at room temperature.
8. Read in Alpha-compatible reader (EnVision® Multilabel Plate Reader or EnSpire® Multimode Plate Reader).

For tagged protein:protein interaction events, the first experiment will involve evaluating lysis buffer and amount of lysate. Cell lysates should be diluted in assay buffer to reduce lysis buffer interference.

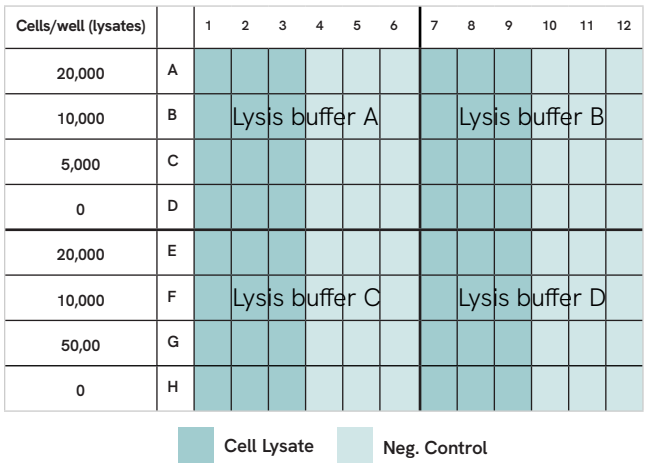


Figure 28: First experiment for cell-based assays using tagged proteins: Titration of cell lysates.

For endogenous protein:protein interaction events, the first experiment will involve evaluating lysis buffer and antibody selection. We recommend using 20,000 cells/well as the lysate volume.

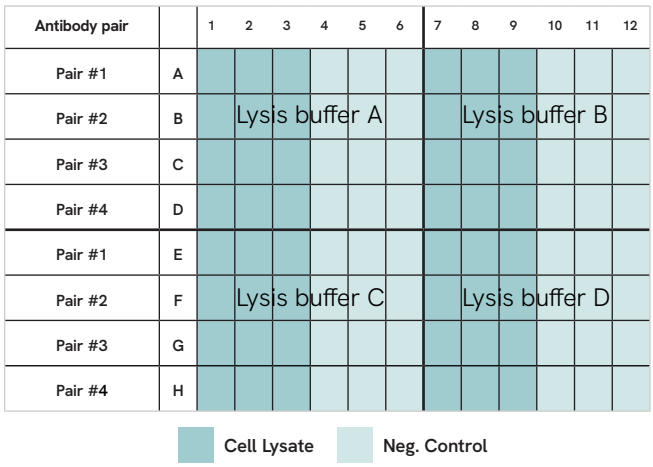


Figure 29: First experiment for cell-based assays using endogenous proteins: Antibody selection.

Negative control: single-transfected lysate, RNAi-transfected lysate, lysate from uninduced cells, or where the interaction has been inhibited.

Cell-based Assays, *continued*

Confirmation that a positive signal is a valid protein:protein interaction

- For assays studying tagged proteins: to confirm that the signal observed is due to the presence of the two specific proteins, when possible, perform a competition experiment in which increasing concentrations of an untagged recombinant form of one of the two proteins is added to the cell lysates, or untagged transfected protein is added by transfection.
- For assays studying endogenous proteins: to confirm that the signal observed correlates with the amount of double-transfected cell lysate, perform a titration of the amount of double-transfected lysates added to the assay.
- To further confirm the specificity of the Alpha assay signal, reference compounds or peptides known to interfere or modulate the protein:protein interaction can be tested for their effect on the assay.

Microplates for Alpha assays and recommended volumes

Scaling the assay volume up or down

The development of new assays and optimization of existing assays using Alpha assay technology is typically accomplished in short timeframes, with relatively small investments of labor and cost.

Alpha assays are well suited for a multitude of R&D applications and lower throughput assays, but the technology is also ideal for HTS (high-throughput screening). Following initial assay optimization at a particular final reaction volume, assay volumes are easily scaled up or down without the need for re-optimization or increased reagent concentrations. One can simply increase or reduce all volume additions proportionately without loss in sensitivity or assay performance. Higher signal and S/B ratios are often achieved with higher density microplates (see Table 8 for available microplates). Using low volume assay formats in 384-well (Shallow well ProxiPlates) or 1536-well plates can yield significant savings in cost per well and help preserve precious and scarce reagents. We recommend using a plate seal cover to prevent evaporation of samples and subsequent signal inconsistencies. Revvity TopSeal™-A (Revvity Cat. No. 6050195) is ideal for this purpose and does not interfere with the Alpha signal, permitting the plate to be read without first removing the plate seal.

Table 8: Microplates for Alpha assays and recommended volumes.

Microplate	Catalog	Color	Recommended assay volume	Comments
½ AreaPlate-96	6005560	white	40-50 µL	The 96-well plate that is recommended for the highest sensitivity in a 50 µL reaction
OptiPlate™-96	6005290	white	100 µL	
CulturPlate™-96	6005680	white	100 µL	Coated for use in tissue culture
OptiPlate-384	6007290	white	24-50 µL	The 384-well plate that is recommended for the highest sensitivity in a 50 µL reaction
AlphaPlate®-384	6005350	light-gray	24-50 µL	Light gray color reduces potential for well-to-well crosstalk
ProxiPlate™-384 Plus	6008280	white	20 µL	
AlphaPlate-1536	6004350	light-gray	8-10 µL	Light gray color reduces potential for well-to-well crosstalk

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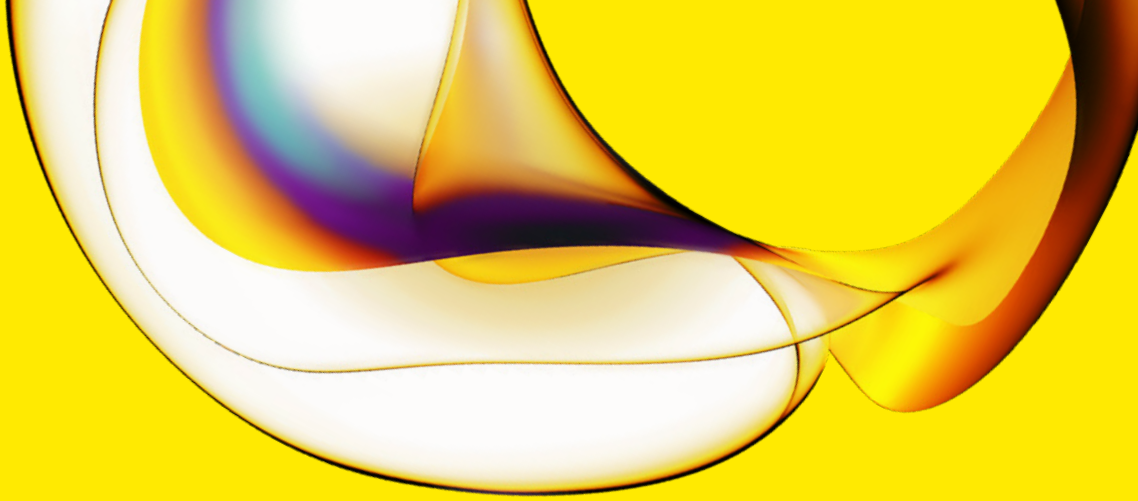

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