

Alpha technology/protein: protein 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.

Introduction

The interactions and binding of proteins are implicated in a large number of biological processes. The need for an efficient, highly sensitive assay to study large protein interactions is increasingly important.

Alpha Technology (Amplified Luminescent Proximity Homo-geneous Assay) is a highly flexible, homogeneous no-wash assay ideal for the measurement of protein interactions and complexes up to 200 nm in size. A bead-based proximity assay, Alpha Technology offers the possibility to assay many biological targets, including enzymes, receptor-ligand interactions, low-affinity interactions, second messenger levels, DNA, RNA, proteins, peptides, sugars and small molecules.

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 μ sec half-life, singlet

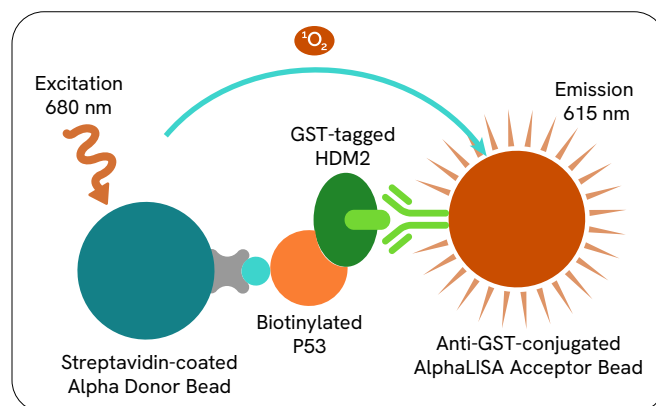


Figure 1. Illustration of an Alpha protein: protein interaction assay, using streptavidin-coated Donor beads, AlphaLISA anti-GST coated Acceptor beads, biotinylated p53, and GST-tagged HDM2.

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).

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 (Figure 1).

The highly versatile beads can be coated with various bio-molecules enabling detection of unique biological events.

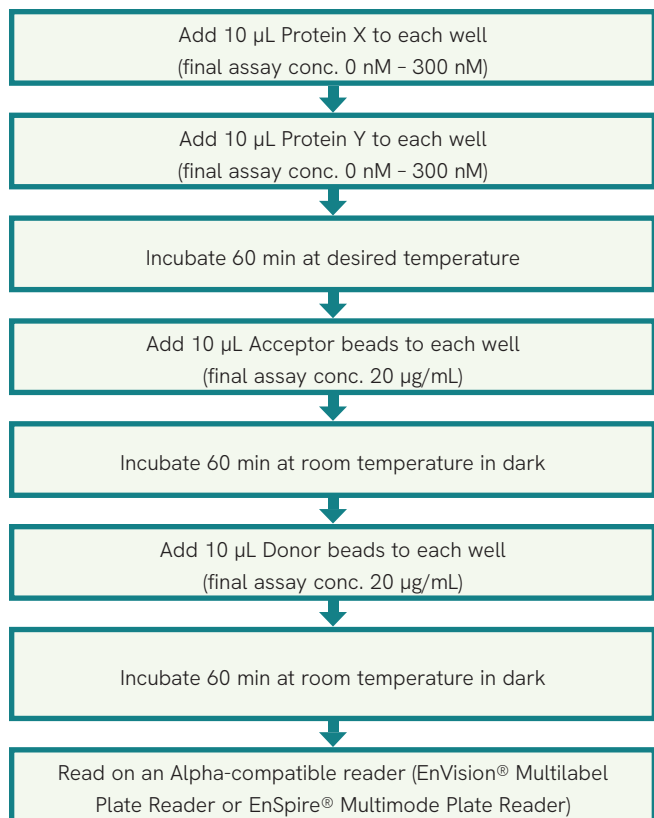
Quick start guide to alpha protein:protein interactions

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).

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



- 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.

assay (Figure 2) 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).

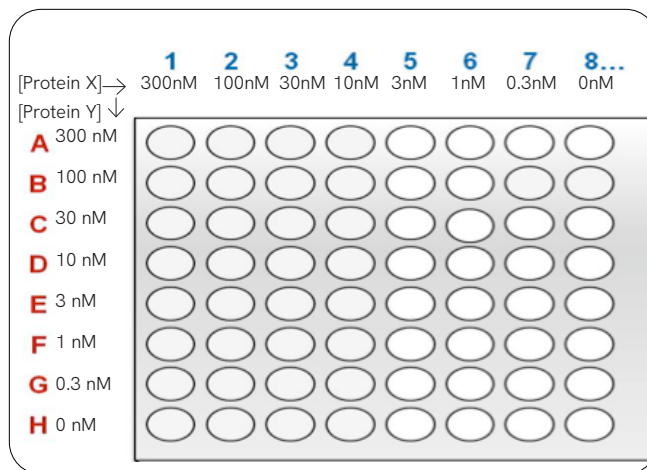


Figure 2. 96-well white 1/2 AreaPlate map (40 µL final assay volume)

Next optimization steps, if desired:

- Order-of-addition (adding both beads at same time, or all proteins and beads at same time, etc.)
- Displacement assay (demonstrating that tagged Protein X or tagged Protein Y can be displaced using untagged protein)
- 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 10⁶ 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)

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.

Cells/well (lysates)		1	2	3	4	5	6	7	8	9	10	11	12
20,000	A	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control
10,000	B	Lysis buffer A				Lysis buffer B				Neg. Control	Neg. Control	Neg. Control	Neg. Control
5,000	C	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control
0	D	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control
20,000	E	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control
10,000	F	Lysis buffer C				Lysis buffer D				Neg. Control	Neg. Control	Neg. Control	Neg. Control
5,000	G	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control
0	H	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control

Legend: Cell lysate (green), Neg. Control (blue)

Figure 3. First experiment for cell-based assays using tagged proteins: Titration of cell lysates.

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

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 3 or 4 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 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.

Antibody pair		1	2	3	4	5	6	7	8	9	10	11	12
Pair #1	A	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control
Pair #2	B	Lysis buffer A				Lysis buffer B				Neg. Control	Neg. Control	Neg. Control	Neg. Control
Pair #3	C	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control
Pair #4	D	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control
Pair #1	E	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control
Pair #2	F	Lysis buffer C				Lysis buffer D				Neg. Control	Neg. Control	Neg. Control	Neg. Control
Pair #3	G	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control
Pair #4	H	Cell lysate	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control	Neg. Control

Legend: Cell lysate (green), Neg. Control (blue)

Figure 4. First experiment for cell-based assays using endogenous proteins: Antibody selection.

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 2 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 2. Microplates for Alpha assays and recommended volumes.

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

