MANUAL

AlphaLISA® Human cIAP1 BIR2 Binding Kit

Product number: AL3166 C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed to assess orthosteric binders of cIAP1 BIR2 protein using a homogeneous no wash AlphaLISA binding assay.

Sensitivity: $IC_{50:}$ 2.4 µM (average, using AlphaLISA Human cIAP1 BIR2 Binding Kit standard (LCL161). To calculate binding affinity (Ki) with the Cheng-Prusoff equation, use Kd_{ligand} of 20 nM.

Signal to background ratio: 690 (average) using 16.8 nM HIS Tagged cIAP1 BIR2 protein and 21 nM biotinylated ligand.

Kit contents: The kit contains 6 components: Streptavidin coated Acceptor beads, Nichel Chelate Donor beads, biotinylated cIAP1 BIR2 ligand, HIS-tagged cIAP1 BIR2 protein, AlphaLISA Human cIAP1 BIR2 Binding Kit standard (LCL161) and AlphaLISA PPI buffer.

Storage: The kit components must be stored at 4 ˚C in the dark. Reconstituted reagents can be aliquoted (aliquot > 10µL) then frozen. Reconstituted HIS-Tagged cIAP1 BIR2 protein can be stored at -80°C for 28 days. Other reconstituted reagents can be stored at ≤ -20°C for 28 days. Avoid multiple freeze-thaw cycles.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging (lyophilized) and the recommended storage conditions (+4°C).

Analyte of Interest

cIAP1, also called BIRC2, is a member of the Inhibitor of Apoptosis Proteins (IAP) family. IAP proteins are involved in multiple biological processes, such as innate immunity, and play an important role in apoptosis inhibition.

cIAP1 overexpression has been associated with cancer resistance, making it an attractive target in cancer therapy where several therapeutic strategies have been investigated, such as SMAC mimetics. Moreover, cIAP1 displays E3 ligase activity and leads to targeted proteins' ubiquitination and their subsequent degradation. This property can be exploited through a Proteolysis-targeting chimera (PROTAC) strategy. SNIPER molecules (Specific and Non-genetic inhibitor of apoptosis protein [IAP]-dependent Protein Erasers) can induce the degradation of both the targeted proteins and IAPs and are expected to harness cancer cell killing. Therefore, new compounds targeting cIAP1 which exhibit dual roles: i) inhibition of cIAP1 anti-apoptotic function and ii) induction of targeted protein degradation, represent a promising therapeutic approach.

cIAP1, like other IAP family proteins, contains BIR domains which interact with the IAP-binding motif of partners such as caspases. This interaction has been suggested to control pro- and anti-apoptotic activities.

cIAP1 compound characterization on the BIR2 and BIR3 domains enables accurate profiling and selectivity studies.

Description of the AlphaLISA Assay

The AlphaLISA Human cIAP1 BIR2 Binding assay uses Nickel Chelate Donor beads to capture the HIS-tagged cIAP1 BIR2 protein and Streptavidin-coated Acceptor beads to capture the biotinylated ligand. Donor beads and Acceptor beads come into proximity through ligand binding to cIAP1 BIR2 protein. Excitation of the Donor beads leads to the release of singlet oxygen that triggers a cascade of energy transfer reactions in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 1).
Pharmacological compounds (cIAP1 BIR2 binders) characterization is allowed by competition with the biotinylated ligand for binding to HIS-Tagged cIAP1 BIR2 protein resulting in AlphaLISA signal extinction. Standard curve is established using the cIAP1 BIR2 Binding Kit Standard (LCL161), a well-Known cIAP1 binder (Figure 2).

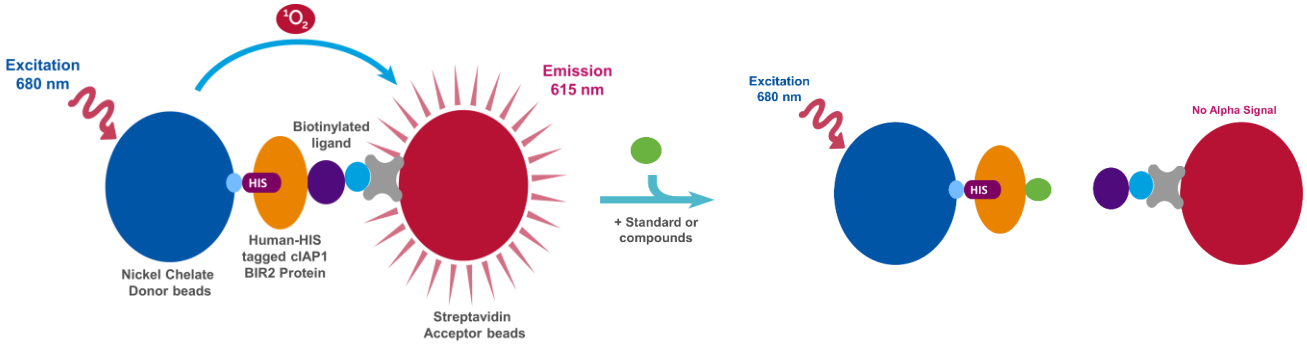


Figure 1. AlphaLISA Human cIAP1 BIR2 Binding Assay Principle.

Precautions

- The Alpha Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- All blood components and biological materials should be handled as potentially hazardous.

Kit Content: Reagents and Materials

Kit components	AL3166C**** (500 assay points)	AL3166F**** (5000 assay points)
Streptavidin (SA) Acceptor beads stored in PBS, 0.05% Kathon CG/ICP II, pH 7.2	80 µL @ 5 mg/mL (1 brown tube, white cap)	800 µL @ 5 mg/mL (1 brown tube, white cap)
Nickel Chelate Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon CG/ICP II, pH 7.4	80 µL @ 5 mg/mL (1 brown tube, black cap)	800 µL @ 5 mg/mL (1 brown tube, black cap)
cIAP1 BIR2 Ligand (Biotinylated)*	300 ng lyophilized (1 tube, clear cap)	300 ng lyophilized (10 tubes, clear cap)
cIAP1 BIR2 protein (HIS tagged)*	2.24 µg lyophilized (1 tube, clear cap)	2.24 µg lyophilized (10 tubes, clear cap)
AlphaLISA Human cIAP1 BIR2 Binding Kit, standard (LCL161)**	1.26 mg lyophilized (1 tube, clear cap)	1.26 mg lyophilized (2 tubes, clear cap)
AlphaLISA PPI Buffer (5X)***	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute cIAP1 BIR2 protein and biotinylted ligand in 100 µL Milli-Q® grade H_2O respectively. Mix gently after reconstitution. The reconstituted reagents should be used within 60 minutes. Reconstituted reagents can be stored frozen (cf Storage section page 2). Avoid multiple freeze-thaw cycles.

** Reconstitute AlphaLISA Human cIAP1 BIR2 Binding Kit, Standard in 100 µL anhydrous DMSO. Mix gently after reconstitution. The reconstituted reagent should be used within 60 minutes. Reconstituted reagent can be stored frozen (cf Storage section page 2). Avoid multiple freeze-thaw cycles. Extra Standard can be ordered separately (Cat # AL3166S)

*** Extra buffer can be ordered separately (cat # AL015C: 10 mL, cat # AL015F: 100 mL).

**** The number of assay points is based on an assay volume of 20 µL in 384 well plates using the kit components at the recommended concentrations.

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the AlphaLISA signal.

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Adhesive Sealing Film	Revvity Inc.	6050185
AlphaPlate-384, Shallow Well (ProxiPlate)	Revvity Inc.	6008350 6008359
EnVision®-Alpha Reader	Revvity Inc.	-

Recommendations

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000g, 10-15 sec). Re-suspend the beads by vortexing before use. Do not vortex the proteins.
- Use Milli-Q® grade H_2O to dilute 5X AlphaLISA PPI Buffer and to reconstitute the lyophilized cIAP1 BIR2 protein and biotinylated ligand.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal™-A Plus Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film.
- The AlphaLISA signal is detected with an EnVision Multilabel Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.

Competition Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The manual described below is an ***example*** for generating 1 dose response curve by using the AlphaLISA cIAP1 BIR2 Binding Kit, standard in a 20 µL final assay volume per well (36 wells). These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes. If a different number of samples are tested, the volumes of all reagents must be adjusted accordingly.
- The dilution manual is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.

One Incubation Step Manual described as below:

1. Preparation of 1X AlphaLISA PPI Buffer (for 10 mL):

Add 2 mL of 5X AlphaLISA PPI buffer and 8 mL of MilliQ water.

2. Preparation of AlphaLISA Human cIAP1 BIR2 Binding Kit, standard (LCL161):

a. Reconstitute lyophilized AlphaLISA Human cIAP1 BIR2 Binding Kit, standard (1.261 mg) in 100 µL DMSO to make a 25.2 mM stock solution of cIAP1 BIR2 Binding standard.
b. Prepare serial dilutions of 4X cIAP1 BIR2 Binding Kit, standard in 1x AlphaLISA PPI buffer as mentioned in the table below, (do not forget to change tips between each dilution):

Tube	Volume of standard	Volume of 1X buffer	[standard] (µM) (4X)	[standard] (µM) (1X)
A (STD 12)	10 µL of 25.2 mM stock solution	190 µL	1260	315
B (STD 11)	60 µL of tube A	140 µL	378	94.5
C (STD 10)	60 µL of tube B	120 µL	126	31.5
D (STD 9)	60 µL of tube C	140 µL	37.8	9.5
E (STD 8)	60 µL of tube D	120 µL	12.6	3.15
F (STD 7)	60 µL of tube E	140 µL	3.8	0.94
G (STD 6)	60 µL of tube F	120 µL	1.3	0.31
H (STD 5)	60 µL of tube G	140 µL	0.38	0.09
I (STD 4)	60 µL of tube H	120 µL	0.13	0.031
J (STD 3)	60 µL of tube I	140 µL	0.04	0.009
K (STD 2)	60 µL of tube J	120 µL	0.013	0.0031
L (STD 1)	60 µL of tube K	140 µL	0.0037	0.00094
M (STD 0)	0	200 µL	0	0

3. Preparation of 4X HIS tagged cIAP1 BIR2 protein:

    a. Reconstitute lyophilized HIS tagged cIAP1 BIR2 protein in 100 µL H_2O to make a 1680 nM protein stock solution.
    b. Add 10 µL of the 1680 nM HIS tagged cIAP1 BIR2 protein stock solution to 240 µL of 1X AlphaLISA PPI buffer to obtain a 67.2 nM working solution of HIS cIAP1 BIR2 protein.

Prepare just before use. Unused Stock solution must be stored at -80°C (aliquot > 10 µL). Do not repeat Freeze/Thaws cycles. Do not store working solution.

4. Preparation of 4X biotinylated ligand:

    a. Reconstitute lyophilized biotinylated ligand in 100 µL H_2O to make a 2100 nM stock solution.
    b. Add 10 µL of 2100 nM biotinylated ligand to 240 µL 1X AlphaLISA PPI buffer to obtain a 84 nM working solution

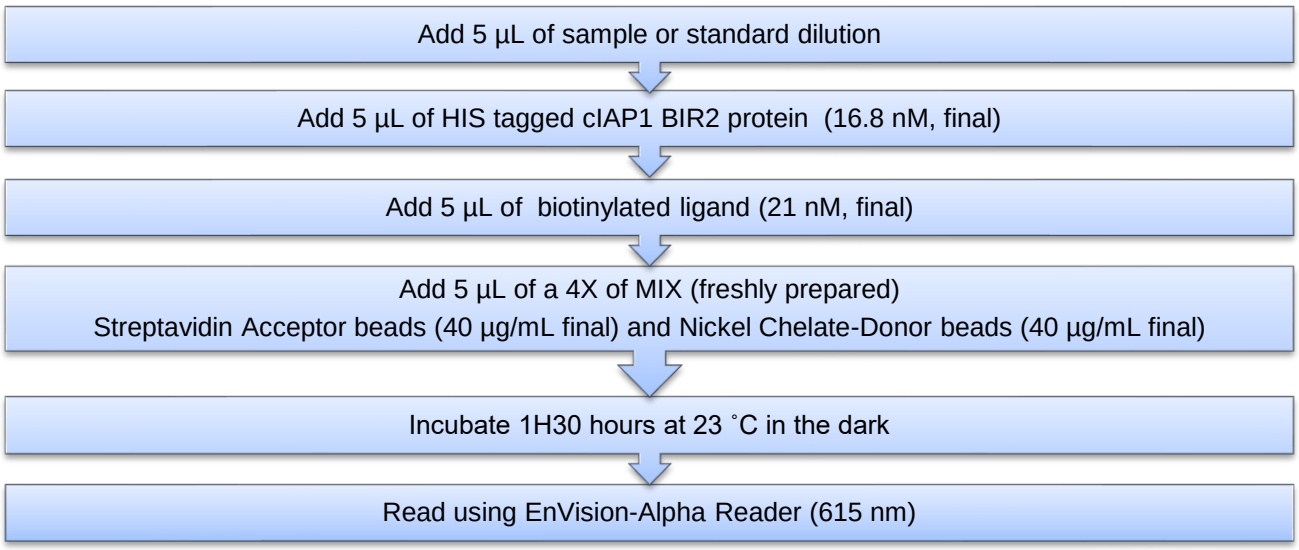
Prepare just before use. Unused Stock solution must be stored at -20°C or below (aliquot > 10 µL). Do not repeat Freeze/Thaws cycles. Do not store working solution.

5. Preparation of the mix of 4X Streptavidin Acceptor beads and Nickel Chelate Donor beads:

    a. Keep the beads under subdued laboratory lighting.
    b. Add 8 µL of 5 mg/mL Streptavidin Acceptor beads and 8 µL of 5 mg/mL Nickel Chelate Donor beads to 234 µL of 1X AlphaLISA PPI buffer to obtain a 160 µg/mL of Streptavidin Acceptor beads and 160 µg/mL of Nickel Chelate Donor beads Mix working solution.

Prepare just before use.

6. Distribute the prepared reagents in a shallow well AlphaPlate (384 wells):



Read Settings: AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).

Typical competitive binding Data:

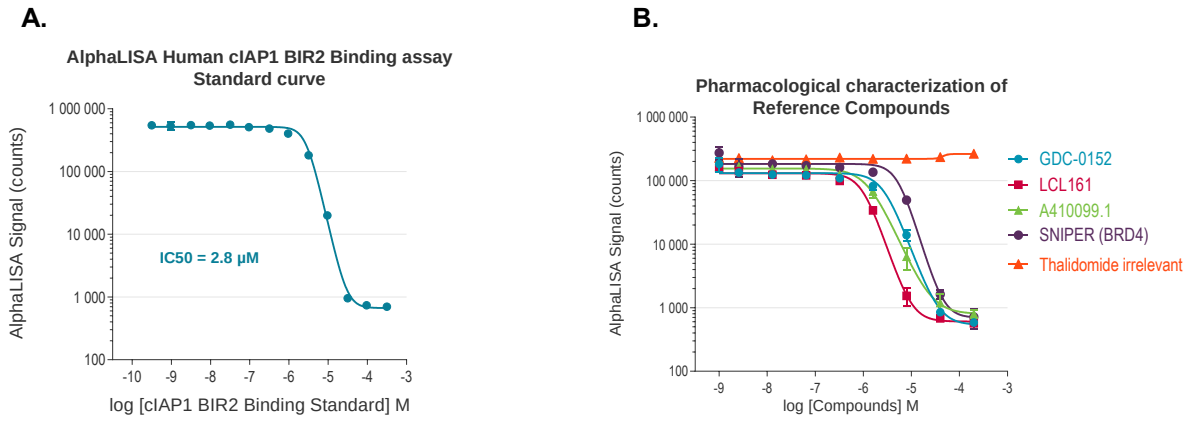


Figure 2. A: Illustration with AlphaLISA Human cIAP1 BIR2 Binding Kit standard which competitively binds to cIAP1 BIR2 protein with IC50 of 2.8 µM. IC50 values were calculated by using a nonlinear regression fitting with GraphPad Prism. B: Pharmacological validation of the ALphaLISA cIAP1 BIR2 Binding Kit using GDC-0152, LCL161, A1400099.1 and BRD4 SNIPER (PROTAC based on LCL161 and JQ1 ligands) They competitively bind to cIAP1 BIR2 domain with IC50 of 2.7, 0.98, 1.3, 3.3 µM respectively. The irrelevant Thalidomide compound (Cereblon E3Ligase ligand) does not compete as expected.

Troubleshooting Guide

You will find below recommendations for common situations that you might encounter with your AlphaLISA binding assay. If further assistance is needed, do not hesitate to contact our technical support team for assistance.

Issue	Recommendations and Comments
High background signal	• Buffer is not freshly made. Make new. • Incubation time is longer than recommended range.
Low AlphaLISA signal	• Optimize EnVision with Plate format.
High variation between replicates or low Z' values	• Make sure that reagents are at the bottom of the well by tapping or swirling the plate gently on a smooth surface after each addition.

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at: www.revvity.com

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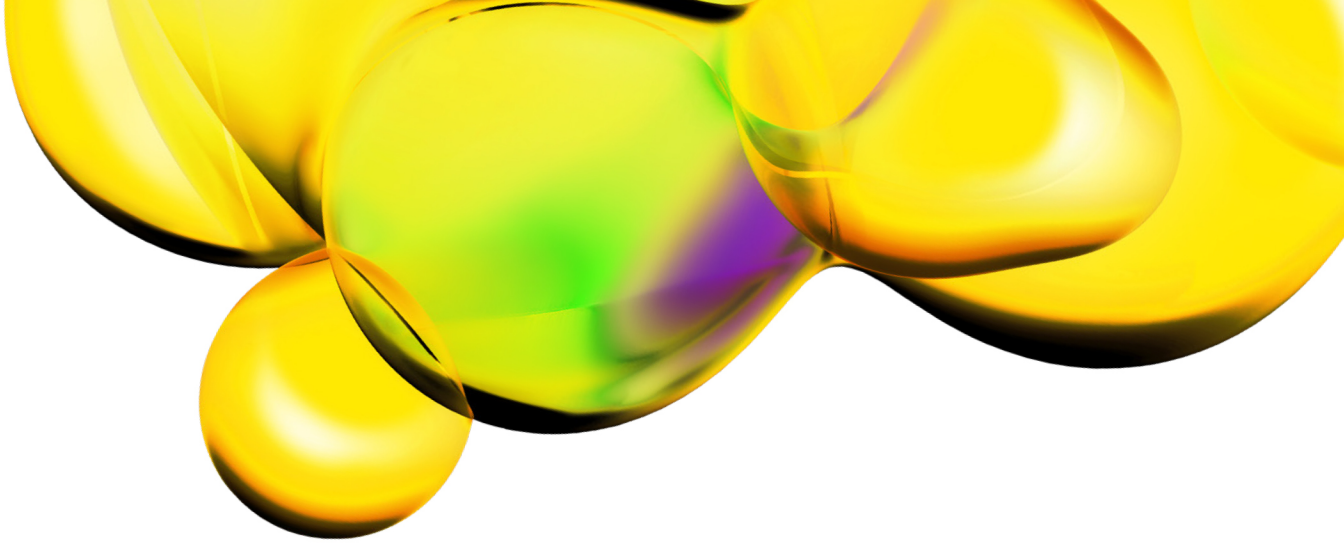

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