MANUAL

# AlphaLISA® Human XIAP BIR3 Binding Kit

**Product number:** AL3173 C/F

Research Use Only. Not for use in diagnostic procedures.

## Product Information

**Application:** This kit is designed to assess orthosteric binders of XIAP BIR3 protein using a homogeneous no wash AlphaLISA binding assay.

**Sensitivity:** $IC_{50}$: 40 nM (average, using AlphaLISA Human XIAP BIR3 Binding Kit standard (LCL161). To calculate binding affinity (Ki) with the Cheng-Prusoff equation, use $Kd_{ligand}$ of 4 nM.

**Signal to background ratio:** 890 (average) using 2 nM GST Tagged XIAP BIR3 protein and 6 nM biotinylated ligand.

**Kit contents:** The kit contains 6 components: Streptavidin coated Acceptor beads, Glutathione Donor beads, biotinylated XIAP BIR3 ligand, GST-tagged Human XIAP BIR3 protein, AlphaLISA Human XIAP BIR3 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 GST-Tagged XIAP BIR3 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

X-linked inhibitor of apoptosis protein (XIAP) protein belongs to the inhibitor of apoptosis protein (IAP) family, which also includes cIAP1. It is the most potent caspase inhibitory IAP family member and a negative regulator of various apoptotic stimuli and death pathways.

XIAP overexpression in tumor cells is a well-described mediator of resistance to chemotherapy in many cancers and has been linked to tumor aggressiveness, making it an attractive target in cancer therapy where several therapeutic strategies have been investigated, such as SMAC mimetics.

Moreover, XIAP 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. Therefore, new compounds targeting XIAP which exhibit dual roles: i) inhibition of XIAP anti-apoptotic function and ii) induction of targeted protein degradation, represent a promising therapeutic approach.

XIAP, 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.

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

## Description of the AlphaLISA Assay

The AlphaLISA Human XIAP BIR3 Binding assay uses Glutathione Donor beads to capture the GST-tagged XIAP BIR3 protein and Streptavidin-coated Acceptor beads to capture the biotinylated ligand. Donor beads and Acceptor beads come into proximity through ligand binding to XIAP BIR3 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 (XIAP BIR3 binders) characterization is allowed by competition with the biotinylated ligand for binding to GST-Tagged XIAP BIR3 protein resulting in AlphaLISA signal extinction. Standard curve is established using the XIAP BIR3 Binding Kit Standard (LCL161), a well-known XIAP binder (Figure 2).

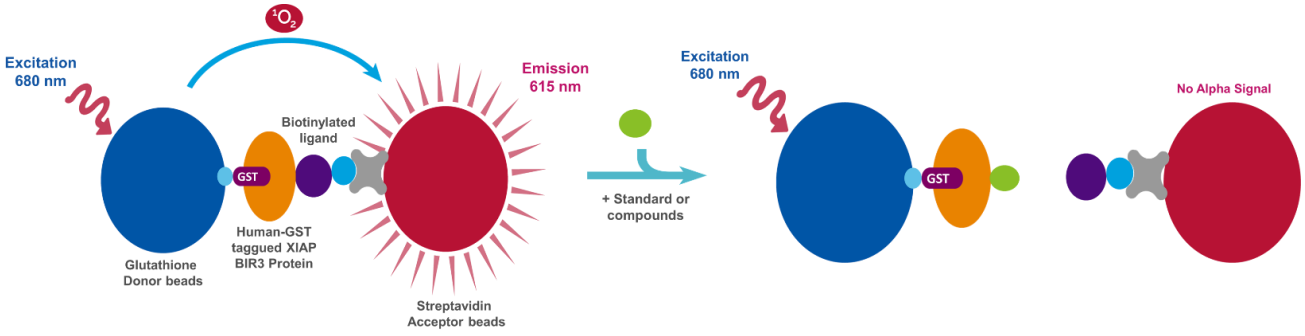



Figure 1. AlphaLISA Human XIAP BIR3 Binding Assay Principle.

## 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 | AL3173C**** (500 assay points) | AL3173F**** (5000 assay points) |
|---|---|---|
| Streptavidin AlphaLISA Acceptor beads stored in PBS pH 7.2 supplemented with 0.05% Kathon as a preservative. | 40 µL @ 5 mg/mL (1 brown tube, white cap) | 400 µL @ 5 mg/mL (1 brown tube, white cap) |
| Glutathione Alpha Donor beads stored In 25 mM Hepes, 0.1% Tween-20, pH 7.4 with 0.05% Kathon as a preservative. | 40 µL @ 5 mg/mL (1 brown tube, black cap) | 400 µL @ 5 mg/mL (1 brown tube, black cap) |
| Ligand (Biotinylated)* | 79 ng lyophilized (1 tube, clear cap) | 79 ng lyophilized (10 tubes, clear cap) |
| Human XIAP BIR3 protein (GST tagged)* | 806 ng lyophilized (1 tube, clear cap) | 806 ng lyophilized (10 tubes, clear cap) |
| XIAP BIR3 Binding Kit standard** | 200 µg lyophilized (1 tube, clear cap) | 200 µg lyophilized (2 tubes, clear cap) |
| AlphaLISA PPI Buffer (5X)*** | 10 mL, 1 small bottle | 100 mL, 1 large bottle |

* Reconstitute XIAP BIR3 protein and biotinylted ligand 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 XIAP BIR3 Binding Kit standard in 100 µL Milli-Q® grade $H_2O$. 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. Extra Standard can be ordered separately (Cat # AL3173S).

AlphaLISA XIAP BIR3 Binding Kit standard reconstitution procedure is different from the one of AlphaLISA XIAP BIR2 Binding Kit standard (included in #AL3172C/F kit). Follow the instruction described for each kit.

*** 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<br>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 XIAP BIR3 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 revvity described below is an ***example*** for generating 1 dose response curve by using the AlphaLISA XIAP BIR3 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 XIAP BIR3 Binding Kit, standard (LCL161):

a. Reconstitute lyophilized AlphaLISA Human XIAP BIR3 Binding Kit, standard (200 µg) in 100 µL DMSO to make a 4 mM stock solution of XIAP BIR3 Binding standard.
b. Prepare serial dilutions of 4X XIAP BIR3 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] (nM) (4X) | [standard] (nM) (1X) |
|---|---|---|---|---|
| A (STD 12) | 10 µL of 4 mM stock solution | 190 µL | 200 000 | 50 000 |
| B (STD 11) | 60 µL of tube A | 140 µL | 60 000 | 15 000 |
| C (STD 10) | 60 µL of tube B | 120 µL | 20 202 | 5 051 |
| D (STD 9) | 60 µL of tube C | 140 µL | 6 061 | 1 515 |
| E (STD 8) | 60 µL of tube D | 120 µL | 2 041 | 510.2 |
| F (STD 7) | 60 µL of tube E | 140 µL | 612.3 | 153.1 |
| G (STD 6) | 60 µL of tube F | 120 µL | 206.0 | 51.5 |
| H (STD 5) | 60 µL of tube G | 140 µL | 61.8 | 15.45 |
| I (STD 4) | 60 µL of tube H | 120 µL | 20.7 | 5.18 |
| J (STD 3) | 60 µL of tube I | 140 µL | 6.3 | 1.58 |
| K (STD 2) | 60 µL of tube J | 120 µL | 2.1 | 0.53 |
| L (STD 1) | 60 µL of tube K | 140 µL | 0.63 | 0.16 |
| M (STD 0) | 0 | 200 µL | 0 | 0 |

3. Preparation of 4X GST tagged XIAP BIR3 protein:

    a. Reconstitute lyophilized GST-tagged XIAP BIR3 protein in 100 µL $H_2O$ to make a 200 nM protein stock solution.
    b. Add 10 µL of the 200 nM GST-tagged XIAP BIR3 protein stock solution to 240 µL of 1X AlphaLISA PPI buffer to obtain a 8 nM working solution of GST XIAP BIR3 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 600 nM stock solution.
    b. Add 10 µL of 600 nM biotinylated ligand to 240 µL 1X AlphaLISA PPI buffer to obtain a 24 nM working solution

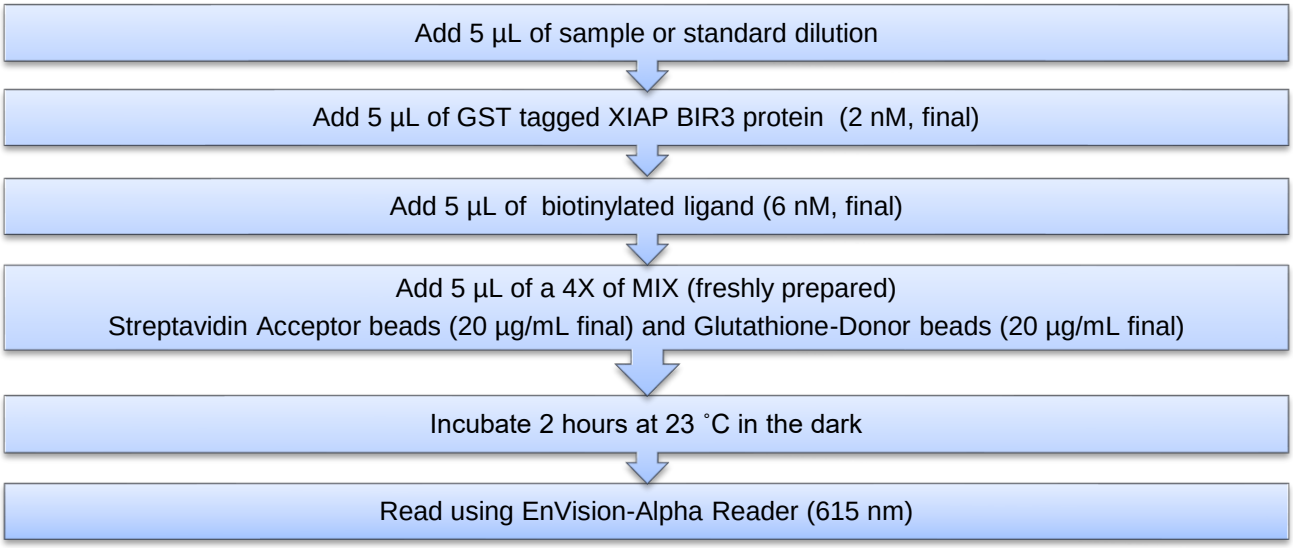
Prepare just before use. Unused Stock solution must be stored at -20°C or below (aliquot > 10 µL). Do not repeat Freeze/Thaws cycles. Do not store working solution.

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

    a. Keep the beads under subdued laboratory lighting.
    b. Add 4 µL of 5 mg/mL Streptavidin Acceptor beads and 4 µL of 5 mg/mL Glutathione Donor beads to 242 µL of 1X AlphaLISA PPI buffer to obtain a 80 µg/mL of Streptavidin Acceptor beads and 80 µg/mL of Glutathione 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:**

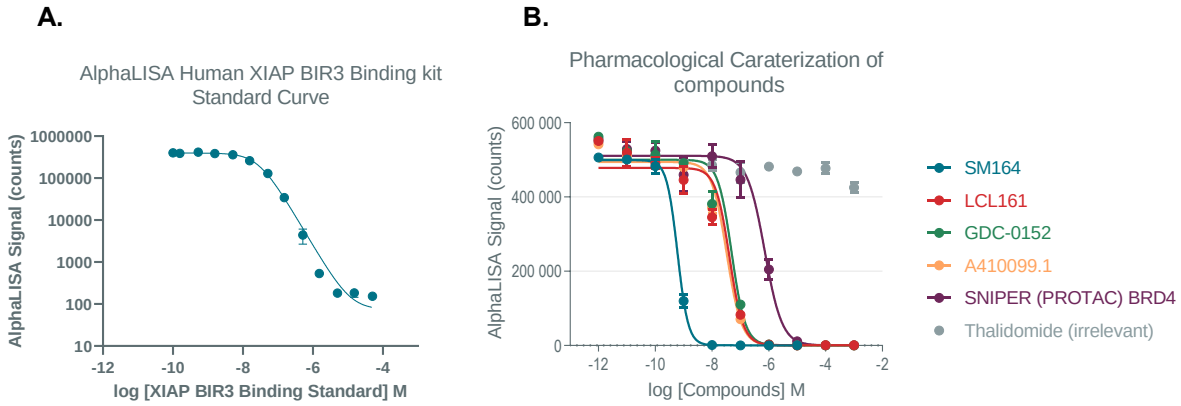



Figure 2. A: Illustration with AlphaLISA Human XIAP BIR3 Binding Kit standard which competitively binds to XIAP BIR3 protein with IC50 of 40 nM. IC50 values were calculated by using a nonlinear regression fitting with GraphPad Prism. B: Pharmacological validation of the ALphaLISA XIAP BIR3 Binding Kit using SM164 (Highly potent bivalent SMAC mimetic), LCL161, GDC-0152, A140099.1 and BRD4 SNIPER (PROTAC based on LCL161 and JQ1 ligands). They competitively bind to XIAP BIR3 domain with IC50 of 0.6, 41, 49, 33, and 712 nM respectively. The irrelevant Thalidomide compound (Cereblon E3Ligase ligand) does not compete as expected.

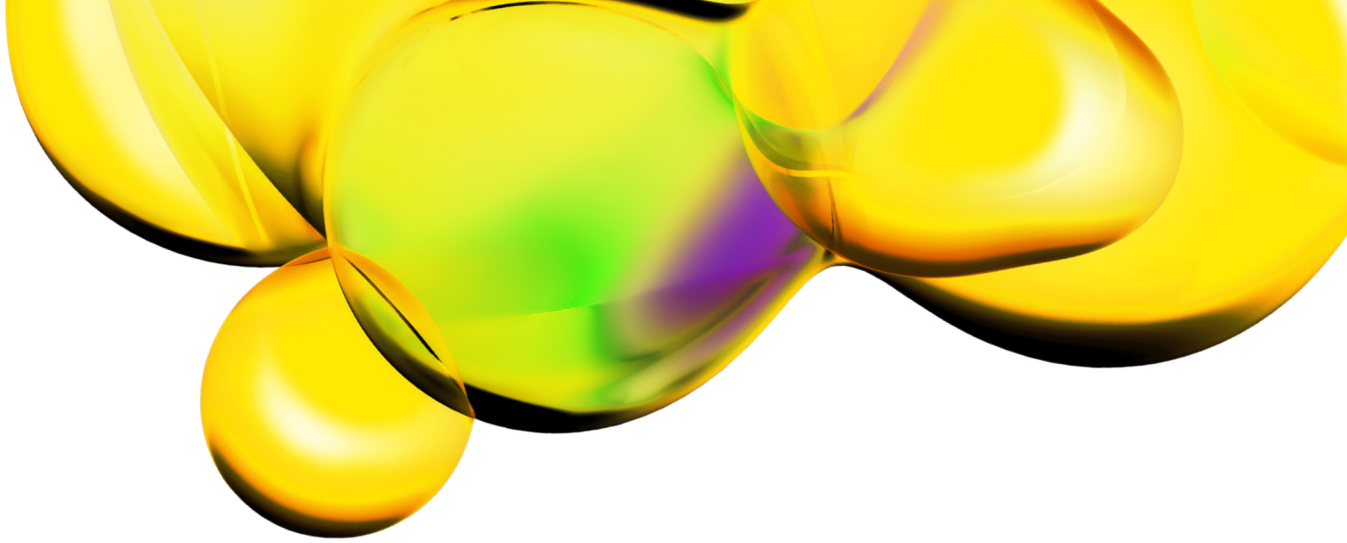
## Troubleshooting Guide

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.<br>• 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

**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES**





**www.revvity.com**

revvity

**Revvity, Inc.**
940 Winter Street
Waltham, MA 02451 USA
**www.revvity.com**

**For a complete listing of our global offices, visit www.revvity.com**