MANUAL

AlphaLISA® Human Cereblon Binding Kit

Product number: AL3147 C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

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

Sensitivity: $IC_{50:}$ 2.2µM (average, using AlphaLISA CRBN Binding kit Standard (Lenalidomide)). To calculate binding affinity (Ki) with the Cheng-Prusoff equation, use Kd_{ligand} of 15nM.

Signal to background ratio: 234 (average) using 3 nM GST-CRBN protein and 20 nM biotinylated ligand

Kit contents: The kit contains 6 components: Glutathione AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, Biotinylated CRBN ligand, GST tagged CRBN protein, AlphaLISA CRBN Binding kit Standard (Lenalidomide) and AlphaLISA PPI buffer.

Storage: The kit components must be stored at 4 ˚C in the dark. Reconstituted GST-tagged CRBN protein can be stored at -80°C and AlphaLISA CRBN Binding kit Standard (Lenalidomide) at -20°C for 14 days. Reconstituted biotinylated CRBN ligand can be stored at -80°C for 14 days. S/B may highly decrease due to a storage at -20°C, or a longer-term storage of the biotinylated CRBN ligand or multiple freeze-thaw cycles.

Stability: This kit is stable for at least 12 months from the manufacturing date when stored in its original packaging (lyophilized) and the recommended storage conditions (+4°C).

Analyte of Interest

Cereblon, also known as CRBN, is involved in many biological processes and is closely associated with proliferation, apoptosis, cell metabolism, and the occurrence of many diseases. Cereblon is also one of the two most popular E3 ligases which is recruited by bifunctional Proteolysis-targeting chimeras (PROTACs) to induce ubiquitination and subsequent proteasomal degradation of a targeted protein. Cereblon interacts with several proteins to form the functional E3 ubiquitin ligase complex, in which CRBN functions as a substrate receptor of the E3 ubiquitin ligase complex and targets proteins for proteolysis.

Identifying new Cereblon ligands is therefore a way to control biological processes involved in oncology, neurodegenerative, or metabolic diseases by inducing the proteasomal-dependent degradation of undesired proteins.

Description of the AlphaLISA Assay

The AlphaLISA detection of CRBN binding uses Glutathione AlphaLisa acceptor beads to capture the GST-tagged CRBN protein and Streptavidin-coated donor beads to capture the biotinylated ligand. Donor beads and acceptor beads come into proximity through ligand binding to CRBN protein. Excitation of the Donor beads provokes 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).

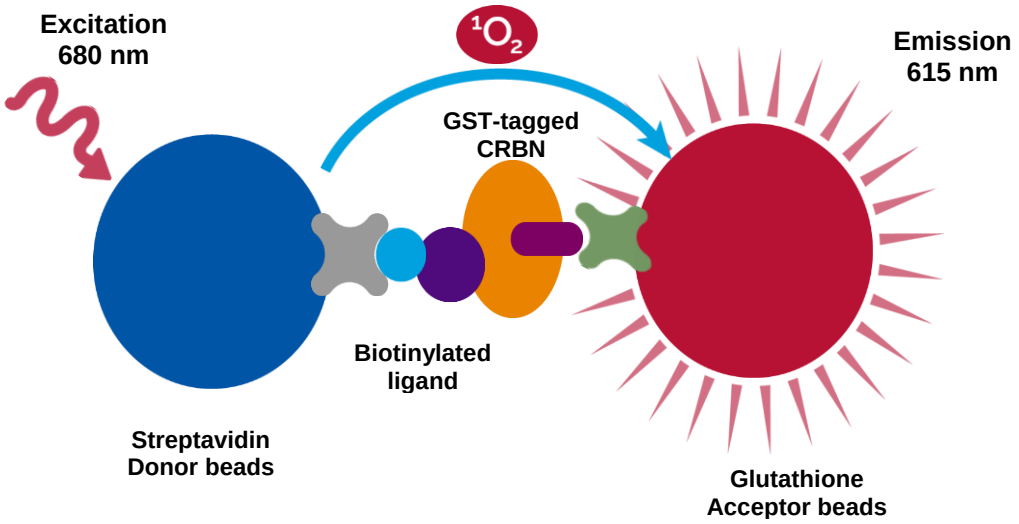


Figure 1. AlphaLISA Assay Principle.

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	AL3147C*** (500 assay points)	AL3147F*** (5000 assay points)
Glutathione AlphaLISA Acceptor beads stored in 50 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween-20, 0.05% Kathon	40 µL @ 5 mg/mL (1 brown tube, white cap)	400 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	400 µL @ 5 mg/mL (1 brown tube, black cap)
Ligand (Biotinylated)*	275 ng lyophilized (1 tube, clear cap)	275 ng lyophilized (10 tubes, clear cap)
CRBN protein (GST tagged)*	2.3 µg lyophilized (1 tube, clear cap)	2.3 µg lyophilized (10 tubes, clear cap)
AlphaLISA CRBN Binding kit Standard (Lenalidomide) *	1.87mg, lyophilized (1 tube, clear cap)	1.87mg, lyophilized (2 tubes, clear cap)
AlphaLISA PPI Buffer (5X)**	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute CRBN protein and ligand in 100 µL Milli-Q® grade H_2O respectively. Reconstitute AlphaLISA CRBN Binding kit Standard (Lenalidomide) in 100µL DMSO. The reconstituted reagents should be used within 60 minutes.

** 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.	-

The following reagents might be required for particular applications:

Item	Supplier	Catalog number
AlphaLISA CRBN Binding kit Standard (Lenalidomide)	Revvity Inc.	AL3147S
Pomalidomide	Tocris	6302
dBRD9	Tocris	6606
VH032	Tocris	6462

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 reagents excepted the AlphaLISA CRBN Binding kit Standard (Lenalidomide). Use DMSO to reconstitute the lyophilized AlphaLISA CRBN Binding kit Standard (Lenalidomide).
- 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 288 assay points in a 20 µL final assay volume per point. 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 to 8 mL H_2O.

2) Preparation of serial dilution of 4X AlphaLISA CRBN Binding kit Standard (Lenalidomide):
   a. Reconstitute lyophilized AlphaLISA CRBN Binding kit Standard (1,87 mg) in 100 µL DMSO to make a 72mM stock solution of AlphaLISA CRBN Binding kit Standard.
   b. Prepare serial dilutions of 4X AlphaLISA CRBN 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	10 µL of 72mM stock solution	170 µL	4000	1000
B	60 µL of tube A	140 µL	1 200	300
C	60 µL of tube B	120 µL	400	100
D	60 µL of tube C	140 µL	120	30
E	60 µL of tube D	120 µL	40	10
F	60 µL of tube E	140 µL	12	3
G	60 µL of tube F	120 µL	4	1
H	60 µL of tube G	140 µL	1	0.3
I	60 µL of tube H	120 µL	0.4	0.10
J	60 µL of tube I	140 µL	0.12	0.03
K	60 µL of tube J	120 µL	0.04	0.01
L	0	140 µL	0	0

3. Preparation of 4X GST tagged CRBN protein:
   a. Reconstitute lyophilized GST-CRBN protein in 100 µL H_2O to make a 300 nM GST-CRBN protein stock solution.
   b. Add 10 µL of the 300nM GST-CRBN protein stock solution to 240 µL of 1X AlphaLISA PPI buffer to obtain a 12nM working solution of GST-CRBN protein.

Prepare just before use.

4. Preparation of 4X biotinylated ligand:

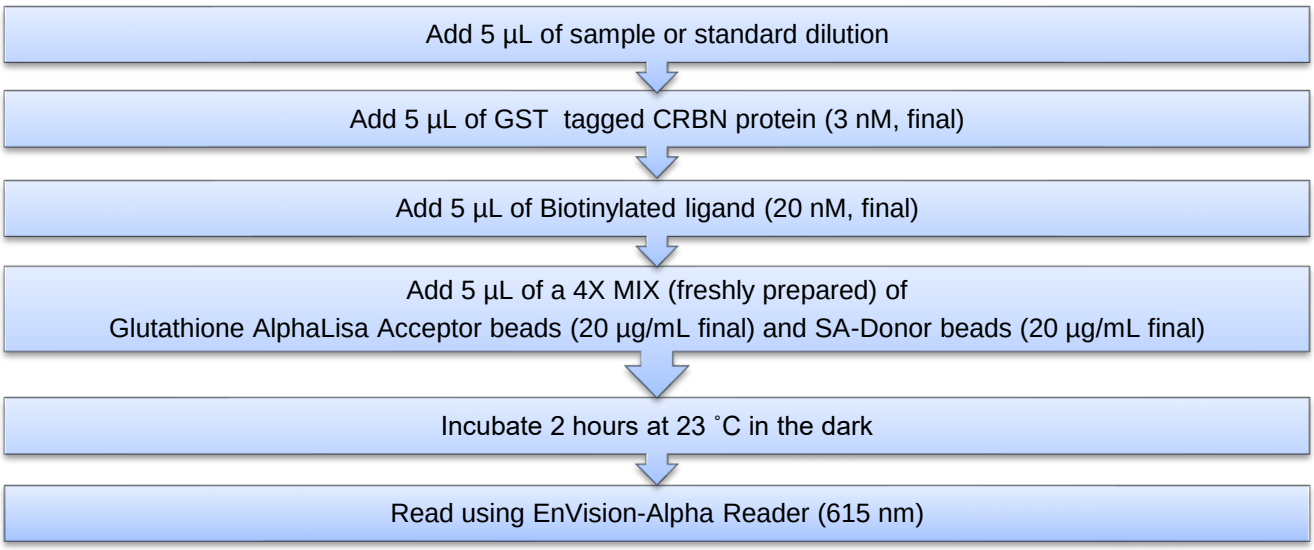
   a. Reconstitute lyophilized biotinylated ligand in 100 µL H_2O to make a 2000 nM stock solution.

   b. Add 10 µL of 2000 nM biotinylated ligand to 240 µL 1X AlphaLISA PPI buffer to obtain a 80nM stock solution of biotinylated ligand.

Prepare just before use.

5. Preparation of the mix of 4X Glutathione AlphaLISA Acceptor beads (20 µg/mL) and 4X Streptavidin (SA) Donor beads (20 µg/mL):
    a. Keep the beads under subdued laboratory lighting.
    b. Add 4 µL of 5 mg/mL Glutathione AlphaLISA Acceptor beads and 4 µL of 5 mg/mL SA-Donor beads to 242 µL of 1X AlphaLISA PPI buffer
    c. 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:

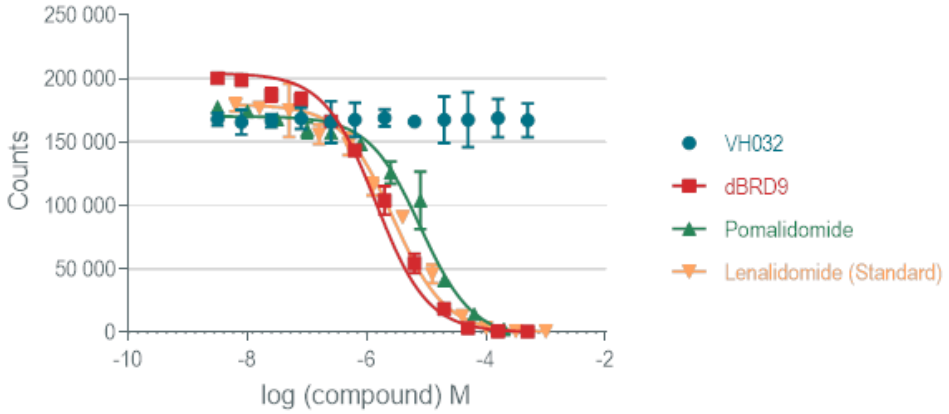


Figure 2. Illustrations with Lenalidomide Standard, Pomalidomide and dBRD9 as competitors, competitively bind to CRBN with Ki =1.5µM, 3.2µM and 0.6µM respectively. VH032 was measured as negative control. All pharmacological values (IC50, Ki) were calculated by using a nonlinear regression fitting with GraphPad Prism 9.

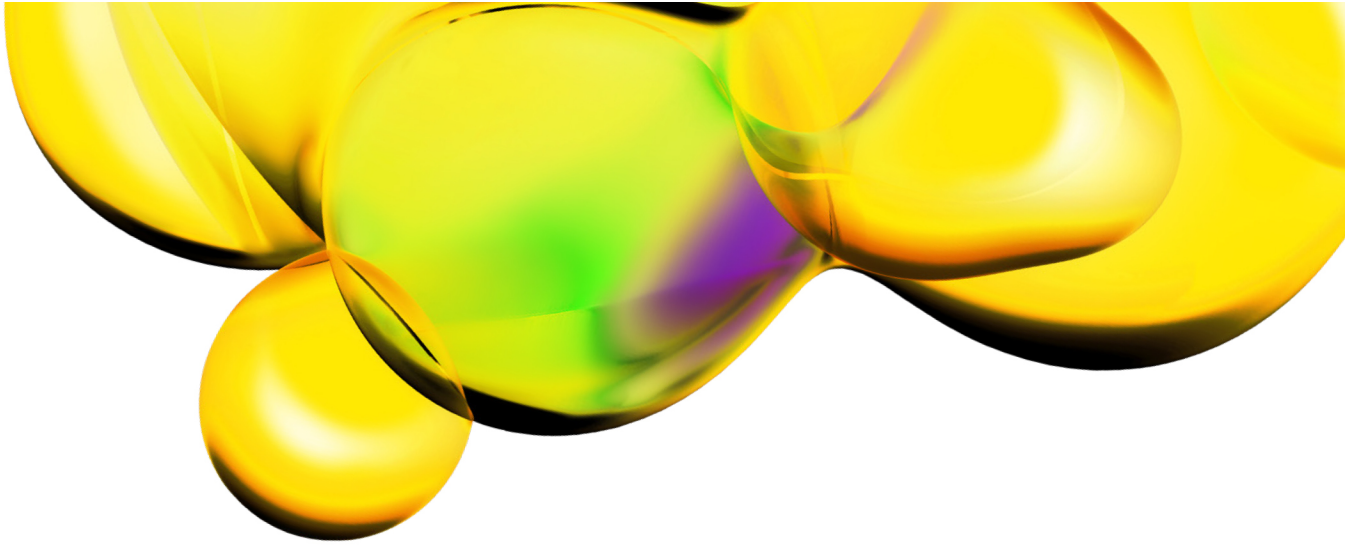
Troubleshooting Guide

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

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



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