MANUAL

AlphaLISA® Human Cereblon Immunoassay Detection Kit

Product number: AL3139HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Material provided:

Format: AL3139HV: 100 assay points AL3139C: 500 assay points AL3139F: 5 000 assay points

The number of assay points is based on an assay volume of 50 µL in 96- or 384-well assay plates using the kit components at the recommended concentrations.

Document version: 1

Product Information

Application: This kit is designed for the quantitative determination of human Cereblon in cell culture/non-human serum, using a homogeneous AlphaLISA assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 30 pg/mL
Lower Limit of Quantification (LLOQ): 102 pg/mL)
EC50: 21 ng/mL

Dynamic Range: 30 – 100 000 pg/mL

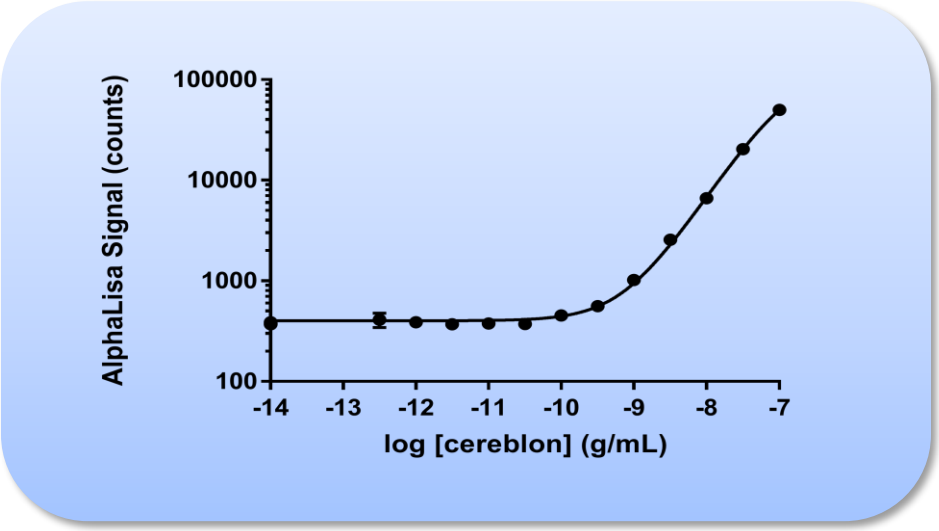


Figure. 1. Typical sensitivity curve in AlphaLISA Immunoassay buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

Storage: Store kit in the dark at 4 °C. For reconstituted analyte aliquot and store at -20 °C. It has been demonstrated that the Cereblon analyte solution is stable for 30 days at -20 °C. Avoid freeze-thaw cycles.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Analyte of Interest

Cereblon (CRBN) is a 49 dDa protein that acts as a ubiquitin E3 ligase. It is involved in development and tissue regulation by regulating amounts of secreted factors such as FGF8 and FGF10. Deregulation of the protein is also involved in cancer. It has also been shown that Cereblon regulate the activity of potassium channels, where deregulation leads to intellectual retardation. Cereblon has been demonstrated as a target for the drug thalidomide. Normally, this would act as an anti-cancer drug. However, serious side effects are present such as altered development of embryos and fetuses leading to malformations.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in buffer, cell culture media, serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in emission at 615 nm (Figure 2).

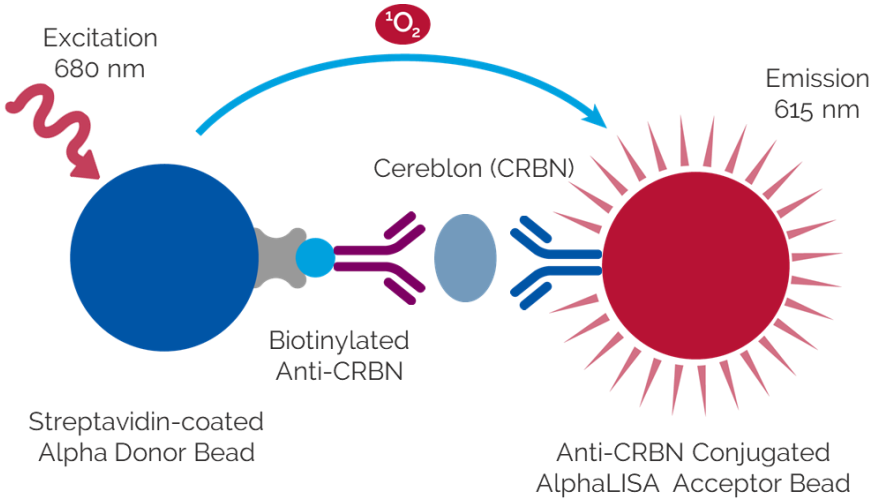


Figure 2. AlphaLISA Cereblon Detection Kit Assay Principle.

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.
- Take precautionary measures to avoid contamination of the reagent solutions.
- The Biotinylated anti-Cereblon antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3139HV (100*** assay points)	AL3139C (500*** assay points)	AL3139F (5000*** assay points)
AlphaLISA Anti-CRBN Acceptor beads stored in PBS, 0.05% Kathon CG/ICP, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, white cap)	50 µL @ 5 mg/mL (1 brown tube, white cap)	500 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 CG/ICP, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	1 000 µL @ 5 mg/mL (2 brown tubes, black cap)
Biotinylated Anti-CRBN Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	20 µL @ 500 nM (1 tube, black cap)	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
Lyophilized CRBN*	0.1 µg (1 tube, clear cap)	0.1 µg (1 tube, clear cap)	0.1 µg (1 tube, clear cap)
AlphaLISA Lysis Buffer (5X)	2 mL, 1 small bottle	2 mL, 1 small bottle	2 mL, 1 small bottle
AlphaLISA Immunoassay Buffer (10X)**	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle

* The thawed analyte should be used within 60 minutes or aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles. It has been demonstrated that the Cereblon analyte solution is stable for at least 1 month at -20°C. One vial contains an amount of Cereblon sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3139S).

** Contains 250 mM HEPES, pH 7.4, 1% Casein, 10 mg/mL Dextran-500, 5% Triton X-100, Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates (AL3139HV) or 50 µL in 96-1/2 area or 384-well assay 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. Note that sodium azide from the Biotinylated anti-Cereblon antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Plus Adhesive Sealing Film	Revvity Inc.	6050185
EnVision®-Alpha Reader	Revvity Inc.	-

Recommendations

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- 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 all reagents by vortexing before use.
- Use Milli-Q® grade H_2O to dilute 5X AlphaLISA Lysis Buffer and to reconstitute the lyophilized analyte.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- 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 Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film in place.
- The AlphaLISA signal is detected with an EnVision Multilabel Plate 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.
- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment in the 1X AlphaLISA Lysis Buffer. Assay Procedure
- The manual described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The manuals also include testing samples in 452 wells. If different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly, as shown in the table below. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.
- The standard dilution manual is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.

		Volume				
Format	**# of data points**	**Final**	**Sample**	**MIX AlphaLISA AccBeads + biotinylated Ab**	**SA-Donor beads**	**Plate recommendation**
AL3139HV	100	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6002290) Light gray ½ AreaPlate-96 (cat # 6002350)
AL3139C	250	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6002290) Light gray ½ AreaPlate-96 (cat # 6002350)
	500	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6002290) Light gray ½ AreaPlate-96 (cat # 6002350) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) Shallow Well AlphaPlate-384 (cat # 6008350) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3139F	5 000	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6005560) Light gray ½ AreaPlate-96 (cat # 6002350) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) Shallow Well AlphaPlate-384 (cat # 6008350) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)

2-step High concentration– Preparation of standards must be done in 1X AlphaLISA Lysis Buffer. Manual described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells).
If different amounts of samples are tested, the volumes of all reagents have to be adjusted accordingly

1) Preparation of AlphaLISA Lysis Buffer 1X:
Add 2 mL of 5X Lysis buffer to 8 mL of Milli-Q® grade H_2O.

2) Preparation of 1X AlphaLISA Immunoassay buffer:
Add 1 mL of 10X Immunoassay buffer to 9 mL of Milli-Q® grade H_2O.

3) Preparation of Cereblon analyte standard dilutions:

   a. Reconstitute lyophilized Cereblon in 100 µL Milli-Q® grade H_2O. The remaining reconstituted analyte should be aliquoted immediately and stored at -20 ºC for future assays.
   b. The reconstituted Cereblon stock solution concentration is 100 000 pg/mL.
   c. Prepare standard dilutions as follows in 1X AlphaLISA **Lysis** Buffer (change tip between each standard dilution):

Tube	Vol. of Human Cereblon (µL)	Vol. of diluent (µL) *	[human Cereblon] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted human Cereblon	90	1.00E-07	100 000
B	60 µL of tube A	140	3.00E-08	30 000
C	60 µL of tube B	120	1.00E-08	10 000
D	60 µL of tube C	140	3.00E-09	3 000
E	60 µL of tube D	120	1.00E-09	1 000
F	60 µL of tube E	140	3.00E-10	300
G	60 µL of tube F	120	1.00E-10	100
H	60 µL of tube G	140	3.00E-11	30
I	60 µL of tube H	120	1.00E-11	10
J	60 µL of tube I	140	3.00E-12	3
K	60 µL of tube J	120	1.00E-12	1
L	60 µL of tube K	140	3.00E-13	0.3
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

* Dilute standards in 1X AlphaLISA Lysis Buffer diluent exclusively
At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

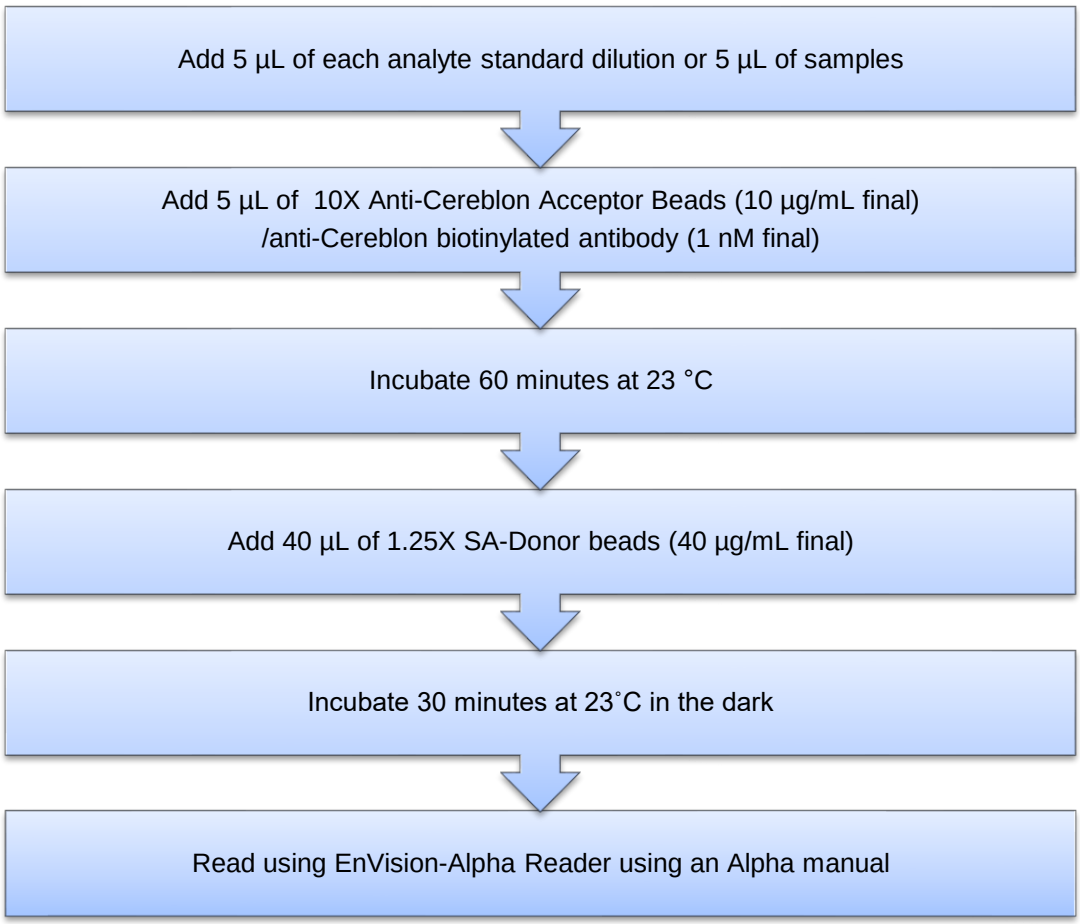
4) Preparation of 10X MIX AlphaLISA Anti-Cereblon Acceptor beads (100 µg/mL) + Biotinylated Anti-Cereblon Antibody (10 nM):

   a. Prepare just before use.
   b. Add 50 µL of 5 mg/mL AlphaLISA Anti-Cereblon Acceptor Bead and 50 µL of 500 nM Biotinylated Anti-Cereblon Antibody to 2400 µL of 1X AlphaLISA Immunoassay Buffer.

5) Preparation of 1.25X Streptavidin (SA) Donor beads (50µg/mL):

   a. Prepare just before use.
   b. Keep the beads under subdued laboratory lighting.
   c. Add 200 µL of 5 mg/mL SA-Donor beads to 19 900 µL of 1X AlphaLISA Immunoassay Buffer.

6) In a recommended plate (here for 384 wells):



Data Analysis

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the **4-Parameter Logistic equation** (sigmoidal dose-response curve with variable slope) and a **$1/Y^2$ data weighting** (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).
- If samples have been diluted, the concentration must be multiplied by the additional dilution factor.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined a 3-step manual using AlphaLISA Immunoassay as assay buffer. The analytes (standards) were prepared in Lysis buffer, DMEM, DMEM + 10% FBS, RPMI, RPMI + 10% FBS and FBS. All other components were prepared in Immunoassay buffer.

Sensitivity:

The LDL and LLOQ were calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL using the recommended assay conditions.

LDL (pg/mL)	Buffer/Media used	# of experiments
30	AlphaLisa Lysis Buffer	6
27	DMEM	6
48	DMEM+ 10% FBS	6
54	RPMI	6
69	RPMI + 10% FBS	6
85	FBS	6

* Note that LDL/ LLOQ can be decreased (i.e. sensitivity increased) by increasing the volume of analyte in the assay (e.g. use 10 µL of analyte in a final assay volume of 50 µL).

- Assay Precision:

The following assay precision data were calculated from the three independent assays using two different kit lots. In each lot, the analytes were prepared in Lysis buffer, DMEM, DMEM + 10% FBS, RPMI, RPMI + 10% FBS and FBS. All other components were prepared in Immunoassay buffer.

Each assay consisted of one standard curve comprising 12 data points (each in triplicate) and 12 background wells (no analytes). The assays were performed in 384-well plate format.

- Intra-assay precision:

The intra-assay precision was determined using a total of 12 independent determinations in triplicate. Shown as CV%.

Human Cereblon (ng/ml)	Lysis	DMEM	DMEM + 10% FBS	RPMI	RPMI + 10% FBS	FBS
CV% intraassay	1.7	4.2	10	13	10.8	6.9

- Inter-assay precision:

The inter-assay precision was determined using a total of 12 independent determinations with 6 measurements for 300 pg/mL sample. Shown as CV%.

Human Cereblon (ng/ml)	Lysis	DMEM	DMEM + 10% FBS	RPMI	RPMI + 10% FBS	FBS
CV% interassay	9.9	3	7	11	6.8	10.8

- Spike Recovery:

Three known concentrations of analyte were spiked in a cell culture media with or without 10% FBS and AlphaLISA Lysis Buffer. All samples, including non-spiked culture media and AlphaLISA Lysis Buffer were measured in the assay. Values calculated for control spiked samples in AlphaLISA Lysis Buffer considered as 100% recovery. The % in cell culture media vs. expected (control spike value) was calculated for each concentration. The average recovery from two independent measurements is reported.

Spike (Cereblon ng/mL)	% Recovery					
	AlphaLISA Lysis Buffer	DMEM	DMEM + 10% FBS	RPMI	RPMI + 10% FBS	FBS
10	92	105	87	108	78	80
3	110	104	85	103	84	77
1	114	89	91	119	88	96

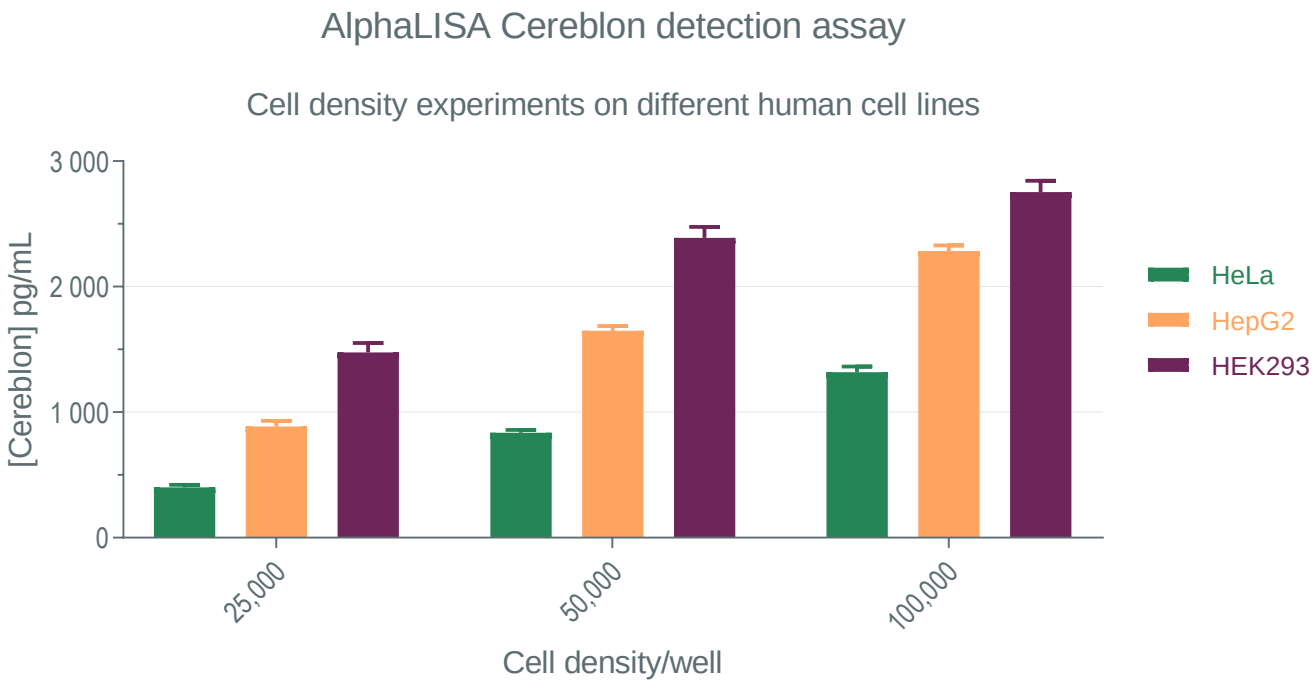
Cell based assays:

Cereblon expression levels in cell lines.

Levels of Cereblon was tested in 25k, 50k and 100k cells from three different human cell lines commonly used in cell research:

- HeLa (cervical cancer)
- HepG2 (liver cancer)
- Hek293 (embryonic kidney)

The human cell lines HEK293, HepG2 and HeLa were seeded in 96-well plates at different cell densities and cultured for 24h. After medium removal, the cells were lysed with 50 µL of AlphaLISA lysis buffer 1X for 30 minutes at RT and 5 µL of cell lysates were transferred into an AlphaPlate-384 light gray (#6005350) before adding AlphaLISA Cereblon detection reagents following the kit's manual. The AlphaLISA signal was recorded on a VICTOR® Nivo™ Multimode Microplate Reader and interpolated on the AlphaLISA Cereblon standard curve performed in parallel to determine Cereblon concentrations into samples.

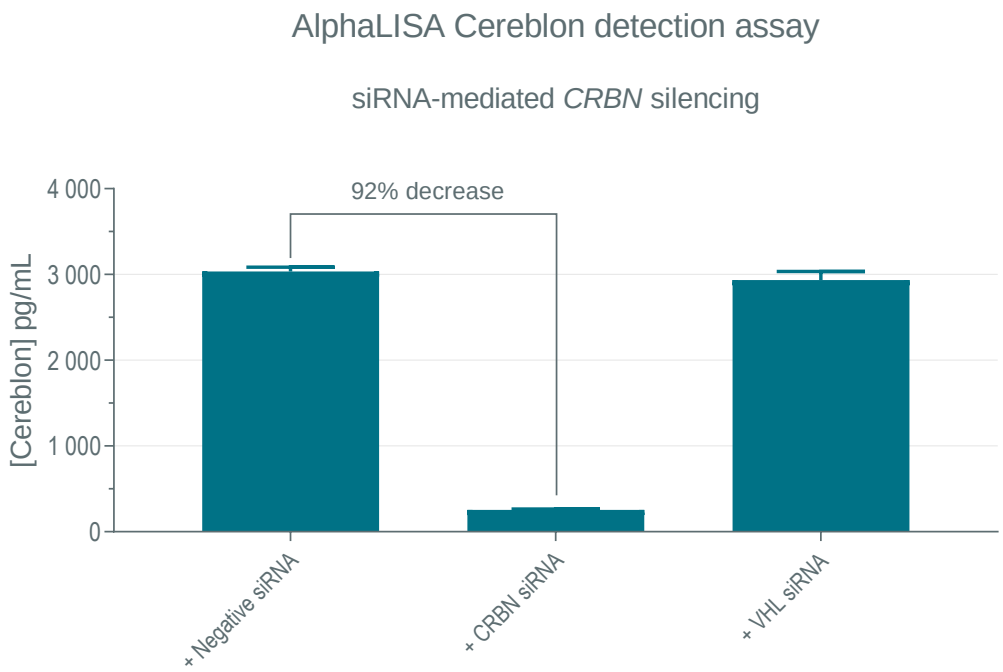


Modulation of expression by siRNA

Expression of Cereblon in HEK293 cells was modulated by incubation of 50 000 cells with siRNA to:

- siRNA to CRBN gene (Cereblon)
- siRNA to VHL gene (von Hippel-Lindau)
- negative control siRNA (random sequence)

HEK293 cells were plated in a 96-well plate (50,000 cells/well) and cultured for 24h. The cells were then transfected with siRNAs specific for the E3 ligases Cereblon or VHL (Von Hippel–Lindau), and with a negative control siRNA. After 48h incubation, the medium was removed, the cells were lysed with 50 µL of AlphaLISA lysis buffer 1X for 30 minutes at RT, and 5 µL of cell lysates were transferred into an AlphaPlate-384 light gray (#6005350) before adding AlphaLISA Cereblon detection reagents following the kit's manual. The AlphaLISA signal was recorded on a VICTOR® Nivo™ Alpha-reade VICTOR® Nivo™ Multimode Microplate Reader r and interpolated on the AlphaLISA Cereblon standard curve performed in parallel to determine Cereblon concentrations into samples.

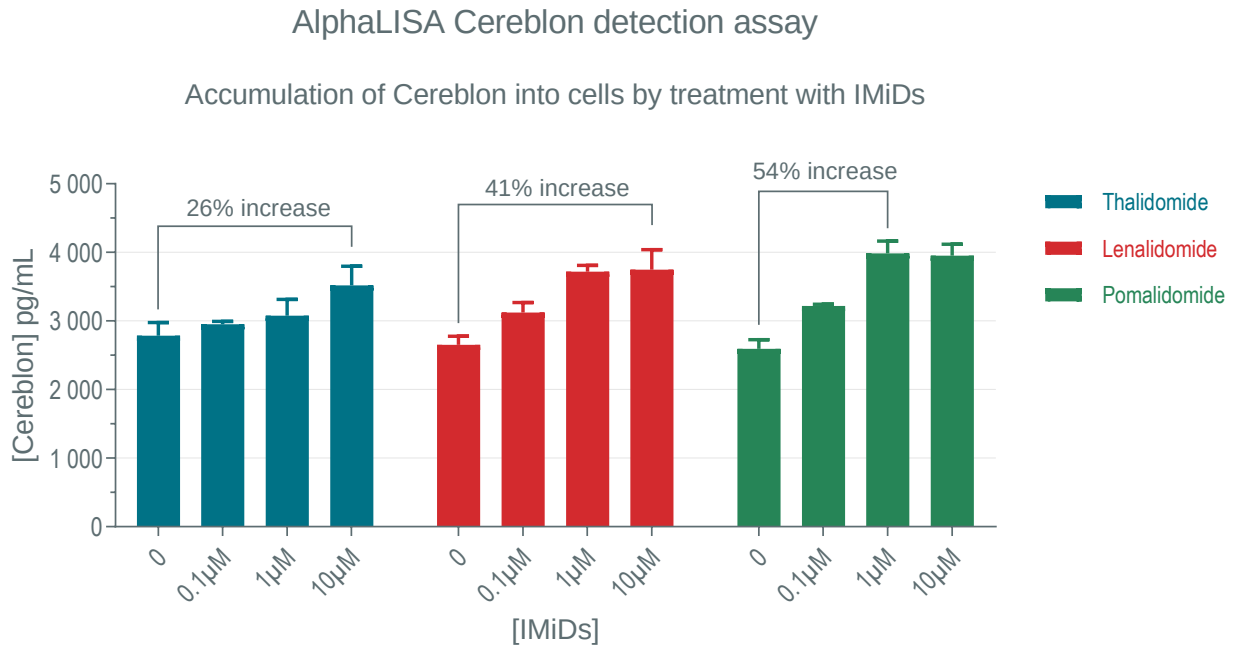


Modulation of Cereblon by drugs of the thalidomide family.

Effects of drugs were tested on 50 000 HEK293 cells with the following drugs:

- Thalidomide
- Lenalidomide
- Pomalidomide

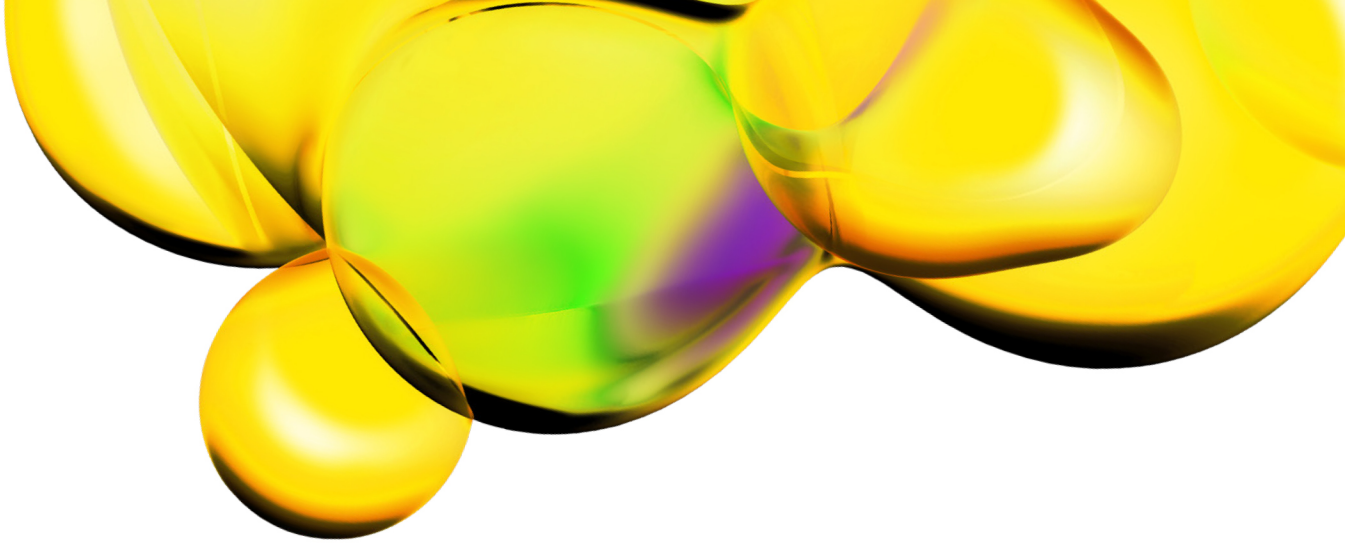
HEK293 cells were seeded in a 96-well plate (50,000 cells/well) and incubated for 6h. The cells were then treated with 0.1, 1 and 10 µM of the IMiDs Thalidomide (TOCRIS, #0652), Lenalidomide (TOCRIS, #6305), and Pomalidomide (TOCRIS, #6302). After an overnight incubation, the medium was removed, the cells were lysed with 50 µL of AlphaLISA lysis buffer 1X for 30 minutes at RT, and 5 µL of cell lysates were transferred into an AlphaPlate-384 light gray (#6005350) before adding AlphaLISA Cereblon detection reagents following the kit's manual. The AlphaLISA signal was recorded on a VICTOR® Nivo™ Multimode Microplate Reader and interpolated on the AlphaLISA Cereblon standard curve performed in parallel to determine Cereblon concentrations into samples.



Troubleshooting Guide

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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