MANUAL

AlphaLISA® Human MDM2 Binding Kit

Product number: AL3168 C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed to assess orthosteric binders of Human MDM2 protein using a homogeneous no wash AlphaLISA binding assay.

Sensitivity: IC_{50}: 3.3nM (average, using AMG-232 as AlphaLISA Human MDM2 Binding Kit, Standard). To calculate binding affinity (Ki) with the Cheng-Prusoff equation, use Kd_{ligand} of 3nM.

Signal to background ratio: 156 (average) using 10 nM of Human GST-MDM2 protein and 5 nM of biotinylated ligand.

Kit contents: The kit contains 6 components: Glutathione AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, Biotinylated MDM2 ligand, Human GST-tagged MDM2 protein, AlphaLISA Binding Assay buffer and the AlphaLISA Human MDM2 Binding Kit, Standard (AMG-232).

Storage: The kit components must be stored at 4 ˚C in the dark. Reconstituted reagents can be aliquoted (not under 10µL) then frozen, and can be stored at -20°C or 80°C for 28 days. Avoid 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

MDM2, also known as murine double minute 2, is involved in many biological processes and is closely associated with DNA repair, cell cycle arrest, apoptosis, and the occurrence of many diseases.

MDM2 is one of the most popular E3 ligases which is recruited by bifunctional Proteolysis-targeting chimeras (PROTACs) to induce ubiquitination and subsequent proteasomal degradation of a targeted protein.

MDM2 interacts with several proteins to form the functional E3 ubiquitin ligase complex, in which MDM2 functions as a substrate receptor of the E3 ubiquitin ligase complex and targets various proteins to proteolysis.

Therefore, identifying new PROTAC MDM2 ligands can improve selective proteasomal-dependent degradation of proteins of interest, involved in the onset of diseases such as cancers, and neurodegenerative and metabolic disorders.

Description of the AlphaLISA Assay

The AlphaLISA detection of MDM2 binders uses Glutathione AlphaLISA acceptor beads to capture the GST-tagged Human MDM2 protein and Streptavidin-coated donor beads to capture the biotinylated ligand. Donor beads and acceptor beads come into proximity through ligand binding to Human MDM2 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).

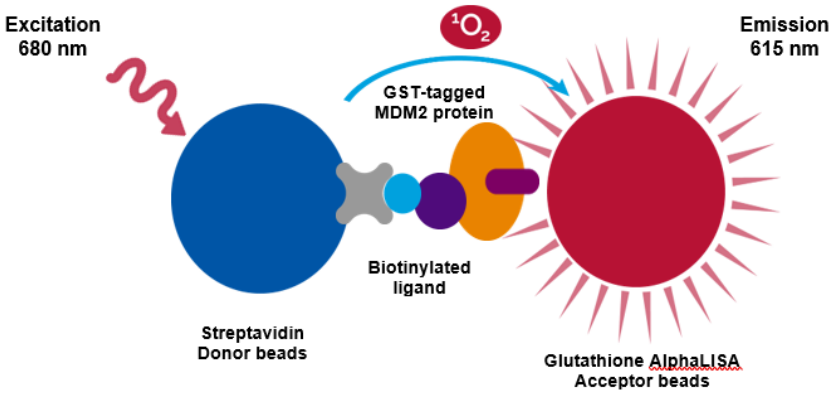


Figure 1. AlphaLISA Human MDM2 Assay Principle.

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	AL3168C*** (500 assay points)	AL3168F*** (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 CG/ICP II	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 CG/ICP II, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	400 µL @ 5 mg/mL (1 brown tube, black cap)
Biotinylated MDM2 ligand*	75,3 ng lyophilized (1 tube, clear cap)	75,3 ng lyophilized (10 tubes, clear cap)
Human MDM2 protein (GST-tagged)*	8,2 µg lyophilized (1 tube, clear cap)	8,2 µg lyophilized (10 tubes, clear cap)
AlphaLISA Human MDM2 Binding Kit, standard (AL3168S)*	4,55 µg lyophilized (1 tube, clear cap)	4,55 µg lyophilized (2 tubes, clear cap)
AlphaLISA Binding Assay Buffer (10X)**	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute Human MDM2 protein, Standard and ligand in 100 µL Milli-Q® grade H_2O respectively. The reconstituted reagents should be used within 60 minutes.

** Extra buffer can be ordered separately (cat # AL018C: 10 mL, cat # AL018F: 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 10X AlphaLISA Binding Assay Buffer and to reconstitute the lyophilized 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 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 Human MDM2 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 Binding Assay Buffer (for 10 mL):

Add 1 mL of 10X AlphaLISA Binding Assay buffer and 9 mL of MilliQ water.

2. Preparation of AlphaLISA Human MDM2 Binding Kit, standard (AL3168S) :

a. Reconstitute lyophilized AlphaLISA Human MDM2 Binding Kit, standard (4,55 µg) in 100 µL H20 to make a 80µM stock solution of AlphaLISA Human MDM2 Binding Kit, standard.
b. Prepare serial dilutions of 4X Standard in 1x AlphaLISA Binding Assay 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 reconstitution standard (80µM)	190 µL	**4**	1
B	60 µL of tube A	140 µL	**1**	0.3
C	60 µL of tube B	120 µL	**0**	0.1
D	60 µL of tube C	140 µL	**0.12**	0.03
E	60 µL of tube D	120 µL	**0.04**	0.01
F	60 µL of tube E	140 µL	**0.01**	0.003
G	60 µL of tube F	120 µL	**0.004**	0.001
H	60 µL of tube G	140 µL	**0.0012**	0.0003
I	60 µL of tube H	120 µL	**0.0004**	0.0001
J	60 µL of tube I	140 µL	**0.00012**	0.00003
K	60 µL of tube J	120 µL	**0.00004**	0.00001
L	0	140 µL	0	0

3. Preparation of Human GST-tagged MDM2 protein:

    a. Reconstitute lyophilized Human MDM2 protein in 100 µL H_2O to make a 1000 nM Human MDM2 protein stock solution.
    b. Add 10 µL of the 1000 nM MDM2 stock solution to 240 µL of 1X AlphaLISA Binding Assay buffer to obtain a 40nM working solution of Human MDM2 protein.

Prepare just before use.

4. Preparation of Biotinylated MDM2 ligand:

    a. Reconstitute the lyophilized biotinylated ligand in 100 µL H_2O to make a 500 nM stock solution.

    b. Add 10 µL of 500 nM biotinylated ligand to 240 µL 1X AlphaLISA Binding Assay buffer to obtain a 20nM stock solution of biotinylated ligand.

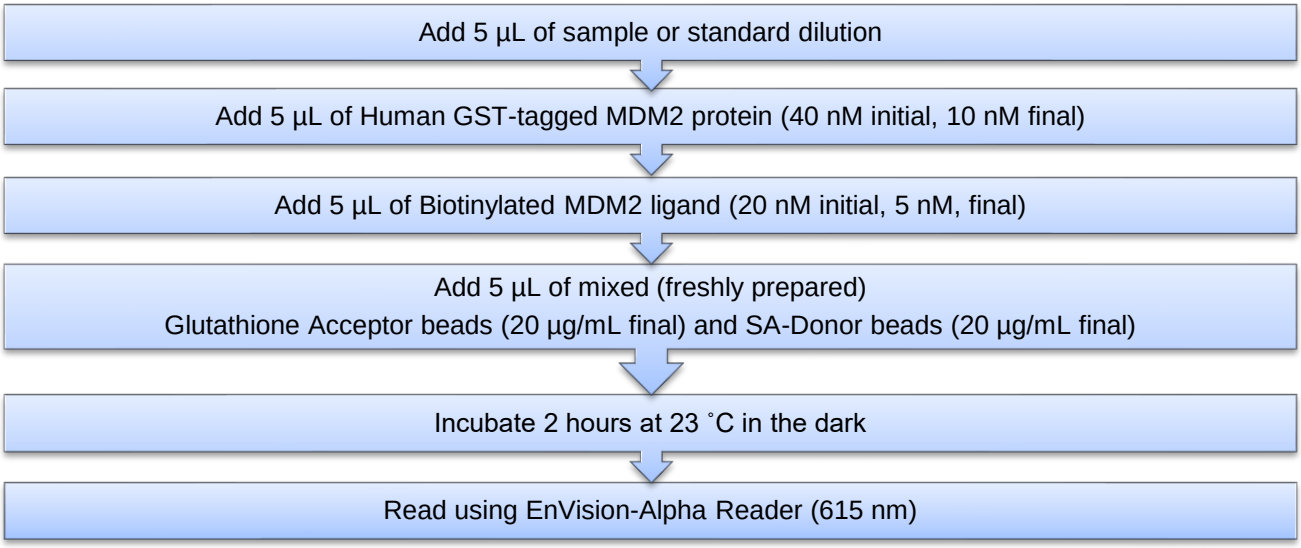
Prepare just before use.

5. Preparation of the mix of Glutathione Acceptor beads and Streptavidin (SA) Donor beads:

    a. Keep the beads under subdued laboratory lighting.
    b. Add 4 µL of 5 mg/mL Glutathione Acceptor beads and 4 µL of 5 mg/mL SA-Donor beads to 242 µL of 1X AlphaLISA Binding Assay buffer

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:

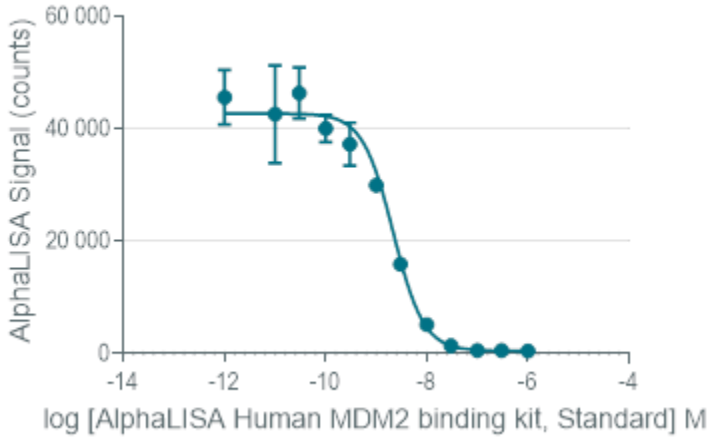


Figure 2. Illustration with AlphaLISA Human MDM2 Binding Kit, standard (ref AL3168S) which competitively binds to Human MDM2 with IC50 =1.95 nM. IC50 value was calculated by using a nonlinear regression fitting with GraphPad Prism.

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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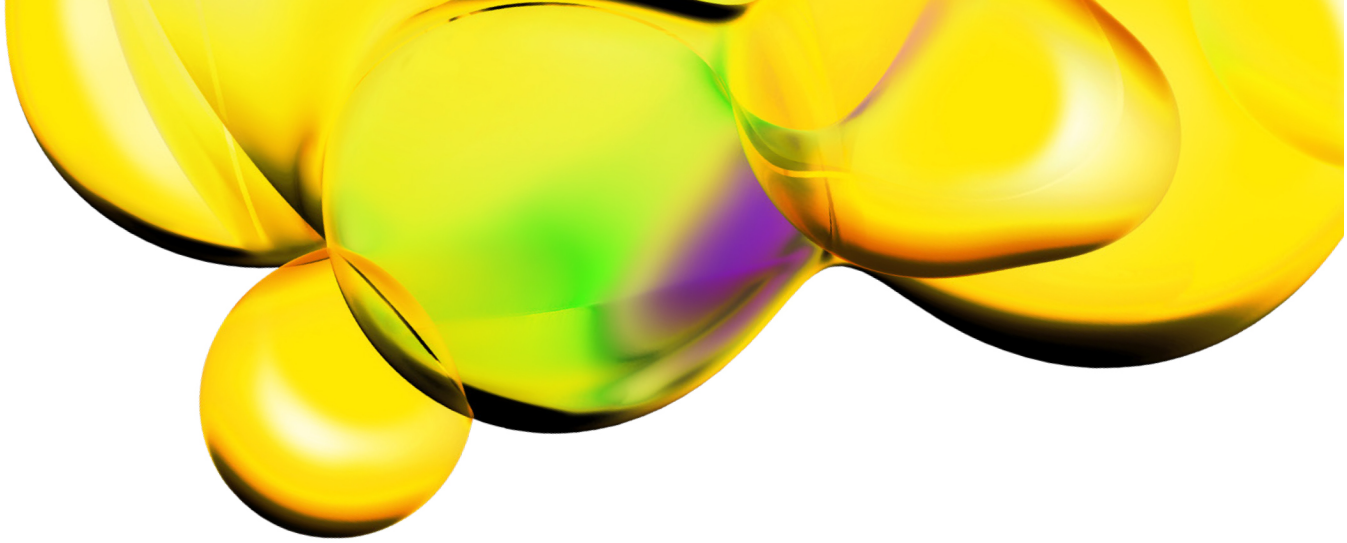

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