Description

The MDM2-Driven p53 Ubiquitination Assay kit is a sensitive AlphaLisa® high-throughput screening (HTS) assay designed to measure MDM2 (mouse double minute 2 homolog) E3 ligase activity in a homogeneous 384 reaction format. The assay kit comes with enough biotinylated ubiquitin, ATP, FLAG-tagged p53, assay buffer, detection buffer, purified UBE1 (E1), UbcH5b (E2), and MDM2 (E3) for 384 reactions. The assay can detect mono-ubiquitination and poly-ubiquitination of p53.

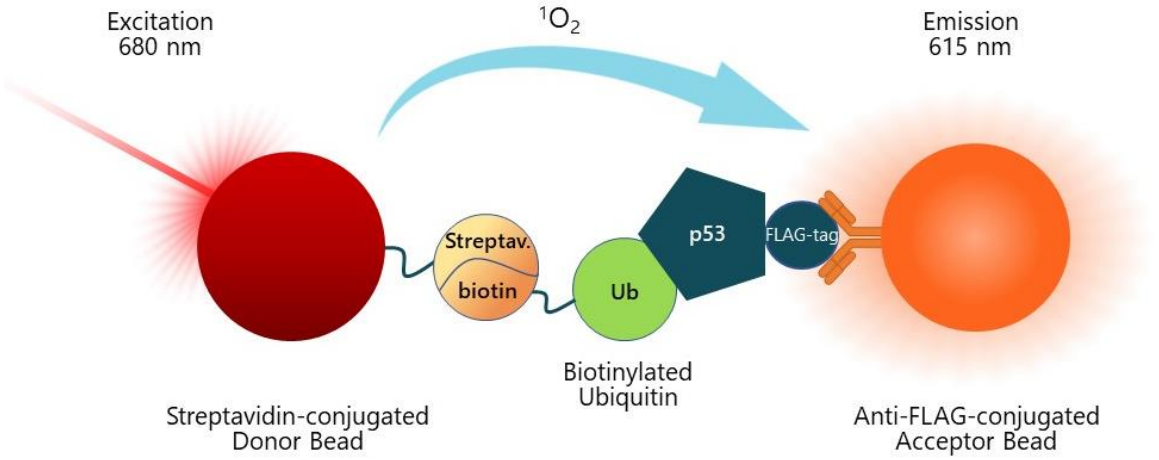

Figure 1: MDM2-Driven p53 Ubiquitination Assay Kit schematic.

E1 and E2 enzymes are incubated with the E3 complex and FLAG-tagged p53, in the presence of biotin-conjugated ubiquitin and ATP. Ubiquitination of p53 occurs in a multistep ubiquitin transfer from E1 to E2 to E3, and E3-mediated conjugation of ubiquitin to p53. Next, acceptor beads are added, followed by streptavidin-conjugated donor beads. Alpha-counts are then measured. The increase in Alpha-counts is proportional to the mono- or poly-ubiquitination of the FLAG-tagged p53.

Background

Covalent conjugation to ubiquitin (Ub) is one of the major post-translational modifications regulating protein stability, function, and localization. Ubiquitination is the concerted action of three enzymes: a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub ligase (E3). The specificity and efficiency of ubiquitination are largely determined by the E3 enzyme, which directs the last step of the Ub-conjugating cascade by binding to both an E2~Ub conjugate and a substrate protein. This step ensures the transfer of Ub from E2~Ub to the substrate, leading to its mono- or poly-ubiquitination.

The p53 tumor suppressor protein is regulated by its interaction with MDM2, which serves as a ubiquitin ligase (E3) to target p53 for degradation. MDM2 ubiquitinates p53, resulting in the rapid degradation of p53 through the Ub–proteasome pathway. MDM2-mediated destabilization and inactivation of p53 are thought to play a critical role in several human cancers. The disruption of the MDM2-p53 interaction has been regarded as an attractive strategy for anticancer drug discovery.

Applications

- Screen molecules that inhibit MDM2 Ubiquitin ligase activity in drug discovery high-throughput screening (HTS) applications,
- Determine compound IC_{50}.
- Perform MDM2-driven ubiquitination studies.

Supplied Materials

Catalog #	Name	Amount	Storage
100402	UBE1 (UBA1), GST-Tag*	25 µg	-80°C
80314	UBCH5b, His-Tag (Human)*	50 µg	-80°C
100409	MDM2, GST-Tag*	1 µg	-80°C
100412	p53, FLAG-Tag*	2 x 2 µg	-80°C
	Biotin-Ubiquitin	400 µl	-80°C
	10 mM ATP	400 µl	-80°C
	U2 Assay Buffer	2 x 10 ml	-80°C
	4x U2 Detection Buffer	2 x 2 ml	-20°C

**The initial concentration of enzyme is lot-specific and will be indicated on the tube containing the protein.*

Materials Required but Not Supplied

Name	Ordering Information
AlphaLISA® anti-FLAG acceptor beads	Perkin Elmer #AL112C
AlphaScreen® Streptavidin-conjugated donor beads	Perkin Elmer #6760002S
Optiplate-384	Perkin Elmer #6007290
AlphaScreen® microplate reader	
DNAse free water	
Orbital Shaker	

Storage Conditions

This assay kit will perform optimally for up to **6 months** from date of receipt when the materials are stored as directed.

Safety

This product is for research purposes only and not for human or therapeutic use. This product should be considered hazardous and is harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly.

Contraindications

- The MDM2-Driven p53 Ubiquitination Assay Kit is compatible with up to 1% final DMSO concentration.
- Green and blue dyes that absorb light in the AlphaScreen® signal emission range (λ=520-620 nm), such as Trypan Blue, interfere with the assay.
- Avoid using potent singlet oxygen quenchers such as sodium azide (NaN_3) or metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Ni^{2+}).
- The presence of the culture medium RPMI 1640 at >1% leads to signal reduction due to the presence of excess biotin and iron in this medium. Media such as MEM, which lacks these components, does not affect AlphaScreen® assays.

Assay Protocol

- All samples and controls should be tested in duplicate.
- The assay should include "Blank", "Positive Control", "Negative Control" and "Test Inhibitor" conditions.
- We recommend maintaining the diluted proteins on ice during use.
- For detailed information on protein handling please refer to Protein FAQs (bpsbioscience.com).
- We recommend preincubating inhibitors with p53 and/or MDM2 as appropriate before initiation of the reaction.

Step 1:

1. Thaw **U2 Assay Buffer**, **ATP, Biotin-Ubiquitin**, **UBE1**, **UbcH5b**, **MDM2,** and **p53** on ice. Briefly spin the tubes to recover their full content.

2. Dilute proteins, as follows, and keep on ice:

   a. Dilute UBE1 with U2 Assay Buffer to 23 ng/µl (160 nM - the final concentration in the reaction will be 40 nM) (2.5 µl/well).

   b. Dilute UbcH5b with U2 Assay Buffer to 90 ng/µl (5 µM – the final concentration in the reaction will be 500 nM) (1 µl/well).

   c. Dilute MDM2 with U2 Assay Buffer to 0.83 ng/µl (10 nM – the final concentration in the reaction will be 1 nM) (1 µl/well).

   d. Dilute p53 with U2 Assay Buffer to 4.6 ng/µl (100 nM – the final concentration in the reaction will be 10 nM) (1 µl/well).

3. Prepare the Test Inhibitor (2.5 µl/well): for a titration prepare serial dilutions at concentrations 4-fold higher than the desired final concentrations. The final volume of the reaction is 10 µl.

   3.1 If the Test Inhibitor is water-soluble, prepare serial dilutions in the U2 Assay Buffer, 4-fold more concentrated than the desired final concentrations.

   For the positive and negative controls, use U2 Assay Buffer (Diluent Solution).

   OR

   3.2 If the Test inhibitor is soluble in DMSO, prepare the test inhibitor at 100-fold the highest desired concentration in 100% DMSO, then dilute the inhibitor 25-fold in U2 Assay Buffer to prepare the highest concentration of the 4-fold intermediate dilutions. The concentration of DMSO is now 4%.

   Prepare serial dilutions of the Test Inhibitor at 4-fold the desired final concentrations using 4% DMSO in U2 Assay Buffer to keep the concentration of DMSO constant.

For positive and negative controls, prepare 4% DMSO in water (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

Note: The final concentration of DMSO should not exceed 1%.

4. Make a Master Mix (5 µl/well, except "Blank" wells): N wells × (1 µl of Biotin-Ubiquitin + 1 µl of 10 mM ATP + 1 µl of diluted UbcH5b + 1 µl of diluted MDM2 + 1 µl of diluted P53).

5. Add 5 µl of Master Mix to the "Negative Control", "Positive Control" and "Test Inhibitor" wells.

6. Make a p53/MDM2-Deficient Master Mix for the "Blank" wells (7.5 µl/ well): N wells x (1 µl of Biotin-Ubiquitin +1 µl 10 mM ATP + 1 µl of diluted UbcH5b + 2.5 µl of Diluent Solution + 2 µl of U2 Assay Buffer).

7. Add 7.5 µl of p53/MDM2-Deficient Master Mix to the "Blank" wells.

8. Add 2.5 µl of inhibitor solution to each well designated "Test Inhibitor".

9. Add 2.5 µl of the Diluent Solution to the "Positive Control" and "Negative Control" wells.

10. Add 2.5 µl of U2 Assay Buffer to the wells designated "Negative Control".

11. Initiate the reaction by adding 2.5 µl of diluted **UBE1** to the wells labeled "Positive Control", "Test Inhibitor" and "Blank."

12. Cover the plate with a plate lid and incubate the reaction at Room Temperature (RT) for one hour.

	Test Inhibitor	Negative Control	Positive Control	Blank
Master Mix	5 µl	5 µl	5 µl	-
P53/MDM2 Deficient Master Mix	-	-	-	7.5 µl
Test Inhibitor	2.5 µl	–	–	-
Diluent Solution	–	2.5 µl	2.5 µl	-
U2 Assay Buffer	–	2.5 µl	–	-
Diluted UBE1 (160 nM)	2.5 µl	–	2.5 µl	2.5 µl
Total	**10 µl**	**10 µl**	**10 µl**	

Note: Protect your samples from direct exposure to light for steps 2 and 3

Step 2:

1. Dilute **4x U2 Detection Buffer** 4-fold with DNAse-free water to make 1x U2 Detection Buffer. Prepare only the amount required for the assay.

2. Dilute AlphaLISA® a-FLAG acceptor beads 250-fold with 1x U2 Detection Buffer and mix well.

3. Add 10 µl of acceptor bead mix to each well.

4. Agitate on an orbital shaker for 30 minutes at RT.

Step 3:

1. Dilute Streptavidin-conjugated donor beads 125-fold with 1x U2 Detection Buffer.
2. Add 10 µl to each well.
3. Agitate on an orbital shaker for 15-30 minutes* at RT.

   **The Signal-to-Noise ratio depends greatly on the performance of the beads from PerkinElmer. Duration of incubation may be extended for some lots of the beads, if necessary.*

4. Read Alpha-counts on an AlphaScreen® microplate reader.
5. The "Blank" control might be important to determine the background A-screen counts in the assay. The blank value should be subtracted from all other values.

Example Results

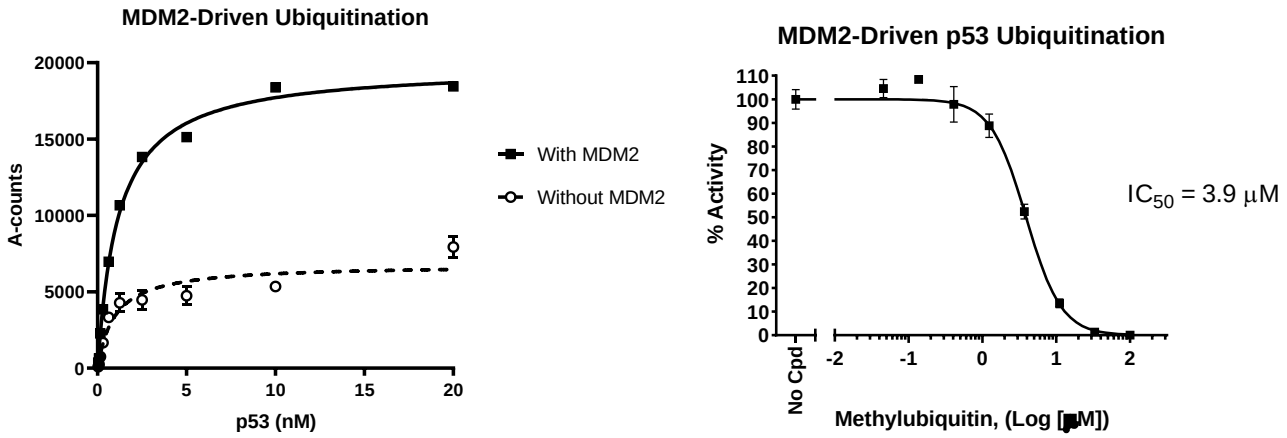

Figure 1: MDM2-driven p53 ubiquitination.
Left: MDM2-driven ubiquitination of p53 was measured with increasing amounts of p53 in the presence and absence of MDM2.
Right: MDM2-dependent ubiquitination of p53 was measured in the presence of increasing concentrations of methylated ubiquitin (R&D Systems #U-501). Results are expressed as percent activity (in which positive control activity in the absence of inhibitor is set at 100%).

Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com.

Troubleshooting Guide
Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For all further questions, please email support@bpsbioscience.com

Related Products

Products	*Catalog #*	*Size*
Cereblon Intrachain TR-FRET Assay Kit	78301	384 reactions
MDM2 Intrachain TR-FRET Assay Kit	78302	384 reactions
VHL Intrachain TR-FRET Assay Kit	78305	384 reactions
XIAP Intrachain TR-FRET Assay Kit	78306	384 reactions
MDM2 TR-FRET Assay Kit	79773	384 reactions
Cereblon Ubiquitination Homogeneous Assay Kit	79881	384 reactions