MSD® Self-Ubiquitylation Demonstration Assay
MULTI-ARRAY™ 384 Glutathione Plates

I. Materials Included

	Storage

MSD Catalog Items

Item	Storage
❑ MULTI-ARRAY 384-well Glutathione plates	4ºC
❑ MSD Read Buffer T (With Surfactant), 4X	RT
❑ MSD Blocker A Kit (shipped separately)	

R&D Custom Materials

Item	Storage
❑ MSD Ubiquitylation Reaction Buffer	4ºC
❑ MSD Ubiquitylation Binding Buffer (4X)	4ºC
❑ EDTA (0.5M)	4ºC
❑ MSD SULFO-TAG™ anti-Ubiquitin detection antibody	4ºC
❑ MSD Blocker A (solid)	4ºC
❑ MDM2 Reagent Pack (2000 Tests) E1 lysate E2 lysate GST-MDM2 lysate Ubiquitin (2.9 mM) DTT (1 M) ATP (200 mM)	-80ºC

II. Other Materials & Equipment (not supplied)

- ❑ Various microcentrifuge tubes for making serial dilutions of test solutions
- ❑ 15 mL tubes
- ❑ DMSO
- ❑ Phosphate Buffered Saline (PBS)
- ❑ Deionized water, if performing washed protocol
- ❑ Automated plate washer, Multidrop®, or other efficient multi-channel pipetting equipment for washing 384 well plates (if performing washed protocol)
- ❑ Appropriate liquid handling equipment for desired throughput that must accurately dispense 1 to 50 µL into a 384-well micro plate

III. Background

Ubiquitin (Ub) is a 76-amino acid protein, highly conserved in all eukaryotes from yeast to humans, that can be covalently attached to other proteins to form multi-Ub chains. Ubiquitylation marks proteins for ATP-dependent proteolytic degradation by 26S proteosomes, and it may influence other protein functions, such as intracellular localization and participation in protein-protein interactions. Ubiquitylation of a variety of cellular proteins regulates many important physiological processes including regulation of the cell cycle and division, response to stress and various extracellular stimuli, DNA repair, transcriptional regulation, and regulation of immune and inflammatory reactions. Deregulation of ubiquitin-dependent proteolysis has been implicated as a key factor in a number of diseases and conditions including cancer, inflammation, neurodegenerative disorders, and inherited diseases.

Ubiquitylation results in formation of an *iso*-peptide bond between the C-terminal Gly-76 of Ub and the ε-amino group of an internal Lys residue in the substrate protein. This reaction involves three sequential enzymatic steps as shown in Figure 1. Ub is first activated in an ATP-dependent manner to form a thioester bond with a specific Cys residue of E1 enzyme. Activated Ub is then transferred to one of a family of Ub-conjugating enzymes (E2). Finally, Ub ligase (E3) selects a substrate protein for ubiquitylation and transfers activated Ub from E2 to a lysine in the substrate protein.

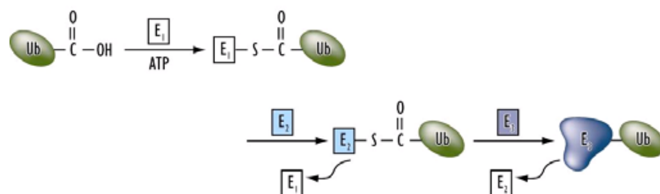

Figure 1. Ubiquitylation of ubiquitin ligase (E3).

IV. Assay Format

The assay for detecting MDM2 self-ubiquitylation involves capturing GST-labeled MDM2 on a glutathione coated MSD MULTI-ARRAY plate. The multi-Ub chain produced on MDM2 is detected through an MSD TAG-labeled anti-Ub antibody. The assay can be performed in washed or non-washed formats. The reagents have been titrated to insure that the levels of E1, E2, ATP, and ubiquitin are not significantly limiting in the assay. The assay operates in the linear range of MDM2 so that it is most sensitive to inhibitors of MDM2. Also the duration of the MDM2 ubiquitylation reaction has been optimized to ensure that the assay operates in the linear kinetic range. As part of this demonstration, the MDM2 concentration will be titrated to demonstrate the linear range of MDM2 ubiquitylation.

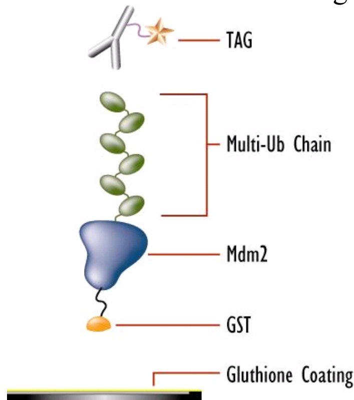

Figure 2. Assay format for detecting MDM2 self-ubiquitylation. The substrate protein (Ub ligase MDM2) is captured on a glutathione plate through a GST moiety. The multi-Ub chain is detected through an MSD TAG-labeled anti-Ub antibody.

V. Preparation and General Notes

Notes:

Read the entire detailed instructions before beginning work.

Preparation of MSD Blocker A solution:

1. Prepare MSD Blocker A solution following the directions enclosed in the MSD Blocker A kit.
2. Store MSD Blocker A solution for up to 14 days at 4 ^{o}C.

Preparation of Buffers (400 tests each):

Note: Solid MSD Blocker A (*not* solution) is used to prepare the buffers listed below.

1. Reaction Buffer
   To 8 mL of Reaction Buffer, add the following:
   - ❑ 16 µL 1M DTT
   - ❑ 40 µL 200 mM ATP
   - ❑ 0.008 g **solid** MSD Blocker A

2. Binding Buffer
   To 8 mL of Binding Buffer (4X), add the following:
   - ❑ 0.032 g **solid** MSD Blocker A

Preparation of MDM2 titrations:

Test Solution titrations of MDM2 are prepared using the amount of lysate per well. This protocol prepares 30 tests per MDM2 concentration.

MDM2 (µL lysate/well)	**Test Solution preparation**
0.3	485 µL Reaction Buffer + 15 µL GST-MDM2 lysate
0.1	200 µL Reaction Buffer + 100 µL 0.3 µL lysate/well
0.03	450 µL Reaction Buffer + 50 µL 0.3 µL lysate/well
0.01	200 µL Reaction Buffer + 100 µL 0.03 µL lysate/well
0.003	450 µL Reaction Buffer + 50 µL 0.03 µL lysate/well
0.001	200 µL Reaction Buffer + 100 µL 0.003 µL lysate/well
0.0003	450 µL Reaction Buffer + 50 µL 0.003 µL lysate/well
0.0001	200 µL Reaction Buffer + 100 µL 0.0003 µL lysate/well
0.00003	450 µL Reaction Buffer + 50 µL 0.0003 µL lysate/well
0.00001	200 µL Reaction Buffer + 100 µL 0.0003 µL lysate/well
0	350 µL Reaction Buffer

Preparation of Pre-Charging E1-E2 Mixtures (125 Negative Wells and 275 Positive Wells):

Note: Prepare these mixtures during STEP 2 of the protocol listed in Section VI, Detailed Instructions.

1. Combine the following to prepare the **Pre-Charging E1-E2 mixture**:
   - ❑ 4 µL E1
   - ❑ 100 µL E2
   - ❑ 4 mL Reaction Buffer
2. Prepare the **Negative Control Pre-Charging E1-E2 mixture**:
   - ❑ 1.25 mL E1-E2 mixture
   - ❑ 125 µL 0.5 M EDTA

VI. Detailed Instructions

Begin with an MSD MULTI-ARRAY Glutathione Plate. No pre-treatment is necessary.

Notes:

Plates may be used unblocked, however blocking can reduce variability.

STEP 1

Add 50 µL/well of MSD Blocking Solution-A.

Incubate at room temperature for 1 hour.

Wash plates three times with PBS.

STEP 2

Dispense 10 µL/well of MDM2 test solution, adding each concentration to the appropriate well of the MSD 384-well Glutathione plate as outlined in Figure 3. Note that each MDM2 concentration will be in a separate row.

Incubate at room temperature for 1 hour**.

Prepare Pre-Charging E1-E2 Mixture and Negative Control Pre-Charging E1-E2 Mixture (see Section V, General Notes) during incubation.

****After 30 minutes of this incubation time have passed, add 2.85 µL of ubiquitin to the Positive Pre-Charging E1-E2 mixture and 1.3 µL of ubiquitin to the Negative Control Pre-Charging E1-E2 Mixture to start the E1-E2 reactions. Proceed to STEP 3 following the completion of the full 1 hour plate incubation in STEP 2.**

STEP 3

Add 1-2 µL/well of 100% DMSO to columns 17-24 of the MSD 384-well Glutathione Plate. This simulates the addition of compound to the appropriate wells.

Add 10 µL/well of the Negative Control Pre-Charging E1-E2 Mixture to columns 1-8.

Add 10 µL/well of the Positive Pre-Charging E1-E2 Mixture.

Incubate at room temperature for 30 minutes. Prepare Antibody Solution during this time.

Prepare Antibody Solution:
Combine the following:

- ❑ 5 mL 4X Binding Buffer
- ❑ 5 mL 4X Read Buffer T, With Surfactant
- ❑ 1.7 µL MSD SULFO-TAG labeled anti-Ubiquitin Antibody (0.5 mg/mL stock)

Note that bubbles in the Read Buffer will interfere with reliable imaging of the plate if carried into the wells.

STEP 4 Add 20 µL/well of Antibody Solution to each well of the MSD 384-well Glutathione plate.

Incubate for 1 hour at room temperature.

Analyze with SECTOR™ instrument.

ADDITIONAL NOTES: The assay protocol can be modified as listed below to achieve a washed assay format:

1. Follow STEPS 1-3 as listed above (Section VI, Detailed Instructions) with the preparation of Antibody Solution as:

- 5 mL 4X Binding Buffer
- 5 mL 4X Read Buffer T, With Surfactant
- 1.7 µL MSD SULFO-TAG labeled anti-Ubiquitin Antibody (0.5 mg/mL stock)
- 10 mL deionized water

2. STEP 4:

Wash plate 3 times with PBS.

Add 40 µL/well Washed Assay Antibody Solution.

Incubate for 1 hour at room temperature.

Analyze with SECTOR instrument.

<table>
<tr><td></td><td colspan="8">Add Negative Control Pre-Charging E1/E2 Mixture</td><td colspan="8">Add Positive Pre-Charging E1/E2 Mixture</td><td colspan="8">Add Positive Pre-Charging E1/E2 Mixture AND DMSO</td></tr>
<tr><td></td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td><td>13</td><td>14</td><td>15</td><td>16</td><td>17</td><td>18</td><td>19</td><td>20</td><td>21</td><td>22</td><td>23</td><td>24</td></tr>
<tr><td>A</td><td colspan="8">0.3 µL MDM2/well</td><td colspan="8">0.3 µL MDM2/well</td><td colspan="8">0.3 µL MDM2/well</td></tr>
<tr><td>B</td><td colspan="8">0.1 µL MDM2/well</td><td colspan="8">0.1 µL MDM2/well</td><td colspan="8">0.1 µL MDM2/well</td></tr>
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<tr><td>I</td><td colspan="8">0.00003 µL MDM2/well</td><td colspan="8">0.00003 µL MDM2/well</td><td colspan="8">0.00003 µL MDM2/well</td></tr>
<tr><td>J</td><td colspan="8">0.00001 µL MDM2/well</td><td colspan="8">0.00001 µL MDM2/well</td><td colspan="8">0.00001 µL MDM2/well</td></tr>
<tr><td>K</td><td colspan="8">0 µL MDM2/well</td><td colspan="8">0 µL MDM2/well</td><td colspan="8">0 µL MDM2/well</td></tr>
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<tr><td>P</td><td colspan="8"></td><td colspan="8"></td><td colspan="8"></td></tr>
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Figure 3. Plate Layout for MDM2 Titration Demonstration

VII. References

1. Wong BR, Parlati F, Qu K, Demo S, Pray T, Huang J, Payan DG, Bennett MK. (2003) Drug discovery in the ubiquitin regulatory pathway. *Drug Discovery Today*, 8, 746-754.

2. Ciechanover A. (2003) The ubiquitin proteolytic system and pathogenesis of human disease: a novel platform for mechanism-based drug targeting. *Biochemical Society Transactions*, 31, 474-481.
3. Pickart CM. (2001) Mechanisms underlying ubiquitination. *Annual Reviews in Biochemistry*, 70, 503-533.
4. Joazeiro CA, Weissman AM. (2000) RING finger proteins: mediators of ubiquitin ligase activity. *Cell*, 102, 549-552.