MSD® 384-Well MULTI-ARRAY®
Human MDM2 Self-Ubiquitination Assay

I. MSD Reagents Supplied

	storage
❑ Read Buffer T, with Surfactant (4X)	RT
❑ Blocker A Kit	RT
❑ MULTI-ARRAY 384-well Glutathione plates	2-8ºC
❑ MSD SULFO-TAG™ Anti-Self-Ubiquitinated Protein Antibody (500 µg/mL)	2-8ºC
❑ Reaction Buffer (1X)	2-8ºC
❑ Binding Buffer (4X)	2-8ºC
❑ Blocker A (Cat.# R93BA-4)	2-8ºC
❑ ATP (200 mM)	-80ºC
❑ DTT (1 M)	-80ºC
❑ EDTA (0.5M)	-80ºC
❑ E1 (Ubiquitin Activating Enzyme)	-80ºC
❑ UbcH5B (E2) lysate	-80ºC
❑ GST-hMDM2 (E3) lysate	-80ºC
❑ Ubiquitin (Ub) (2.9 mM)	-80ºC

II. Materials & Equipment Not Supplied

- ❑ Various microcentrifuge tubes for making serial dilutions of test solutions
- ❑ 15 mL tubes
- ❑ DMSO
- ❑ Tris-Buffered Saline (TBS)
- ❑ Ultrapure water
- ❑ Adhesive plate seals
- ❑ Microtiter plate shaker
- ❑ Automated plate washer or other efficient multi-channel pipetting equipment for washing 384-well plates (if performing washed protocol)
- ❑ Appropriate liquid handling equipment for desired throughput. Must accurately dispense 1 to 50 µL into a 384-well micro plate.

III. Assay Format

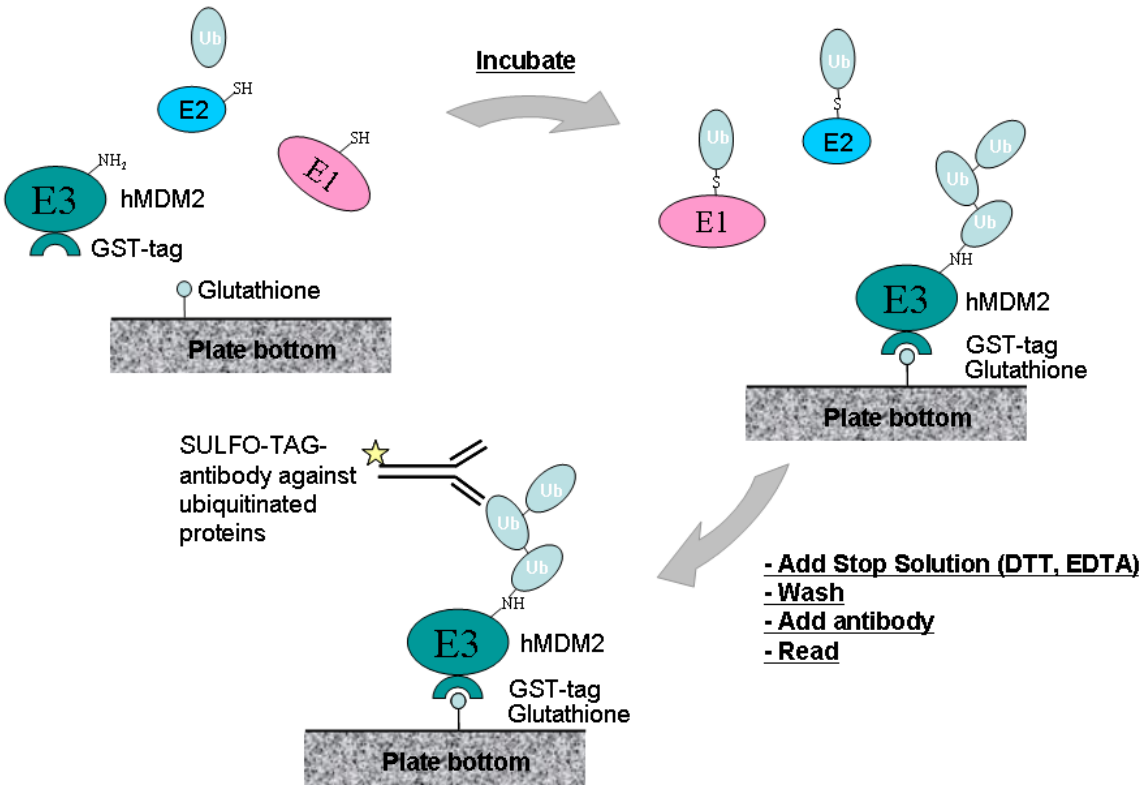

Figure 1. The assay for detecting hMDM2 self-ubiquitination involves capturing GST-tagged hMDM2 on a glutathione coated MSD MULTI-ARRAY plate. The *in vitro* reaction mediated by E1 and E2 enzymes results in conjugation of Ub to Lys residues of MDM2. Multiple Ub moieties can attach to each other through *iso*-peptide bonds to form poly-Ub chains. Stop Solution, containing EDTA and DTT, is added to terminate the reaction. After treatment with Stop Solution the plate is washed to remove E1, E2 and free Ub. Ub covalently linked to hMDM2 is detected by MSD SULFO-TAG Anti-Self-Ubiquitinated Protein Antibody.

IV. Assay Workflow

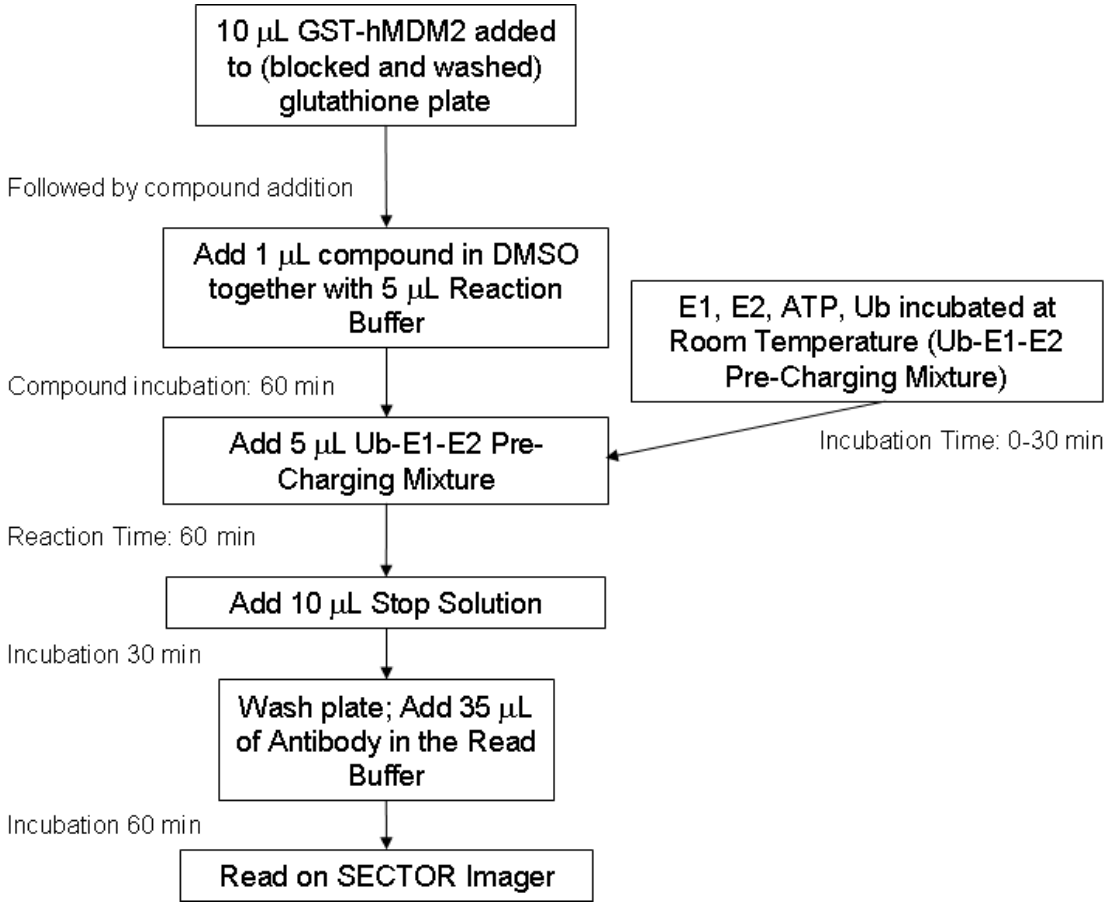

Figure 2. Assay workflow.

V. Reagent Preparation

Notes:

Read the entire detailed instructions before beginning work.

The reagent volumes below are calculated for 400 wells (approx. one 384-well plate) with no overhead. The users should do their own calculations based on the number of plates screened, and should add some extra volumes dictated by their liquid handling HTS instrumentation.

Preparation of MSD Blocker A solution:

Prepare MSD Blocker A solution following the directions enclosed in the MSD Blocker A kit.

Preparation of the Complete Reaction Buffer:

To 10 mL of Reaction Buffer add the following:

- 50 µL 200 mM ATP
- 20 µL 1 M DTT

GST-hMDM2 dilutions, Stop Solution, and Antibody/Read Buffer Solution should be prepared immediately prior to use, and the leftovers discarded.

Preparation of GST-hMDM2 in Complete Reaction Buffer:

a) Make a 1/10 dilution of GST-hMDM2 lysate by adding 2 µL of GST-hMDM2 lysate to 18 µL of the Complete Reaction Buffer. This intermediate dilution will only be used in subsequent dilution step, and not in the reaction.

b) To 4 mL of the Complete Reaction Buffer add the following:

- 12 µL 1/10 diluted GST-hMDM2 lysate

This creates final dilution of GST-hMDM2 (3 nL/well) in Complete Reaction Buffer for use in the ubiquitination reaction.

Preparation of Pre-Charging Ub-E1-E2 Mixture:

Note: Prepare these mixtures ≤ 30 min before use during STEP 3 of the protocol listed in Section VI, Detailed Instructions.

To 2 mL of the Complete Reaction Buffer add the following:

- 4 µL E1 (10 nL/well)
- 24 µL UbcH5B lysate (60 nL/well)
- 13.8 µL 2.9 mM Ub (5 µM final concentration)

Preparation of Stop Solution:

To 3.4 mL of water add the following:

- 480 µL 0.5 M EDTA
- 120 µL 1M DTT

Preparation of Antibody/Read Buffer Solution:

a) To 3.5 mL 4X Binding Buffer add the following:

- 14 mg Blocker A (cat.# R93BA-4)

Mix for ≤ 30 min to dissolve. Use 4X Binding Buffer with Blocker A at step b) and c) only, and not in the assay.

b) Dilute the stock solution of SULFO-TAG Anti-Self-Ubiquitinated Protein Antibody 10-fold in 4X Binding Buffer with Blocker A from a)

- 90 µL 4X Binding Buffer with Blocker A from a)
- 10 µL SULFO-TAG Anti-Self-Ubiquitinated Protein Antibody (500 µg/mL)

c) Mix the following to prepare Antibody/Read Buffer Solution:

- 3.5 mL 4X Binding Buffer with Blocker A from a)
- 7 mL ultrapure water
- 3.5 mL 4X Read Buffer T
- 16 µL SULFO-TAG Anti-Self-Ubiquitinated Protein Antibody diluted in step b) (2 ng/well)

VI. Detailed Instructions

Notes:

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

STEP 1 Add 50 µL/well of MSD Blocking Solution-A to each well of the MSD 384-well Glutathione plate.
Incubate at room temperature for 2 hours.
Wash plates three times with TBS.

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

STEP 2 Dispense 10 µL/well of GST-hMDM2 in Complete Reaction Buffer.
Immediately proceed to Step 3.

STEP 3 Add 1 µL/well of compound in 100% DMSO pre-mixed with 5 µL/well Complete Reaction Buffer (for a total added volume of 6 µL).
Incubate at room temperature for 60 minutes.

Prepare Pre-Charging Ub-E1-E2 Mixture (see Section V, Reagent Preparation) during this incubation.

STEP 4 Add 5 µL/well of Pre-Charging Ub-E1-E2 Mixture.
Incubate at room temperature for 60 min.

STEP 5 **(Optional)**
Add 10 µL/well of Stop Solution.
Incubate at room temperature for 30 min.

STEP 6 Wash the plate with TBS.

STEP 7 Add 35 µL/well of Antibody/Read Buffer Solution.
Leave at room temperature for 60 min.

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

STEP 8 Read on SECTOR® Imager.