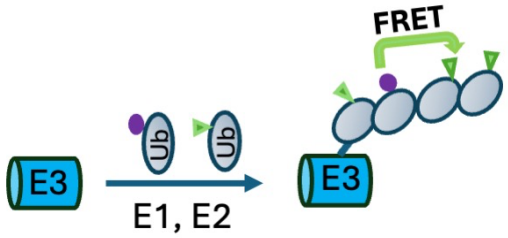
RELAY^{TR} MDM2 Activity Assay Kit (Catalog # T4060)

400 x 20 µL reactions

Description

RELAY^{TR} MDM2 Activity Assay Kit utilizes the TR-FRET technology to measure the ubiquitin ligase activity of MDM2 by detecting its autoubiquitination (see schematic below). The kit is based on the terbium/fluorescein pair for FRET detection. It is easy to use, sensitive, and stable under room temperature operation.

RELAY^{TR} FRET Assay - E3 Autoubiquitination



Implications

1) Screening inhibitors of MDM2.
2) Profiling ubiquitin ligases.
3) Assessing MDM2 ubiquitin ligase activity.

Materials Included

Component	Stock (dilu. factor*, final [C])	Quantity
UbE1	2 µM (200X, 10 nM)	40 µl
6xHis-UbE2D4	20 µM (200X, 100 nM)	40 µl
6xHis-MDM2	5 µM (100X, 50 nM)	80 µl
ATP	500 mM (500X, 1 mM)	25 µl
RELAY^{TR} Terbium/Fluorescein Ubiquitin Mix	100X (100X, 1X)	80 µl
RELAY^{TR} Ubiquitination Buffer	10X (10X, 1X)	1.25 ml

* Dilution factor in parentheses is referred to the final reaction concentration of each component.

Notes

Aliquot protein components in the kit if planning to use for multiple times.
Use liquid nitrogen to snap freeze protein stocks. Avoid multiple freeze/thaw cycles.
Do not reuse diluted components.

Materials Not Included

Plate reader capable of measuring the terbium/fluorescein-based TR-FRET; Low volume and non-binding 384-well black plate; Single-/muti-channel pipets and tips; Clear adhesive film for 384-well plate; 1.5 ml microfuge tubes; MilliQ water; plate shaker; and plate centrifuge.

A Four-Step Reaction Scheme

Assay Instruction

1. The experiment below is to set up 4 x 20 µl reactions for **Blank** (with all components but E3, recording TR-FRET signal of E1/E2/Ubiquitin Mix with ATP), **Positive** (with all components, recording TR-FRET signal of ubiquitination), and **Negative** (with all components but ATP, recording TR-FRET signal without ubiquitination). Similar reactions can be used for compound screening/profiling. Triplicates or quadruplicates are recommended.

[Plate Reader Setup]

2. The terbium and fluorescein pair can be excited at 320-340 nm with dual emission detections centered at 490 nm and 520 nm. A delay time of 50 - 100 µs with the integration time of 200 - 400 µs is often used. The number of flashes should follow plate reader manufacturer's recommendation. Using the kit to perform test experiments is highly recommended.

[Reagent Preparation]

3. Thaw provided kit components and 2 ml MilliQ water on ice.
4. *Preparation of 250 µl **1X** buffer.* In a 1.5 ml microfuge tube, add 25 µl 10X RELAY^{TR} Ubiquitination Buffer and 225 µl MilliQ water. Pipette to mix well. Keep under room temperature.
5. *Preparation of 25 µl **10X** ATP*. In a 1.5 microfuge tube, add 24.5 µl 1X buffer prepared in STEP 4 and 0.5 ul 500X ATP. Pipette to mix well. Keep under room temperature.
6. *Preparation of 20 µl **5X** Blank Master Mix*. In a 1.5 microfuge tube, add 2 µl 10X RELAY^{TR} Ubiquitination Buffer and 16 µl cold MilliQ water. Tab the tube to mix well. Then add 0.5 µl 200X UbE1, 0.5 µl 200X 6xHis-UbE2D4 and 1 µl 100X RELAY^{TR} Terbium/Fluorescein Ubiquitin Mix. Pipette to mix well. Keep under room temperature.
7. *Preparation of 40 µl **5X** Positive/Negative Master Mix*. In a 1.5 microfuge tube, add 4 µl 10X RELAY^{TR} Ubiquitination Buffer and 30 µl cold MilliQ water. Tab the tube to mix well. Then add 1 µl 200X UbE1, 1 µl 200X 6xHis-UbE2D4, 2 µl 100X 6xHis-MDM2, and 2 µl 100X RELAY^{TR} Terbium/Fluorescein Ubiquitin Mix. Pipette to mix well. Keep under room temperature.
8. Centrifuge solutions prepared in STEPS 4-7 at 12,000 xg for 2 min to collect them to tube bottoms.

[Reaction Setup]

9. In a low volume and non-binding 384-well black plate (Corning, Catalog #4514, or other vendor's plate), add 14 µl 1X buffer into each well of A2-A5 (**Negative reactions**), B2-B5 (**Positive reactions**) and C2-C5 (**Blank reactions**). See plate layout below. Use an electronic pipettor with repeat dispense is highly recommended for reaction setup. This is STEP 1 in the Reaction Scheme above.

	1	2	3	4	5
A		Negative →	→	→	→
B		Positive →	→	→	→
C		Blank →	→	→	→
D		Compound →	→	→	→
E		Compound →	→	→	→
F		Compound →	→	→	→
G		Compound →	→	→	→
H		Compound →	→	→	→
I		Compound →	→	→	→

Note: In compound screening (not conducted here), if a compound stock is in DMSO, prepare 20 µL of 5X compound stock in 1X buffer, with a final DMSO concentration of 5%. Similarly, prepare 1X buffer containing 5% DMSO for Blank, Positive, and Negative reactions.

Add 4 µL of 1X buffer containing 5% DMSO to the Blank, Positive, and Negative reaction wells, and add 4 µL of 5X compound in 1X buffer containing 5% DMSO to the Compound reaction wells. Then, add 10 µL of 1X buffer to each well. Mix well using a plate shaker.

The final DMSO concentration will be 1% after all components being added.

10. Add 4 µl 5X Positive/Negative Master Mix into each well of A2-A5 and B2-B5, and 4 µl 5X Blank Master Mix into each well of C2-C5. Use a plate shaker to thoroughly mix (300-500 rpm, 2 minutes). This is STEP 2 in the Reaction Scheme above.

Note: In compound screening, incubate STEP 2 mixtures under room temperature for 20 min to allow enzyme/compound interaction.

11. In A2-A5, add 2 µl 1X buffer/well (**Negative**). In B2-B5 (**Positive**) and C2-C5 (**Blank**), add 2 µl 10X ATP/well to initiate ubiquitination. This is STEP 3 in the Reaction Scheme above.

12. Seal the plate with a clear adhesive film. Shake the plate to thoroughly mix (300-500 rpm, 2 min), then centrifuge the plate at 1,000 xg for 2 min. Transfer the plate into a plate reader to record TR-FRET signals. This is STEP 4 in the Reaction Scheme above.

Note: We use a PHERAstar FS plater reader with the 337nm/520nm/490nm filter set to record both 490 nm and 520 nm fluorescence under a kinetic mode of 600 seconds/reading up to three hours. Integration starts at 50 µs and integration time is 400 µs. Shaking the plate at 300 rpm for 10 seconds before each reading.

In end point assays, read the plate at designated time points up to three hours, such as at 0, 15, 30, 60, 90, 120, 150 and 180 min. Keep the plate inside the instrument during readings. Incubate the plate under 30 ^{0}C or 37 ^{0}C can facilitate ubiquitination reactions.

[Data Processing]

13. TR-FRET signal is calculated as the product of the ratio of acceptor emission intensity (λ = 520 nm) to donor emission intensity (λ = 490 nm) multiplies a "convenience constant" of 10^4. FRET ratio (R) = (Fluorescence Intensity$_{520nm}$/Fluorescence Intensity$_{490nm}$) X 10^4.
14. Signal-to-Blank (S/B) ratio can be used to compare results conducted at different times. FRET ratios (R) of Positive reactions and Blank reactions serve as Signal Ratios (R_S) and Blank Ratios (R_B), respectively. The signal-to-blank ratio is calculated using the formula S/B = (R_S – R_B)/R_B. In compound screening assays, the S/B value of reactions without a compound can be referred to as 100% of MDM2 activity.
15. Data below were mean ± S.D. of quadruplicates from the experiment described above. Time 0 was ~5 min after ATP addition.