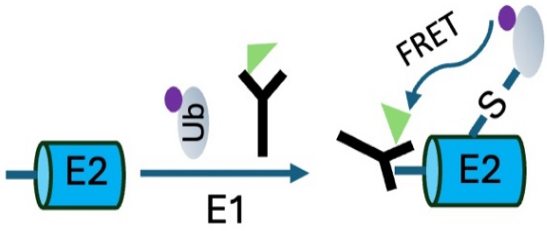
RELAY^{TR} Ubiquitin E2 Profiling Kit (Catalog # T5000)

(23 E2 enzymes, 50 x 20 µl reactions per E2)

Description

The RELAY^{TR} Ubiquitin E2 Profiling Kit is based on the TR-FRET technology that offers a simple, sensitive, homogeneous, and stable readout to measure the activity of ubiquitin-conjugating enzymes (E2s) by detecting formation of the E2-ubiquitin thioester conjugates (see schematic below). This assay detects as low as 10 nM E2 activity with a robust signal/background ratio and stable readout up to three hours.

Schematic



Implications

1) E2 profiling.
2) Hit/lead compound discovery and validation.

23 Ubiquitin E2s Included

	E2 Name	Reactivity
1	6XHis-UbE2A	Ub
2	6XHis-UbE2B	Ub
3	6XHis-UbE2C	Ub
4	6XHis-UbE2D1	Ub
5	6XHis-UbE2D2	Ub
6	6XHis-UbE2D3	Ub
7	6XHis-UbE2D4	Ub
8	6XHis-UbE2E1	Ub
9	6XHis-UbE2E2	Ub
10	6XHis-UbE2E3	Ub
11	6XHis-UbE2G1	Ub
12	6XHis-UbE2G2	Ub

	E2 Name	Reactivity
13	6XHis-UbE2H	Ub
14	6XHis-UbE2J1$_{(1-282)}$	Ub
15	6XHis-UbE2L3	Ub
16	6XHis-UbE2N	Ub
17	6XHis-UbE2Q2	Ub
18	6XHis-UbE2R1	Ub
19	6XHis-UbE2R2	Ub
20	6XHis-UbE2S	Ub
21	6XHis-UbE2T	Ub
22	6xHis-Ubc13/UbE2V2	Ub
23	6XHis-UbE2W	Ub

Components in the kit*

• 1,000X UBE1 (2.5 µM)	25 µl
• 500X E2 Ub-conjugating Enzymes (5 µM each, 23 total E2s)	5 µl each
• 500X RELAY^{TR} Terbium Ubiquitin/Fluorescein-anti-His Antibody Mix	50 µl
• 10X RELAY^{TR} Ubiquitination Buffer	3 x 1.25 ml
• 500X ATP (500 mM)	100 µl

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

* Aliquot components if needed. Use liquid nitrogen to snap freeze proteins.

Materials not included

Plate reader capable of measuring terbium/fluorescein-based FRET; Low volume and non-binding 96-well or 384-well black plate; Single or muti-channel pipets and tips; Clear plate sealing film; 1.5 ml microfuge tubes; MilliQ water

A general guide using the RELAY^{TR} Ubiquitin E2 Profiling Kit

The experiment described below was used to set up 8 x 20 µl reactions for each tested ubiquitin E2 (6xHis-UbE2D2, 6xHis-UbE2E2 and 6xHis-Ubc13/UbE2V2), in which 4 x 20 µl reactions do not have ATP (defined as Background), and 4 x 20 µl reactions have ATP (defined as Signal).

Reaction Schematic of 20 µl/well Reaction

[Plate Reader Setup]

1. Users should use the kit to adjust plate reader settings to achieve a desirable signal-to-background ratio. 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 instrument manufacturer's recommendation.

[Reagent Preparation]

2. Thaw components and chill 1 ml MilliQ water on ice.

   Note: Diluting the provided stocks are necessary as described below. Do not dilute components to low concentrations and then stock them.

3. *Preparation of 600 µl 1X RELAY^{TR} Ubiquitination Buffer (referred to as 1X buffer).* In a 1.5 ml microfuge tube, add 60 µl 10X RELAY^{TR} Ubiquitination Buffer and 540 µl cold MiliQ water. Invert the tube to mix well. Keep under room temperature.

4. *Preparation of 50 µl 10X ATP*. In a 1.5 microfuge tube, add 1 µl 500 mM ATP stock and 49 µl 1X Buffer. Tab the tube to mix well. 10X ATP concentration is 10 mM. Keep under room temperature.

5. *Preparation of 100X protein stocks*. According to the experimental use in this assay, make 10 µl 100X UbE1, 100X E2, and 100X RELAY^{TR} Terbium-Ubiquitin/Fluorescein-anti-His Antibody Mix by diluting the provided stocks at 1,000X or 500X using 1X Buffer.

6. *Preparation of 40 µl **5X** Master Mix for each E2-mediated reaction*. 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 2 µl 100X UbE1, 2 µl 100X E2, 2 µl 100X RELAY^{TR} Terbium-Ubiquitin/Fluorescein-anti-His Antibody Mix. Tab the tube to mix well. Keep under room temperature.

[Reaction Setup]

7. 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 A1-A4, A7-A10, B1-B4, B7-B10, C1-C4 and C7-C10. This is STEP 1 in the Reaction Schematic above.

   Note: In compound profiling assays, add 0.5 µl chemical at 40X stock in DMSO into each well, then add 13.5 µl room temperature 1X buffer and mix well by shaking the plate at 300 rpm for 2-5 min. For control reactions without a chemical, add 0.5 µl DMSO and 13.5 µl 1X buffer. The final DMSO concentration should not be more than 2.5%.

8. Add 4 µl 5X Master Mix into each well. A1-A4 and A7-A10 are for UbE2D4, B1-B4 and B7-B10 are for UBE2E2, and C1-C4 and C7-C10 are for Ubc13/UbE2V2. Use a plate shaker to mix well at 300 rpm for 2 min. This is STEP 2 in the Reaction Schematic above.

   Note: In compound profiling assays, once 5X Master Mix being added, mix well and incubate 15-30 min under room temperature prior to adding ATP to initiate ubiquitination. This is to ensure chemicals react with E2s.

9. In A1-A4, B1-B4, and C1-C4, add 2 µl 1X buffer (Background wells). In A7-A10, B7-B10, and C7-C10, add 2 µl 10X ATP to initiate ubiquitination (Signal wells). This is STEP 3 in the Reaction Schematic above.

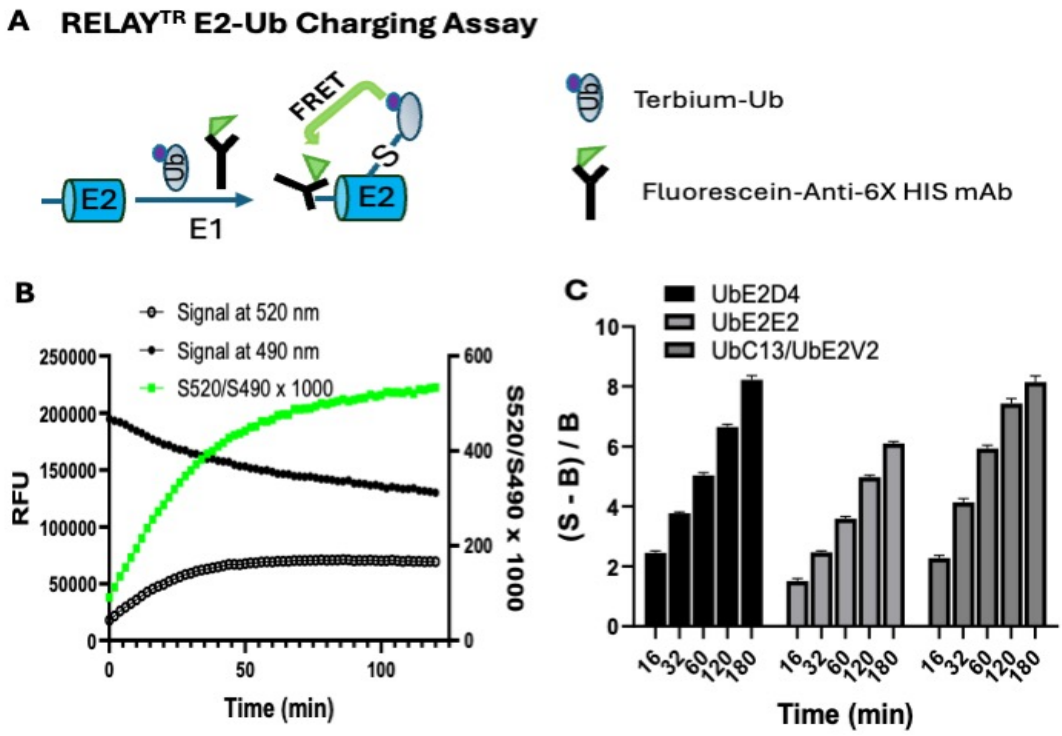
10. Seal the plate with a clear adhesive film. For kinetic mode detection, transfer the plate to your plate reader to record FRET signals. We use a PHERAstar FS plater reader with the 337nm/520nm/490nm filter set to record both 490 nm and 520 nm fluorescence under a kinetic mode up to three hours. Integration starts at 50 µs and integration time is 400 µs. This is STEP 4 in the Reaction Schematic above.

    Note: End point assay is preferred as it may reduce photobleaching. In end point assays, use a plate shaker to mix well at 300 rpm for 2 min, then keep the plate under dark (such as in a drawer) under room temperature. Read the plate at your designated time points up to three hours, such as at 15, 30, 60, 90, 120, 150 and 180 min. Shake the plate before each reading using plate reader's shaking function.

[Data Processing]

11. 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 10^3 (green line in Fig. B). FRET ratio (R) = (Fluorescence Intensity$_{520nm}$/Fluorescence Intensity$_{490nm}$) X 10^3.

12. For a specific E2-Ub charging assay, FRET ratios (R) of reactions with ATP or without ATP serve as Signals (R_{signal}) or Backgrounds ($R_{backgound}$), respectively. The Signal/Background ratio is calculated using the formula (S-B)/B = (R_{signal} – $R_{background}$)/$R_{background}$ (Fig. C), which corrects background variations among experiments. For compound profiling assays, the mean (S-B)/B value from reactions without a compound can be referred to as 100% of E2 activity.



A. Schematic of the RELAYTR E2-Ub Charging Assay using 6xHis-tagged E2, fluorescein-Anti-6XHIS antibody and terbium-Ub.

B. Representative kinetic curves and the FRET ratio (S520/S490) of the RELAY^{TR} E2-Ub charging assay with ATP.

C. Signal-to-background ratios of each tested E2-Ub charging assay. Data were shown as mean ± S.D. of four reactions.