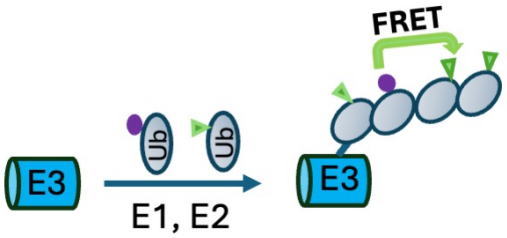
RELAY^{TR} NEDD4 Activity Assay Kit (Catalog # T4040)

400 x 20 µL reactions

Description

RELAY^{TR} NEDD4 Activity Assay Kit utilizes the TR-FRET technology to measure the ubiquitin ligase activity of NEDD4 by detecting its autoubiquitination (see schematic below). The kit is easy to use and sensitive with high signal-to-background ratio readout.

RELAY^{TR} FRET Assay - E3 Autoubiquitination



Implications

1) Screening inhibitors/activators of NEDD4.
2) Profiling ubiquitin ligases.
3) Assessing the ubiquitin ligase activity of NEDD4.

Materials Included

Component	Stock (dilution factor*)	Quantity
UbE1	2 µM (200X)	40 µl
6xHis-UbE2D4	20 µM (200X)	40 µl
GST-NEDD4	5 µM (200X)	40 µl
ATP	500 mM (500X)	25 µl
RELAY^{TR} Terbium/Fluorescein Ubiquitin Mix	100X (100X)	80 µl
RELAY^{TR} Ubiquitination Buffer	10X (10X)	1.25 ml

* Dilution factor in parentheses is referred to the final reaction concentration of each component.
We recommend to aliquot protein components when needed. Use liquid nitrogen to snap freeze protein.

Materials Not Included

Plate reader capable of measuring the terbium/fluorescein-based 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

An Easy-to-use Assay – Reaction Scheme

Assay Instruction

The experiment described below is to set up 8 x 20 µl reactions, 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).

[Plate Reader Setup]

1. Users should use the kit to run test experiments and 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 manufacturer's recommendation.

[Reagent Preparation]

2. Thaw provided or aliquoted components on ice, and chill 1 ml MilliQ water on ice as well.
3. *Preparation of 200 µl 1X buffer.* In a 1.5 ml microfuge tube, add 20 µl 10X RELAY^{TR} Ubiquitination Buffer and 180 µl MilliQ water. Tap 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 500X 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 40 µl **5X** Master Mix*. In a 1.5 microfuge tube, add 4 µl 10X RELAY^{TR} Ubiquitination Buffer and 31 µl cold MilliQ water. Tab the tube to mix well. Then add 1 µl 200X UbE1, 1 µl 200X UbE2D4, 1 µl 200X GST-NEDD4, and 2 µl 100X RELAY^{TR} Terbium/Fluorescein Ubiquitin Mix. Tab the tube to mix well. Keep under room temperature.

[Reaction Setup]

6. 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 (Background wells) and A7-A10 (Signal wells). This is STEP 1 in the Reaction Scheme above.

   Note: In compound screening assays, add 0.5 µl chemical at the concentration of 40X stock in DMSO into corresponding wells, then add 13.5 µl 1X buffer and mix well using a plate shaker. For control reactions without a chemical, add 0.5 µl DMSO and 13.5 µl 1X buffer. Usually, the final DMSO concentration should not be more than 2.5%.
7. Add 4 µl 5X Master Mix into each well. Use a plate shaker to shake the plate at 300 rpm for 2 min. This is STEP 2 in the Reaction Scheme above.

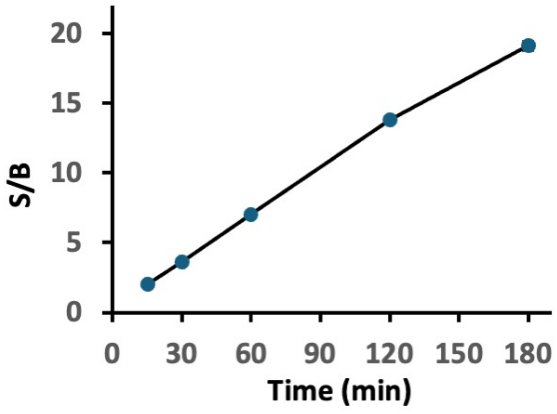
Note: In compound screening assays, once 5X Master Mix being added, mix well and incubate 15-30 min under room temperature prior to adding ATP to initiate ubiquitination.

8. In A1-A4, add 2 µl 1X buffer (Background). In A7-A10, add 2 µl 10X ATP to initiate ubiquitination (Signal). This is STEP 3 in Reaction Scheme above.
9. Seal the plate with a clear adhesive film. Transfer the plate to 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 Scheme above.

   Note: End point assay is preferred as it may reduce photobleaching. In end point assays, shake the sealed plate using a plate shaker at 300 rpm for 2 min, then keep the plate in dark (such as in a drawer) under room temperature. Read the plate at 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 if your plate reader doesn't have a shaking function. Incubate the plate under 30 ^{0}C or 37 ^{0}C can facilitate the reaction.

[Data Processing]

10. 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 or 10^4. FRET ratio (R) = (Fluorescence Intensity$_{520nm}$/Fluorescence Intensity$_{490nm}$) X 10^3 that can be calculated by instrument software.
11. Signal-to-background (S/B) ratio can be used to compare results conducted at different times or conditions. FRET ratios (R) of reactions with ATP or without ATP serve as Signal Ratios (R_S) or Background Ratios (R_B), respectively. The signal-to-background ratio is calculated using the formula $S/B = (R_S - R_B)/R_B$. In compound screening assays, the S/B of reactions with ATP but without a compound can be referred to as 100% of NEDD4 activity.



S/B ratios of 25 nM NEDD4 autoubiquitination. Data were shown as mean ± S.D. of four reactions.