Description

The NEDD4 Intrachain TR-FRET Assay Kit is a sensitive high-throughput screening (HTS) TR-FRET (Time Resolved Fluorescence Resonance Energy Transfer) assay kit, designed to measure NEDD4 (neural precursor cell expressed developmentally down-regulated protein 4) auto-ubiquitination activity in a homogeneous 384 reaction format. It utilizes a Europium-labeled ubiquitin (Ub) donor as well as Cy5-labeled Ub acceptor to complete the TR-FRET pairing. Since both the TR-FRET donor and acceptor are incorporated into poly-ubiquitin chains formed on NEDD4, this assay measures poly-ubiquitination. As a homogeneous assay, it requires no time-consuming washing steps, making it especially suitable for HTS applications as well as real-time analyses. This kit contains enough recombinant human NEDD4 (amino acids 150-end) and reagents for 384 reactions.

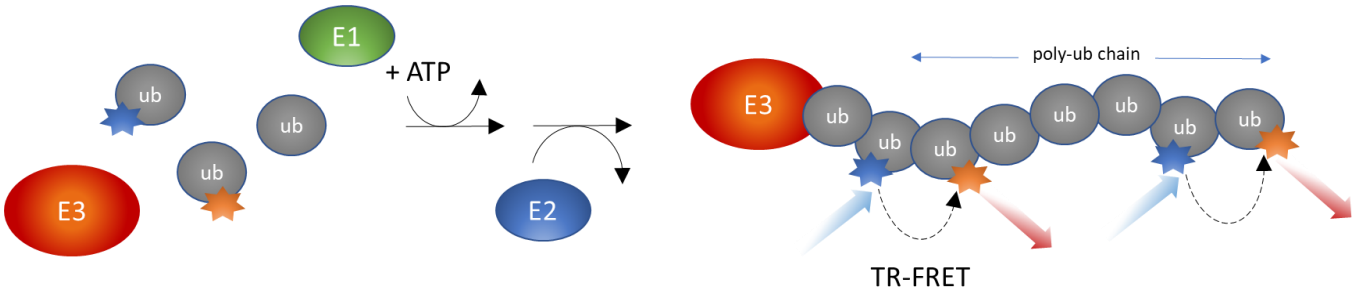

Figure 1. E3 ligase NEDD4 Intrachain TR-FRET Assay Kit schematic.

Background

Covalent conjugation to ubiquitin (Ub) is one of the major post-translational modifications that regulates protein stability, function, and localization. Ubiquitination is the concerted action of three enzymes: a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub ligase (E3). The specificity and efficiency of ubiquitination are largely determined by the E3 enzyme, which directs the last step of the Ub-conjugating cascade by binding to both an E2~Ub conjugate and a substrate protein. This step ensures the transfer of Ub from E2~Ub to the substrate, leading to its mono- or poly-ubiquitination.

NEDD4 (neural precursor cell expressed developmentally down-regulated protein 4) is an E3 ligase, member of the HECT (Homologous to the E6-AP carboxyl terminus) ubiquitin ligase family which target client proteins to the proteasome system for degradation. The protein contains an N-terminal calcium and phospholipid-binding C2 domain, three tryptophan-rich WW domains (that bind to proline-rich peptide motifs), and a C-terminal HECT catalytic domain.

NEDD4 regulates the expression levels of various receptor tyrosine kinases, notably growth factor receptors IGF1R (Insulin-like growth factor 1 receptor), FGFR1 (fibroblast growth factor receptor 1), EGFR (Epidermal growth factor receptor), and VEGFR2 (Vascular endothelial growth factor receptor 2), thereby playing a role in growth factor signaling and regulating cell proliferation. The E3 ligase also controls the expression levels of ion channels and is part of a signaling complex involved in dendrite extension and neuron architecture. It is an essential protein during development, including neural development. NEDD4 is a potential therapeutic target for the treatment of various types of cancer, cardiovascular disease, and neuro-degenerative diseases such as Parkinson's disease, Alzheimer's disease or Amyotrophic Lateral Sclerosis.

Applications

- Screen molecules that inhibit NEDD4 Ub ligase activity in drug discovery HTS applications.
- Determine compound IC_{50}.
- Perform NEDD4 real-time kinetics.

Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), FLAG-tag*	50 µg	-80°C
80314	UbcH5b, His-Tag*	300 µg	-80°C
80404	NEDD4, FLAG-tag*	2 x 20 µg	-80°C
78307	Ubiquitin Mix (200x)	50 µl	-80°C
	ATP (4 mM)	2 x 1 ml	-80°C
	U1 Assay Buffer	2 x 10 ml	-80°C
	White, nonbinding, low volume microtiter plate		Room Temp

**The initial concentration of enzyme is lot-specific and will be indicated on the tube containing the protein.*

The Ubiquitin Mix is sourced from South Bay Bio LLC.

Materials Required but Not Supplied

- Fluorescent microplate reader capable of measuring Time Resolved Fluorescence Resonance Energy Transfer (TR-FRET)
- Adjustable micropipettor and sterile tips
- Rotating or rocker platform

Storage Conditions

This assay kit will perform optimally for up to 6 months from date of receipt when the materials are stored as directed. ***Avoid multiple freeze/ thaw cycles!***

Safety

This product is for research purposes only and not for human or therapeutic use. This product should be considered hazardous and is harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly.

Contraindications

The NEDD4 Intrachain TR-FRET Assay Kit is compatible with up to 1% final DMSO concentration. We recommend preparing the inhibitor in a solution containing no higher than 5% DMSO and using 4 µl per well.

Assay Protocol

- All samples and controls should be performed in triplicate.
- The assay should include "Blank", "Positive Control", "Negative Control" and "Test Inhibitor" conditions.
- If the assay plate is going to be used more than once, prepare enough of each protein and aliquot the remaining undiluted proteins into single-use aliquots depending on how many times the assay plate will be used. Store the aliquots at -80°C.

1) Thaw **UBE1**, **UbcH5b**, **NEDD4**, **Ubiquitin Mix**, **U1 assay buffer**, and **ATP** on ice. Briefly spin the tubes to recover their full content.

2) Prepare 5x TRF Ubiquitin Mix in U2 Assay Buffer by making a 40-fold dilution of the stock TRF Ubiquitin Mix (200x).

3) Calculate the amount of protein required for the assay and prepare the appropriate amounts of diluted proteins. The concentration of each protein is lot-specific and is indicated on the tube. Verify the initial concentration and dilute accordingly.

    a) Dilute **UBE1** in U1 Assay Buffer to 96 ng/µl (800 nM - final concentration in the reaction will be 40 nM) (1 µl/well).

    b) Dilute **UbcH5b** in U1 Assay Buffer to 720 ng/µl (10 µM - final concentration in the reaction will be 500 nM) (1 µl/well).

    c) Dilute **NEDD4** in U1 Assay Buffer to 17.2 ng/µl (200 nM - final concentration in the reaction will be 50 nM) (5 µl/well).

    Note: UBE1, UBCH5b, NEDD4, Ubiquitin Mix, and U1 assay buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles. Keep all diluted proteins on ice until use. Do not freeze and re-use diluted proteins.

4) Prepare the Test Inhibitor (4 µl/well): for a titration, prepare serial dilutions at concentrations 5-fold higher than the desired final concentrations. The final volume of the reaction is 20 µl.

    a) If the Test Inhibitor is water-soluble, prepare serial dilutions in the U1 Assay Buffer, 5-fold more concentrated than the desired final concentrations. For the positive and negative controls, use U1 Assay Buffer (Diluent Solution).

    OR

    b) If the Test inhibitor is soluble in DMSO, prepare the test inhibitor at a concentration 100-fold higher than the highest desired concentration in 100% DMSO, then dilute the inhibitor 20-fold in U1 Assay Buffer to prepare the highest concentration of the 5-fold intermediate dilutions. The concentration of DMSO is now 5%.

Prepare serial dilutions of the Test Inhibitor at 5-fold the desired final concentrations using 5% DMSO in U1 Assay Buffer to keep the concentration of DMSO constant.

For positive and negative controls, prepare 5% DMSO in U1 Assay Buffer (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

Note: The final concentration of DMSO should not exceed 1%.

5) For the "Blank" wells prepare the following mix: N wells x (4 µl of 5x Ubiquitin Mix + 1 µl of diluted UBE1 + 1 µl of diluted UbcH5b + 4 µl of Diluent Solution + 5 µl of U1 Assay Buffer).

6) Add 15 µl of mix to each "Blank" well.

	Blank
TRF Ubiquitin Mix (5x)	4 µl
Diluted UBE1	1 µl
Diluted UbcH5b	1 µl
Diluted NEDD4	-
Test Inhibitor	-
Diluent Solution	4 µl
U1 Assay Buffer	5 µl
ATP (4 mM)	5 µl
Total	**20 µl**

7) Prepare a Master Mix: N wells × (4 µl 5x Ubiquitin Mix + 1 µl diluted UBE1 + 1 µl diluted UbcH5b + 5 µl diluted NEDD4).

8) Add 11 µl of Master Mix to each well designated for the "Negative Control", "Positive Control" and "Test Inhibitor".

9) Add 4 µl of inhibitor solution to each well designated "Test Inhibitor".

10) Add 4 µl of the Diluent Solution to the "Positive Control" and "Negative Control".

11) Initiate the reaction by adding 5 µl of ATP to the wells labeled "Positive Control", "Test Inhibitor" and "Blank".

12) Add 5 µl of U1 Assay Buffer to the wells designated "Negative Control".

	Test Inhibitor	Negative Control	Positive Control
Master Mix	11 µl	11 µl	11 µl
Test Inhibitor	4 µl	–	–
Diluent Solution	–	4 µl	4 µl
U1 Assay Buffer	–	5 µl	–
ATP (4 mM)	5 µl	–	5 µl
Total	**20 µl**	**20 µl**	**20 µl**

13) Read the fluorescence intensity in a microtiter-plate reader capable of measuring TR-FRET in kinetic mode for up to 1 hour. An end point readout can be done in 20-40min.

14) "Blank" value should be subtracted from all other values.

Instrument Settings

Two sequential measurements should be conducted. Eu-donor emission should be measured at 620 nm followed by dye-acceptor emission at 665 nm. Data analysis is performed using the TR-FRET ratio (665 nm emission/620 nm emission).

Reading Mode	Time Resolved
Excitation Wavelength	317±20 nm
Emission Wavelength	620±10 nm
Lag Time	60 µs
Integration Time	500 µs
Excitation Wavelength	317±20 nm
Emission Wavelength	665±10 nm
Lag Time	60 µs
Integration Time	500 µs

CALCULATING RESULTS:

Data analysis is performed using the TR-FRET ratio (665 nm emission/620 nm emission). "Blank" value is subtracted from all other values.

When percentage activity is calculated, the FRET value from the Blank (it is expected that Blank and Negative Control have a similar value) can be set as zero percent activity and the FRET value from the positive control can be set as one hundred percent activity.

$$\% \text{ Activity} = \frac{FRET_s - FRET_{blank}}{FRET_p - FRET_{blank}} \times 100\%$$

Where FRETs = Sample FRET, $FRET_{blank}$ = Blank FRET, and $FRET_P$ = Positive control FRET.

Example Results

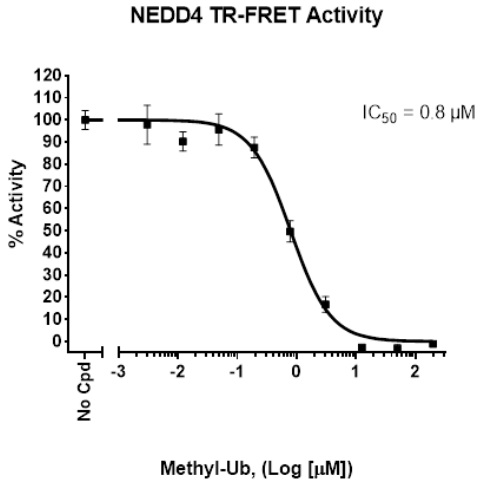

Figure 2: Inhibition of NEDD4 auto-ubiquitination by Methylated Ubiquitin. NEDD4 auto-ubiquitination was measured in the presence of increasing concentrations of Methylated Ubiquitin. Results are expressed as percent activity, in which absence of inhibitor is set to 100%.

Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com.

Troubleshooting Guide

Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For all further questions, please email support@bpsbioscience.com

Related Products

Products	*Catalog #*	*Size*
Cereblon intrachain TR-FRET Assay Kit	78301	384 reactions
MDM2 intrachain TR-FRET Assay Kit	78302	384 reactions
SMURF1 intrachain TR-FRET Assay Kit	78303	384 reactions
SMURF2 intrachain TR-FRET Assay Kit	78304	384 reactions
VHL intrachain TR-FRET Assay Kit	78305	384 reactions
XIAP intrachain TR-FRET Assay Kit	78306	384 reactions
MDM2 TR-FRET Assay Kit	79773	384 reactions
VHL/CUL2/ELOB/ELOC/RBX1 Complex Recombinant	100373	10 µg