Description

The TRAF6 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 TRAF6 (TNF receptor associated factor 6) 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 TRAF6, 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.

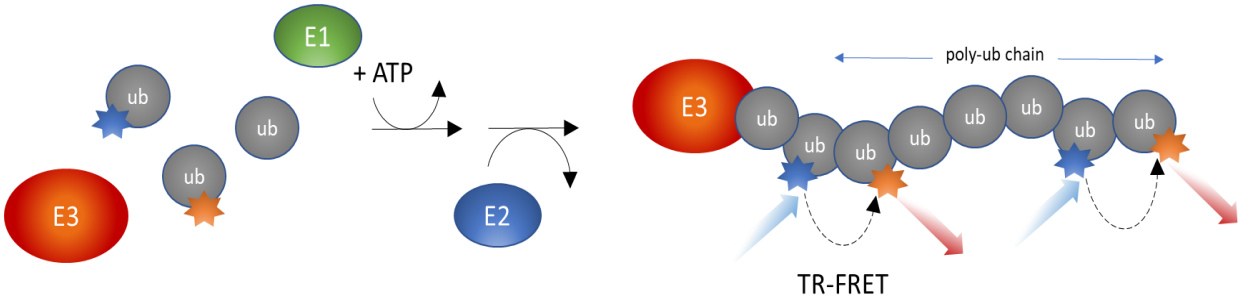

Figure 1. E3 ligase TRAF6 intrachain TR-FRET Assay Kit schematic.

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.

Application(s)

- Screen molecules that inhibit TRAF6 Ub ligase activity in drug discovery HTS applications.
- Determine compound IC_{50}.
- Perform TRAF6 ubiquitination real-time kinetics.

Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), FLAG-Tag*	50 µg	-80°C
80314	UbcH5b, His-Tag*	60 µg	-80°C
101597	TRAF6, GST-Tag*	16 µg	-80°C
78307	Ubiquitin Mix (200x)	50 µl	-80°C
	ATP (4 mM)	2 x 1 ml	-80°C
	U2 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 TRAF6 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.
- Calculate the amount needed for the desired number of wells for each protein, assay buffer, and ATP. Aliquot the remaining into 3-4 single-use aliquots depending on how many times the plate will be used and immediately store at -80°C.

1. Thaw **UBE1**, **UbcH5b**, **TRAF6**, **Ubiquitin Mix**, **U2 Assay Buffer**, and **ATP** on ice. Briefly spin the tubes to recover their full content.

2. Prepare a 5x Ubiquitin Mix in U2 Assay Buffer by making a 40-fold dilution of 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 U2 Assay Buffer to 96 ng/µl (800 nM - final concentration in the reaction will be 40 nM) (1 µl/well).

    b) Dilute UbcH5b in U2 Assay Buffer to 144 ng/µl (8 µM - final concentration in the reaction will be 400 nM) (1 µl/well).

    c) Dilute TRAF6 in U2 Assay Buffer to 8 ng/µl (200 nM - final concentration in the reaction will be 50 nM) (5 µl/well).

    Note: Keep all diluted proteins on ice until use. Do not freeze and re-use diluted proteins. UBE1, UbcH5b, TRAF6, Ubiquitin Mix, and U2 Assay Buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles.

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 U2 Assay Buffer, 5-fold more concentrated than the desired final concentrations. For the positive and negative controls, use U2 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 DMSO, then dilute the inhibitor 20-fold in U2 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 U2 Assay Buffer to keep the concentration of DMSO constant.

   For positive and negative controls, prepare 5% DMSO in water (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 U2 Assay Buffer).

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

Component	µl
Ubiquitin Mix (5x)	4 µl
Diluted UBE1	1 µl
Diluted UbcH5b	1 µl
Diluted TRAF6	-
Test Compound	-
Diluent Solution	4 µl
U2 Assay Buffer	5 µl
ATP (4 mM)	5 µl
Total	**20 µl**

7. Prepare a Master Mix: N wells × (4 µl of 5x Ubiquitin Mix + 1 µl diluted UBE1 + 1 µl diluted UbcH5b + 5 µl diluted TRAF6).

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 Diluent Solution to the "Positive Control" and "Negative Control" wells.

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 U2 Assay Buffer to the wells designated "Negative Control".

Component	Test Inhibitor	Negative Control	Positive Control
Master Mix	11 µl	11 µl	11 µl
Test compound	4 µl	–	–
Diluent Solution	–	4 µl	4 µl
U2 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 20-40 minutes.

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 similar values) 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

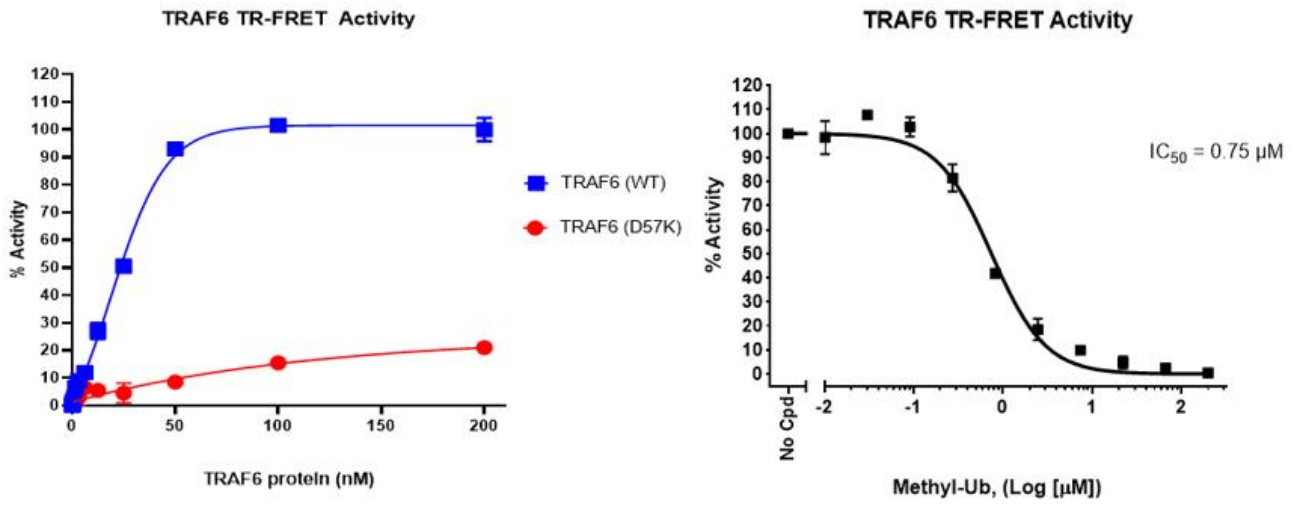

Figure 2: TRAF6 activity measured by TR-FRET.
Left Figure: Activity of TRAF6 wild type (WT) protein and TRAF6(D57K) mutant was tested at different concentrations. Right Figure: Inhibition of TRAF6 auto-ubiquitination in the presence of increasing concentrations of Methylated Ubiquitin.

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*
DCAF11 Intrachain TR-FRET Assay Kit	78542	384 reactions
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
VHL Intrachain TR-FRET Assay Kit	78305	384 reactions
XIAP Intrachain TR-FRET Assay Kit	78306	384 reactions