Description

The TRAF6 intrachain TR-FRET Assay Kit is a sensitive high-throughput screening (HTS) TR-FRET Assay Kit, designed to measure TRAF6 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.

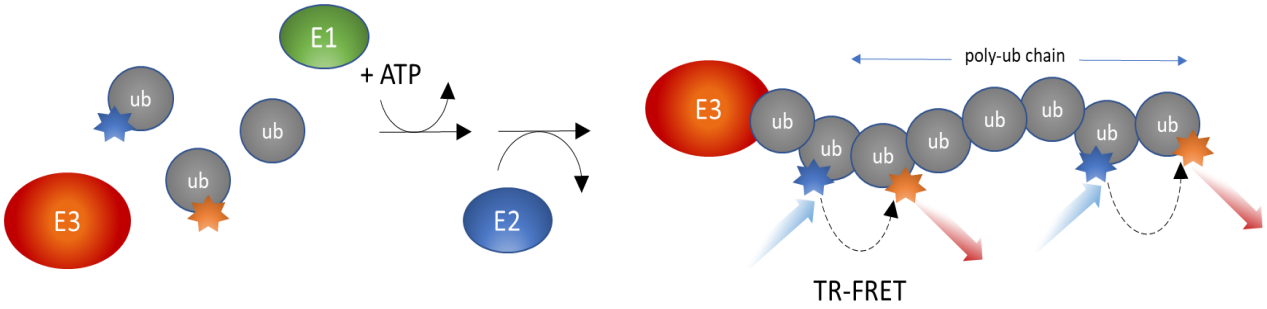

Figure 1. E3 ligase TRAF6 intrachain TR-FRET Assay Kit schematic.

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 real-time kinetics

Supplied Materials

Catalog #	Name	Amount	Storage	
80301	UBE1 (UBA1), FLAG-Tag (E1)*	50 µg	-80°C	***Avoid multiple freeze/ thaw cycles***
80314	UbcH5b, His-Tag (E2)*	300 µg	-80°C	
101597	TRAF6, GST-Tag (E3)*	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.*

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 no higher than 5% DMSO solution in buffer and using 4 µl per well.

Assay Protocol

- All samples and controls should be performed in triplicates
- The assay should include a "Blank", a "Positive control", and a "Negative control"

1. Thaw **UBE1**, **UBCH5b**, **TRAF6**, **Ubiquitin Mix**, **U2 Assay Buffer**, and **ATP** on ice. Briefly spin the tubes to recover their full contents.

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

   Example: Add 1 volume of stock Ubiquitin Mix to 39 volumes of U2 Assay Buffer.

3. Calculate the amount of protein required for the assay and dilute enough for the assay. Refer to the protocol below to calculate how much of each protein is needed.

4. 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 assay buffer at 96 ng/µl (800 nM - final concentration in the reaction will be 40 nM);
   b. Dilute **UBCH5b** in assay buffer at 144 ng/µl (8 µM - final concentration in the reaction will be 400 nM);
   c. Dilute **TRAF6** in assay buffer at 8 ng/µl (200 nM - final concentration in the reaction will be 50 nM);

   Note: Keep all diluted proteins on ice until use. Do not freeze and re-use diluted proteins.

5. Aliquot the remainder of each protein, U2 Assay Buffer, and the ATP into 2-4 single-use aliquots as may be necessary and immediately store at -80°C.

   Note: UBE1, UBCH5b, TRAF6, Ubiquitin Mix, and U2 Assay Buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles.

6. 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.

   Without DMSO

   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 1x Assay Buffer (Diluent Solution).

   Or

   With DMSO

   a. 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%.

   b. 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.

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

      The final concentration of DMSO should not exceed 1%.

7. To the wells designated as "Blank", add 4 µl of **5x Ubiquitin Mix** + 1 µl of **UBE1** + 1 µl of **UBCH5b** + 4 µl of **diluent solution** (for example 5% DMSO in U2 Assay Buffer) + 5 µl of **U2 Assay Buffer**.

Component	µl
Ubiquitin Mix (5x)	4 µl
UBE1	1 µl
UBCH5b	1 µl
TRAF6	-
Test Compound	-
Diluent solution* (no inhibitor)	4 µl
U2 Assay Buffer	5 µl
ATP (4 mM)	5 µl
Total	**20 µl**

**The diluent solution contains the assay buffer with the same concentration of solvent (e.g. DMSO) as the test compound solution.*

8. Prepare Master Mix (using diluted reagents prepared in steps 2 and 3): N wells × (4 µl of **5x Ubiquitin Mix** + 1 µl **UBE1** + 1 µl **UBCH5b** + 5 µl **TRAF6**).

9. Add 11 µl of Master Mix to each well designated for the "Negative Control", "Positive Control", "Test Sample".

10. Add 4 µl of inhibitor solution to each well designated "Test Inhibitor". For "Positive Control" and "Negative Control", add 4 µl of the diluent solution without inhibitor.

11. Initiate the reaction by adding 5 µl of **ATP** to the wells labeled "Positive Control" and "Test Inhibitor," and "Blank". Add 5 µl of **U2 Assay Buffer** to the wells designated "Negative Control." Cover the plate with a plate sealer. Incubate the reaction at room temperature for two hours or at 30°C for one hour.

Component	Test Sample	Negative Control	Positive Control
Master Mix	11 µl	11 µl	11 µl
Test compound	4 µl	–	–
Diluent solution* (no inhibitor)	–	4 µl	4 µl
U2 Assay Buffer	–	5 µl	–
ATP (4 mM)	5 µl	–	5 µl
Total	**20 µl**	**20 µl**	**20 µl**

**The diluent solution contains the assay buffer with the same concentration of solvent (e.g. DMSO) as the test compound solution.*

12. Read the fluorescent intensity in a microtiter-plate reader capable of measuring TR-FRET. "Blank" value is subtracted from all other values.

Instrument Settings

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:

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).

When percentage activity is calculated, the FRET value from the Blank (it is expected that Blank and Negative Control represent 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

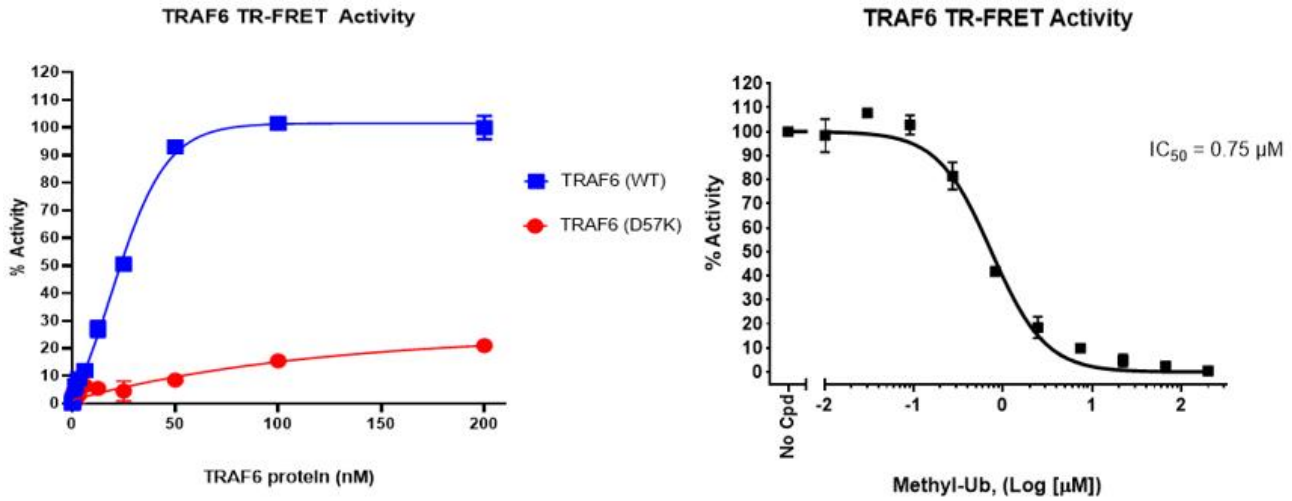

Figure 2: TRAF6 TR-FRET Activity.
TRAF6 E3 ligase activity was measured using the TRAF6 intrachain TR-FRET Assay Kit (BPS Bioscience #78598). Left Figure: Titration of TRAF6 wild type (WT) protein and TRAF6(D57K) mutant. Right Figure: Inhibition of TRAF6 auto-ubiquitination. TRAF6 auto-ubiquitination was measured in the presence of increasing concentrations of Methylated Ubiquitin.

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