Description

The UbcH13 TR-FRET Assay Kit is a homogeneous, sensitive TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) assay kit designed to measure UbcH13 (Ubiquitin-conjugating enzyme E2N) ubiquitination activity. It utilizes biotin-labeled Ubiquitin and a Terbium-labeled antibody recognizing the His-tagged UbcH13 protein to complete the TR-FRET pairing. The kit contains enough purified UbcH13, purified UBE1 (ubiquitin activating enzyme 1), Biotin-Ubiquitin, anti-His Tb-labeled donor, dye-labeled acceptor, and assay buffer for 400 reactions.

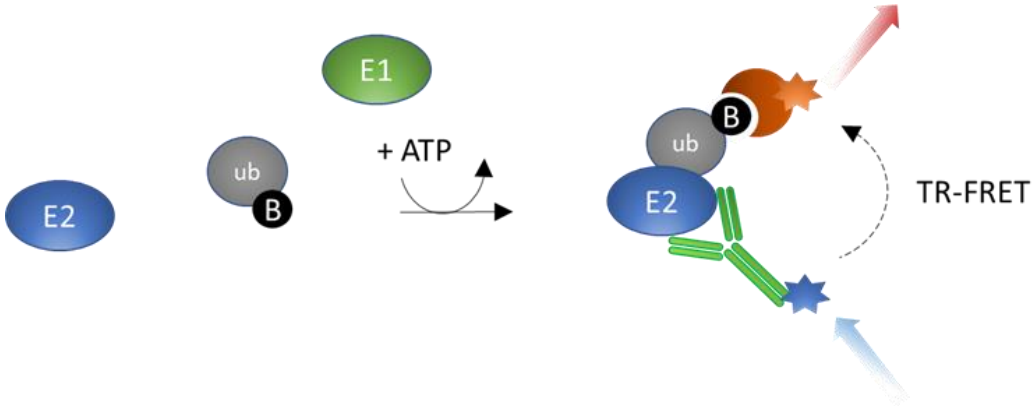

Figure 1: UbcH13 TR-FRET Assay Kit schematic.

The Terbium-labeled anti-His antibody binds to the His-tagged E2 conjugating protein, while the Dye-labeled streptavidin acceptor will bind to Biotin-Ubiquitin. The complex forms when ubiquitin is transferred to the E2 enzyme, and the TR-FRET signal can be measured using a fluorescence plate reader capable of measuring Time Resolved-Fluorescence Resonance Energy Transfer. The TR-FRET signal is proportional to UbcH13 activity.

Background

UbcH13, also known as Ubiquitin Conjugating Enzyme E2N, or UBE2N, also known as UEV1A, is a noncanonical ubiquitin conjugating enzyme (E2). E2 conjugating ubiquitin proteins receive Ubiquitin from Ubiquitin-activating E1 in an ATP-dependent fashion and transfer it to E3 ligases. UbcH13 has been implicated in a variety of cellular signaling processes due to its ability to catalyze the formation of lysine 63-linked polyubiquitin chains on various substrates, such as the TNF Receptor-Associated Factor 6 (TRAF6). UbcH13 is also critical for double-strand DNA repair. UbcH13 inhibition blocks NF-κB and p53 signaling in Diffuse-Large B-Cell lymphoma cell lines.

Applications

Screen molecules that inhibit UbcH13 activity in drug discovery High-Throughput Screening (HTS) applications.

Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), FLAG-Tag*	12 µg	-80°C
80323	UbcH13 (UBE2N), His-Tag*	>2.5 µg	-80°C
78294	Biotin-Ubiquitin	80 µl	-80°C
82509	4 mM ATP	1 ml	-80°C
78856	U2 Assay Buffer	2 x 10 ml	-80°C
30017	Anti-His Tb-Labeled Donor	10 µl	-20°C
82510	Dye-Labeled Acceptor	10 µl	-20°C
79969	White, nonbinding, low volume microtiter plate	1	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
- Adjustable micropipettor and sterile tips
- Orbital Shaker

Storage Conditions

This assay kit will perform optimally for up to **6 months** from date of receipt when the materials are stored as directed.

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 final concentration of DMSO in the assay should not exceed 1%.
- Compounds that are fluorescent may interfere with the results, depending on their spectral excitation and emission properties.
- It is recommended that the compound alone is tested to determine any potential interference of the compound on the assay results.

Assay Protocol

- All samples should be run in duplicate while controls should be performed in quadruplicate.
- The assay should include “Blank”, “Positive Control”, “Negative Control” and “Test Inhibitor” conditions.
- It is recommended all controls are run side by side as they may be necessary for result calculation.
- We recommend using Bay 11-7821 as an internal control for the assay. If not running a dose response curve for the control inhibitor, run at 0.1X, 1X, and 10X the IC_{50} value shown in the validation data below.

- We recommend maintaining the diluted protein on ice during use.
- For detailed information on protein handling please refer to Protein FAQs (bpsbioscience.com).
- For instructions on how to prepare reagent dilutions please refer to Serial Dilution Protocol (bpsbioscience.com).

1. Thaw **UBE1**, **UbcH13**, **U2 Assay Buffer**, **Biotin-Ubiquitin**, and **ATP** on ice. Briefly spin the tubes to recover their full content.

2. Dilute **UBE1** to 20 ng/µl with U2 Assay Buffer (1.5 µl/well).

3. Dilute **UbcH13** to 4 ng/µl with U2 Assay Buffer (1.5 µl/well).

4. Dilute **Biotin-Ubiquitin** 5-fold with U2 Assay Buffer (1 µl/well).

5. Prepare a **Master Mix** as follows (5.5 µl/well, except "Blank" wells): N wells x (1.5 µl of diluted UBE1 + 1.5 µl of diluted UbcH13 + 1 µl of diluted Biotin-Ubiquitin + 1.5 µl of U2 Assay Buffer).

6. Add 5.5 µl of **Master Mix** to the "Positive Control", "Negative Control" and "Test Inhibitor" wells.

7. Add 5.5 µl of **U2 Assay Buffer** to the "Blank" wells.

8. Prepare the Test Inhibitor (2 µl/well): for a titration, prepare serial dilutions at concentrations 10-fold higher than the desired final concentrations. The final volume of the reaction is 20 µl.

   8.1 If the Test Inhibitor is water-soluble, prepare serial dilutions in U2 Assay Buffer, 10-fold more concentrated than the desired final concentrations.

   For the positive and negative controls, use U2 Assay Buffer (Diluent Solution).

   OR

   8.2 If the Test inhibitor is soluble in DMSO, prepare it in 100% DMSO at a concentration 100-fold higher than the highest desired concentration, then dilute 10-fold in U2 Assay Buffer to prepare the highest concentration of the 10-fold intermediate dilutions. The concentration of DMSO is now 10%.

   Prepare serial dilutions of the Test Inhibitor at 10-fold the desired final concentrations using 10% DMSO in U2 Assay Buffer to keep the concentration of DMSO constant.

   For controls prepare 10% DMSO in U2 Assay Buffer (Diluent Solution) so that all wells contain the same amount of DMSO.

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

9. Add 2 µl of inhibitor solution to each well designated "Test Inhibitor" wells.

10. Add 2 µl of Diluent Solution to the "Positive Control", "Negative Control" and "Blank" wells.

11. Dilute together **Anti-His Tb-Labeled Donor** 400-fold and **Dye-Labeled Acceptor** 400-fold with U2 Assay Buffer (10 µl of mix/well). This makes the **Donor/Acceptor Mix**.

12. Add 10 µl of **Donor/Acceptor Mix** to each well.

    Note: Consider a preincubation step for 30 min in Room Temperature prior to adding the substrate (ATP).

13. Initiate the reaction by adding 2.5 µl of **4 mM ATP** to the "Blank", "Test Inhibitor", and "Positive Control" wells.

14. Add 2.5 µl **U2 Assay Buffer** to the "Negative Control" wells.

15. Protect from light and incubate the reaction at RT for 15-20 minutes or perform kinetic analysis for up to 1 hour.

16. Read the fluorescent intensity in a microtiter-plate reader capable of measuring TR-FRET.

17. The "Blank" value should be subtracted from all other values.

	Blank	**Test Sample**	**Positive Control**	**Negative Control**
Master Mix	-	5.5 µl	5.5 µl	5.5 µl
U2 Assay Buffer	5.5 µl	-	-	2.5 µl
Test Inhibitor	-	2 µl	-	
Diluent Solution	2 µl	–	2 µl	2 µl
Donor/Acceptor Mix	10 µl	10 µl	10 µl	10 µl
ATP (4 mM)	2.5 µl	2.5 µl	2.5 µl	-
Total	**20 µl**	**20 µl**	**20 µl**	**20 µl**

Instrument Settings

Two sequential measurements should be conducted. Tb-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	340 (20 nm bandwidth)
Emission Wavelength	620 (10 nm bandwidth)
Lag Time	60 µs
Integration Time	500 µs
Excitation Wavelength	340 (20 nm bandwidth)
Emission Wavelength	665 (10 nm bandwidth)
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).

$$FRET = \frac{S_{665}}{S_{620}}$$

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

$$\%\ Activity = \frac{FRET_S - FRET_{blank}}{FRET_P - FRET_{blank}} \times 100\%$$

$FRET_S$ =FRET value for samples of Test Inhibitor, $FRET_{blank}$ = FRET value for the Blank, and $FRET_p$ = FRET value for the Positive Control (no inhibitor).

Example Results

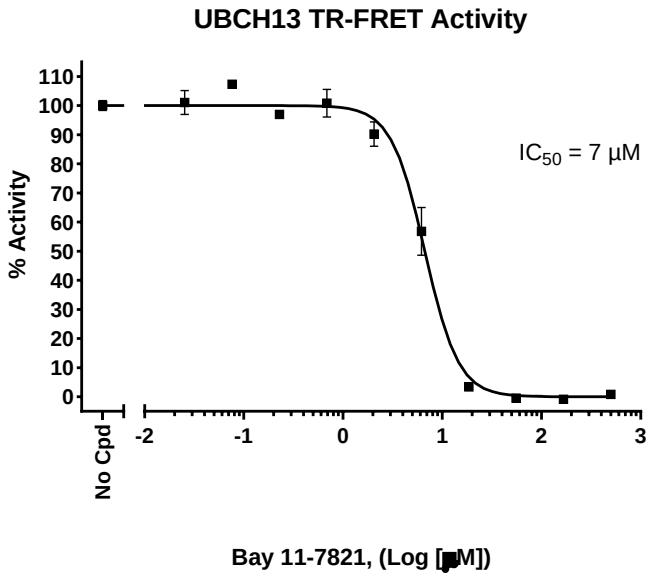

Figure 2: UbcH13 activity is inhibited by Bay 11-7821.
UbcH13 activity was measured in the presence of increasing concentrations of Bay 11-7821 (Tocris #1744). Results are expressed as percentage of activity relative to positive control (measured in the absence of inhibitor and set at 100%).

Data shown is representative.

Troubleshooting Guide

Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For lot-specific information and all other questions, please visit https://bpsbioscience.com/contact.

Related Products

Products	*Catalog #*	*Size*
ChoosE3-Freedom™ Intrachain TR-FRET Assay Kit	78560	384 reactions
ChoosE2-Opti™ Intrachain TR-FRET Assay Kit	78561	384 reactions
UbcH7 TR-FRET Assay Kit	78861	384 reactions
UbcH5a TR-FRET Assay Kit	79900	384 reactions
UbcH5b TR-FRET Assay Kit	79896	384 reactions
UbcH5c TR-FRET Assay Kit	79901	384 reactions

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