

Description

The Ubch5b TR-FRET Assay Kit is a homogeneous, sensitive TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) assay kit designed to measure Ubch5b (Ubiquitin-conjugating enzyme E2 D2) ubiquitination activity. It utilizes biotin-labeled Ubiquitin and a Terbium-labeled antibody recognizing the His-tagged Ubch5b protein to complete the TR-FRET pairing. The kit contains enough purified Ubch5b, purified UBE1, Biotin-Ubiquitin, anti-His Tb-labeled donor, dye-labeled acceptor, and assay buffer for 400 reactions.

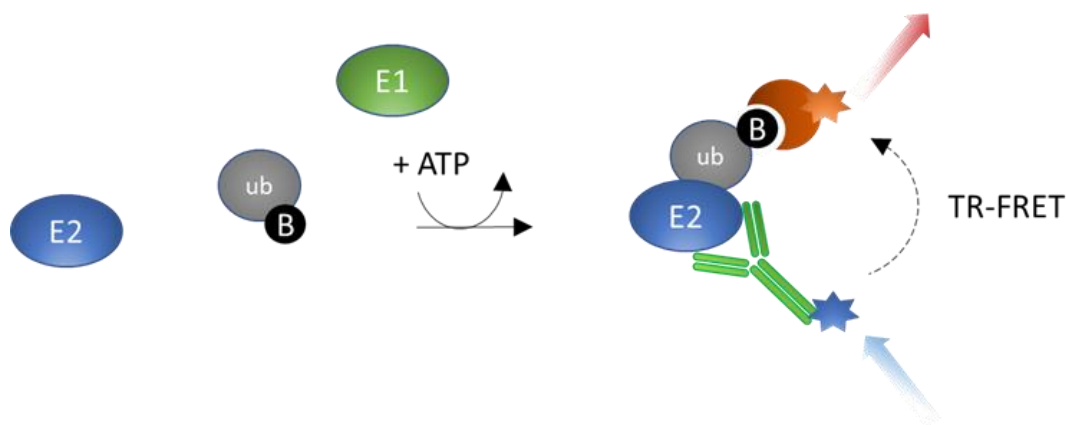


Figure 1: Ubch5b TR-FRET Assay Kit schematic.

The Terbium-labeled anti-His antibody binds to the His-tagged E2 conjugating protein, while the Dye-labeled streptavidin acceptor binds to Biotin-Ubiquitin. The complex forms when ubiquitin is transferred to the E2 enzyme, and the TR-FRET signal is measured using a fluorescence plate reader capable of measuring Time Resolved-Fluorescence Resonance Energy Transfer. The TR-FRET signal is proportional to Ubch5b activity.

**NOTE: As of January 2024, this protocol has been re-optimized for performance. Previous versions of this kit are available upon request.*

Background

Ubch5b (also known as Ubiquitin-conjugating Enzyme E2 D2, UBE2D2) is an E2 conjugating ubiquitin protein. E2 conjugating proteins receive Ubiquitin from E1 in an ATP dependent fashion and transfer it to an E3 ligase. Ubch5b is overexpressed in certain cancer types. It functions in the ubiquitination of tumor-suppressor proteins p53 and p62. Ubch5b-p62 complex confers therapeutic resistance in triple negative breast cancer cells. Ubch5b is also involved in endocytosis-mediated degradation of MHC class I molecules, and overexpression has been found in inflammatory bowel disease. Inhibitors of Ubch5b may prove beneficial in cancer and auto-immune disease treatment.

Applications

Screen molecules that inhibit UBCH5b activity in drug discovery High-Throughput Screening (HTS) applications.

Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), FLAG-Tag*	12 µg	-80°C
80314	Ubch5b, His-Tag (Human)*	>2.5 µg	-80°C
	Biotin-Ubiquitin	80 µl	-80°C
	4 mM ATP	1 ml	-80°C
	U2 Assay Buffer	2 x 10 ml	-80°C
30017	Anti-His Tb-Labeled Donor	10 µl	-20°C
	Dye-Labeled Acceptor	10 µl	-20°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
- 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 Ubch5b TR-FRET Assay Kit is compatible with up to 1% final DMSO concentration.

Assay Protocol

- All samples and controls should be performed in triplicate.
- The assay should include “Blank”, “Positive Control”, “Negative Control” and “Test Inhibitor” conditions.
- We recommend maintaining the diluted protein on ice during use.
- For detailed information on protein handling please refer to Protein FAQs (bpsbioscience.com).
- We recommend using Bay11-7821 as internal control. If not running a dose response curve for the control inhibitor, we recommend running the control inhibitor at 0.1X, 1X and 10X the IC₅₀ value shown in the validation data below.

1. Thaw **UBE1**, **UbcH5b**, **U2 Assay Buffer**, **Biotin-Ubiquitin**, and **ATP** on ice. Briefly spin the tubes to recover their full content.
2. Dilute **UBE1** to 20 ng/ μ l with U2 Assay Buffer (1.5 μ l/well).
3. Dilute **UbcH5b** to 4 ng/ μ l with U2 Assay Buffer (1.5 μ l/well).
4. Dilute Biotin-Ubiquitin 5-fold with U2 Assay Buffer (1 μ l/well).
5. Prepare a **Master Mix** as follows (5.5 μ l/well): N wells x (1.5 μ l of diluted UBE1 + 1.5 μ l of diluted UbcH5b + 1 μ l of diluted Biotin-Ubiquitin + 1.5 μ l of U2 Assay Buffer).
6. Add 5.5 μ l of **Master Mix** to the “Positive Control”, “Negative Control” and “Test Inhibitor” wells.
7. Add 5.5 μ l of U2 Assay Buffer to the “Blank” wells.
8. Prepare the Test Inhibitor (2 μ l/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 μ l.
 - 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 μ l of inhibitor solution to each well designated “Test Inhibitor”.
10. Add 2 μ l of the 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 μ l/well). This makes the **Donor/Acceptor Mix**.

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

Note: Consider preincubation step for 30 minutes in Room Temperature (RT) prior 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
4 mM ATP	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 \pm 20 nm
Emission Wavelength	620 \pm 10 nm
Lag Time	60 μs
Integration Time	500 μs
Excitation Wavelength	340 \pm 20 nm
Emission Wavelength	665 \pm 10 nm
Lag Time	60 μs
Integration Time	500 μs

CALCULATING RESULTS: Calculate the FRET value by using the following formula:

$$FRET = \frac{S_{665} - \left(\frac{Tb_{665}}{Tb_{620}} \times S_{620} \right)}{S_{620}} \times 1000$$

S_{665} = Sample value measured at 665 nm, S_{620} = Sample value measured at 620 nm, Tb_{665} = Tb only or Blank value measured at 665 nm, Tb_{520} = Tb only or Blank value measured at 520 nm.

The FRET value calculated for the negative control should be subtracted from all other measurements and can be set as 0%. The FRET value from the "Positive Control" can be set as 100% activity.

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

$FRET_S$ = FRET value for samples of Test Inhibitor, $FRET_{Sub}$ = FRET value for the Substrate Control, and $FRET_P$ = FRET value for the Positive Control (no inhibitor).

Example Results

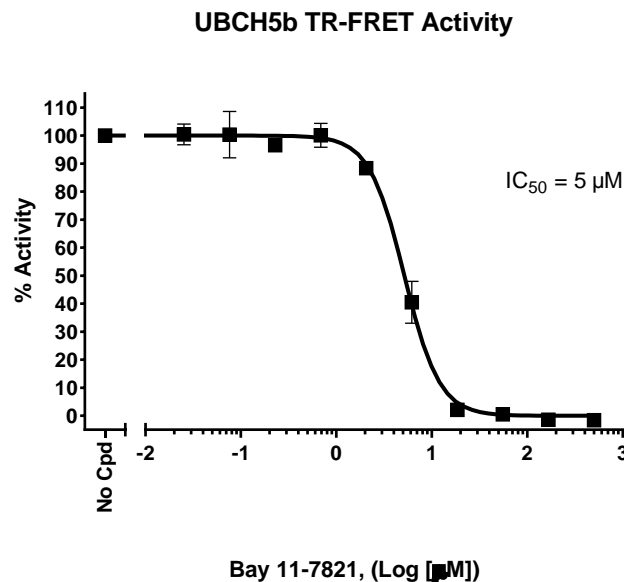


Figure 2: UbcH5b activity is inhibited by Bay11-7821.

UbcH5b activity was measured in the presence of increasing concentrations of Bay11-7821 (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. 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 further questions, email support@bpsbioscience.com

Related Products

<i>Products</i>	<i>Catalog #</i>	<i>Size</i>
ChoosE3-Freedom™ Intrachain TR-FRET Assay Kit	78560	384 reactions
ChoosE2-Opti™ Intrachain TR-FRET Assay Kit	78561	384 reactions
Ubch6 TR-FRET Assay Kit	78862	384 reactions
Ubch6 TR-FRET Assay Kit	78861	384 reactions
Ubch5a TR-FRET Assay Kit	79900	384 reactions
Ubch5c TR-FRET Assay Kit	79901	384 reactions

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