Description

The UbcH6 TR-FRET Assay Kit is a homogeneous, sensitive TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) assay kit designed to measure UbcH6 (Ubiquitin-conjugating enzyme E2) ubiquitination activity. It utilizes biotin-labeled Ubiquitin and a Terbium-labeled antibody recognizing the His-tagged UbcH6 protein to complete the TR-FRET pairing. The kit contains enough purified UbcH6, purified UBE1, Biotin-Ubiquitin, anti-His Tb-labeled donor, dye-labeled streptavidin acceptor, and assay buffer for 400 reactions.

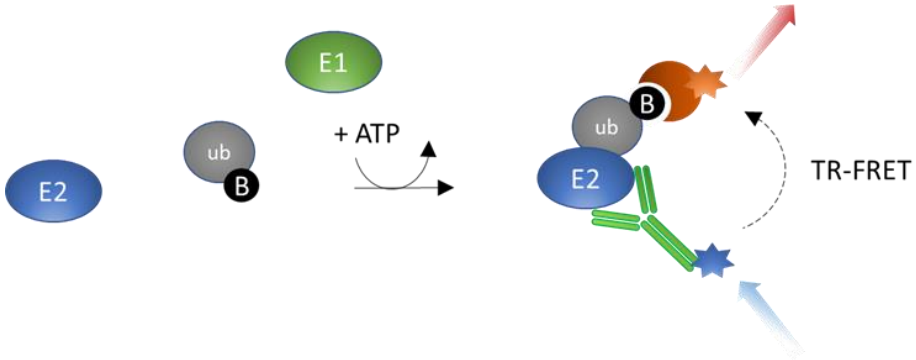

Figure 1: UbcH6 TR-FRET Assay Kit schematic.

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 can be measured using a fluorescence plate reader capable of measuring Time Resolved-Fluorescence Resonance Energy Transfer. The TR-FRET signal is proportional to UbcH6 activity.

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

Background

UbcH6 (also known as Ubiquitin-conjugating enzyme E2 E1, UBE2E1) is an E2 ubiquitin-conjugating protein that receives Ubiquitin from a Ubiquitin-activating enzyme (E1) in an ATP-dependent fashion and transfers it to an E3 ligase. UbcH6 is regulated by Ubiquitin-specific protease 7 (USP7) and it is part of PRC1 (Polycomb Repressive Complex 1), an E3 complex that ubiquitinates histone H2 and it is thus involved in the proliferation of stem cells and cancer cells. It also interacts with and ubiquitinates ataxin-1, regulating its activity. Ataxin-1 is involved in spinocerebellar ataxia type 1 (SCA1), an autosomal-dominant neurodegenerative disease. Targeting UbcH6 may have therapeutical potential for the treatment of cancer and SCA1.

Applications

Screen molecules that inhibit UbcH6 activity in drug discovery and high throughput screening (HTS) applications.

Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), FLAG-Tag*	12 µg	-80°C
80316	UbcH6 (UBE2E1), His-Tag*	>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 UbcH6 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 IC_{50} value shown in the validation data below.

1. Thaw **UBE1**, **UbcH6**, **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 **UbcH6** 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): N wells x (1.5 µl of diluted UBE1 + 1.5 µl of diluted UbcH6 + 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 the 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 "Test Inhibitor" wells.

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

11. Dilute together the Anti-His Tb-labeled donor 400-fold and Dye-labeled acceptor 400-fold with U2 Assay Buffer (10 µl/well). This is the **Donor/Acceptor Mix**.

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

    Note: Consider preincubation step for 30 min 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".

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. 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	340±20 nm
Emission Wavelength	620±10 nm
Lag Time	60 µs
Integration Time	500 µs
Excitation Wavelength	340±20 nm
Emission Wavelength	665±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.

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

$FRET_S$ =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

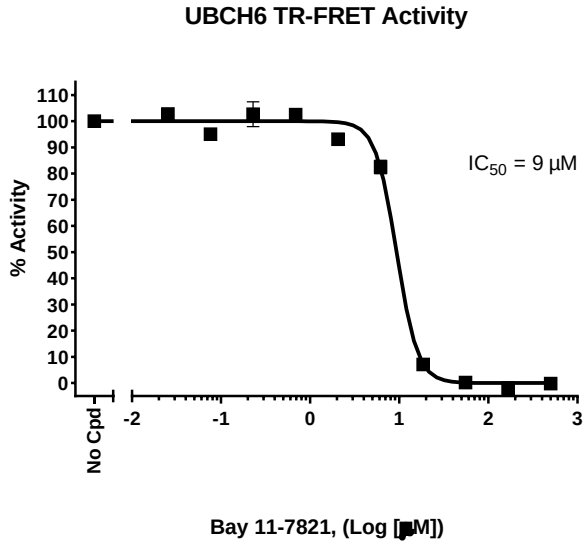

Figure 2: UbcH6 activity is inhibited by Bay11-7821.
UbcH6 activity was measured in the presence of increasing concentrations of Bay11-7821 (Tocris #1744). Results are expressed as percentage of activity relative to positive control (measured in the absence of inhibitor and set at 100%).

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

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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