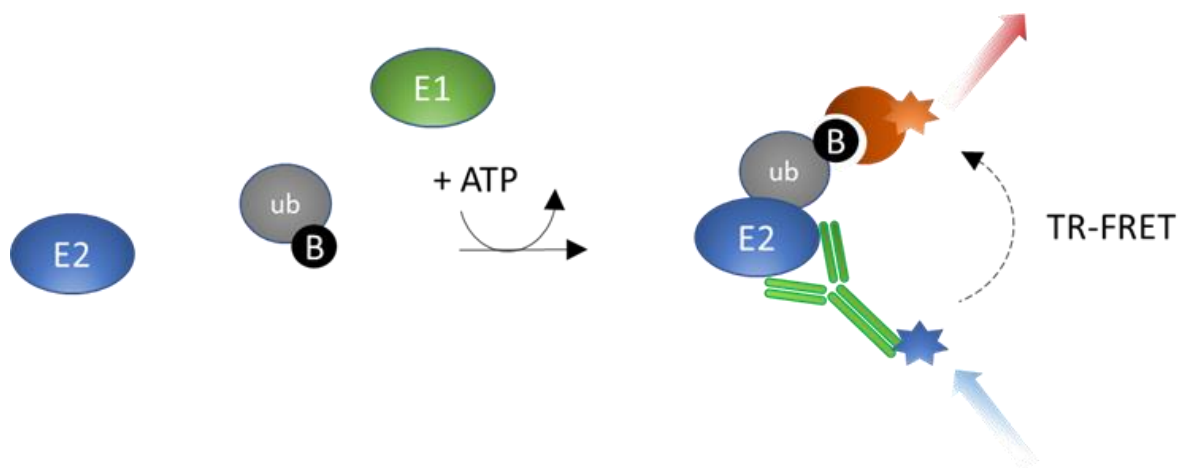


## Description

The Ubch5c TR-FRET Assay Kit is a homogeneous, sensitive TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) assay kit designed to measure Ubch5c (Ubiquitin-conjugating enzyme E2 D3) ubiquitination activity. It utilizes a biotin-labeled Ubiquitin and a Terbium-labeled antibody recognizing the His-tagged Ubch5c protein to complete the TR-FRET pairing. The kit contains enough purified Ubch5c, purified UBE1, Biotin-Ubiquitin, anti-His Tb-labeled donor, dye-labeled acceptor, and assay buffer for 400 reactions.



*Figure 1: Ubch5c 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 Ubch5c activity.

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

## Background

Ubch5c (also known as Ubiquitin-conjugating enzyme E2 D3, UBE2D3) is an E2 conjugating ubiquitin protein of the Ubch5 protein family. E2 conjugating ubiquitin proteins receive Ubiquitin from E1 in an ATP-dependent manner and transfer it to E3. Ubch5c regulates the ubiquitination of transcription factors such as nuclear factor kappa-B (NF-κB) and p53 tumor suppressor, and it thus involved in diseases such as rheumatic arthritis (RA) and cancer. Small molecule inhibitors of Ubch5c alter expression levels and activity of p53 and are thus promising therapeutical candidates in cancer therapy.

## Applications

Screen molecules that inhibit Ubch5c activity in drug discovery high-throughput screening (HTS) applications.

**Supplied Materials**

| Catalog # | Name   | Amount    | Storage   |
|-----------|--|-----------|-----------|
| 80301     | UBE1 (UBA1), FLAG-Tag*                         | 12 µg     | -80°C     |
| 80313     | UbcH5c, 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     |
| 79969     | 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 UbcH5c 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](http://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<sub>50</sub> value shown in the validation data below.

1. Thaw **UBE1**, **UbcH5c**, **U2 Assay Buffer**, **Biotin-Ubiquitin**, and **ATP** on ice. Briefly spin the tubes to recover their full content.
2. Dilute **UBE1** to 20 ng/ $\mu$ l with U2 Assay Buffer (1.5  $\mu$ l/well).
3. Dilute **UbcH5c** to 4 ng/ $\mu$ l with U2 Assay Buffer (1.5  $\mu$ l/well).
4. Dilute Biotin-Ubiquitin 5-fold with U2 Assay Buffer (1  $\mu$ l/well).
5. Prepare a **Master Mix** as follows (5.5  $\mu$ l/well): N wells x (1.5  $\mu$ l of diluted UBE1 + 1.5  $\mu$ l of diluted UbcH5c + 1  $\mu$ l of diluted Biotin-Ubiquitin + 1.5  $\mu$ l of U2 Assay Buffer).
6. Add 5.5  $\mu$ l of **Master Mix** to the “Positive Control”, “Negative Control” and “Test Inhibitor” wells.
7. Add 5.5  $\mu$ l of U2 Assay Buffer to the “Blank” wells.
8. Prepare the **Test Inhibitor** (2  $\mu$ 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  $\mu$ 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  $\mu$ l of Inhibitor dilutions to each well designated “Test Inhibitor” wells.
10. Add 2  $\mu$ l 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  $\mu$ l/well). This makes the **Donor/Acceptor Mix**.

12. Add 10  $\mu$ l 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  $\mu$ l of **4 mM ATP** to the “Blank”, “Test Inhibitor”, and “Positive Control” wells.

14. Add 2.5  $\mu$ l U2 Assay Buffer to the “Negative Control” wells.

15. Protect from light and incubate the reaction at RT for 30 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 Inhibitor              | Positive Control            | Negative Control            |
|--------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Master Mix         | -                           | 5.5 $\mu$ l                 | 5.5 $\mu$ l                 | 5.5 $\mu$ l                 |
| U2 Assay Buffer    | 5.5 $\mu$ l                 | -                           | -                           | 2.5 $\mu$ l                 |
| Test Inhibitor     | -                           | 2 $\mu$ l                   | -                           | -                           |
| Diluent Solution   | 2 $\mu$ l                   | -                           | 2 $\mu$ l                   | 2 $\mu$ l                   |
| Donor/Acceptor Mix | 10 $\mu$ l                  | 10 $\mu$ l                  | 10 $\mu$ l                  | 10 $\mu$ l                  |
| 4 mM ATP           | 2.5 $\mu$ l                 | 2.5 $\mu$ l                 | 2.5 $\mu$ l                 | -                           |
| <b>Total</b>       | <b>20 <math>\mu</math>l</b> | <b>20 <math>\mu</math>l</b> | <b>20 <math>\mu</math>l</b> | <b>20 <math>\mu</math>l</b> |

### **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 $\mu$ s      |
| Integration Time      | 500 $\mu$ s     |
| Excitation Wavelength | 340 $\pm$ 20 nm |
| Emission Wavelength   | 665 $\pm$ 10 nm |
| Lag Time              | 60 $\mu$ s      |
| Integration Time      | 500 $\mu$ s     |

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

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

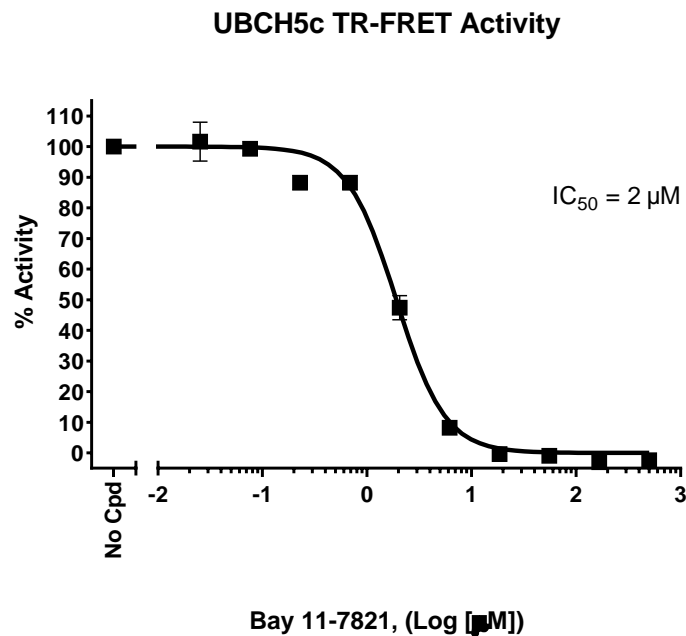
$S_{665}$  = Sample value measured at 665 nm,  $S_{620}$  = Sample value measured at 620 nm,  $Tb_{665}$  = Tb only or Blank value measured at 665 nm,  $Tb_{520}$  = Tb only or Blank value measured at 520 nm.

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: UbcH5c activity is inhibited by Bay11-7821.*

UbcH5c 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](https://bpsbioscience.com/assay-kits-faq) for detailed troubleshooting instructions. For further questions, email [support@bpsbioscience.com](mailto: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 |
| Ubch7 TR-FRET Assay Kit                       | 78861            | 384 reactions |
| Ubch5b TR-FRET Assay Kit                      | 79896            | 384 reactions |
| Ubch5a TR-FRET Assay Kit                      | 79900            | 384 reactions |

*Version 010324*