Description

The WWP1 Intrachain TR-FRET Assay Kit is a sensitive high-throughput screening (HTS) TR-FRET (Time Resolved Fluorescence Resonance Energy Transfer) assay kit, designed to measure WWP1 (WW Domain Containing E3 Ubiquitin Protein Ligase 1) auto-ubiquitination activity in a homogeneous 384 reaction format. This assay measures poly-ubiquitination of WWP1. The kit contains enough recombinant human WWP1 and reagents for 384 reactions.

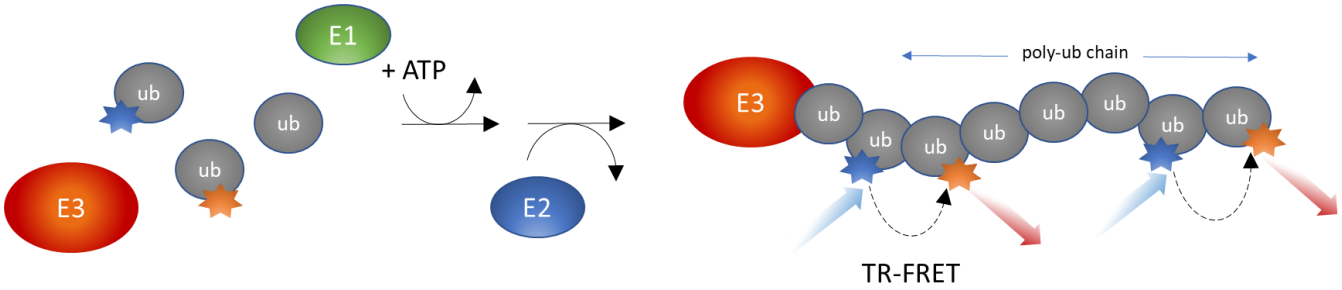

Figure 1. WWP1 Intrachain TR-FRET Assay Kit schematic.
E1 and E2 enzymes, Europium cryptate-labeled Ubiquitin (TR-FRET donor) and Cy5-labeled Ubiquitin (TR-FRET acceptor) are incubated with WWP1. The donor and acceptor are incorporated into poly-ubiquitin chains formed on WWP1, allowing energy transfer to occur. The TR-FRET signal is proportional to WWP1 activity.

Background

Covalent conjugation to ubiquitin (Ub) is one of the major post-translational modifications regulating 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.

WW Domain Containing E3 Ubiquitin Protein Ligase 1 (WWP1) is a HECT-type E3 Ub ligase belonging to the NEDD4 family of E3 ligases. WWP1 interacts with and ubiquitinates many substrates, including transcription factor ΔNp63 as well as phosphatase and tensin homolog (PTEN), and regulates the degradation of sodium channels and membrane receptors. Genetic mutations or regulatory defects involving WWP1 are associated with neurological disorders and cancer. Aberrant expression of WWP1 in gastric, prostate, and breast cancer, for instance, is an area of high interest, and therefore, WWP1 represents an excellent therapeutic candidate multiple cancer types.

Application(s)

- Screen molecules that inhibit WWP1 Ub ligase activity in HTS applications.
- Determine Inhibitor IC_{50}.
- Perform WWP1 real-time kinetic studies.

Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), FLAG-Tag*	50 µg	-80°C
80314	UbcH5b, His-Tag*	60 µg	-80°C
80405	WWP1, FLAG-Tag*	45 µg	-80°C
82185	200x Ubi-Mix™	40 µl	-80°C
82509	4 mM ATP	2 x 1 ml	-80°C
78856	U2 Assay Buffer	2 x 10 ml	-80°C
79969	White, nonbinding Corning, 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 (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 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 Methyl Ubiquitin 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**, **UbcH5b**, **WWP1**, **200x Ubi-Mix™**, **U2 Assay Buffer**, and **ATP** on ice. Briefly spin the tubes to recover their full content.

2. Dilute **200x Ubi-Mix™** 40-fold with U2 Assay Buffer to make a 5x Ubiquitin Mix.

3. Dilute proteins, as follows, and keep on ice:

   a) Dilute **UBE1** with U2 Assay Buffer to 96 ng/µl (800 nM - the final concentration in the reaction is 40 nM) (1 µl/well).

   b) Dilute **UbcH5b** with U2 Assay Buffer to 144 ng/µl (8 µM - the final concentration in the reaction is 400 nM) (1 µl/well).

   c) Dilute **WWP1** with U2 Assay Buffer to 21.2 ng/µl (200 nM - the final concentration in the reaction is 50 nM) (5 µl/well).

4. 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.

   4.1 If the Test Inhibitor is soluble in water, prepare a solution of the compound in U2 Assay Buffer that is 5-fold higher than the final desired concentration.

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

   OR

   4.2 If the Test Inhibitor is dissolved in DMSO, prepare a solution of the compound in 100% DMSO that is 100-fold higher than the highest concentration of the serial dilution. Then dilute 20-fold in U2 Assay Buffer (at this step the compound concentration is 5-fold higher than the desired final concentration). The concentration of DMSO in the dilution is now 5%.

   Prepare serial dilutions of the Test Inhibitor at concentrations 5-fold higher than the desired final concentrations using 5% DMSO in U2 Assay Buffer to keep the concentration of DMSO constant.

   For positive and negative controls, prepare 5% DMSO in U2 Assay Buffer (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

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

5. Prepare a **Master Mix** (11 µl/well, except "Blank" wells): N wells × (4 µl of 5x Ubi-Mix™ + 1 µl of diluted UBE1 + 1 µl of diluted UbcH5b + 5 µl of diluted WWP1).

6. Add 11 µl of **Master Mix** to the "Positive Control", "Negative Control" and "Test Inhibitor" wells.

7. For the "Blank" wells prepare a **WWP1 Deficient Master Mix** (11 µl/well): N wells x (4 µl of 5x Ubi-Mix™ + 1 µl of diluted UBE1 + 1 µl of diluted UbcH5b + 5 µl of U2 Assay Buffer).

8. Add 11 µl of **WWP1 Deficient Mix** to every "Blank" well.

9. Add 4 µl of **Test Inhibitor** to each well designated "Test Inhibitor".

10. Add 4 µl of the **Diluent Solution** to the "Blank", "Positive Control" and "Negative Control" wells.

11. Initiate the reaction by adding 5 µl of 4 mM **ATP** to the wells labeled "Positive Control", "Test Inhibitor" and "Blank" wells.

12. Add 5 µl of **U2 Assay Buffer** to the well designated "Negative Control".

13. Read the fluorescence intensity in a microtiter-plate reader capable of measuring TR-FRET in kinetic mode for up to 2 hours. An end point readout can be done in 70-90 min.

14. "Blank" value should be subtracted from all other values.

Component	Blank	Negative Control	Positive Control	Test Inhibitor
Master Mix	-	11 µl	11 µl	11 µl
WWP1 Deficient Master Mix	11 µl	-	-	-
Test Inhibitor	-	–	–	4 µl
Diluent Solution	4 µl	4 µl	4 µl	–
U2 Assay Buffer	-	5 µl	–	–
4 mM ATP	5 µl	–	5 µl	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	317 (20 nm bandwidth)
Emission Wavelength	620 (10 nm bandwidth)
Lag Time	60 µs
Integration Time	500 µs
Excitation Wavelength	317 (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

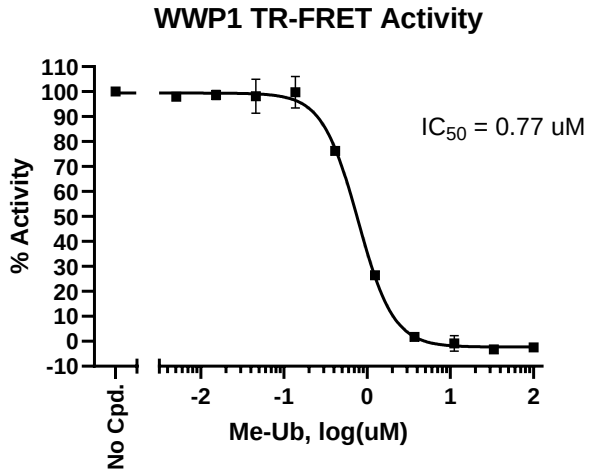

Figure 2: Inhibition of WWP1 auto-ubiquitination by Methyl Ubiquitin.
WWP1 auto-ubiquitination was measured in the presence of increasing concentration of Methyl Ubiquitin. Results are expressed as percent activity, in which absence of inhibitor is set to 100%.

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*
MDM2 Intrachain TR-FRET Assay Kit	78302	384 reactions
MDM2 TR-FRET Assay Kit	79773	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
UBCH5a TR-FRET Assay Kit	79900	384 reactions

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