Description

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

The von Hippel–Lindau protein (VHL) interacts with Elongins B and C, Cullin 2, and Ring Box Protein 1 (Rbx1) to form the functional E3 Ub ligase complex where it functions as a substrate recognition entity. Most of the tumor-derived mutations within VHL disrupt its tumor suppressor role by compromising its substrate receptor function and generally whole VHL complex Ub ligase activity. VHL complex not only targets the alpha subunits of the heterodimeric transcription factor hypoxia inducible factor (HIF) for ubiquitylation and proteasomal degradation, but it is involved in many other biological processes related to tumor growth. That is why it is an attractive potential drug target in cancer immunotherapy. Like most E3 ligases, VHL complex can ubiquitinate itself.

The VHL intrachain TR-FRET Assay Kit is a sensitive high-throughput screening (HTS) TR-FRET Assay Kit, designed to measure VHL auto-ubiquitination activity in a homogeneous 384 reaction format. It utilizes a Europium cryptate-labeled Ub (donor) as well as Cy5-labeled Ub (acceptor) to complete the TR-FRET pairing. Since both the TR-FRET donor and acceptor are incorporated into poly-ubiquitin chains formed on VHL, this FRET-based assay requires no time-consuming washing steps, making it especially suitable for HTS applications as well as real-time kinetics analyses of polyubiquitination.

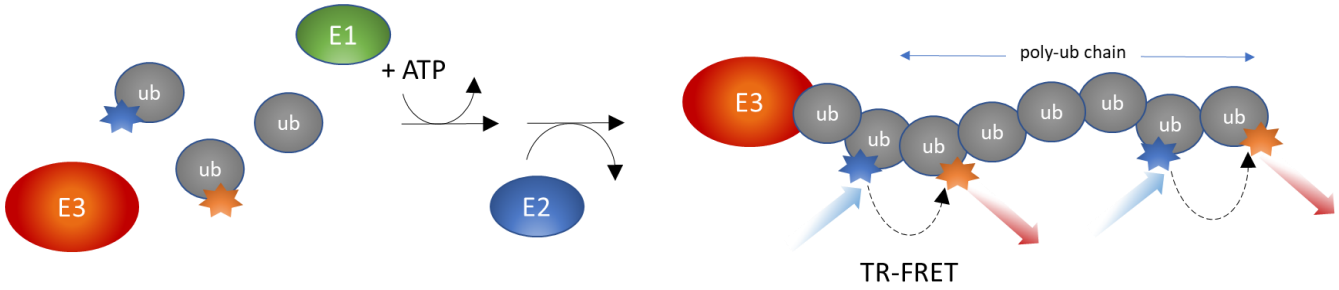

Figure 1. VHL intrachain TR-FRET Assay Kit schematic

Applications

Great for screening molecules that inhibit VHL Ub ligase activity in drug discovery HTS applications, for determination of compound IC_{50}, and for VHL real-time kinetics analyses.

Supplied Materials

Catalog #	Name	Amount	Storage	
80301	UBE1 (E1)*	40 µg	-80°C	***Avoid multiple freeze/ thaw cycles***
80314	UBCH5b (E2)*	60 µg	-80°C	
100373	VHL/CUL2/ELOB/ELOC/RBX1 Complex (E3)*	30 µg	-80°C	
78307	TRF Ubiquitin Mix (200x)	40 µl	-80°C	
	ATP (4 mM)	2 x 1 ml	-80°C	
	U2 Assay Buffer	2 x 10 ml	-80°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 (TR-FRET)
Adjustable micropipettor and sterile tips
Rotating or rocker platform

Storage Conditions

This assay kit will perform optimally for up to 6 months from date of receipt when the materials are stored as directed. ***Avoid multiple freeze/ thaw cycles!***

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 VHL intrachain TR-FRET Assay Kit is compatible with up to 1% final DMSO concentration. We recommend preparing the inhibitor in no higher than 5% DMSO solution in buffer and using 4 µl per well.

Assay Protocol

All samples and controls should be performed in triplicates
The assay should include a “Blank”, a “Positive control”, and a “Negative control”

1) Thaw **UBE1**, **UBCH5b**, **VHL complex**, **TRF Ubiquitin Mix**, **U2 Assay Buffer**, and **ATP** on ice. Aliquot each protein, U2 Assay Buffer, and ATP into single-use aliquots and immediately store at -80°C. Note: UBE1, UBCH5b, VHL complex, TRF Ubiquitin Mix, and U2 Assay Buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles.

2) Carefully calculate the amount of each protein needed and prepare appropriate amounts of diluted proteins:

Prepare 5x TRF Ubiquitin Mix in U2 Assay Buffer (40-fold dilution of the 200x TRF Ubiquitin Mix);
Dilute the UBE1 in U2 Assay Buffer at 800 nM (96 ng/µl) (final concentration in reaction 40 nM);
Dilute the UBCH5b in U2 Assay Buffer at 8 µM (144 ng/µl) (final concentration in reaction 400 nM);
Dilute the VHL complex in U2 Assay Buffer at 100 nM (15 ng/µl) (final concentration in reaction 25 nM);

Keep all diluted proteins on ice until use.

3) Prepare the compound solution.

If the compound is dissolved in DMSO, make a 100-fold higher concentration of the compound in DMSO than the highest concentration you want to test in the assay. Then dilute 20-fold in U2 Assay Buffer (at this step the compound concentration is 5-fold higher than the desired final concentration). If you want to run an IC_{50} or test lower concentrations of the compound, prepare serial dilutions using U2 Assay Buffer containing 5% DMSO, so the final concentration of DMSO will be 1% in all samples.

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

4) To the wells designated as "Blank", add 4 µl of **5x TRF Ubiquitin Mix** + 1 µl of **UBE1** + 1 µl of **UBCH5b** + 4 µl of **diluent solution** (for example DMSO 5%) + 5 µl of **U2 Assay Buffer**.

	Blank
TRF Ubiquitin Mix (5x)	4 µl
UBE1	1 µl
UBCH5b	1 µl
VHL complex	-
Test Compound	-
Diluent solution* (no inhibitor)	4 µl
U2 Assay Buffer	5 µl
ATP (4 mM)	5 µl
Total	**20 µl**

**The diluent solution contains the assay buffer with the same concentration of solvent (e.g. DMSO) as the test compound solution.*

5) Make the master mixture using diluted reagents:

N wells × (4 µl **5x TRF Ubiquitin Mix** + 1 µl **UBE1** + 1 µl **UBCH5b** + 5 µl **VHL complex**).

6) Add 11 µl of master mixture to each well designated for the "Negative Control", "Positive Control", "Test Sample".

7) Add 4 µl of inhibitor solution to each well designated "Test Inhibitor". For all other wells: "Positive Control", "Negative Control", add 4 µl of the diluent solution without inhibitor.

8) Initiate the reaction by adding 5 µl of **ATP** to the wells labeled "Positive Control," "Test Inhibitor," and "Blank." Add 5 µl of **U2 Assay Buffer** to the well designated "Negative Control." Cover the plate with a plate sealer. Incubate the reaction at room temperature for two hours or at 30°C for one hour.

	Test Sample	Negative Control	Positive Control
Master Mix	11 µl	11 µl	11 µl
Test compound	4 µl	–	–
Diluent solution* (no inhibitor)	–	4 µl	4 µl
U2 Assay Buffer	–	5 µl	–
ATP (4 mM)	5 µl	–	5 µl
Total	**20 µl**	**20 µl**	**20 µl**

**The diluent solution contains the assay buffer with the same concentration of solvent (e.g. DMSO) as the test compound solution.*

9) Read the fluorescent intensity in a microtiter-plate reader capable of measuring TR-FRET. "Blank" value is subtracted from all other values.

Instrument Settings

Reading Mode	Time Resolved
Excitation Wavelength	317±20 nm
Emission Wavelength	620±10 nm
Lag Time	60 µs
Integration Time	500 µs
Excitation Wavelength	317±20 nm
Emission Wavelength	665±10 nm
Lag Time	60 µs
Integration Time	500 µs

CALCULATING RESULTS:

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).

When percentage activity is calculated, the FRET value from the Blank (it is expected that Blank and Negative Control represent similar value) can be set as zero percent activity and the FRET value from the positive control can be set as one hundred percent activity.

$$\% \text{ Activity} = \frac{FRET_s - FRET_{blank}}{FRET_p - FRET_{blank}} \times 100\%$$

Where FRETs = Sample FRET, $FRET_{blank}$ = Blank FRET, and $FRET_P$ = Positive control FRET.

Example Results

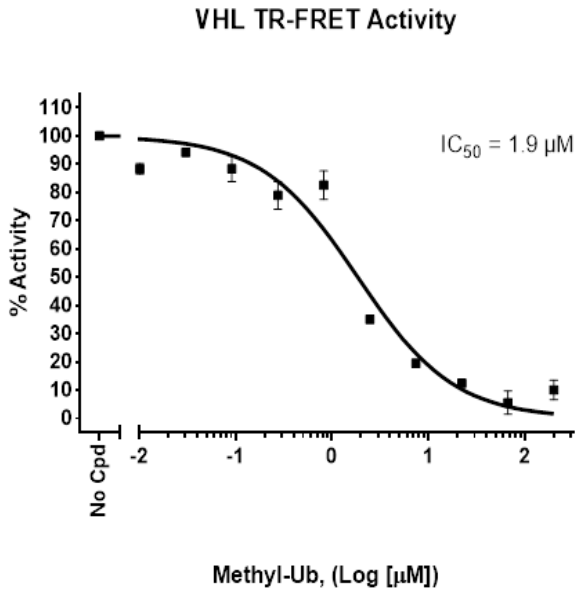

Figure 1: *Inhibition of VHL complex auto-ubiquitination by Methylated Ubiquitin, measured using the VHL intrachain TR-FRET Assay Kit, BPS Bioscience #78305. Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com.*

Troubleshooting Guide

Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For all further questions, please email support@bpsbioscience.com

Related Products

Products	*Catalog #*	*Size*
Cereblon intrachain TR-FRET Assay Kit	78301	384 rxns.
MDM2 intrachain TR-FRET Assay Kit	78302	384 rxns.
SMURF1 intrachain TR-FRET Assay Kit	78303	384 rxns.
SMURF2 intrachain TR-FRET Assay Kit	78304	384 rxns.
XIAP intrachain TR-FRET Assay Kit	78306	384 rxns.
MDM2 TR-FRET Assay Kit	79773	384 rxns.
CBL-B TR-FRET Assay Kit	79575	384 rxns.
c-CBL TR-FRET Assay Kit	79786	384 rxns.
Cereblon Ubiquitination Homogenous Assay Kit	79881	384 rxns.
UBCH13 TR-FRET Assay Kit	79741	384 rxns.
UBCH5a TR-FRET Assay Kit	79900	384 rxns.
UBCH5c TR-FRET Assay Kit	79901	384 rxns.
UBCH5b TR-FRET Assay Kit	79896	384 rxns.
MDM2, GST-Tag (Human)	80751	20 µg
UBE1 (UBA1), FLAG-tag	80301	100 µg
UBE1, GST-Tag	100402	100 µg
UBE2A, His-Tag	79368	20 µg
UBE2C, His-Tag	79369	20 µg
UBE2D2, His-Tag	79370	20 µg
UBE2E3 (UBCH9), His-Tag	79371	20 µg
UBE2G1 (UBC7), His-Tag	79372	20 µg
UBE2K (UBC1), His-Tag	79373	20 µg
UBE2O, GST-Tag	79374	20 µg
UbcH5a (UBE2D1), His-tag	80315	100 µg
UbcH5b, His-Tag (Human)	80314	100 µg
UbcH6 (UBE2E1), His-tag	80316	100 µg
UbcH7, His-tag (E. coli-derived)	80317	100 µg
UbcH7, His-tag (Sf9-derived)	80318	50 µg
UbcH13 (UBE2N), His-tag	80323	100 µg
CBL-B, GST-Tag (Human)	80415	100 µg
c-CBL, GST-Tag (Human)	100370	100 µg
XIAP, FLAG-tag	80401	20 µg
SMURF1, FLAG-tag	80402	20 µg
SMURF2, FLAG-tag	80403	20 µg
Cereblon/DDB1/Cul4A/Rbx1 Complex	100329	10 µg
VHL/CUL2/ELOB/ELOC/RBX1 Complex	100373	10 µg
Ubiquitin, His-Tag	79293	2 mg
Ubiquitin, His-Avi-Tag, Biotin Labeled	11236	50 µg