Description

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.

Casitas B-lineage lymphoma proto-oncogene-b (CBL-B) is the RING-type E3 ligase that functions as a negative regulator of T cell activation and of growth factor receptor and non-receptor tyrosine kinase signaling. It contains an N-terminal tyrosine kinase binding (TKB) domain comprised of a four-helix bundle, a calcium binding EF-hand and a Src homology (SH2) domain, followed by a linker helical region and the RING domain, responsible for its catalytic function. Additionally, CBL-B contains proline-rich regions mediating the association with tyrosine- and serine phosphorylation sites, and a ubiquitin-associated (UBA)/leucine zipper domain for dimerization. CBL-B interacts with a large number of target proteins implicated in the control of cell proliferation, differentiation, and cell morphology. The ubiquitin ligase activity of CBL-B is up-regulated by the phosphorylation of Tyrosine (Tyr) 363, which is located in the helix linker between the TKB and RING domains. Phosphorylation of Tyr363 opens CBL-B from its auto-inhibitory confirmation, allowing E2 and substrates to bind to CBL-B. CBL-B is phosphorylated for example by receptor-type tyrosine kinase Tyro3, which also serves as a substrate for CBL-B ubiquitylation both in vitro and in vivo.

The CBL-B-driven Tyro3 ubiquitination intrachain TR-FRET Assay Kit is a sensitive high-throughput screening (HTS) TR-FRET Assay Kit, designed to measure CBL-B E3 ligase activity in a homogeneous 384 reaction format. It utilizes a Europium cryptate-labeled Ub (donor) and a Cy5-labeled Ub (acceptor) to complete the TR-FRET pairing. Since both the TR-FRET donor and acceptor are incorporated into poly-ubiquitin chains, 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. Of note, the assay kit does not detect mono-ubiquitination.

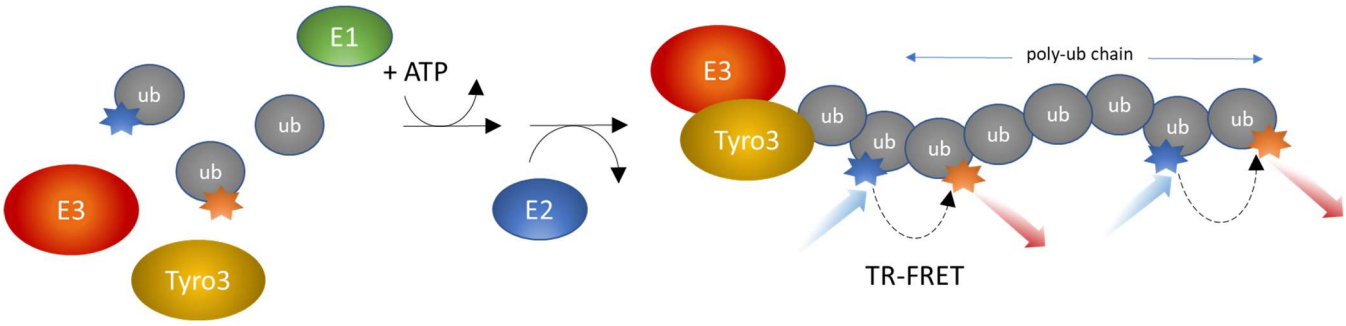

Figure 1. CBL-B-driven Tyro3 ubiquitination intrachain TR-FRET Assay Kit schematic

Applications

Screen molecules that inhibit CBL-B Ub ligase activity in drug discovery HTS applications, determine compound IC_{50} and perform CBL-B real-time kinetics analyses.

BPS Bioscience
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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	
80415	CBL-B, GST-tag*	8 µg	-80°C	
40293	TYRO-3, GST-tag*	16 µg	-80°C	
78307	TRF Ubiquitin Mix (200x)	50 µl	-80°C	
	ATP (4 mM)	2 x 1 ml	-80°C	
78269	CBL assay buffer 2	2 x 10 ml	-80°C	
79699	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
- 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 CBL-B-driven Tyro3 Ubiquitination 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.

Tyro3 kinase inhibitors may inhibit the ubiquitination reaction. It is recommended to confirm if the Test compounds explicitly affect CBL-B ligase activity and not Tyro3 kinase activity by determining the effect of compounds on Tyro3 activity in the TYRO3 Assay Kit (BPS Bioscience#79593).

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**, **CBL-B, TYRO3**, **TRF Ubiquitin Mix**, **CBL assay buffer 2**, and **ATP** on ice. Briefly spin the tube to recover its full contents. Calculate the amount of protein required for the assay and dilute enough for the assay. Refer to step 6 (preparing the master mix) to calculate how much of each protein is needed.

   Aliquot unused protein into 2-4 aliquots as may be necessary (single use aliquots) and store them at -80°C. Aliquot assay buffer and ATP and store at -80°C.

   Note: UBE1, UBCH5b, CBL-B, TYRO3, TRF Ubiquitin Mix, and assay buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles.

2) Prepare 5x TRF Ubiquitin Mix in assay buffer (i.e. make a 40-fold dilution of the 200x TRF Ubiquitin Mix)

3) Prepare appropriate amounts of diluted proteins. The concentration of each protein is lot-specific and is indicated on the tube. Verify the initial concentration and dilute accordingly.

   Dilute UBE1 in assay buffer at 96 ng/µl (800 nM - the final concentration in the reaction will be 40 nM);
   Dilute UBCH5b in assay buffer at 144 ng/µl (2 µM – the final concentration in the reaction will be 100 nM);
   Dilute CBL-B in assay buffer at 7.2 ng/µl (100 nM – the final concentration in the reaction will be 12.5 nM);
   Dilute TYRO3 in assay buffer at 15.4 ng/µl (200 nM – the final concentration in the reaction will be 25 nM);

   Keep all diluted proteins on ice until use. Do not freeze and re-use diluted proteins.

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.

   a) If the Test Inhibitor is water-soluble, prepare serial dilutions in the Assay Buffer, 5-fold more concentrated than the desired final concentrations. For the positive and negative controls, use 1x Assay Buffer (Diluent Solution).

   b) If the Test inhibitor is soluble in DMSO, prepare the test inhibitor at 100-fold the highest desired concentration in DMSO, then dilute the inhibitor 20-fold in Assay Buffer to prepare the highest concentration of the 5-fold intermediate dilutions. The concentration of DMSO is now 5%.

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

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

      The final concentration of DMSO should not exceed 1%.

5) 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 **assay buffer**.

	Blank
TRF Ubiquitin Mix (5x)	4 µl
UBE1	1 µl
UBCH5b	1 µl
CBL-B/TYRO3	-
Test Compound	-
Diluent solution* (no inhibitor)	4 µl
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.

6) Make the Master Mix using diluted reagents:

   N wells × (4 µl **5x TRF Ubiquitin Mix** + 1 µl **UBE1** + 1 µl **UBCH5b** + 2.5 µl **CBL-B** + 2.5 µl **TYRO3**).

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

8) 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.

9) Initiate the reaction by adding 5 µl of **ATP** to the wells labeled "Positive Control," "Test Inhibitor," and "Blank." Add 5 µl of **assay buffer** to the well designated "Negative Control." Cover the plate with a plate sealer. Incubate the reaction at 30°C for 40 minutes or at room temperature 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
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.

10) 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	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

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

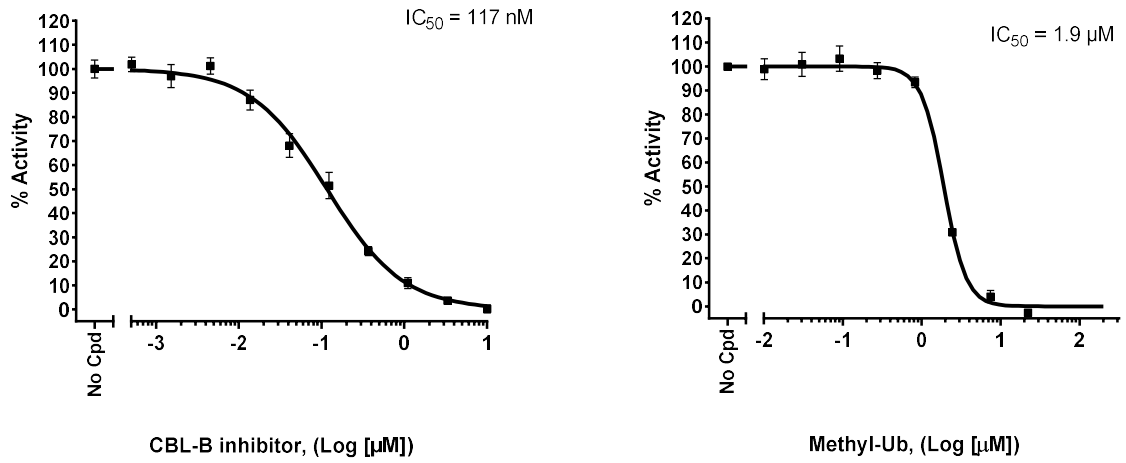

Figure 1: Inhibition of CBL-B-driven Tyro3 ubiquitination. CBL-B-dependent ubiquitination of Tyro3 was measured in the presence of increasing concentrations of CBL-B-IN-1 inhibitor (MedChem Express, #HY-136339) or methylated Ubiquitin using the CBL-B-driven Tyro3 Ubiquitination Intrachain TR-FRET Assay Kit (BPS Bioscience #78388). 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 reactions
MDM2 intrachain TR-FRET Assay Kit	78302	384 reactions
SMURF1 intrachain TR-FRET Assay Kit	78303	384 reactions
SMURF2 intrachain TR-FRET Assay Kit	78304	384 reactions
VHL intrachain TR-FRET Assay Kit	78305	384 reactions
XIAP intrachain TR-FRET Assay Kit	78306	384 reactions
MDM2 TR-FRET Assay Kit	79773	384 reactions
Cereblon Ubiquitination Homogeneous Assay Kit	79881	384 reactions
UBCH13 TR-FRET Assay Kit	79741	384 reactions
UBCH5a TR-FRET Assay Kit	79900	384 reactions
UBCH5c TR-FRET Assay Kit	79901	384 reactions

UBCH5b TR-FRET Assay Kit	79896	384 reactions
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	100370	100 µg
XIAP, FLAG-tag	80401	20 µg
SMURF1, FLAG-tag	80402	20 µg
SMURF2, FLAG-tag	80403	20 µg
NEDD4, FLAG-tag	80404	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