Description

The CBL-B-Driven Tyro3 Ubiquitination 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 CBL-B (Casitas B-lineage lymphoma proto-oncogene-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. This kit contains enough recombinant human CBL-B (amino acids 39-426), recombinant human TYRO3 (tyrosine-protein kinase receptor 3) (amino acids 455-end) and remaining reagents for 384 reactions.

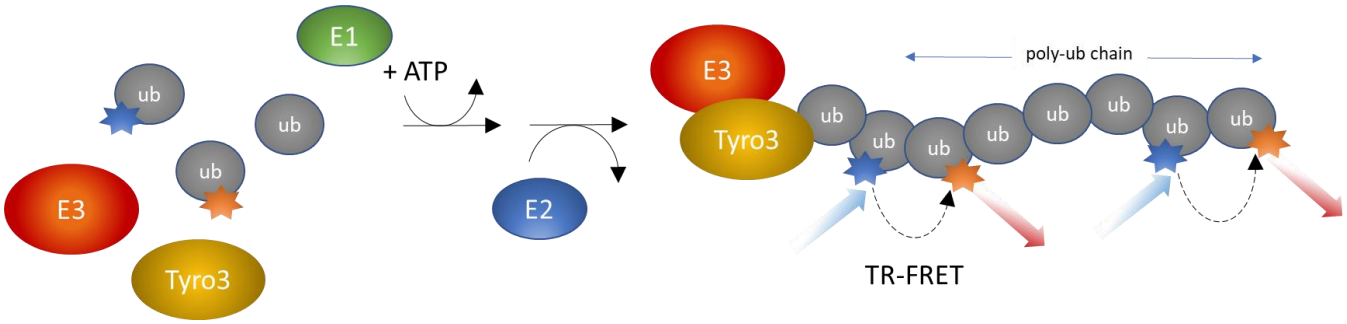

Figure 1. CBL-B-Driven Tyro3 ubiquitination Intrachain TR-FRET Assay Kit schematic.

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.

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*.

Applications

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

Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), FLAG-tag*	40 µg	-80°C
80314	UbcH5b, His-Tag*	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)	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 384-well microtiter plate		Room Temp

**The initial concentration of enzyme is lot-specific and will be indicated on the tube containing the protein.*

The Ubiquitin Mix is sourced from South Bay Bio LLC.

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 a solution containing no higher than 5% DMSO 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 by using the TYRO3 Assay Kit (BPS Bioscience #79593).

Assay Protocol

- All samples and controls should be performed in triplicate.
- The assay should include "Blank", "Positive Control", "Negative Control" and 'Test Inhibitor".
- If the assay plate is going to be used more than once, prepare enough of each protein and aliquot the remaining undiluted proteins into single-use aliquots depending on how many times the assay plate will be used. Store the protein aliquots at -80°C and store aliquots of U2 Assay Buffer and ATP at -20°C.

1) Thaw **UBE1**, **UbcH5b**, **CBL-B, TYRO3**, **TRF Ubiquitin Mix**, **U2 Assay Buffer**, and **ATP** on ice. Briefly spin the tube to recover its full content.

2) Prepare 5x TRF Ubiquitin Mix in U2 Assay Buffer by making a 40-fold dilution of the stock TRF Ubiquitin Mix (200x).

3) Calculate the amount of protein required for the assay and prepare the 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.

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

    b) Dilute UbcH5b in U2 Assay Buffer to 144 ng/µl (8 µM – the final concentration in the reaction will be 400 nM) (1 µl/well).

    c) Dilute CBL-B in U2 Assay Buffer to 7.2 ng/µl (100 nM – the final concentration in the reaction will be 12.5 nM) (2.5 µl/well).

    d) Dilute TYRO3 in U2 Assay Buffer to 15.4 ng/µl (200 nM – the final concentration in the reaction will be 25 nM) (2.5 µl/well).

    Note: UBE1, UbcH5b, CBL-B, TYRO3, TRF Ubiquitin Mix, and U2 Assay Buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles. 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 U2 Assay Buffer, 5-fold more concentrated than the desired final concentrations. For the positive and negative controls, use U2 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 U2 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 U2 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).

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

5) For the "Blank" wells prepare the following mix: N wells x (4 µl of 5x TRF Ubiquitin Mix + 1 µl of diluted UBE1 + 1 µl of diluted UbcH5b + 4 µl of Diluent Solution + 5 µl of U2 Assay Buffer).

6) Add 15 µl of mix to each "Blank" well.

	Blank
TRF Ubiquitin Mix (5x)	4 µl
Diluted UBE1	1 µl
Diluted UBCH5b	1 µl
Diluted CBL-B/TYRO3	-
Test Inhibitor	-
Diluent Solution	4 µl
U2 Assay Buffer	5 µl
ATP (4 mM)	5 µl
Total	**20 µl**

7) Make a Master Mix: N wells × (4 µl 5x TRF Ubiquitin Mix + 1 µl diluted UBE1 + 1 µl diluted UbcH5b + 2.5 µl diluted CBL-B + 2.5 µl diluted TYRO3).

8) Add 11 µl of Master Mix to each well designated for the "Negative Control", "Positive Control" and "Test Inhibitor".

9) Add 4 µl of inhibitor solution to each well designated "Test Inhibitor".

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

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

12) Add 5 µl of U2 Assay Buffer to the wells designated "Negative Control".

	Test Sample	Negative Control	Positive Control
Master Mix	11 µl	11 µl	11 µl
Test Inhibitor	4 µl	–	–
Diluent Solution	–	4 µl	4 µl
U2 Assay Buffer	–	5 µl	–
ATP (4 mM)	5 µl	–	5 µl
Total	**20 µl**	**20 µl**	**20 µl**

13) Read the fluorescence intensity in a microtiter-plate reader capable of measuring TR-FRET in kinetic mode for up to 1 hour. An end point readout can be done in 20-40 min.

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

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
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

Data analysis is performed using the TR-FRET ratio (665 nm emission/620 nm emission). "Blank" value is subtracted from all other values.

When percentage activity is calculated, the FRET value from the Blank (it is expected that Blank and Negative Control have a 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

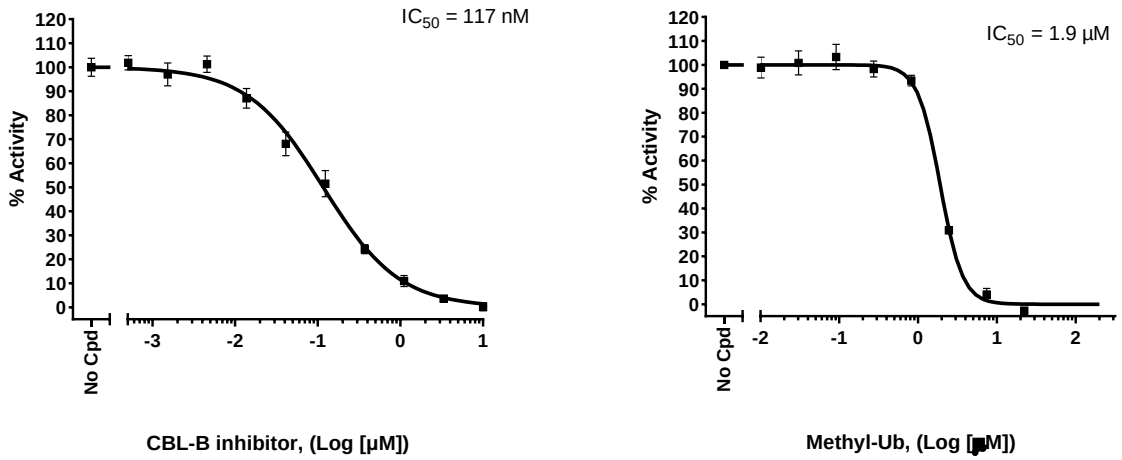

Figure 2: Inhibition of CBL-B-driven Tyro3 ubiquitination.
CBL-B-dependent ubiquitination of Tyro3 was measured in the presence of increasing concentrations of CBL-B-IN-1 inhibitor (MedChem Express #HY-136339) or methylated Ubiquitin. Results are expressed as percent activity, in which absence of inhibitor is set to 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 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