Description

The CBL-B-driven AXL ubiquitination intrachain TR-FRET Assay Kit is a sensitive high-throughput screening (HTS) TR-FRET Assay Kit designed to measure the E3 ligase activity of CBL-B toward tyrosine kinase AXL in a homogeneous 384-reaction format. It utilizes a Europium cryptate-labeled Ubiquitin Donor and a Cy5-labeled Ubiquitin Acceptor to complete the TR-FRET pairing. Since both the TR-FRET donor and acceptor are incorporated into poly-ubiquitin chains, this assay does not detect mono-ubiquitination. The FRET-based format requires no time-consuming washing steps, making it especially suitable for HTS applications as well as real-time analyses of polyubiquitination.

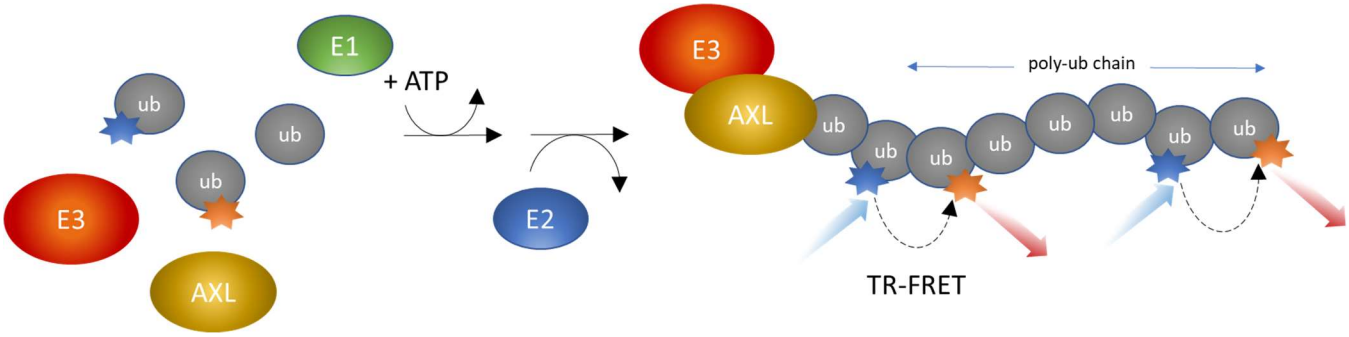

Figure 1: CBL-B-driven AXL ubiquitination intrachain TR-FRET Assay Kit schematic.

Background

Covalent conjugation to ubiquitin (Ub) 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.

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. It contains an N-terminal tyrosine kinase binding domain, a SRC homology domain, and the RING domain responsible for its catalytic function. Additionally, CBL-B contains proline-rich regions mediating the association with phosphorylated proteins, and a dimerization domain. 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 363, which opens CBL-B from its auto-inhibitory conformation, allowing binding of E2 and substrates. Kinases that phosphorylate CBL-B, such as AXL, Tyro3, and SRC, also serve as substrates for ubiquitination.

Applications

- Screen inhibitors of CBL-B Ubiquitin ligase activity in drug discovery and HTS applications
- Determine compound IC_{50}
- Perform real-time kinetic analyses

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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	
100174	AXL, FLAG-Tag*	10 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 each 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 AXL 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.

AXL kinase inhibitors may inhibit the ubiquitination reaction. It is recommended to confirm if the Test compounds explicitly affect CBL-B ligase activity and not AXL kinase activity by determining the effect of compounds on AXL activity in the AXL Kinase Assay Kit (BPS Bioscience#79711).

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, AXL**, **TRF Ubiquitin Mix**, **U2 Assay Buffer**, and **ATP** on ice. Briefly spin the tubes to recover their full contents. If the assay plate is going to be used more than once, prepare enough of each protein for this portion of the assay and aliquot the remaining undiluted proteins into single-use aliquots depending on how many times the assay plate will be used. Store the aliquots at -80°C. Refer to step 3 (dilutions) and to step 6 (preparing the master mix) to calculate how much of each protein is needed.

   Note: UBE1, UBCH5b, CBL-B, AXL, TRF Ubiquitin Mix, and U2 Assay Buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles.

2) Prepare 5x TRF Ubiquitin Mix in U2 Assay Buffer by making a 40-fold dilution of the 200x TRF Ubiquitin Mix (e.g. add 1 volume of stock TRF Ub Mix to 39 volumes of U2 Assay Buffer).

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 all proteins in **U2 Assay Buffer:**

   - Dilute UBE1 to 96 ng/µl (800 nM - the final concentration in the reaction will be 40 nM)
   - Dilute UBCH5b to 144 ng/µl (8 µM – the final concentration in the reaction will be 400 nM)
   - Dilute CBL-B to 7.2 ng/µl (100 nM – the final concentration in the reaction will be 12.5 nM)
   - Dilute AXL to 10 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 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 in 100% DMSO at a concentration 100-fold higher than the highest desired concentration, 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 U2 Assay Buffer (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 5% DMSO) + 5 µl of **U2 Assay Buffer** + 5 µl of **ATP (4 mM)**.

	Blank
TRF Ubiquitin Mix (5x)	4 µl
UBE1	1 µl
UBCH5b	1 µl
CBL-B/AXL	-
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.*

6) Prepare a Master Mix using diluted reagents:

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

7) Add 11 µl of Master Mix to each well designated "Negative Control", "Positive Control", "Test Inhibitor".

8) Add 4 µl of Test Inhibitor to each well designated "Test Inhibitor". For "Positive Control" and "Negative Control", add 4 µl of the diluent solution.

9) Initiate the reaction by adding 5 µl of **ATP** to the wells labeled "Positive Control" and "Test Inhibitor." Add 5 µl of **U2 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 Inhibitor	**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**

**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	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

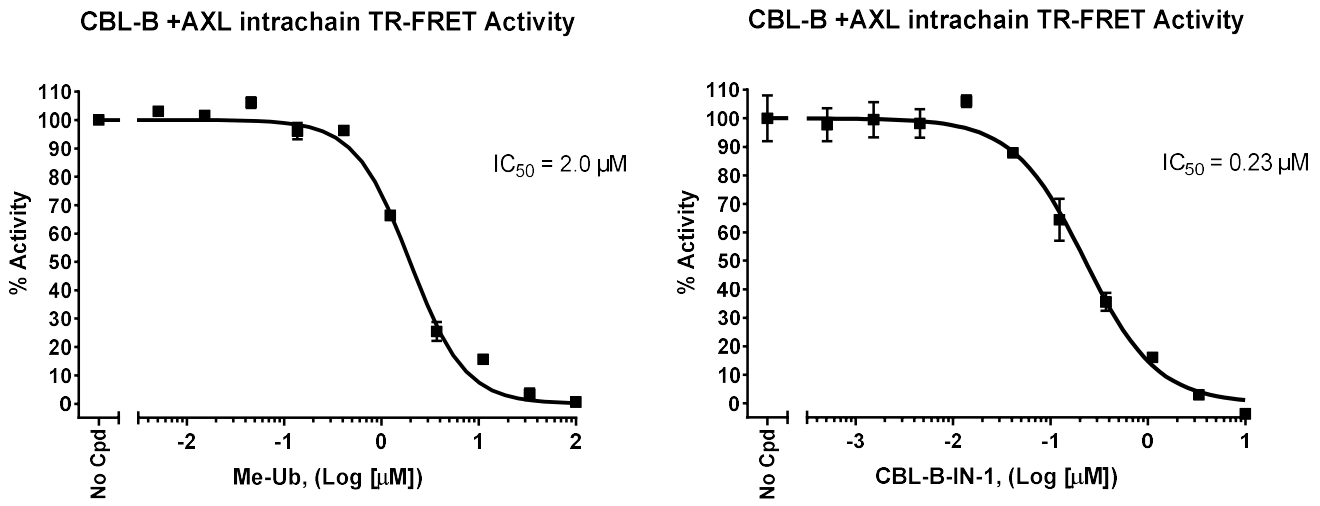

Figure 2: Inhibition of CBL-B-driven AXL ubiquitination.
CBL-B-dependent ubiquitination of AXL was measured in the presence of increasing concentrations of CBL-B-IN-1 inhibitor (MedChem Express #HY-136339) or methylated Ubiquitin.

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*
ChoosE3-Freedom™ Intrachain TR-FRET Assay Kit	78560	384 reactions
ChoosE2-Opti™ Intrachain TR-FRET Assay Kit	78561	384 reactions
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
CBL-B-driven Tyro3 Ubiquitination Intrachain TR-FRET Assay Kit	78388	384 reactions
C-CBL-driven Tyro3 Ubiquitination Intrachain TR-FRET Assay Kit	78408	384 reactions
C-CBL-driven SRC Ubiquitination Intrachain TR-FRET Assay Kit	78822	384 reactions
C-CBL-driven Axl Ubiquitination Intrachain TR-FRET Assay Kit	78823	384 reactions
CBL-B-driven SRC Ubiquitination Intrachain TR-FRET Assay Kit	78820	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
VHL/CUL2/ELOB/ELOC/RBX1 Complex	100373	10 µg
Ubiquitin, His-Tag	79293	2 mg
Ubiquitin, His-Avi-Tag, Biotin Labeled	11236	50 µg