C-CBL-Driven AXL Ubiquitination Intrachain TR-FRET Assay Kit

#78823
384 reactions

Description

The C-CBL-driven AXL 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 the E3 ligase activity of C-CBL (Casitas B-lineage lymphoma) toward tyrosine kinase AXL (tyrosine-protein kinase receptor UFO) 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 assay requires no time-consuming washing steps, making it especially suitable for HTS applications as well as real-time analyses of polyubiquitination.

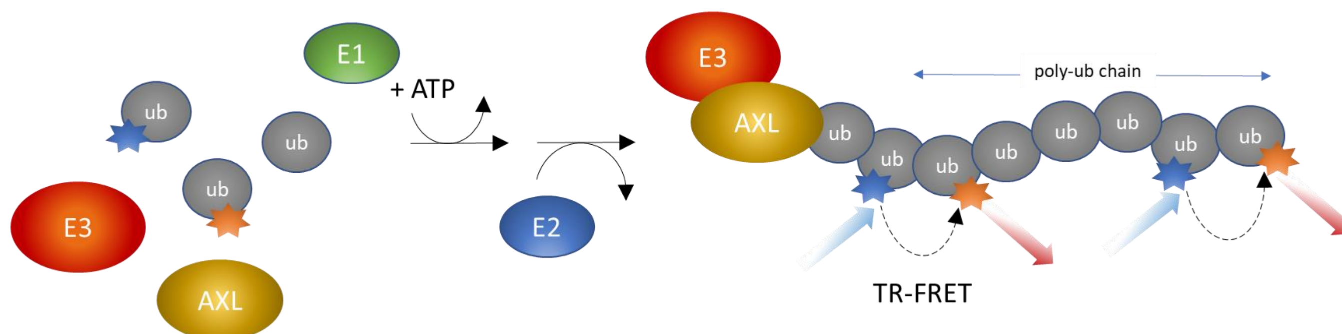

Figure 1: C-CBL-Driven AXL Ubiquitination Intrachain TR-FRET Assay Kit schematic.

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 (C-CBL) 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, C-CBL contains proline-rich regions mediating the association with various targets, as well as a ubiquitin-associated (UBA) domain for ubiquitin binding and dimerization. C-CBL interacts with phosphorylated proteins. C-CBL interacts with a large number of target proteins implicated in the control of cell proliferation and differentiation. The ubiquitin ligase activity of C-CBL is regulated by the phosphorylation of Tyrosine 371, which C-CBL from its auto-inhibitory conformation, allowing binding to E2 and substrates. Kinases that phosphorylate C-CBL, such as AXL, Tyro3 (tyrosine-protein kinase receptor 3), and SRC (non-receptor tyrosine kinase), also serve as substrates for ubiquitination.

Applications

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

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Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), FLAG-Tag*	40 µg	-80°C
80314	UbcH5b, His-Tag*	60 µg	-80°C
100370	C-CBL, GST-Tag*	8 µg	-80°C
100174	AXL, FLAG-Tag*	10 µg	-80°C
78307	TRF Ubiquitin Mix (200x)	50 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		Room Temp

**The initial concentration of protein 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 C-CBL-Driven AXL 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.

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

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Assay Protocol

- All samples and controls should be performed in triplicate.
- The assay should include “Blank”, “Positive Control”, “Negative Control” and “Test Inhibitor” conditions.
- 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**, **C-CBL, AXL**, **TRF Ubiquitin Mix**, **U2 Assay Buffer**, and **ATP** on ice. Briefly spin the tubes to recover their full content.

   Note: UBE1, UbcH5b, C-CBL, AXL, TRF Ubiquitin Mix, and U2 Assay Buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles. Do not re-use the diluted proteins.

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

   c) Dilute C-CBL U2 Assay Buffer to 7.1 ng/µl (100 nM – the final concentration in the reaction will be 12.5 nM) (2.5 µl/well).

   d) Dilute AXL U2 Assay Buffer to 10 ng/µl (200 nM – the final concentration in the reaction will be 25 nM) (2.5 µl/well).

   Note: 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).

   OR

   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

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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).

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 + 5 µl of ATP (4 mM)).

6) Add 20 µl of mix to each "Blank" well.

	Blank
TRF Ubiquitin Mix (5x)	4 µl
Diluted UBE1	1 µl
Diluted UbcH5b	1 µl
Diluted C-CBL/AXL	-
Test Compound	-
Diluent Solution	4 µl
U2 Assay Buffer	5 µl
ATP (4 mM)	5 µl
Total	**20 µl**

7) Prepare a Master Mix: N wells × (4 µl of 5x TRF Ubiquitin Mix + 1 µl of diluted UBE1 + 1 µl of diluted UbcH5b + 2.5 µl of diluted C-CBL + 2.5 µl of diluted AXL).

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

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

10) A dd 4 µl of 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" and "Test Inhibitor".

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

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	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**

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 20-40min.

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.

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Example Results

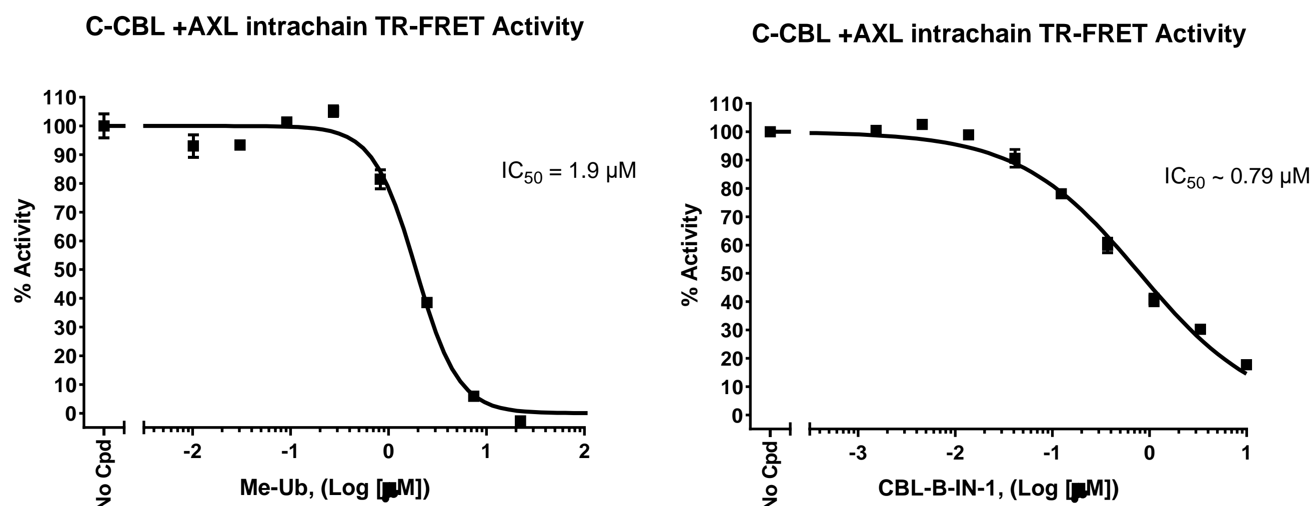

Figure 2: Inhibition of C-CBL-driven AXL ubiquitination by Methylated Ubiquitin and CBL-B-IN-1.
C-CBL-dependent ubiquitination of AXL was measured in the presence of increasing concentrations of Methylated Ubiquitin or CBL-B-IN-1 inhibitor (MedChem Express #HY-136339). Results are expressed as percent 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*
ChoosE3-Freedom™ Intrachain TR-FRET Assay Kit	78560	384 reactions
ChoosE2-Opti™ Intrachain TR-FRET Assay Kit	78561	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
CBL-B-driven SRC Ubiquitination Intrachain TR-FRET Assay Kit	78820	384 reactions
C-CBL-driven Axl Ubiquitination Intrachain TR-FRET Assay Kit	78823	384 reactions