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Data Sheet

CBL-B TR-FRET Assay Kit

Catalog # 79575
Size: 384 reactions

DESCRIPTION:

Human Casitas B-lineage lymphoma proto-oncogene b (CBL-B) is an E3 ubiquitin-protein ligase that functions as a negative regulator of T-cell activation. It is a promising drug target in cancer immunotherapy. The *CBL-B TR-FRET Assay Kit* is designed to measure CBL-B auto-ubiquitination activity in a homogeneous 384 reaction format. It utilizes biotin-labeled Ubiquitin and a terbium-labeled antibody recognizing the GST-tagged CBL-B protein to complete the TR-FRET pairing. This FRET-based assay requires no time-consuming washing steps, making it especially suitable for high throughput screening applications.

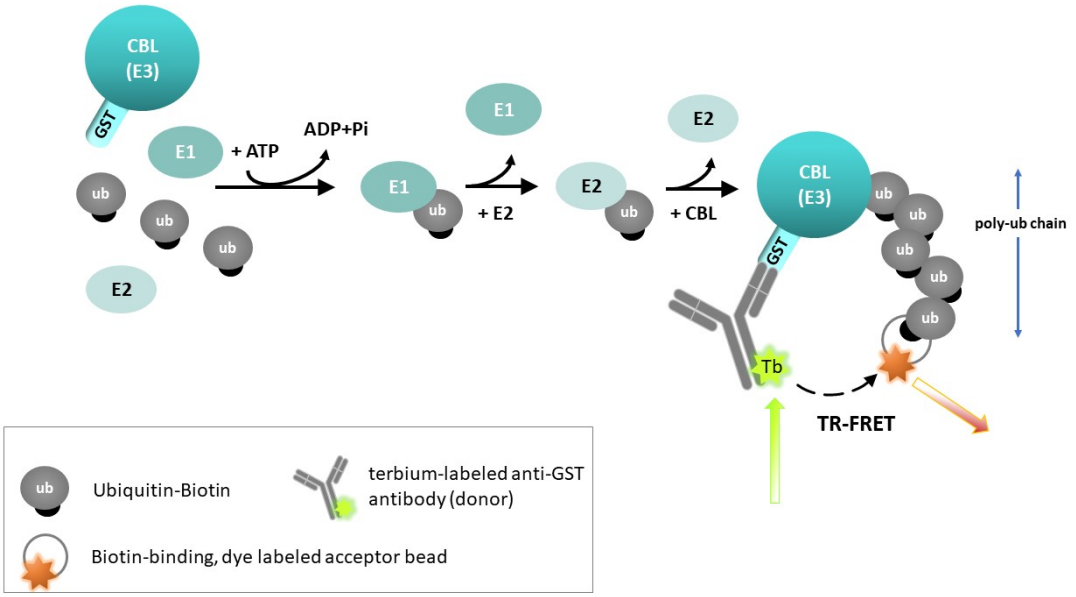

Figure: Illustration of Assay Principle

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COMPONENTS:

Catalog #	Component	Amount	Storage	
80301	UBE1 (E1)*	10 µg	-80°C	***Avoid freeze/ thaw cycles!***
80314	UBCH5b (E2)*	100 µg	-80°C	
80415	Human CBL-B (E3), GST-tag*	10 µg	-80°C	
	Biotin-Ubiquitin	400 µl	-80°C	
	ATP (400 µM)	150 µl	-80°C	
	U2 assay buffer**	2 x 10 ml	-80°C	
	Tb-labeled donor	10 µl	-20°C	
	Dye-labeled acceptor	10 µl	-20°C	
79969	White 384-well microtiter plate	1	Room temp.	

**The concentrations of E1, E2, and E3 enzymes are lot-specific and will be indicated on the tubes containing the enzyme.*

***In February 2023, buffer was re-named, but formulation unchanged.*

MATERIALS OR INSTRUMENTS REQUIRED BUT NOT SUPPLIED:

Fluorescent microplate reader capable of measuring Time Resolved Fluorescence Resonance Energy Transfer (TR-FRET)
Adjustable micropipettor and sterile tips

APPLICATIONS: Great for screening small molecular inhibitors for drug discovery and HTS applications.

STABILITY: At least 6 months from date of receipt when stored as directed.

REFERENCE: 1. Jeon, M.S., *et al., Immunity*. 2004; **21(2):** 167-177.
2. Chiang, Y.J. *et al., Nature.* 2000; **403:** 216-220.

ASSAY PROTOCOL:

All samples and controls should be tested in triplicates.

1) Thaw **UBE1, UBCH5b, CBL, Biotin-Ubiquitin, U2 assay buffer**, and **ATP** on ice. Aliquot each protein, **U2 assay buffer**, and **ATP** into single-use aliquots and stored at -80°C immediately. *Note: **UBE1, UBCH5b, CBL-B, Biotin-Ub, U2 assay buffer**, and **ATP** are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles.*

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2) Carefully calculate the amount of proteins needed. Prepare appropriate amounts of diluted proteins; dilute only the amount required for the assay. Do not store diluted proteins

   Dilute the **UBE1** in **U2 assay buffer** at 25 ng/µl
   Dilute the **UBCH5b** in **U2 assay buffer** at 220 ng/µl
   Dilute the **CBL-B** in **U2 assay buffer** at 3 ng/µl
   Keep the diluted reagents on ice until use.

3) Prepare the master mixture using diluted reagents: N wells × (1 µl **Biotin-Ub** + 1 µl diluted **UBE1** + 1 µl diluted **UBCH5b** + 2.5 µl diluted **CBL-B**). Add 5.5 µl of master mixture to each well designated for the "Substrate Control," "Positive Control," and "Test Inhibitor." For the "blank," add 1 µl **Biotin-Ub**+ 1 µl diluted **UBE1** + 1 µl diluted **UBCH5b** + 2.5 µl **U2 assay buffer**.

4) Add 2 µl of inhibitor solution of each well designated "Test Inhibitor." For the "Positive Control," "Substrate Control," and "Blank," add 2 µl of 5% DMSO in water (inhibitor buffer). Preincubate the test inhibitor for 1 hour at room temperature.

5) Dilute the **ATP stock** (400 µM) 10-fold using **U2 assay buffer** to 40 µM; dilute only the amount required for the assay. Initiate the reaction by adding 2.5 µl of diluted **ATP** to the wells labeled "Positive Control," "Test Inhibitor," and "Blank." Add 2.5 µl of assay buffer to the well designated "Substrate Control." Incubate the reaction at 30°C for four hours. Cover the plate with a plate sealer if necessary.

	Blank	Substrate Control	Positive Control	Test Inhibitor
Biotin-Ub	1 µl	1 µl	1 µl	1 µl
UBE1	1 µl	1 µl	1 µl	1 µl
UBCH5b	1 µl	1 µl	1 µl	1 µl
CBL-B	–	2.5 µl	2.5 µl	2.5 µl
Test Inhibitor/Activator	–	–	–	2 µl
5% DMSO in water (Inhibitor buffer)	2 µl	2 µl	2 µl	–
U2 assay buffer	2.5 µl	2.5 µl	–	–
ATP (40 µM)	2.5 µl	–	2.5 µl	2.5 µl
Total	**10 µl**	**10 µl**	**10 µl**	**10 µl**

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6) Dilute **Tb-labeled donor** (1:400) and **Dye-labeled acceptor** (1:400) in one step using U2 Assay Buffer. Prepare only the amount required for the assay. Add 10 µl of diluted donor/acceptor mixture into each well. Incubate at room temperature for one hour*.

**Alternatively, incubation overnight with the plate sealed can give a slightly greater signal to noise ratio.*

7) 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 negative control (Blank or Substrate Control) can be set as zero percent activity and the FRET value from the positive control can be set as one hundred percent activity.

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

Where $FRET_s$ = Sample FRET, $FRET_{neg}$ = negative control FRET, and $FRET_P$ = Positive control FRET.

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Example of Assay Results:

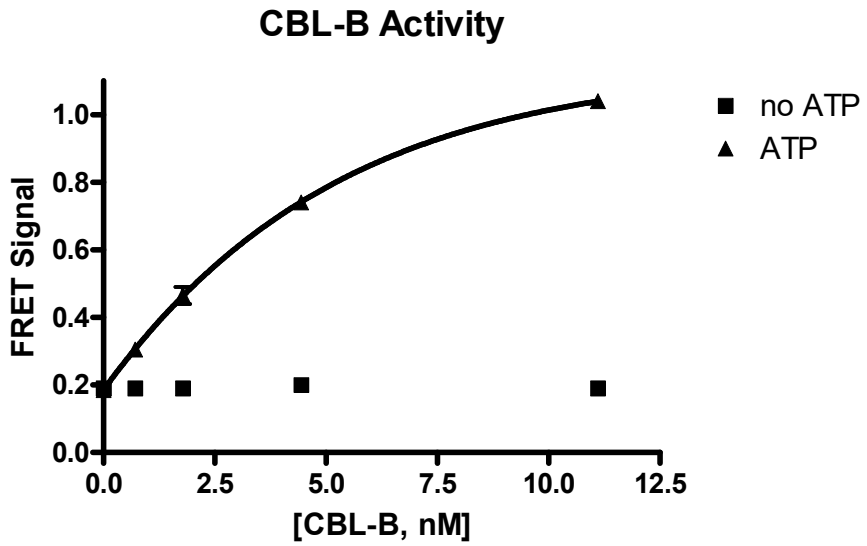

Figure 1: Titration of CBL-B activity using the *CBL-B TR-FRET Assay Kit*, BPS Bioscience #79575. *Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com.*

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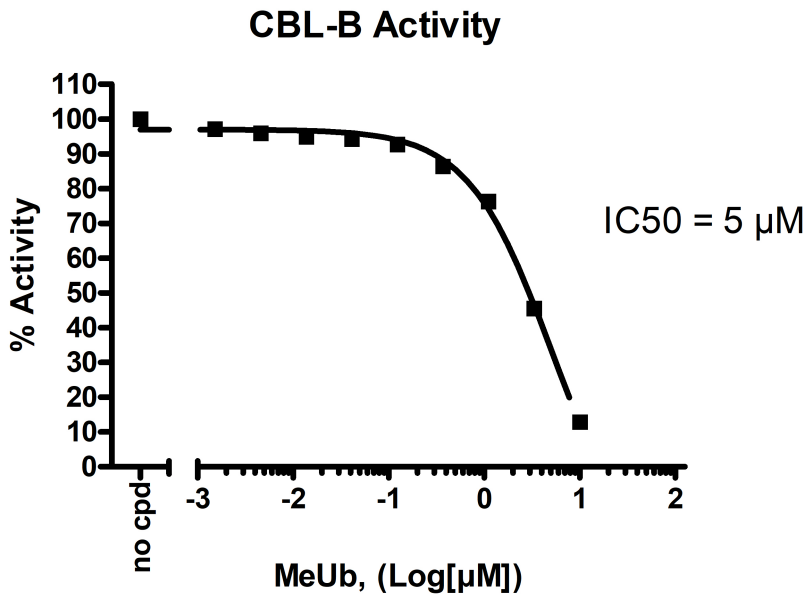

Figure 2: Inhibition of CBL-B Assay FRET signal by Methylated Ubiquitin, measured using the *CBL-B TR-FRET Assay Kit*, BPS Bioscience #79575. *Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com.*

RELATED PRODUCTS

Product Name	**Catalog #**	**Size**
CBL-B, GST-Tag (Human)	#80415	100 µg
CBL-B, His-Avi-Tag	#80414	100 µg
CBL-B, Biotin-labeled (Human)	#80412	50 µg
CBL-B (Y363F), Biotin-labeled (Human)	#80413	50 µg
UBE1 (UBA1), FLAG-tag	#80301	100 µg
UBCH5b	#80314	100 µg
UbcH5a, His-Tag (Human)	#80315	100 µg
UbcH5c, His-Tag (Human)	#80313	100 µg
UBA6 (UBE1L2), FLAG-tag	#80303	100 µg