Description

The DCAF11 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 DCAF11 (DDB1 and CUL4-associated Factor 11) auto-ubiquitination activity in a homogeneous 384-reaction format. It utilizes Europium-labeled Ubiquitin (donor) and Cy5-labeled Ubiquitin (acceptor) to complete the TR-FRET pairing. This assay measures poly-ubiquitination since both the TR-FRET donor and acceptor are incorporated into poly-ubiquitin chains formed on DCAF11. As a homogenous assay, it requires no time-consuming washing steps, making it especially suitable for HTS applications as well as real-time analyses.

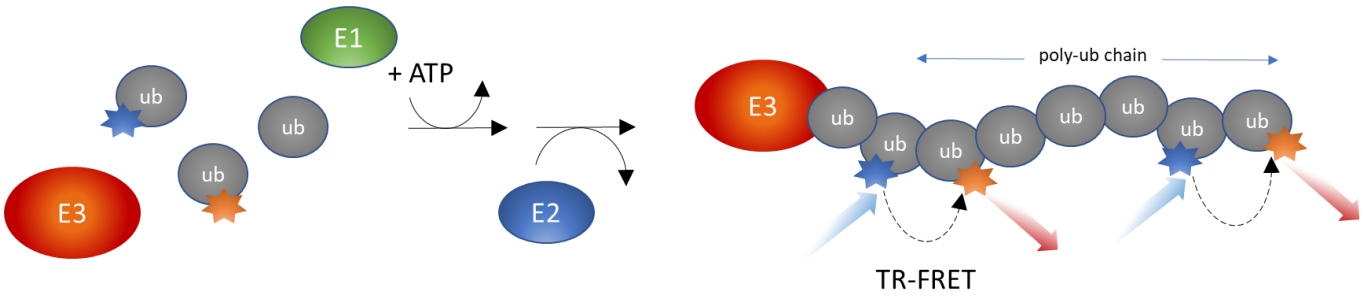

Figure 1. DCAF11 Intrachain TR-FRET Assay Kit schematic.

Background

Covalent conjugation to ubiquitin (Ub) is one of the major post-translational modifications that 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. This step ensures the transfer of Ub from E2~Ub to the substrate, leading to its mono- or poly-ubiquitination.

DDB1 and CUL4-associated Factor 11 (DCAF11) is a protein that associates with CUL4A or CUL4B to form a complex with E3 ligase activity. DCAF11 interacts with and ubiquitinates nuclear factor E2-related factor 2 (Nrf2), which is known to regulate cellular stress response genes, including those associated with oxidative stress. Additionally, DCAF11 activity is repressed by transcriptional factor EB (TFEB), allowing for stabilization of proteins such as Nrf2. Because Nrf2 and TFEB are involved in misfolded protein clearance, and oxidative stress is known to contribute to diseases such as Alzheimer's, Huntington's, and Parkinson's disease, DCAF11 is a potential target for the treatment of multiple neurodegenerative disorders. Like most E3 ligases, DCAF11 ubiquitinates itself.

Application(s)

- Screen molecules that inhibit DCAF11 Ubiquitin ligase activity in drug discovery HTS applications.
- Determine Inhibitor IC_{50}.
- Perform DCAF11 activity real-time kinetics.

Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), FLAG-Tag*	50 µg	-80°C
80314	UbcH5b, His-Tag*	60 µg	-80°C
101495	DCAF11/Rbx1/CUL4B/DDB1 Complex*	62 µg	-80°C
78307	TRF Ubiquitin Mix (200x)	40 µl	-80°C
	ATP (4 mM)	2 x 1 ml	-80°C
	CBL Assay Buffer 2	2 x 10 ml	-80°C
79969	White, nonbinding Corning, 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 DCAF11 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.

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 CBL Assay Buffer 2 and ATP at -80°C.

1. Thaw **UBE1**, **UbcH5b**, **DCAF11**, **Ubiquitin Mix**, **Assay Buffer**, and **ATP** on ice. Briefly spin the tubes to recover their full content.

2. Prepare a 5x TRF Ubiquitin Mix in CBL Assay Buffer 2 by making a 40-fold dilution of 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 CBL Assay Buffer 2 to 96 ng/µl (800 nM - the final concentration in the reaction will be 40 nM) (1 µl/ well).

    b) Dilute UbcH5b in CBL Assay Buffer 2 to 144 ng/µl (8 µM - the final concentration in the reaction will be 400 nM) (1 µl/ well).

    c) Dilute the DCAF11 complex in CBL Assay Buffer 2 to 31 ng/µl (100 nM – the final concentration in the reaction will be 25 nM) (5 µl/ well).

    Note: Keep all diluted proteins on ice until use. Do not freeze and re-use diluted proteins. UBE1, UbcH5b, DCAF11, TRF Ubiquitin Mix, and Assay Buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles.

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 CBL Assay Buffer 2, 5-fold more concentrated than the desired final concentrations. For the positive and negative controls, use CBL Assay Buffer 2 (Diluent Solution).

    OR

    b) If the Test inhibitor is soluble in DMSO, prepare the test inhibitor at a concentration 100-fold higher than the highest desired concentration in DMSO, then dilute the inhibitor 20-fold in CBL Assay Buffer 2 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 CBL Assay Buffer 2 to keep the concentration of DMSO constant.

    For positive and negative controls, prepare 5% DMSO in CBL Assay Buffer 2 (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 CBL Assay Buffer 2).

6. Add 15 µl of mix to the “Blank” wells.

Component	Blank
TRF Ubiquitin Mix (5x)	4 µl
Diluted UBE1	1 µl
Diluted UbcH5b	1 µl
Diluted DCAF11 complex	-
Test Inhibitor	-
Diluent Solution	4 µl
CBL Assay Buffer 2	5 µl
ATP (4 mM)	5 µl
Total	**20 µl**

7. Prepare a Master Mix: N wells × (4 µl 5x TRF Ubiquitin Mix + 1 µl diluted UBE1 + 1 µl diluted UbcH5b + 5 µl diluted DCAF11).

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 solution to each well designated "Test Inhibitor".

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

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 CBL Assay Buffer 2 to the well designated "Negative Control".

Component	Test Inhibitor	Negative Control	Positive Control
Master Mix	11 µl	11 µl	11 µl
Test Inhibitor	4 µl	–	–
Diluent Solution	–	4 µl	4 µl
CBL Assay Buffer 2	–	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).

Eu Donor Emission	Time Resolved	Dye-acceptor Emission	Time Resolved
Excitation Wavelength	317±20 nm	Excitation Wavelength	317±20 nm
Emission Wavelength	620±10 nm	Emission Wavelength	665±10 nm
Lag Time	60 µs	Lag Time	60 µs
Integration Time	500 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

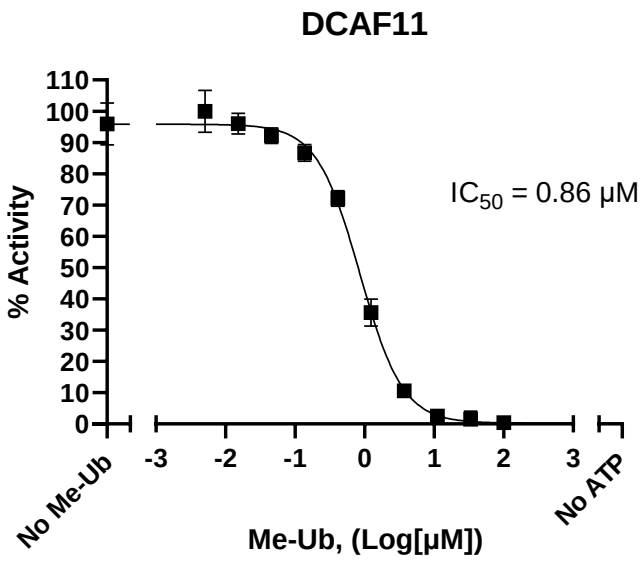

Figure 2: Inhibition of DCAF11 auto-ubiquitination by Methylated Ubiquitin.
DCAF11 auto-ubiquitination was measured in presence of increasing concentrations of Methylated Ubiquitin. Results are expressed as percent activity, in which absence of inhibitor is set to 100%.

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
VHL Intrachain TR-FRET Assay Kit	78305	384 reactions
XIAP Intrachain TR-FRET Assay Kit	78306	384 reactions
DCAF15 Intrachain TR-FRET Assay Kit	78543	384 reactions