Description

The CUL1 Intrachain TR-FRET Assay Kit is a homogeneous, sensitive TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) assay kit designed to measure CUL1 (cullin 1) auto-ubiquitination. It utilizes a Europium-labeled ubiquitin (Ub) donor as well as Cy5-labeled Ub acceptor to complete the TR-FRET pairing. Since both the TR-FRET donor and acceptor are incorporated into poly-ubiquitin chains formed on CUL1, this assay measures poly-ubiquitination. The kit comes in a convenient 384-well format and contains enough purified CUL1/SKP1/SKP2/RBX1 Complex, purified UBE1 (ubiquitin-like modifier activating enzyme 1), UbcH5b (Ubiquitin-conjugating enzyme E2 D2), Ubi-Mix™, ATP, and assay buffer for 400 reactions.

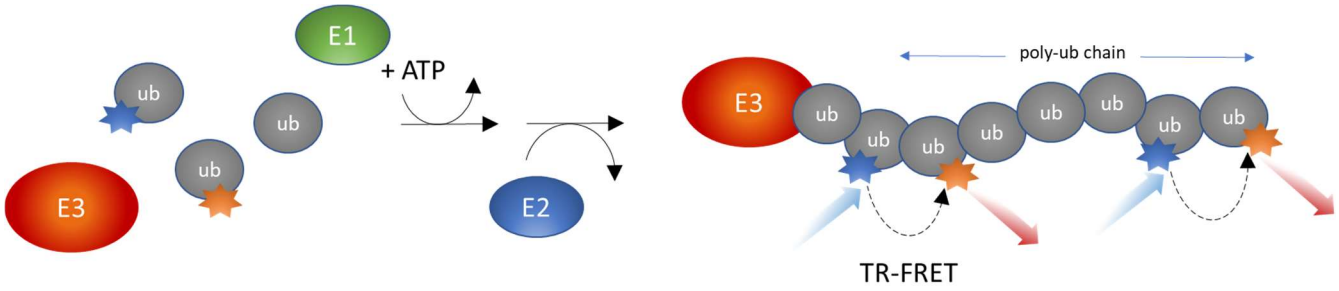

Figure 1. CUL1 Intrachain TR-FRET Assay Kit schematic.
E1 and E2 enzymes, Europium cryptate-labeled Ubiquitin (TR-FRET donor) and Cy5-labeled Ubiquitin (TR-FRET acceptor) are incubated with CUL1 complex. The donor and acceptor are incorporated into poly-ubiquitin chains formed on CUL1, allowing energy transfer to occur. The TR-FRET signal is proportional to CUL1 activity.

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.

CUL1, or cullin 1, is part of an E3 ligase complex, the SCF (SKP1 (s-phase kinase associated protein 1)- CUL1- F-box protein) complex, that also involves SKP2 and RBX1 (RING-box protein 1). The SCF complex is involved in the ubiquitination of proteins that act on cell cycle, signal transduction and transcription, with CUL1 serving as a structural scaffold. Cullins can be used in the context of targeted protein degradation in cancer therapy.

Applications

- Screen molecules that inhibit CUL1 Ub ligase activity in drug discovery high throughput screening (HTS) applications.
- Determine compound IC_{50}.
- Perform CUL1 real-time kinetic studies.

Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), FLAG-Tag*	40 µg	-80°C
80314	UbcH5b, His-Tag*	60 µg	-80°C
101755	CUL1/SKP1/SKP2/RBX1 Complex*	70 µg	-80°C
	200x Ubi-Mix™	40 µl	-80°C
	4 mM ATP	2 x 1 ml	-80°C
	U2 Assay Buffer	2 x 10 ml	-80°C
	White, nonbinding, low volume microtiter plate		Room Temp

** The initial concentration of 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
- Orbital Shaker

Storage Conditions

This assay kit will perform optimally for up to **6 months** from date of receipt when the materials are stored as directed.

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 CUL1 Intrachain TR-FRET Assay Kit is compatible with up to 1% final DMSO concentration.

Assay Protocol

- All samples and controls should be performed in triplicate.
- The assay should include “Blank”, “Positive Control”, “Negative Control” and “Test Inhibitor” conditions.
- We recommend maintaining the diluted protein on ice during use.
- For detailed information on protein handling please refer to Protein FAQs (bpsbioscience.com).
- We recommend using Methylated Ubiquitin as internal control. If not running a dose response curve for the control inhibitor, we recommend running the control inhibitor at 0.1X, 1X and 10X the IC_{50} value shown in the validation data below.

1. Thaw **UBE1**, **UbcH5b**, **CUL1 Complex**, **Ubi-Mix™, U2 Assay Buffer**, and **ATP** on ice. Briefly spin the tubes to recover their full content.

   Note: Ubi-Mix™ and U2 Assay Buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles. Aliquot not used Assay Buffer, ATP and Ubi-Mix™ in single use aliquots (minimum volume of 5 µl) and store at -80°C.

2. Dilute 200x Ubi-Mix™ 40-fold with U2 Assay Buffer to make a 5x Ubi-Mix™.

3. Dilute proteins, as follows, and keep on ice:

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

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

   c) Dilute **CUL1 Complex** with U2 Assay Buffer to 34.4 ng/µl (200 nM - final concentration in the reaction will be 50 nM) (5 µl/well).

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.

   4.1 If the Test Inhibitor is soluble in water, prepare a solution of the compound in U2 Assay Buffer that is 5-fold higher than the final desired concentration.

   For the positive and negative controls, use U2 Assay Buffer (Diluent Solution).

   OR

   4.2 If the Test Inhibitor is dissolved in DMSO, prepare a solution of the compound in 100% DMSO that is 100-fold higher than the highest concentration of the serial dilution. Then dilute 20-fold in U2 Assay Buffer, to prepare the highest concentration of the 5-fold intermediate dilutions. The concentration of DMSO in the dilution is now 5%.

   Prepare serial dilutions of the Test Inhibitor at concentrations 5-fold higher than 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. Prepare a Master Mix (11 µl/well, except "Blank" wells): N wells × (4 µl of 5x Ubi-Mix™ + 1 µl of diluted UBE1 + 1 µl of diluted UbcH5b + 5 µl of diluted CUL1 Complex).

6. Add 11 µl of Master Mix to the "Positive Control", "Negative Control" and "Test Inhibitor" wells.

7. For the "Blank" wells prepare a CUL1 Deficient Master Mix (11 µl/well): N wells x (4 µl of 5x Ubi-Mix™ + 1 µl of diluted UBE1 + 1 µl of diluted UbcH5b + 5 µl of U2 Assay Buffer).

8. Add 11 µl of CUL1 Deficient Mix to every "Blank" well.

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

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

11. Initiate the reaction by adding 5 µl of 4 mM ATP to the wells labeled "Positive Control", "Test Inhibitor" and "Blank" wells.

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

Component	Test Inhibitor	Blank	Negative Control	Positive Control
Master Mix	11 µl	-	11 µl	11 µl
CUL1 Deficient Master Mix	-	11 µl	-	-
Test Inhibitor	4 µl	-	–	–
Diluent Solution	–	4 µl	4 µl	4 µl
U2 Assay Buffer	–	-	5 µl	–
4 mM ATP	5 µl	5 µl	–	5 µl
Total	**20 µl**	**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 25-35 minutes.

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: Calculate the FRET value by using the following formula:

$$FRET = \frac{S_{665} - \left(\frac{Tb_{665}}{Tb_{620}} \times S_{620}\right)}{S_{620}} \times 1000$$

S_{665} = Sample value measured at 665 nm, S_{620} = Sample value measured at 620 nm, Tb_{665} = Tb only or Blank value measured at 665 nm, Tb_{520} = Tb only or Blank value measured at 520 nm.

The FRET value calculated for the negative control should be subtracted from all other measurements and can be set as 0%. The FRET value from the "Positive Control" can be set as 100% activity.

$$\%\ Activity = \frac{FRET_S - FRET_{neg}}{FRET_P - FRET_{neg}} \times 100\%$$

$FRET_S$ =FRET value for samples of Test Inhibitor, $FRET_{Sub}$ = FRET value for the Substrate Control, and $FRET_p$ = FRET value for the Positive Control (no inhibitor).

Example Results

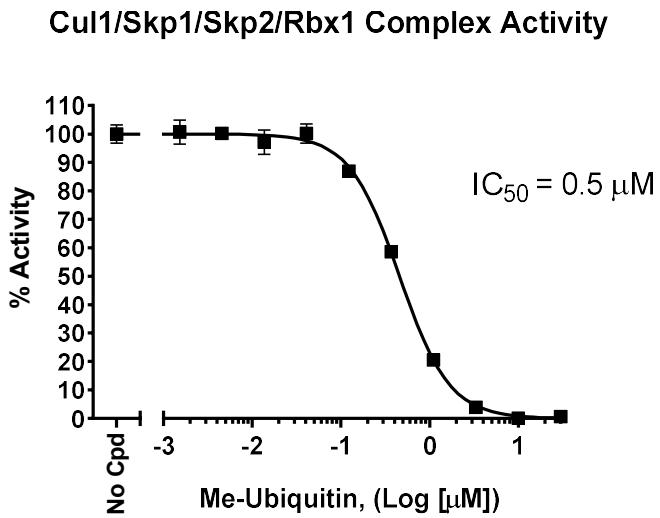

Figure 2: Inhibition of CUL1 auto-ubiquitination by Methylated Ubiquitin (Me-Ubiquitin).
CUL1 auto-ubiquitination was measured in the presence of increasing concentrations of Methylated Ubiquitin (BPS Bioscience #102075). The "Blank" value was subtracted from all other values. Results are expressed as the percent of control (activity in the absence of inhibitor, set at 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

References

Sweeney M., *et al.*, 2020 *Scientific Reports* 10:13942.

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

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