**Description**

The DCAF15 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 DCAF15 (DDB1 and CUL4-associated factor 15) 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 DCAF15. As a homogenous assay, it requires no time-consuming washing steps, making it especially suitable for HTS applications as well as real-time kinetics analyses.

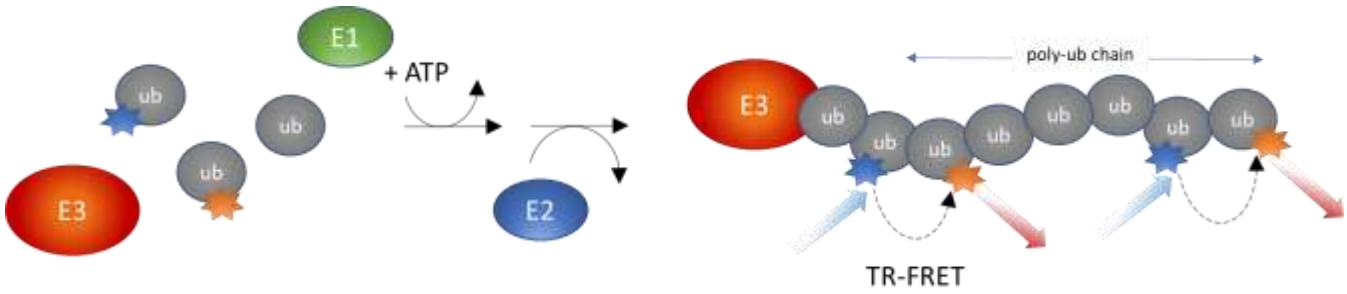


*Figure 1. DCAF15 Intrachain TR-FRET Assay Kit schematic.*

**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 15 (DCAF15) is a protein that associates with CUL4A or CUL4B to form a complex with E3 ligase activity. Like most E3 ligases, DCAF15 ubiquitinates itself. DCAF15 interacts with and ubiquitinates the major transcription factor ZEB1, which is involved in the activation of the epithelial-mesenchymal transition (EMT) in metastatic cancer cells. Furthermore, DCAF15 is necessary for ubiquitination of the RNA splicing factor RBM39, the degradation of which leads to enhanced sensitivity of hematopoietic and lymphoid cancer cells to drug-induced cytotoxicity. Therefore, DCAF15 is an attractive potential drug target for cancer therapy.

**Applications**

- Screen molecules that inhibit DCAF15 Ubiquitin ligase activity in drug discovery HTS applications.
- Determine Inhibitor $IC_{50}$.
- Perform DCAF15 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 |
| 101497 | DCAF15/Rbx1/CUL4B/DDB1/DDA1 Complex* | 130 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 |
| | White, nonbinding Corning, 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
- 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 DCAF15 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 U2 Assay Buffer and ATP at -20°C.

1. Thaw **UBE1**, **UbcH5b**, **DCAF15**, **TRF Ubiquitin Mix**, **U2 Assay Buffer**, and **ATP (4 mM)** on ice. Briefly spin the tubes to recover their full content.

2. Prepare a 5x Ubiquitin Mix in U2 Assay Buffer by making a 40-fold dilution of Ubiquitin Mix (200x).

3. Calculate the amount of each 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 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 in U2 Assay Buffer to 144 ng/µl (8 µM - the final concentration in the reaction will be 400 nM) (1 µl/ well).

   c) Dilute the DCAF15 complex in U2 Assay Buffer to 64.8 ng/µl (200 nM - the final concentration in the reaction will be 50 nM) (5 µl/ well).

   *Note: Keep all diluted proteins on ice until use. UBE1, UbcH5b, DCAF15, TRF Ubiquitin Mix, and U2 Assay Buffer are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles. Do not freeze and re-use the 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 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 at a concentration 100-fold higher than the highest desired concentration in 100% DMSO, 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 water (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

   *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 Ubiquitin Mix + 1 µl of diluted UBE1 + 1 µl of diluted UbcH5b + 4 µl of Diluent Solution + 5 µl of U2 Assay Buffer).

6. Add 15 µl to each “Blank” well.

| Component | µl |
|---|---|
| Ubiquitin Mix (5x) | 4 µl |
| Diluted UBE1 | 1 µl |
| Diluted UbcH5b | 1 µl |
| Diluted DCAF15 | - |
| Test Inhibitor | - |
| 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 Ubiquitin Mix + 1 µl diluted UBE1 + 1 µl diluted UbcH5b + 5 µl diluted DCAF15).

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

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

10. Add 4 µl of the 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," "Test Inhibitor," and "Blank".

12. Add 5 µl of U2 Assay Buffer 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 |
| 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 the 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**

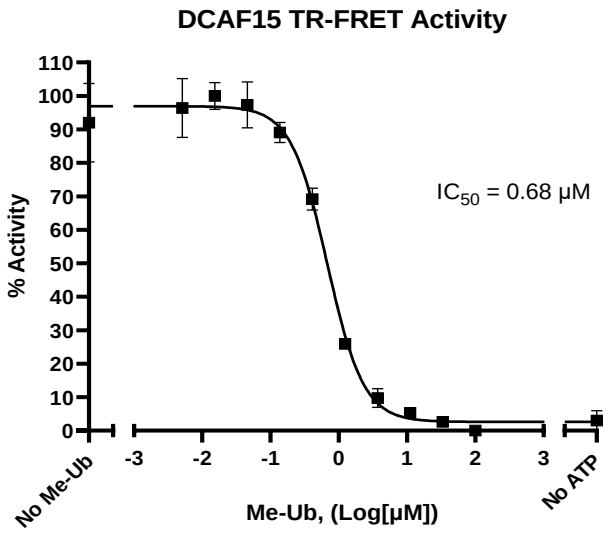


*Figure 1: Inhibition of DCAF15 auto-ubiquitination by Methylated Ubiquitin.*
DCAF15 auto-ubiquitination was measured in the presence of increasing concentrations of Methylated Ubiquitin. Results are expressed as percent activity, in which absence of inhibitor is set to 100%.

*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* |
|---|---|---|
| DCAF11 Intrachain TR-FRET Assay Kit | 78542 | 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 |
| VHL Intrachain TR-FRET Assay Kit | 78305 | 384 reactions |
| XIAP Intrachain TR-FRET Assay Kit | 78306 | 384 reactions |