**Description**

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.

The SMAD ubiquitination regulatory factor 2 (SMURF2) is a HECT-type E3 Ub ligase that regulates TGF-β/BMP pathways via ubiquitination of key signal transducers (SMAD1, SMAD2, or SMAD5), or TGF-β receptor I. SMURFs play a critical role in cell-type specification, tissue and organ development by regulating planar cell polarity signaling and convergent extension. SMURFs can also accelerate tumor progression, invasion, and metastasis as they regulate ubiquitination and subsequent proteasomal degradation of tumor-suppressing proteins including p53 as well as various cell signaling proteins. That is why SMURF2 and especially its Ub ligase activity is an attractive potential drug target in cancer immunotherapy. Like most E3 ligases, SMURF2 ubiquitinates itself.

The SMURF2 intrachain TR-FRET Assay Kit is a sensitive high-throughput screening (HTS) TR-FRET Assay Kit, designed to measure SMURF2 auto-ubiquitination activity in a homogeneous 384 reaction format. It utilizes a Europium cryptate-labeled 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 SMURF2, this FRET-based assay requires no time-consuming washing steps, making it especially suitable for HTS applications as well as real-time kinetics analyses of polyubiquitination.

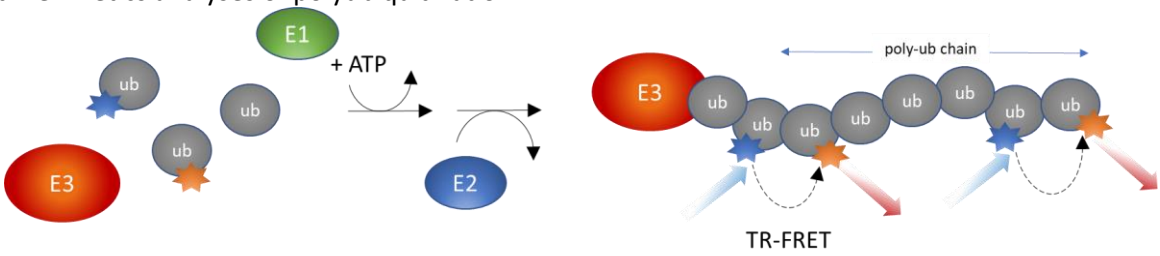


**Figure 1.** SMURF2 intrachain TR-FRET Assay Kit schematic

**Applications**

Great for screening molecules that inhibit SMURF2 Ub ligase activity in drug discovery HTS applications, for determination of compound $IC_{50}$ and for SMURF2 real-time kinetics analyses.

**Supplied Materials**

| Catalog # | Name | Amount | Storage | |
|---|---|---|---|---|
| 80301 | UBE1 (E1)* | 50 µg | -80°C | ***Avoid multiple freeze/ thaw cycles*** |
| 80314 | UBCH5b (E2)* | 300 µg | -80°C | |
| 80403 | SMURF2, FLAG-tag (E3)* | 20 µg | -80°C | |
| 78307 | TRF Ubiquitin Mix (200x) | 50 µl | -80°C | |
| | ATP (4 mM) | 150 µl | -80°C | |
| 78269 | CBL assay buffer 2 | 2x10ml | -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.***



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**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 SMURF2 intrachain TR-FRET Assay Kit is compatible with up to 1% final DMSO concentration. We recommend preparing the inhibitor in no higher than 5% DMSO solution in buffer and using 4 µl per well.

**Assay Protocol**

- All samples and controls should be performed in triplicates
- The assay should include a "Blank", a "Positive control", and a "Negative control"

1) Thaw **UBE1**, **UBCH5b**, **SMURF2**, **TRF Ubiquitin Mix**, **CBL assay buffer 2**, and **ATP** on ice. Aliquot each protein, assay buffer, and ATP into single-use aliquots and immediately store at -80°C. Note: UBE1, UBCH5b, SMURF2, TRF Ubiquitin Mix, and CBL assay buffer 2 are sensitive to freeze/thaw cycles. Avoid multiple freeze-thaw cycles.

2) Carefully calculate the amount of each protein needed and prepare appropriate amounts of diluted proteins:

   Prepare 5x TRF Ubiquitin Mix in CBL assay buffer 2 (40-fold dilution of the 200x TRF Ubiquitin Mix);
   Dilute the UBE1 in CBL assay buffer 2 at 800 nM (96 ng/µl) (final concentration in reaction 40 nM);
   Dilute the UBCH5b in CBL assay buffer 2 at 10 µM (720 ng/µl) (final concentration in reaction 500 nM);
   Dilute the SMURF2 in CBL assay buffer 2 at 100 nM (7.2 ng/µl) (final concentration in reaction 25 nM);

   Keep all diluted proteins on ice until use.

3) Prepare the compound solution.



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If the compound is dissolved in DMSO, make a 100-fold higher concentration of the compound in DMSO than the highest concentration you want to test in the assay. Then dilute 20-fold in CBL assay buffer 2 (at this step the compound concentration is 5-fold higher than the desired final concentration). If you want to run an $IC_{50}$ or test lower concentrations of the compound, prepare serial dilutions using 1X assay buffer containing 5% DMSO, so the final concentration of DMSO will be 1% in all samples.

If the compound is soluble in water, prepare a solution of the compound in CBL assay buffer 2 that is 5-fold higher than the final assay concentration.

4) To the wells designated as "Blank", add 4 µl of **5x TRF Ubiquitin Mix** + 1 µl of **UBE1** + 1 µl of **UBCH5b** + 4 µl of **diluent solution** (for example DMSO 5%) + 5 µl of **CBL assay buffer 2**.

| | **Blank** |
|---|---|
| TRF Ubiquitin Mix (5x) | 4 µl |
| UBE1 | 1 µl |
| UBCH5b | 1 µl |
| SMURF2 | - |
| Test Compound | - |
| Diluent solution* (no inhibitor) | 4 µl |
| CBL assay buffer 2 | 5 µl |
| ATP (4 mM) | 5 µl |
| **Total** | **20 µl** |

***The diluent solution contains the assay buffer with the same concentration of solvent (e.g. DMSO) as the test compound solution.***

5) Make the master mixture using diluted reagents:

N wells × (4 µl **5x TRF Ubiquitin Mix** + 1 µl **UBE1** + 1 µl **UBCH5b** + 5 µl **SMURF2**).

6) Add 11 µl of master mixture to each well designated for the "Negative Control", "Positive Control", "Test Sample".

7) Add 4 µl of inhibitor solution to each well designated "Test Inhibitor". For all other wells: "Positive Control", "Negative Control", add 4 µl of the diluent solution without inhibitor.

8) Initiate the reaction by adding 5 µl of **ATP** to the wells labeled "Positive Control," "Test Inhibitor," and "Blank." Add 5 µl of **CBL assay buffer 2** to the well designated "Negative Control." Cover the plate with a plate sealer. Incubate the reaction at room temperature for two hours or at 30°C for one hour.


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| | Test Sample | Negative Control | Positive Control |
|---|---|---|---|
| Master Mix | 11 µl | 11 µl | 11 µl |
| Test compound | 4 µl | – | – |
| Diluent solution* (no inhibitor) | – | 4 µl | 4 µl |
| CBL assay buffer 2 | – | 5 µl | – |
| ATP (4 mM) | 5 µl | – | 5 µl |
| **Total** | **20 µl** | **20 µl** | **20 µl** |

***The diluent solution contains the assay buffer with the same concentration of solvent (e.g. DMSO) as the test compound solution.***

9) 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 Blank (it is expected that Blank and Negative Control represent 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**

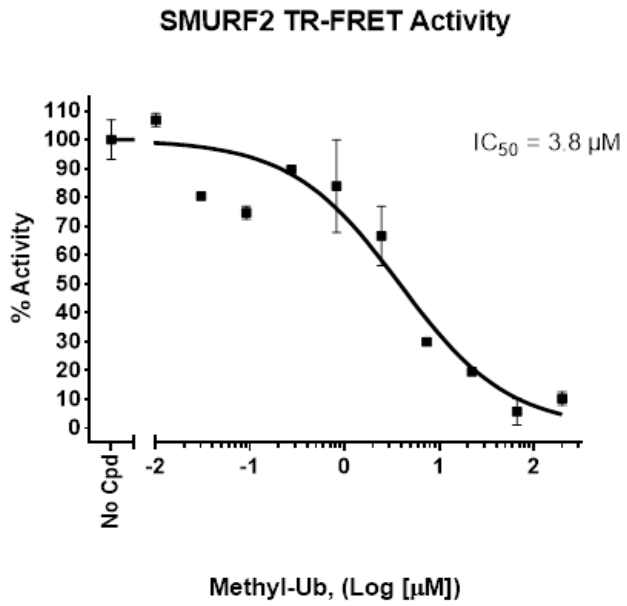


***Figure 1:*** *Inhibition of SMURF2 auto-ubiquitination by Methylated Ubiquitin, measured using the SMURF2 intrachain TR-FRET Assay Kit, BPS Bioscience #78304. Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com.*

**Troubleshooting Guide**

Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For all further questions, please email support@bpsbioscience.com



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**Related Products**

| *Products* | *Catalog #* | *Size* |
|---|---|---|
| Cereblon intrachain TR-FRET Assay Kit | 78301 | 384 rxns. |
| MDM2 intrachain TR-FRET Assay Kit | 78302 | 384 rxns. |
| SMURF1 intrachain TR-FRET Assay Kit | 78303 | 384 rxns. |
| VHL intrachain TR-FRET Assay Kit | 78305 | 384 rxns. |
| XIAP intrachain TR-FRET Assay Kit | 78306 | 384 rxns. |
| MDM2 TR-FRET Assay Kit | 79773 | 384 rxns. |
| CBL-B TR-FRET Assay Kit | 79575 | 384 rxns. |
| c-CBL TR-FRET Assay Kit | 79786 | 384 rxns. |
| Cereblon Ubiquitination Homogenous Assay Kit | 79881 | 384 rxns. |
| UBCH13 TR-FRET Assay Kit | 79741 | 384 rxns. |
| UBCH5a TR-FRET Assay Kit | 79900 | 384 rxns. |
| UBCH5c TR-FRET Assay Kit | 79901 | 384 rxns. |
| UBCH5b TR-FRET Assay Kit | 79896 | 384 rxns. |
| MDM2, GST-Tag (Human) | 80751 | 20 µg |
| UBE1 (UBA1), FLAG-tag | 80301 | 100 µg |
| UBE1, GST-Tag | 100402 | 100 µg |
| UBE2A, His-Tag | 79368 | 20 µg |
| UBE2C, His-Tag | 79369 | 20 µg |
| UBE2D2, His-Tag | 79370 | 20 µg |
| UBE2E3 (UBCH9), His-Tag | 79371 | 20 µg |
| UBE2G1 (UBC7), His-Tag | 79372 | 20 µg |
| UBE2K (UBC1), His-Tag | 79373 | 20 µg |
| UBE2O, GST-Tag | 79374 | 20 µg |
| UbcH5a (UBE2D1), His-tag | 80315 | 100 µg |
| UbcH5b, His-Tag (Human) | 80314 | 100 µg |
| UbcH6 (UBE2E1), His-tag | 80316 | 100 µg |
| UbcH7, His-tag (E. coli-derived) | 80317 | 100 µg |
| UbcH7, His-tag (Sf9-derived) | 80318 | 50 µg |
| UbcH13 (UBE2N), His-tag | 80323 | 100 µg |
| CBL-B, GST-Tag (Human) | 80415 | 100 µg |
| c-CBL, GST-Tag (Human) | 100370 | 100 µg |
| Great for screening molecules that inhibit SMURF2 Ub ligase activity in drug discovery HTS applications, for determination of compound $IC_{50}$ and for SMURF1 real-time kinetics analyses. | 80401 | 20 µg |
| | 80402 | 20 µg |
| | 80403 | 20 µg |
| , FLAG-tag | 100329 | 10 µg |
| SMURF1, FLAG-tag | 100373 | 10 µg |
| SMURF2, FLAG-tag | 79293 | 2 mg |
| Cereblon/DDB1/Cul4A/Rbx1 Complex | 11236 | 50 µg |
| VHL/CUL2/ELOB/ELOC/RBX1 Complex | | |
| Ubiquitin, His-Tag | | |
| Ubiquitin, His-Avi-Tag, Biotin Labeled | | |



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