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# Produktinformation

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- Trockeneiszuschlag
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# ChoosE3-Freedom Intrachain TR-FRET Assay Kit

#78560
384 reactions

**Description**

The ChoosE3-Freedom Intrachain TR-FRET Assay Kit is a sensitive high-throughput screening (HTS) TR-FRET assay kit, designed to measure the auto-polyubiquitination of any ***purified*** E3 ligase of interest in a homogeneous 384 reaction format. The kit contains E1 and E2 enzymes, ATP, an optimized TRF Ubiquitin Mix, and a universal buffer. Purified E3 ligase MDM2 is also provided as an internal quality control.

The assay was designed with a Europium-labeled Ubiquitin donor and a Cy5-labeled Ubiquitin acceptor to complete the TR-FRET pairing. Since both the TR-FRET donor and acceptor are incorporated into poly-ubiquitin chains formed on the E3 ligase, the assay measures only poly-ubiquitination and not mono-ubiquitination. This FRET-based assay requires no time-consuming washing steps, making it especially suitable for HTS applications as well as real-time kinetics.

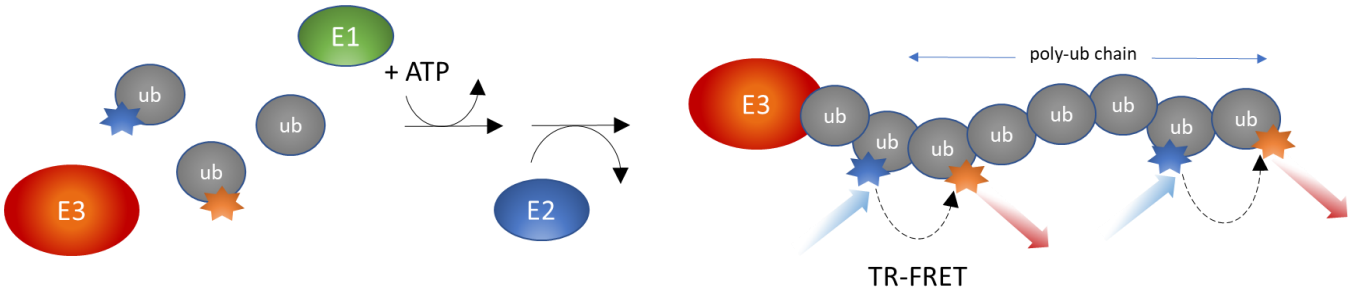


*Figure 1: ChoosE3-Freedom Intrachain TR-FRET Assay Kit schematic.*

**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 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.

**Application(s)**

- Measure the auto-polyubiquitination of any E3 ligase of interest
- Identify novel E3 ligases or variants of known ligases
- Screen inhibitors or activators of an E3 ligase of interest in HTS applications
- Determine compound $IC_{50}$
- Perform E3 ligase real-time analyses

**Supplied Materials**

| Catalog # | Name | Amount | Storage | |
|---|---|---|---|---|
| 80301 | UBE1 (UBA1), FLAG-Tag (E1)* | 50 µg | -80°C | ***Avoid multiple freeze/ thaw cycles*** |
| 80314 | UbcH5b, His-Tag (E2)* | 60 µg | -80°C | |
| 100409 | MDM2, GST-Tag (E3)* | 10 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 384-well 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 intrachain TR-FRET Assay Kit is not suitable for measuring mono-ubiquitination. Weak signals may be obtained for multi-mono-ubiquitination or for short poly-ubiquitin chains.
- This kit is compatible with up to 1% final DMSO concentration. We recommend preparing the inhibitor in no higher than 5% DMSO solution in assay buffer and using 4 µl per well.
- The E3 ligase used in the assay should be purified. The assay will not perform well when using cell lysates due to the presence of other E3 ligases and ubiquitin.

**Assay Protocol**

**Protocol 1 - Screen or assay E3 ligase(s) of interest**

- All samples and controls should be performed in triplicate
- The assay should include a "Blank", "Internal control", and "Negative control"
- Calculate the amount needed for the desired number of wells for each protein, assay buffer, and ATP Aliquot the remaining into 3-4 single-use aliquots depending on how many times the plate will be used and immediately store at -80°C.

1. Thaw UBE1, UBCH5b, MDM2 ligase (internal control), TRF Ubiquitin Mix, U2 Assay Buffer, and ATP on ice. Briefly spin the tubes to recover their full contents.

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

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 U2 Assay Buffer at 96 ng/µl (corresponding to a concentration of 800 nM - the final concentration in the reaction is 40 nM, the volume needed is 1 µl/well)
    b. Dilute UBCH5b in U2 Assay Buffer at 144 ng/µl (8 µM - the final concentration in the reaction is 400 nM, the volume needed is 1 µl/well)
    c. Dilute MDM2 E3 ligase control in U2 Assay Buffer at 8.3 ng/µl (100 nM - the final concentration in the reaction is 25 nM, the volume needed is 5 µl/well)

4. Prepare appropriate dilutions(s) of the desired purified E3 ligase(s) in U2 Assay Buffer. You will need 5 µl/well.

   *Note 1: To screen unknown E3 ligases, BPS Bioscience recommends initially using final concentrations of 25 - 50 nM of each E3 ligase in the reaction as a starting point.*

   *Note 2: Keep all diluted proteins on ice until use*

5. Prepare a Master Mix using diluted reagents: N wells × (4 µl of **5x TRF Ubiquitin Mix** + 1 µl of **UBE1** + 4 µl of **U2 Assay Buffer** + 5 µl of **4 mM ATP**).

6. Add 14 µl of Master Mix to all the wells

7. Add 1 µl of **UBCH5b** to each well described as "Blank", "Internal Control", "Test E3 Ligase"

8. Add 1 µl of **U2 Assay Buffer** to the wells described as "Negative Control" and 5 µl to the wells designated as "Blank"**.**

9. To the wells designated as "Internal Control," add 5 µl of **E3 ligase control (MDM2).**

10. For wells designated for "**Test E3 ligase" and "Negative control"**, initiate the reaction by adding 5 µl of the diluted individual **E3 ligase**.

    *Note: If multiple E3 ligases are being tested, a corresponding negative control is needed for each E3 ligase.*

| Component | Blank | Internal Control | Negative Control | Test E3 ligase |
|---|---|---|---|---|
| Master Mix | 14 µl | 14 µl | 14 µl | 14 µl |
| UBCH5b | 1 µl | 1 µl | - | 1 µl |
| Internal Control E3 ligase (MDM2) | - | 5 µl | - | - |
| Test E3 ligase | - | - | 5 µl | 5 µl |
| U2 Assay Buffer | 5 µl | - | 1 µl | - |
| **Total** | **20 µl** | **20 µl** | **20 µl** | **20 µl** |

11. Cover the plate and incubate at room temperature for two hours or at 30°C for one hour. Alternatively, directly start real-time kinetics analysis.

12. Read the fluorescent intensity in a microtiter-plate reader capable of measuring TR-FRET (go the end of the "Protocols" section for **Instrument Settings**).

**Assay Protocol 2- Inhibitor/activator screening**

- All samples and controls should be performed in triplicates
- The assay should include a "Blank", "Internal Control", "Positive control", and "Negative control"
- Calculate the amount needed for the desired number of wells for each protein, assay buffer, and ATP. Aliquot the remaining into 3-4 single-use aliquots depending on how many times the plate will be used and immediately store at -80°C

1. Thaw UBE1, UBCH5b, E3 ligase control (MDM2), TRF Ubiquitin Mix, U2 Assay Buffer, and ATP on ice.

*Note: UBE1, UBCH5b, MDM2 E3 ligase control, TRF Ubiquitin Mix, and U2 Assay Buffer are sensitive to freeze/thaw cycles.* ***Avoid multiple freeze-thaw cycles***

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

3. Carefully calculate the amount of each protein needed and prepare the appropriate amounts of diluted proteins:
    a. Dilute **UBE1** in U2 Assay Buffer at 96 ng/µl (corresponding to a concentration of 800 nM - the final concentration in the reaction is 40 nM, the volume needed is 1 µl/well)
    b. Dilute the **UBCH5b** in U2 Assay Buffer at 144 ng/µl (8 µM - the final concentration in the reaction is 400 nM, the volume needed is 1 µl/well)
    c. Dilute **E3 ligase control (MDM2)** in U2 Assay Buffer at 8.3 ng/µl (100 nM - the final concentration in the reaction is 25 nM, the volume needed is 5 µl/well)
    d. Prepare the appropriate dilution(s) of the purified desired E3 ligase(s) in U2 Assay Buffer

    *Note 1: We suggest titrating the desired E3 ligase to determine its optimal concentration prior to screening compounds.*

    ⚠ *Note 2: Keep all diluted proteins on ice until use.*

4. Prepare the compound solution:
    *Without DMSO*
    a. If the compound is soluble in water, prepare a solution of the compound in U2 Assay Buffer at a concentration 5-fold higher than the final desired concentration.
    b. To determine the $IC_{50}$ or $EC_{50}$ of the compound, prepare serial dilutions using U2 Assay Buffer at concentrations 5-fold higher than the desired final concentrations.

    ***Or***

    *With DMSO*
    a. If the compound is dissolved in DMSO, prepare a solution of the compound in DMSO at a concentration that is 100-fold higher than the highest desired concentration. Then dilute 20-fold in U2 Assay Buffer (at this step the compound concentration is 5-fold higher than the desired final concentration). The concentration of DMSO in the dilution is now 5%.
    b. To determine the $IC_{50}$ or $EC_{50}$ of the compound, prepare serial dilutions using U2 Assay Buffer containing 5% DMSO, so the final concentration of DMSO will be 1% in all samples.

5. Prepare a Master Mix using diluted reagents: N wells × (4 µl of **5x TRF Ubiquitin Mix** + 1 µl of **UBE1** + 1 µl of **UBCH5b**).

6. Add 6 µl of Master Mix to all the wells.

7. Add 4 µl of Test Compound dilutions to each well designated “Test Compound.”

8. For all other wells: “Blank”, “Internal Control”, “Negative Control”, and “Positive Control”, add 4 µl of the diluent solution without inhibitor.

9. Add 5 µl of **U2 Assay Buffer** to the well designated as “Blank” and “Negative Control.”

10. Add 5 µl of MDM2 to the “Internal Control” wells a

11. Add 5 µl of the Test E3 ligase to "Positive control" and "Test compound" wells.

12. Initiate the reaction by adding 5 µl of **ATP** to all the wells.

| Component | Blank | Internal Control | Negative Control | Positive Control | Test Compound |
|---|---|---|---|---|---|
| Master Mix | 6 µl | 6 µl | 6 µl | 6 µl | 6 µl |
| Diluent solution* | 4 µl | 4 µl | 4 µl | 4 µl | - |
| Test Compound | - | - | - | - | 4 µl |
| MDM2 | - | 5 µl | - | - | - |
| Test E3 ligase | - | - | - | 5 µl | 5 µl |
| U2 Assay Buffer | 5 µl | – | 5 µl | - | - |
| ATP (4 mM) | 5 µl | 5 µl | 5 µl | 5 µl | 5 µl |
| **Total** | **20 µl** | **20 µl** | **20 µl** | **20 µl** | **20 µl** |

**The diluent solution contains the assay buffer with the same concentration of solvent (i.e., DMSO) as the test compound solution but does not contain the compound*

13. Cover the plate with a plate sealer and incubate the reaction at room temperature for two hours or at 30°C for one hour.

14. Read the fluorescent intensity in a microtiter-plate reader capable of measuring TR-FRET.

**Instrument Settings**

| Eu-donor emission | | Dye-acceptor emission | |
|---|---|---|---|
| Reading Mode | Time Resolved | Reading Mode | 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 the 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.

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

Where FRETs = Sample FRET, $FRET_{blank}$ = Blank FRET, and $FRET_P$ = Positive control FRET.

**Example Results**

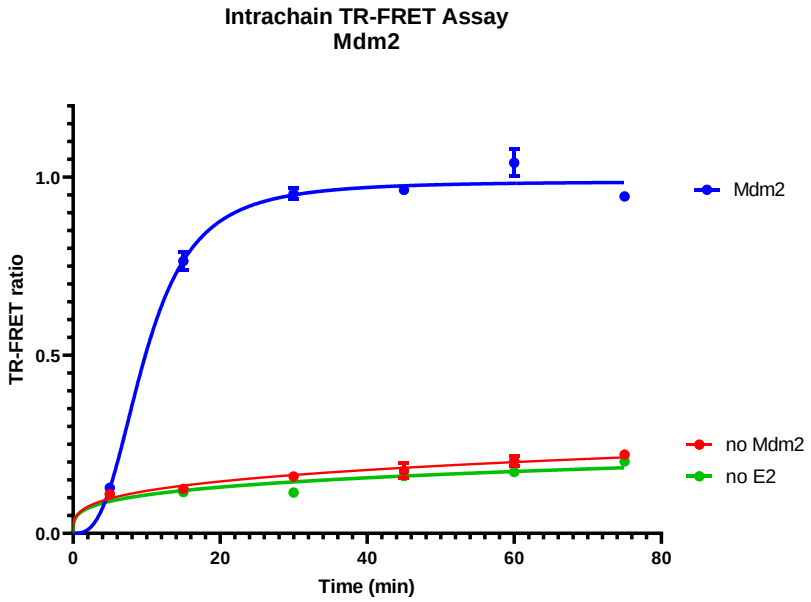


*Figure 1: Real-time analysis of MDM2 E3 ligase activity.*
Polyubiquitination activity of MDM2 (BPS Bioscience #100409) was measured using the Intrachain TR-FRET Assay Kit (BPS Bioscience #78560) as a function of time. Negative controls included a "no E2" and "no MDM2" conditions.

*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 |
| Cereblon Ubiquitination Homogenous Assay Kit | 79881 | 384 reactions |
| MDM2 intrachain TR-FRET Assay Kit | 78302 | 384 reactions |
| SMURF1 intrachain TR-FRET Assay Kit | 78303 | 384 reactions |
| CBL-B TR-FRET Assay Kit | 79575 | 384 reactions |
| c-CBL TR-FRET Assay Kit | 79786 | 384 reactions |
| UBCH13 TR-FRET Assay Kit | 79741 | 384 reactions |