Description

The ChoosE3-Freedom 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 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 (mouse double minute 2 homolog) 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.

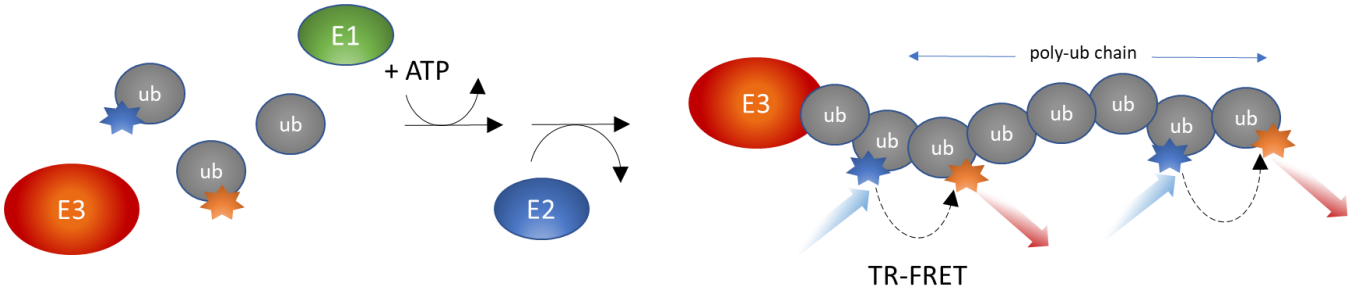

Figure 1: ChoosE3-Freedom Intrachain TR-FRET Assay Kit schematic.

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*	50 µg	-80°C
80314	UbcH5b, His-Tag*	60 µg	-80°C
100409	MDM2, GST-Tag*	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		

** 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)
- Purified E3 ligase to be tested
- 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 a solution containing no higher than 5% DMSO 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 “Blank”, “Internal Control”, “Negative Control” and “Test E3 Ligase” 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**, **MDM2** (internal control), **TRF Ubiquitin Mix**, **U2 Assay Buffer**, and **ATP** 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 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 (corresponding to a concentration of 800 nM - the final concentration in the reaction is 40 nM) (1 µl/well).

    b) Dilute UbcH5b in U2 Assay Buffer to 144 ng/µl (8 µM - the final concentration in the reaction is 400 nM) (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) (5 µl/well).

    d) Dilute the E3 ligase to be tested in U2 Assay Buffer (5 µl/well).

    Note: To screen unknown E3 ligases, BPS Bioscience recommends using final concentrations of 25-50 nM of each E3 ligase in the reaction as a starting point. Keep all diluted proteins on ice until use.

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

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

6. Add 1 µl of UbcH5b to each well described as “Blank”, “Internal Control” and “Test E3 Ligase”.

7. Add 1 µl of U2 Assay Buffer to the “Negative Control” wells.

8. Add 5 µl of U2 Assay Buffer to the “Blank” wells**.**

9. Add 5 µl of diluted E3 ligase control (MDM2) to the “Internal Control” wells.

10. Initiate the reaction by adding 5 µl of the diluted individual E3 ligase to be tested to the “Test E3 ligase” and “Negative Control” wells.

    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
Diluted UbcH5b	1 µl	1 µl	-	1 µl
Diluted MDM2	-	5 µl	-	-
Diluted Test E3 ligase	-	-	5 µl	5 µl
U2 Assay Buffer	5 µl	-	1 µl	-
Total	**20 µl**	**20 µl**	**20 µl**	**20 µl**

11. 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 20-40 minutes.

Assay Protocol 2- Inhibitor/activator screening

- All samples and controls should be performed in triplicate.
- The assay should include "Blank", "Internal Control", "Positive Control", "Negative control" and "Test Compound" conditions.
- 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** (internal control), **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 a 5x Ubiquitin Mix in U2 Assay Buffer by making a 40-fold dilution of 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 U2 Assay Buffer to 96 ng/µl (corresponding to a concentration of 800 nM - the final concentration in the reaction is 40 nM) (1 µl/well).

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

   c) Dilute MDM2 E3 ligase control in U2 Assay Buffer to 8.3 ng/µl (100 nM - the final concentration in the reaction is 25 nM) (5 µl/well).

   d) Prepare the appropriate dilution(s) of the purified desired E3 ligase(s) in U2 Assay Buffer (5 µl/well).

      Note: We suggest titrating the desired E3 ligase to determine its optimal concentration prior to screening compounds. Keep all diluted proteins on ice until use.

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 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.

      OR

   b) 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 (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%.

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: N wells × (4 µl of 5x TRF Ubiquitin Mix + 1 µl of diluted UBE1 + 1 µl of diluted UbcH5b).

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

7. Add 4 µl of the test compound dilutions to each well designated "Test Compound".

8. Add 4 µl of Diluent Solution to all other wells.

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

10. Add 5 µl of diluted MDM2 to the "Internal Control" wells.

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
Diluted MDM2	-	5 µl	-	-	-
Diluted 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**

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		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 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.

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

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

Example Results

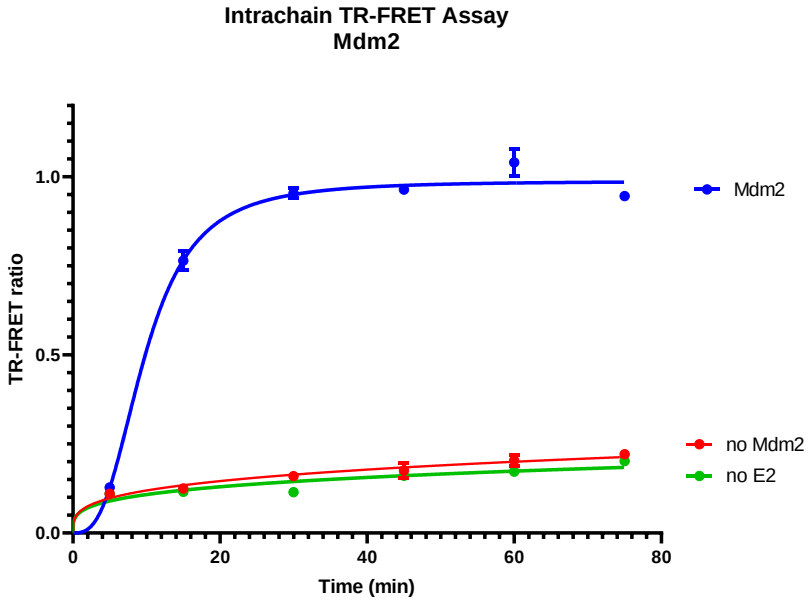

Figure 2: Real-time analysis of MDM2 E3 ligase activity.
Polyubiquitination activity of MDM2 was measured as a function of time. Negative controls included a "no E2" and "no MDM2" conditions.

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