Description

ChoosE2-Opti™ Intrachain TR-FRET Assay Kit is a sensitive high-throughput screening (HTS) TR-FRET Assay Kit, designed to identify E2 enzyme(s) that are functional partners for a ***purified*** E3 ubiquitin ligase of interest and lead to its self-polyubiquitination. The assay kit contains five E2 enzymes to be tested with the E3 ligase of interest, and two E3 ligases (SMURF1 and MDM2) to use as internal controls. It also contains an E1 enzyme, ATP, an optimized TRF Ubiquitin Mix, and a universal buffer.

ChoosE2-Opti™ Intrachain TR-FRET Assay Kit comes in a convenient 384-well reaction format. It utilizes a Europium-labeled Ubiquitin Donor as well as Cy5-labeled Ubiquitin Acceptor to complete the TR-FRET pairing. Since both the TR-FRET donor and acceptor are incorporated into polyubiquitin chains, this FRET-based assay requires no time-consuming washing steps, making it especially suitable for HTS applications as well as real-time kinetics analyses.

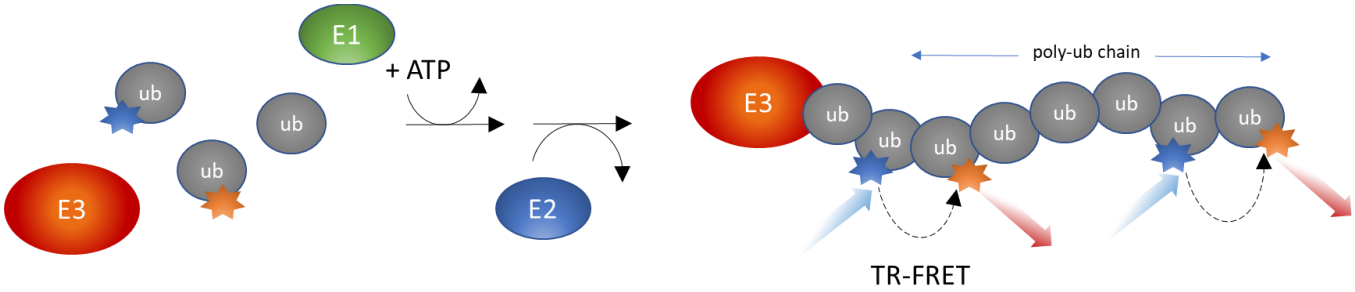


Figure 1: ChoosE2-Opti intrachain TR-FRET Assay Kit schematic

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 E2-E3 complex where the E3 enzyme directs the last step of the Ub-conjugating cascade by binding to both an E2~Ub conjugate and a substrate protein.

Application(s)

- Identify E2 enzymes that are partners of an E3 ligase of interest
- Assess the activity of new E2-E3 protein pairs
- Screen new E3 ligases and variants in HTS applications
- Screen molecules that inhibit/activate E2 enzyme activity in HTS applications,
- Determine compound IC_{50}
- Analyze E3 ligase activity in real-time

Supplied Materials

Catalog #	Name	Amount	Storage	
80301	UBE1 (UBA1), FLAG-Tag (E1)*	50 µg	-80°C	***Avoid multiple freeze/ thaw cycles***
80315	UbcH5a (UBE2D1), His-Tag (E2)*	15 µg	-80°C	
80314	UbcH5b, His-Tag (E2)*	15 µg	-80°C	
80313	UbcH5c, His-Tag (E2)*	15 µg	-80°C	
80316	UbcH6 (UBE2E1), His-Tag (E2)*	20 µg	-80°C	
80318	UbcH6 (UBE2E1), His-Tag (E2)*	15 µg	-80°C	
100409	MDM2, GST-Tag (E3)*	5 µg	-80°C	
80402	SMURF1, FLAG-Tag (E3)*	5 µg	-80°C	
78307	TRF Ubiquitin Mix (200x)	50 µl	-80°C	
	ATP (4 mM)	2 x 1 ml	-80°C	
78269	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.

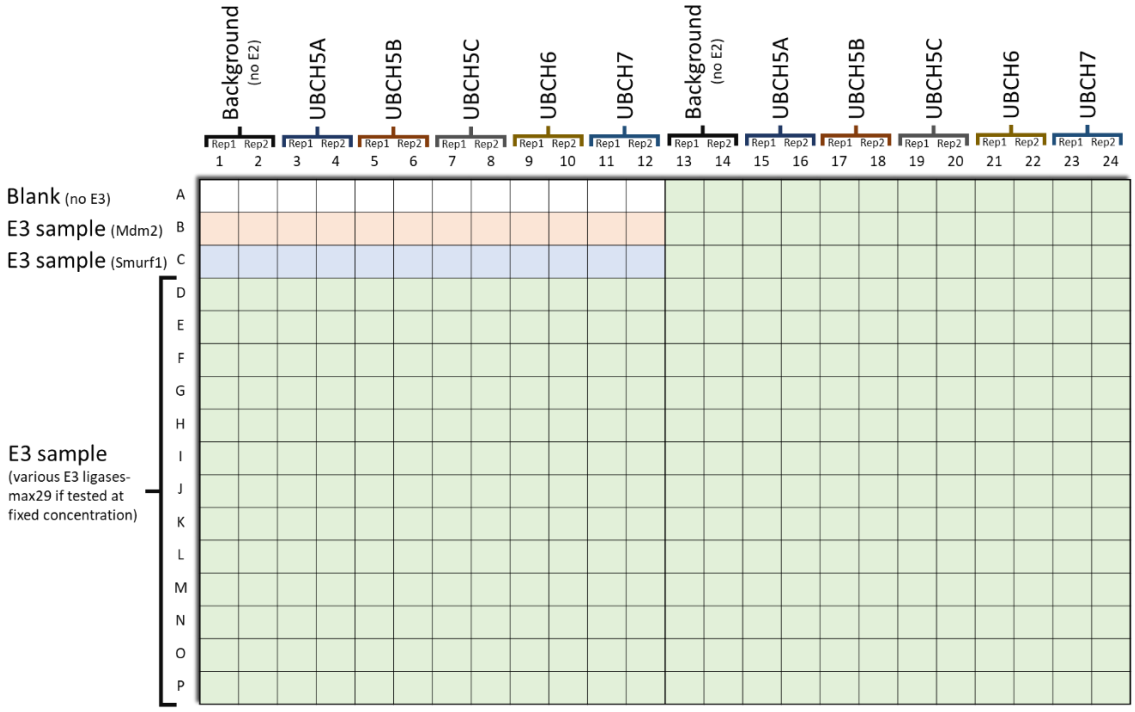
Contraindications

- ChoosE2-Opti™ 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

- All samples and controls should be performed in duplicate
- The assay should include a "Blank" for each E2 used in the assay, a "Negative control" for each E3 ligase, and at least one "Internal control" consisting of MDM2 or SMURF1 (or both separately if two internal controls are desired).
- A "Positive control" should also be included when assessing an inhibitor.
- 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.
- The assay can accommodate various experimental designs. A few examples are presented below with the corresponding suggested protocols.

Protocol 1: Identification of functional E2-E3 complexes using an E3 ligase of interest



1. Thaw **UBE1, E2 enzymes, E3 ligases MDM2 and SMURF1, TRF Ubiquitin Mix (200x), U2 Assay Buffer**, and **ATP** on ice. Briefly spin the tubes to recover their full contents.

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

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 the E2 ligases in U2 Assay Buffer (the volume needed is 1 µl/well):
   i. **UBCH5a**: 136 ng/µl
   ii. **UBCH5b**: 144 ng/µl
   iii. **UBCH5c**: 144 ng/µl
   iv. **UBCH6**: 176 ng/µl
   v. **UBCH7**: 144 ng/µl

   These dilutions correspond to a concentration of 8 µM for each E2 enzyme. The final concentration in the reaction is 400 nM.

c. Dilute MDM2 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).

d. Dilute SMURF1 ligase control in U2 Assay Buffer at 14 ng/µl (200 nM - the final concentration in the reaction is 50 nM, the volume needed is 5 µl/well).

   Note: To profile novel E3 ligases, BPS Bioscience recommends an initial screening using 25-50 nM final concentration of each E3 ligase in reaction, prior to further optimization.

   Note: Keep all diluted proteins on ice until use

4. Prepare the appropriate dilution(s) of the purified E3 ligase(s) of interest in U2 Assay Buffer.

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 mixture to all wells

7. Add 1 µl of **U2 Assay Buffer** to the wells designated as "Negative Control" and 5 µl of **U2 Assay Buffer** to the wells designated "Blank". Since the "Blank" is determined in the absence of E2, there should be a "Blank" for each E2 used in the assay.

8. Add 5 µl of the diluted Test **E3 ligase(s)** to each well designated as "Negative Control" and "E3 sample". Ideally, there will be a "Negative control" for each Test E3 ligase being used in the assay.

9. Add 5 µl of diluted MDM2 ligase to wells designated as "Internal control #1".

10. Add 5 µl of diluted SMURF1 ligase to wells designated as "Internal control #2".

11. Initiate the reaction by adding 1 µl of the desired **E2 enzyme** to each well designated as "Blank", "Internal control" and "E3 Sample"

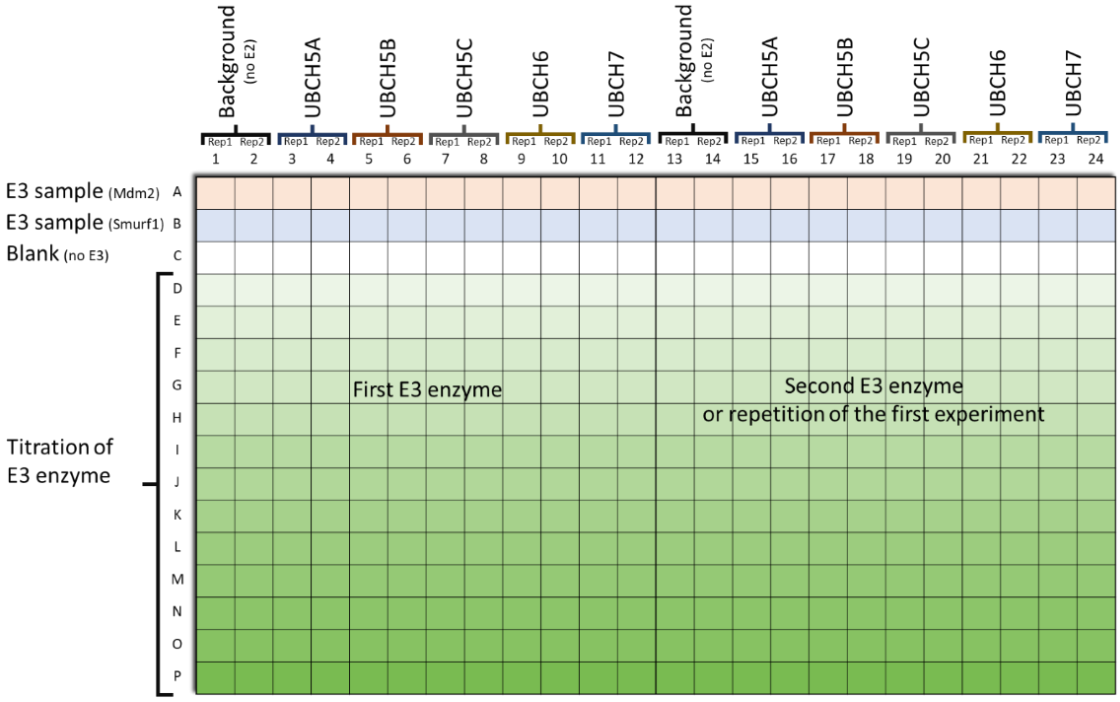
12. Cover the plate with a plate sealer and incubate at room temperature for two hours or at 30°C for one hour. Alternatively, directly start a real-time kinetics analysis.

Component	Blank	Internal control	E3 sample	Negative control
Master Mix	14 µl	14 µl	14 µl	14 µl
U2 Assay Buffer	5 µl	–	–	1 µl
MDM2 or SMURF1	-	5 µl	-	-
Test E3 ligase****	-	-	5 µl	5 µl
E2 enzyme***	1 µl	1 µl	1 µl	–
Total	**20 µl**	**20 µl**	**20 µl**	**20 µl**

**** For each E2 enzyme prepare separate "Blank" reactions.*
***** For each tested E3 ligase prepare separate "E3 sample" and "Negative control" reactions.*

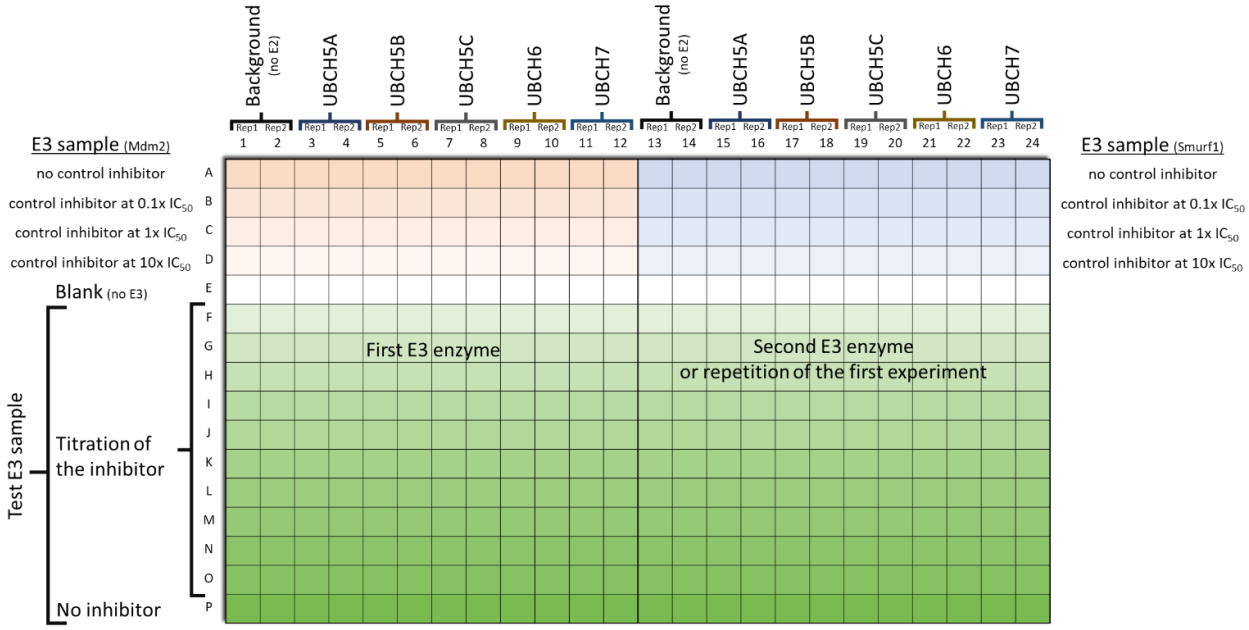
13. Read the fluorescent intensity in a microtiter-plate reader capable of measuring TR-FRET (go the end of the "Protocols" section for **Instrument Settings**).

Optimization of E3 concentration for individual E2 enzymes:



This design uses **Protocol 1**, in which the E3 ligase of interest is serially diluted to optimize its concentration for screening purposes. For serial dilutions, dilute the E3 ligase(s) of interest in U2 Assay Buffer at concentrations 4-fold higher than the desired final concentrations (5µl/well will be used in a final volume of 20 µl).

Protocol 2: Inhibitor or activator screening or titration/Compound IC_{50} determination



1. Thaw UBE1, E2, E3 ligase control (MDM2 or SMURF1), TRF Ubiquitin Mix, U2 Assay Buffer, and ATP on ice.

   Note: UBE1, E2, 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. 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 the E2 ligases in U2 Assay Buffer (the volume needed is 1 µl/well):
      i. **UBCH5a**: 136 ng/µl
      ii. **UBCH5b**: 144 ng/µl
      iii. **UBCH5c**: 144 ng/µl
      iv. **UBCH6**: 176 ng/µl
      v. **UBCH7**: 144 ng/µl

      These dilutions correspond to a concentration of 8 µM for each E2 enzyme. The final concentration in the reaction is 400 nM.

   c. Dilute MDM2 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).

d. Dilute SMURF1 ligase control in U2 Assay Buffer at 14 ng/µl (200 nM - the final concentration in the reaction is 50 nM, the volume needed is 5 µl/well).

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

Note: 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**).

6. Add 5 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 wells designated as "Blank" and "Negative Control."

10. Add 5 µl of diluted MDM2 to the wells designated "Internal control MDM2" and 5 µl of SMURF1 to the wells designated "Internal control SMURF1".

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

12. Initiate the reaction by adding 5 µl of **ATP** (4mM) to all the wells.

Component	Blank	Internal Control	Negative Control	Positive Control	Test Compound
Master Mix	5 µl	5 µl	5 µl	5 µl	5 µl
Diluent solution*	4 µl	4 µl	4 µl	4 µl	-
Test Compound	-	-	-	-	4 µl
E2 Enzyme**	1 µl	1 µl	1 µl	1 µl	1 µl
MDM2 or SMURF1	-	5 µl	-	-	-
Test E3 ligase	-	-	-	5 µl	5 µl
U2Assay 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*
*** For each E2 enzyme prepare separate "Blank" reactions.*

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 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

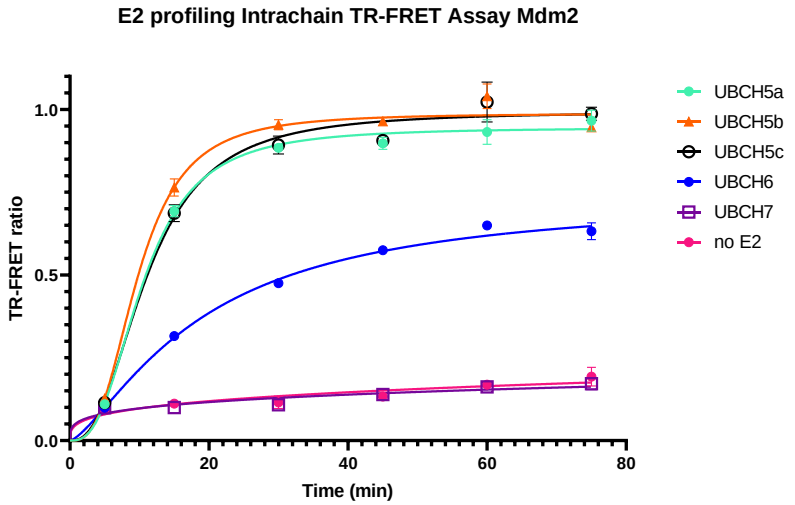


Figure 2: Identification of functional E2 partners for Mdm2 E3 ligase.
Real-time kinetics of MDM2 with the designated panel of E2 enzymes from the ChoosE2-Opti™ Intrachain TR-FRET Assay Kit (BPS Bioscience #78561)

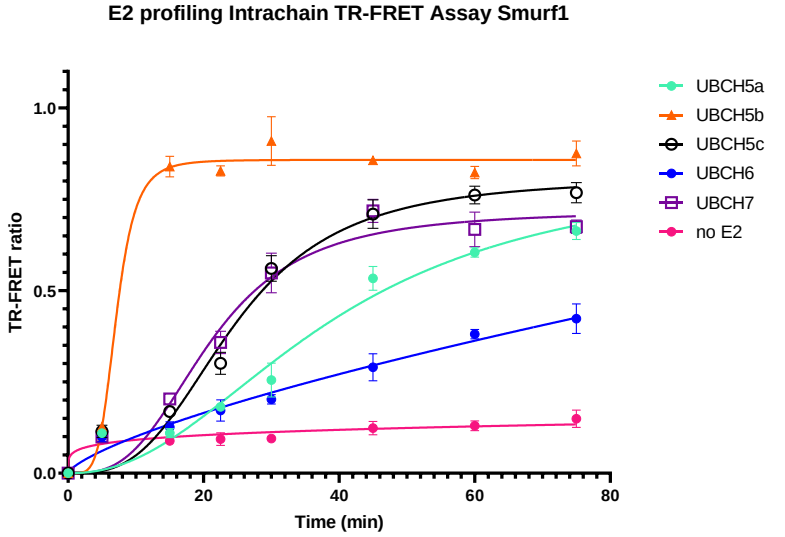


Figure 3: Identification of functional E2 partners for SMURF1 E3 ligase.
Real-time kinetics of SMURF1 with the designated panel of E2 enzymes from the ChoosE2-Opti™ Intrachain TR-FRET Assay Kit (BPS Bioscience #78561)

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
E3 Freedom intrachain TR-FRET Assay Kit	78560	384 rxns.
Cereblon intrachain TR-FRET Assay Kit	78301	384 rxns.
Cereblon Ubiquitination Homogenous Assay Kit	79881	384 rxns.
MDM2 intrachain TR-FRET Assay Kit	78302	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.
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.