MANUAL

TR-FRET *In Vitro* E3 Ligase Activity Kit

Catalog Number UC-108

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BACKGROUND

Ubiquitin and Ubiquitin Conjugation Machinery

Ubiquitin is a small (~8.6 kDa) polypeptide that can be conjugated via its C-terminus to amine groups of lysine residues on target proteins. This conjugation is referred to as monoubiquitylation. Additional ubiquitin moieties can be conjugated to this initial ubiquitin utilizing any one of the seven lysine residues present in ubiquitin. The formation of these ubiquitin chains is referred to as polyubiquitylation. The most well characterized of this polyubiquitylation is chain formation via lysine at position 48 of ubiquitin (K48- linked chains). Monoubiquitylation has been shown to alter the localization, activity, and/or function of the target protein. The most prevalent consequence of polyubiquitylation is the proteasome-mediated degradation of the target protein.

The conjugation of ubiquitin to a target protein requires the coordinated function of three distinct enzymes, **E1** (ubiquitin activating enzyme), **E2** (ubiquitin conjugating enzyme), and **E3** (ubiquitin ligase) resulting in isopeptide bond formation between the C-terminus of ubiquitin and the ε-amino group of the lysine residue on target proteins. Ubiquitin E3 ligases act as scaffold proteins, providing docking sites for an ubiquitin-conjugating enzyme (E2), and a target substrate. Typically, E3 ligases mediate the transfer of ubiquitin from an E2 thioester intermediate to an amide linkage with a substrate protein (Hershko and Ciechanover, 1998). In addition to the ubiquitylation of substrates, E3 ligases can also "auto ubiquitylate" themselves and this feature is utilized in *in vitro assays* to monitor the E3 ligase activity.

Importance

E3 ligases interface with numerous aspects of regulating cellular processes important to human health and disease. Recent research implicates E3s in neurodegenerative diseases[5], the development and progression of cardiovascular diseases, cancer and metabolic diseases[6].

ABOUT THE ASSAY

The TR-FRET *In vitro* E3 activity kit has been developed to be simple, fast, and easy to use while being robust and sensitive. As E3 ligases autoubiquitinate they build long polyubiquitin chains; our unique assay will detect these chains as they are being built. This assay is homogenous and compatible for High-throughput Screening (HTS).

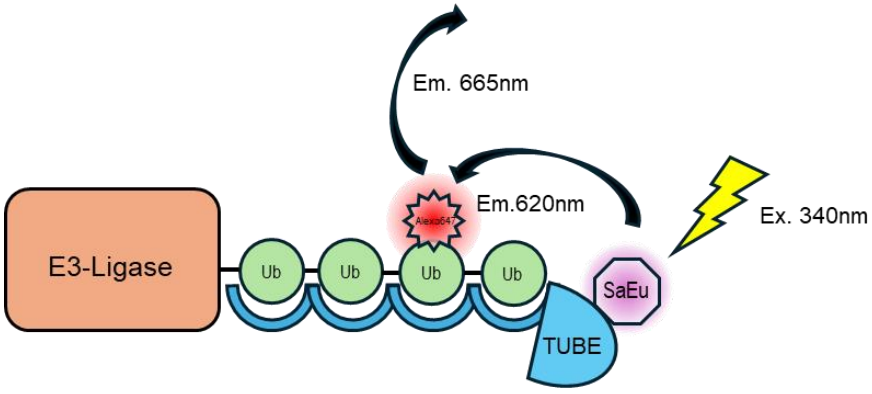

Figure 1. Schematic of TR-FRET E3 Ligase activity kit

TR-FRET *In Vitro* E3 Ligase Activity Kit

BENEFITS

1. Sensitive and robust signal
2. Homogeneous Assay allows for simple “Add-Add-Read” experimentation thereby reducing the experimental variability.
3. TR-FRET based detection has low background signal and reduced fluorescent interference when screening compound libraries.
4. Low volume, high-density plate format (384-well or 1534-well) allows for many samples to be tested simultaneously.

SUGGESTED USES

1. Testing E3 ligases for Autoubiquitination activity.
2. Testing different E2s to determine effective E2 for your E3 ligase.
3. HTS screen to identify E3 inhibitors or activators.
4. Easily generate IC50s/EC50s and conduct mechanism of action studies *in vitro*.

COMPONENTS

Store all materials at -80ºC, avoid cycles of freezing and thawing.

1. **10X Assay Buffer**

   Size: 1.0 mL
   Note: Dilute 10x assay buffer in water and add β-mercaptoethanol fresh to final concentration of 1mM in 1X assay buffer.
2. **Your Favorite E3**

   Quantity and concentration product dependent
3. **Your Favorite E2**

   Quantity and concentration product dependent
4. **Ubiquitin Activating Enzyme E1 (20x)**

   Size:1 vial, 250 µl
5. **Ubiquitin-Mixture (20x)**

   Size: 1 vial, 250 µl (20x)
6. **ATP (100mM)**
   Size: 1 vial, 100 µl
7. **TR-FRET Detection reagent (100x)**

   Size: 1 vial 50 µl (100x)
8. **Positive Control E3 (Carp2 with UBE2D3, 20x)**

ADDITIONAL ITEMS REQUIRED BUT NOT PROVIDED

1. **TR-FRET capable plate reader with UV (320nm +/- 20nM), 620nm, and 665nm optical filters such as BMG Clariostar Plus**
2. **β-mercaptoethanol**
3. **384-shallow well assay plate such as Nunc Cat#267461**
4. **Multi-channel Pipets appropriate for 5 µl and 0.5 µl volumes**
5. **Centrifuge with microplate adaptor**

6. **Streptavidin Europium Chelate W-1024 (Sa-Eu) purchased from Columbia Biosciences (Cat# D17-2212-50).**
   - **Note: Streptavidin Europium should be stored according to manufacturer recommendations, typically at 2 ° C - 8 °C.**
7. **(Optional) 96 or 384 well polypropylene plate for preparing compound dilutions**

Protocol Overview

1. Prepare 1x assay buffer.
2. (optional) dispense 0.5uL compound(s) to assay plate.
3. Prepare Mix A (E3 mix) at a 2x concentration and dispense 5 µl per well to assay plate.
4. Prepare Mix B (E1, E2, Ubiquitin Mix, ATP, FRET detection reagent, and Streptavidin Europium) and dispense 5 µl per well to assay plate.
5. Read assay plate(s).

Suggested Protocol

Volumes listed below are sufficient for one 384-well plate

1. Dilute 1 ml 10x Assay buffer in 9 ml Ultra pure water and add fresh β-mercaptoethanol to final concentration of 1.0 mM in 1x assay buffer.
2. If testing compounds, prepare compounds at a 21x concentration (for example if you want to test a compound at a final concentration of 30 µM, prepare your compound at 630 µM). Dispense 0.5 µl Vehicle/compound to assay plate.
   - DMSO tolerance of each E3 should be determined by the end user
   - Preincubation of E3 with compounds for 30-60 min prior to initiating the E3 reaction may increase activity of certain compounds (e.g time-dependent covalent inhibitors).
3. Prepare 2.5 ml Mix A (E3 mix) at a 2x concentration in assay buffer. For Example, if you would like to test your E3 at a final concentration of 100nM prepare a 200nM solution. Dispense 5 µl Mix A per well into assay plate. (See Table 1 for example volume calculations)
   - Be sure to include a “minus E3” control sample to ensure the observed ubiquitin chains are E3 dependent. You can use 5 µl per well assay buffer for this control .
4. Briefly centrifuge assay plate. If testing compounds, consider an incubation time and cover assay plate until next step.
5. Prepare 2.5 ml Mix B at a 2x concentration in assay buffer. Dilute your E2, E1, Ubiquitin Mixture, TR-FRET Detection Reagent, Streptavidin Europium, and ATP into assay buffer. Gently mix and dispense 5 µl Mix B per well. (See Table 1 for example volume calculations)
   - Including a Blank control (minus ATP) may be appropriate. Some E2s have been demonstrated to build ubiquitin chains independent of E3s.
   - If pre-charging of E1/E2 with ubiquitin is desired, the Mix B can be incubated for 30-60 min before adding to Mix A.
   - E3 ligase reaction will start immediately after the addition of Mix B.
6. Briefly centrifuge assay plate.
7. Read assay plate in plate reader. See table 2 for suggested reader parameters.
   - E3 ligases have varying levels of activity; appropriate E3 concentration and reaction time for your E3 should be empirically determined.

Table 1. Example Volume Calculations for 384-well plate

	Final Concentration	2x Concentration	Volume Required
2.5mL Mix A			
Example E3 (20 μM)	100 nM	200 nM	25 μl
1x Assay buffer			2475 μl
2.5 mL Mix B			
Example E2 (40 μM)	100 nM	200 nM	12.5 μl
E1 (20x)	1x	2x	250 μl
Ubiquitin Mixture (20x)	1x	2x	250 μl
ATP (100 mM)	400 μM	800 μM	20 μl
TR-FRET Detection Reagent (100x)	1x	2x	50 μl
Streptavidin Europium (1.85 μM)	1 nM	2 nM	2.7 μl
1x Assay buffer			2176 μl

Table 2. Suggested Parameters for Time-Resolved FRET Detection

	Wavelength	Integration Time Start	Integration Time Stop
Excitation	320nM, +/- 20nM		
Channel 1 Emission	620, +/-10nM	60ms	400ms
Channel 2 Emission	665, +/-10nM	60ms	400ms

Example Data

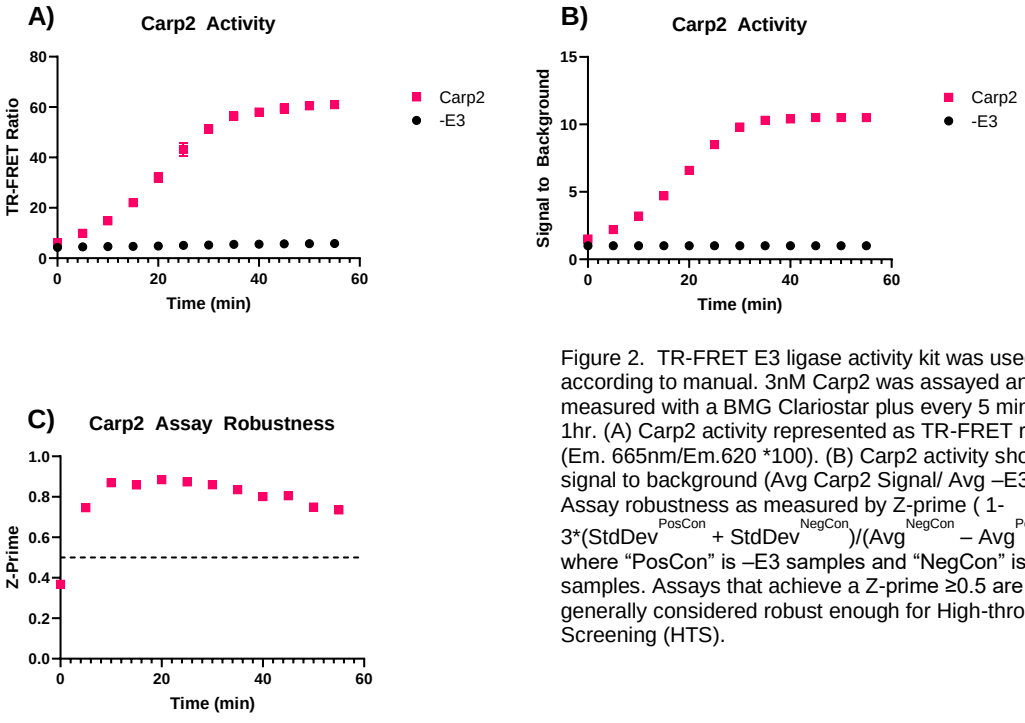

Figure 2. TR-FRET E3 ligase activity kit was used according to manual. 3nM Carp2 was assayed and measured with a BMG Clariostar plus every 5 minutes for 1hr. (A) Carp2 activity represented as TR-FRET ration (Em. 665nm/Em.620 *100). (B) Carp2 activity shown as signal to background (Avg Carp2 Signal/ Avg –E3). (C) Assay robustness as measured by Z-prime (1- $3*(StdDev^{PosCon} + StdDev^{NegCon})/(Avg^{NegCon} – Avg^{PosCon})$) where “PosCon” is –E3 samples and “NegCon” is Carp2 samples. Assays that achieve a Z-prime ≥0.5 are generally considered robust enough for High-throughput Screening (HTS).

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