MANUAL

E3LITE Customizable Ubiquitin Ligase Kit (384-well format)

Catalog Number UC101-384

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BACKGROUND

Ubiquitin and Ubiquitin Conjugation Machinery

Ubiquitin is a small 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 co-ordinated function of three distinct ligases, **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 "autoubiquitylate" themselves. There are two classes of E3 ligases: RING E3s, which act as scaffolds to bring the components of the ubiquitylation machinery together in close contact with the substrate, and HECT E3s that form intermediates with ubiquitin before transferring it to the substrate.

ABOUT THE ASSAY

The E_3LITE Customizable Ubiquitin Ligase Kit has been developed for the exploration of the mechanistic basis underlining the activity of E3 ligase enzyme of choice. At the core of the assay, 384-well plates pre-coated with a proprietary reagent are used for the capture of polyubiquitin chains formed in an E3 ligase-dependent reaction. For the assay, an E1-E2 enzyme cocktail is added first, in the presence of ubiquitin, to the coated microtiter plate wells. An E3 ligase under consideration is then added to the wells, and the reaction is initiated with ATP. During the reaction, polyubiquitin chains generated by the E1-E2-E3 machinery are recognized and captured in the wells. Following the reaction and subsequent wash steps, the isolated polyubiquitylated product is incubated with Detection Reagent 1 and streptavidin-HRP (not included) allowing for detection by chemiluminescence. Thus, the signal generated by captured polyubiquitylated product in this "sandwich" ELISA-like assay is a quantitative measure of E3 ligase activity. **Furthermore, this detection strategy does not require additional non-native tagging or labeling of ubiquitin, which could lead to experimental artifact.**

The E_3LITE Customizable Ubiquitin Ligase Kit is flexible by design; essentially providing a singular platform for the focused investigation of any E2/E3 enzyme pair, as well as any particular polyubiquitylation linkage type. In support of this flexible platform, LifeSensors offers a wide array of reagents to meet your research needs. The kit itself can be assembled with any one of more than twenty E2 conjugation enzymes, with a selection representing members from each enzyme class. LifeSensors also offers the ability to assemble a panel of (4) E2 enzymes through E2 Selection Panel (Cat. No. UB200). The recombinant ubiquitin component of this kit is available as wild-type or variants that have been engineered to form linkages only through one of the seven possible surface exposed lysines (e.g. K6, K11, K27, K29, K33, K48, K63).

BENEFITS

1. Monitor E3 activity in solution phase, with the E2 of your choice.
2. Detection system provides robust readout for E3 ligase activity.
3. 384-well format is amenable to high-throughput screening (HTS).
4. E_3LITE kit utilizes non-radioactive reporter substrates.
5. E_3LITE kit does not require excitation in the UV range (reducing false positive rate).

SUGGESTED USES

1. Testing of E3 ligase activity.
2. Demonstration of novel activity from an E2 or E3 with it's cognate enzyme.
3. High-throughput screening (HTS) of agonist/antagonists of either E2 or E3 activity.
4. Testing of E2 conjugating activity with cognate E3 ligase.

COMPONENTS (Store all materials at -80ºC, avoid cycles of freezing and thawing)

1. **Ubiquitin E1 Activating Enzyme**

   Size: 1 x 70µl (2µM)

   Buffer: 20mM Tris (pH 8.0), 150mM NaCl, 10% glycerol

2. **Ubiquitin E2 Conjugating Enzyme**

   Size: 1 x 70µl (40µM)

   Buffer: 20mM Tris (pH 8.0), 150mM NaCl, 10% glycerol

3. **Control E1-E2-E3 Control Solution**

   Size: 1 x 100µl (20X)

   Buffer: 20mM Tris (pH 8.0), 150mM NaCl, 10% glycerol

4. **Recombinant Human Ubiquitin**

   Size: 1 x 250µl (4mg/ml)

   Buffer: 20mM Tris (pH 8.0), 150mM NaCl, 10% glycerol

5. **Detection Reagent 1**

   Size: 1 x 35µl (1000X)

   Buffer: 20mM Tris (pH 8.0), 150mM NaCl, 10% glycerol

6. **Poly-Ubiquitin Linear Chain (Control)**

   Size: 1 x 200µl (3X)

   Buffer: 20mM Tris (pH 8.0), 150mM NaCl, 10% glycerol

7. **2 x 384-well microtiter plates**

   Plates are pre-coated with LifeSensors' proprietary polyubiquitin capture reagent, prior to shipment in a storage solution. This solution must be removed prior to assay.

ADDITIONAL ITEMS REQUIRED

1. **Assay Buffer Components**

   Tris-HCl (pH 8.0), Recommended: Stock 1M

   $MgCl_2$, Recommended: Stock 0.5M

   Reducing Agents: β-mercaptoethanol or DTT

2. **Wash Buffer(s)**

   Phosphate Buffered Saline (PBS)

   Phosphate Buffered Saline, 0.1% Tween (PBST)

   5% Bovine Serum Albumin (BSA) in Phosphate Buffered Saline w/ 0.1% Tween (PBST)

   Low quality BSA can result in high background in the assay. For best results, we recommend using high grade BSA (>98%), such as SIGMA (catalog # A3059).

3. **Luminescence capable plate reader**

4. **Streptavidin Secondary Detection Reagent**

   The selection of appropriate streptavidin reagent is critical to generating a robust signal. We do not recommend using avidin-horseradish peroxidase (HRP) conjugates. We strongly suggest using one of the following streptavidin-HRP reagents which have been used successfully with the assay:

   Anaspec (catalog # 60668)

   Jackson ImmunoResearch Laboratories, Inc. (catalog # 016-030-084)

   Rockland, Inc. (catalog # S000-03)

   Sigma-Aldrich (catalog # S-2438)

5. **Enhanced Chemiluminescent Reagent**

   Recommended: Millipore Western Immobilon ECL is recommended.

   Catalog numbers WBKLS0050 (50ml), WBKLS0100 (100ml), WBKLS0500 (500ml)

5. **Adenosine triphospate (ATP)**

   Recommended: Stock of 0.1M.

6. **1.5ml snap cap tubes**

7. **15ml centrifuge tubes**

SOLUTIONS FOR E3 LIGASE REACTION

Volumes listed below are sufficient for a single 384-well plate

Assay Buffer, 10ml

1. Prepare 10ml of 100mM Tris·HCl pH 8.0, 10mM $MgCl_2$, 2mM β-Mercaptoethanol (or 0.2mM DTT).

Enzyme Cocktail (4x), 3ml

1. Add 30µl **E1 activating enzyme** for a (4x) concentration of 20nM to 2.88ml of **assay buffer**:
2. Add 30µl **E2 conjugating enzyme** for a (4x) concentration of 400nM.
3. Add 60µl supplied **recombinant human ubiquitin**.

E3 Ligase Solution (4x), 3ml

In a final volume of 3ml, dilute E3 ligase of interest to (4x) optimized concentration in assay buffer. Refer to **Optimization of E3 Ligase Concentration** section below for more information.

Control E1-E2-E3 Solution (2x), 30µl

1. Add 3µl of supplied **Control E1-E2-E3 Solution** to 27µl of **assay Buffer** for a final concentration of 2X.

ATP Start Solution (2x), 5ml

Prepare 0.4mM ATP (2x) in 5ml of water.

Wash Buffers

Prepare 75ml phosphate buffered saline (PBS)

Prepare 250ml phosphate buffered saline, 0.1% Tween (PBST)

PBST with 5% BSA, 25ml

Add 1.25g of BSA to 25ml of PBST.

Detection Solution 1, 12ml

Add 12µl of **Detection Reagent 1** to 12ml of PBST with BSA **immediately before use (Step 9)**.

Streptavidin Secondary Solution, 12ml

Dilute **Streptavidin Secondary Detection Reagent** into 12ml of PBST with BSA. **Use immediately (Step 11)**.

A dilution of 1:10,000 is recommended for Streptavidin-HRP.

PolyUbiquitin Linear Chain (Optional Control) Solution, 90µl

Add 30µl **PolyUbiquitin Linear Chain** to 60µl of PBS.

PROTOCOLS

Optimization of E3 Ligase Concentration

We recommend to test serial dilutions of the E3 ligase of interest to optimize its concentration/activity for the E_3LITE Customizable Ligase Activity Assay. For comparison, an illustrative dose response for CARP2 with UBE2D3 is included on page 7. The following protocol is recommended for performing serial dilutions and will allow each point to be measured in triplicate.

1. Label seven 1.5ml snap cap tubes T1 through T7.
2. Aliquot 25µl of assay buffer in tubes T2 through T7.
3. Dilute the **E3 ligase** to a concentration of 400nM in 50µl of assay Buffer in tube T1.
4. Vortex tube T1 and perform a 2-fold dilution by transferring 25µl of solution from tube T1 into tube T2.
5. Perform another 2-fold dilution by transferring 25µl from tube T2 into tube T3. Vortex to mix thoroughly.
6. Repeat Step 4 for tubes T4 through T7.
7. Each tube now contains (4x) E3 ligase for optimizing concentration/activity in triplicate.
8. Add 6.25µl of serially diluted (4x) **E3 ligase** to the **coated plate wells** containing premixed **enzyme cocktail**, as detailed in the suggested protocol below.
9. Proceed to Step 6 of the suggested protocol below to initiate the reaction with ATP.

E3 Ligase Activity Assay (Suggested Protocol)

1. As directed in previous sections, prepare all reagents, standards, and samples.
2. Allow coated plate wells to equilibrate to room temperature.
3. Discard storage solution by inverting the plate frame and blotting against a clean paper towel. Optionally, aspirate each well and wash, repeating the process 1-3 times. Wash by filling each well with PBS (50µl) (do not use PBST) using a multichannel pipette, manifold dispenser or autowasher. After the final wash, remove excess PBS by inverting the plate and blotting against a clean paper towel. **NOTE: When washing the plate wells and adding reagents, be sure to pipet onto the side of the well avoid contact with the bottom.**
4. Add 6.25µl of (4x) **enzyme cocktail** to each well.
5. Add 6.25µl of (4x) **E3 ligase** solution to each well containing enzyme cocktail. For the background signal, add 6.25µl assay buffer (containing no E3 ligase).
6. Add 12.5µl of (2x) **Control E1-E2-E3 Solution** to separate well(s) as a positive control.
7. To start the enzymatic reaction, add 12.5µl of (2x) **ATP Start Solution** to each well and incubate for 30-60 minutes at room temperature.
8. Remove and discard well contents and wash each well, repeating the process for a total of three washes. Wash each well by filling each well with **PBST** (50µl).
9. Add 25µl of **Detection Solution 1** (1:1000 in PBST + 5% BSA) to each well. Incubate for 1 hour at room temperature.
10. Repeat the removal/wash as in step 8.

11. Add 25µl of **Streptavidin Secondary Solution** (1:10,000 in PBST + 5% BSA) to each well. Incubate for 1 hour at room temperature.

12. Repeat the removal/wash, for a total of four washes.

13. For Streptavidin-HRP detection, add 25µl of **Enhanced Chemiluminescent** (ECL) reagent to each well, and incubate for 1-5 minutes, protecting the plate from light.

14. Determine the Relative Luminescence Units (RLUs) using a luminometer. Ensure the plate reader is preset with parameters recommended by the manufacturer.

Control for Detection Using PolyUbiquitin Linear Chain Reagent

1. Add 25µl of **PolyUbiquitin Linear Chain Solution** to **coated plate** wells in triplicate.

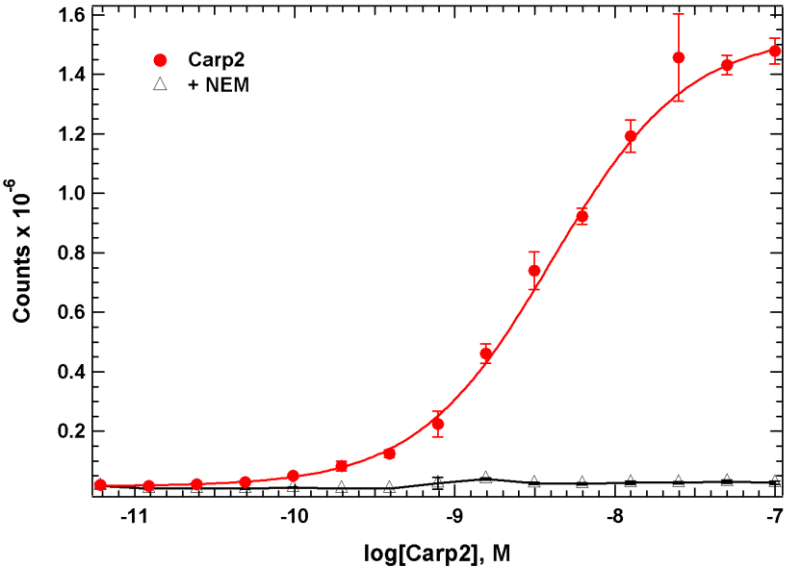
2. Incubate at room temperature for 30-60min. This step can be performed during the incubation step of the E3 Ligase Activity Assay to control for detection of polyubiquitin.

3. Remove and discard contents of each well and wash with PBST three times.

4. Proceed with detection as in Steps 9 through 14 of the protocol above.

EXAMPLE DOSE RESPONSE OF CARP2 WITH UBIQUITIN E3 LIGASE ACTIVITY ASSAY

Premixed **enzyme cocktail** was added to serial 2-fold dilutions of control E3 ligase **(CARP2)** in **coated wells** in the presence (Δ) or absence (•) of N-ethylmaleimide (NEM). NEM is a thiol-reactive agent that blocks the active site of ubiquitylation enzymes. Subsequent autoubiquitylation of **CARP2** was allowed to progress prior to detection by luminescence as detailed above.



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