LifeSensors from genomics to proteomics

E3LITE Customizable Ubiquitin ligase Kit

Instruction Manual

Cat. No. UC101C

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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, microtiter plate strips, 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 colorimetry. 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. Plate-based 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. All components (except for microtiter plates) are stable for at least 2 months.

1. **Ubiquitin E1 Activating Enzyme**

   Size: 1 x 70 µl (2µM)

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

2. **Ubiquitin E2 Conjugating Enzyme**

   Size: 1 x 70 µl (40µM)

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

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

   Size: 1 x 100 µl (20X)

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

4. **Recombinant Human Ubiquitin**

   Size: 1 x 250 µl (4mg/ml)

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

5. **Detection Reagent 1**

   Size: 1 x 35 µl (1000X)

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

6. **Colorimetric Reagent**

   Size: 2 x 12 mL

7. **Stop Solution**

   Size: 2 x 12 mL

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

   Size: 1 x 200 µl (3X)

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

9. **2 x 96-well microtiter plates (modular)**

   Plates are pre-coated with LifeSensors' proprietary polyubiquitin capture reagent, prior to shipment in a storage solution. Handling of modular plate strips most e achieved while storage solution is frozen. This solution must be removed prior to assay. **(Plates must be used within 30 days of receiving kit)**

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 solution, 0.1% Tween (PBST)

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

3. **Absorbance capable plate reader**

   After adding Colorimetric reagent the solution will change to blue color and can be detected at Absorbance of 650 nm. After adding the Stop solution, it will change color from blue to yellow and can be measured at Absorbance 450 nm.

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. **Colorimetric Reagent**
   Recommended: Neogen Diagnostics Enhanced K-Blue TMB substrate, 308177.

6. **Adenosine triphospate (ATP)**

   Recommended: Stock of 0.1M.

7. **1.5 ml snap cap tubes**

8. **15 ml centrifuge tubes**

SOLUTIONS FOR E3 LIGASE REACTION

Volumes listed below are sufficient for 8 reaction wells, or 1 modular strip (scale accordingly).

Assay Buffer

1. Prepare 5ml of 100mM Tris·HCl pH 8.0, 10mM $MgCl_2$, 2mM β-Mercaptoethanol (or 0.2mM DTT).

Enzyme Cocktail (4x), 250µl

1. Add 2.5 µl **E1 activating enzyme** for a (4x) concentration of 20nM to 240 µl of **assay buffer**:
2. . Add 2.5 µl **E2 conjugating enzyme** for a (4x) concentration of 400nM.
3. Add 5 µl supplied **recombinant human ubiquitin**.

E3 Ligase Solution (4x), 250µl

In a final volume of 250µl, 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), 50µl

1. Add 5 µl of supplied **Control E1-E2-E3 Solution** to 45 µl of **assay Buffer** for a final concentration of 2X (adjust total volume based on number of wells.)

ATP Start Solution (2x), 500µl

Prepare 0.4mM ATP (2x) in 0.5ml of water.

PBST with 5% BSA, 3ml

Add 150mg of BSA to 3ml of PBST.

Detection Solution 1, 1 ml

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

Streptavidin Secondary Solution, 1 ml

Dilute **Streptavidin Secondary Detection Reagent** into 1ml 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, 360µl

Add 120 µl **PolyUbiquitin Linear Chain** to 240 µl 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 Ubch5 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 1.5 ml snap cap tubes T1 through T7.
2. Aliquot 100 µl of assay buffer in tubes T2 through T7.
3. Dilute the **E3 ligase** to a concentration of 400nM in 200 µl of assay Buffer in tube T1.
4. Vortex tube T1 and perform a 2-fold dilution by transferring 100 µl of solution from tube T1 into tube T2.
5. Perform another 2-fold dilution by transferring 100 µl 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 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. Determine the number of **coated microplate wells** required (based upon the number of reactions to be run), cut and remove aluminum seal from wells and transfer strips to user plate frame. **Transfer of strips may be easier with frozen wells.** 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 (200 µl) 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 25 µl of (4x) **enzyme cocktail** to each well.
5. Add 25 µl of (4x) **E3 ligase** solution to each well containing enzyme cocktail. For the background signal, add 25µl assay Buffer (containing no E3 ligase).
6. Add 50 µl of (2) **Control E1-E2-E3 Solution** to separate well(s) as a positive control.
7. To start the enzymatic reaction, add 50 µl 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** (200 µl).
9. Add 100 µl 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 100 µl 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 colorimetric detection, add 100 µl of **Colorimetric Reagent** to each well, and incubate for 1-30 minutes. A blue color will develop and can be read at Absorbance 650 nm using the plate reader. When the solution has turned to a bright blue color (absorbance 650 = ~1.0) proceed to step 14.

a. *Note: prolonged incubation which makes the color darker blue might saturate the signal after adding stop solution. Do not let the signal saturate.*

14. To stop the reaction, add 100 µl of **Stop Solution.** . The solution will change color from blue to yellow. Measured the Absorbance at 450nm within 30 minutes of adding the stop solution.

Control for Detection Using PolyUbiquitin Linear Chain Reagent

The Polyubiquitin Linear Chain Reagent is titrated to give a positive, but minimal signal in the assay, therefore serving as a sensitive control for capture of polyubiquitinated proteins. One should expect to see a 2- to 5-fold signal to background with the control reagent. Absence of a positive signal using the Polyubiquitin Linear Chain Reagent indicates a problem with the detection steps (steps 9-14 of the assay). If the Polyubiquitin Linear Chain Reagent fails to give a signal in the assay, check that the streptavidin-HRP and Colorimetric detection reagents are working and contact LifeSensors technical services (info@lifesensors.com or 610-644-8845) for assistance.

1. Add 100 µl of **PolyUbiquitin Linear Chain Solution** to **coated plate** wells in triplicate.

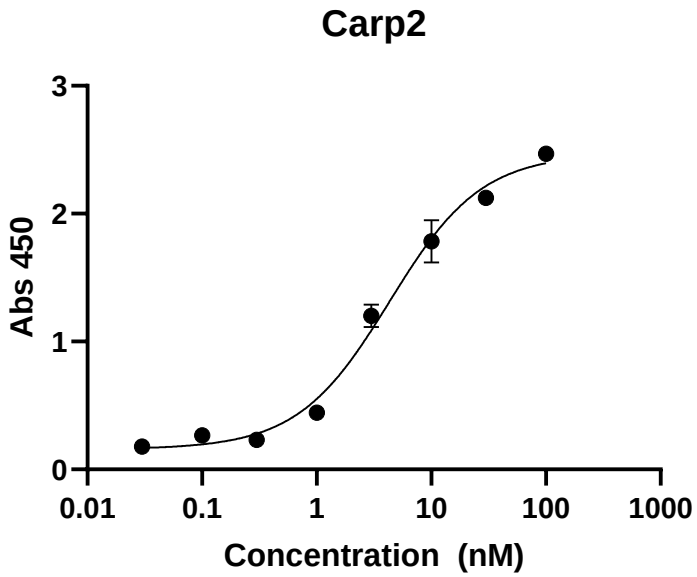
2. Incubate at room temperature for 30-60min. This step can be performed during the incubation step of the E3 Ligase Activity Assay to control for detection of polyubiquitin.

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 3-fold serial dilutions of control E3 ligase **(CARP2).** Subsequent autoubiquitylation of **CARP2** was allowed to progress prior to detection by colorimetry as detailed above.



REFERENCES

1. Hershko, A. and A. Ciechanover, The ubiquitin-proteasome pathway. *Annu Rev Biochem*, 1998. **67**: p. 425-479.
2. Welchman, R.L., C. Gordon, and R.J. Mayer, Ubiquitin and ubiquitin-like proteins as multifunctional signals. **Nat Rev Mol Cell Biol**, 2005. **6**(8): p. 599-609.
3. Ciechanover, A., The ubiquitin-proteasomal pathway: on protein death and cell life. Embo J, 1998. **17**(24): p. 7151-7160.
4. Raasi, S., *et al.*, Diverse polyubiquitin interaction properties of ubiquitin-associated domains. Nat Struct Mol Biol, 2005. **12**(8): p. 708-14.

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