

MANUAL

E2 Profiling Kit

Catalog Number UC102

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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 proteins: E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase). This results 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 a 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 E2 Profiling Kit has been developed for the exploration of the mechanistic basis underlining the activity of partnering E2-E3 enzymes. 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 a E3 ligase-dependent reactions. Here, an E1 and E3 enzyme cocktail is first added, in the presence of ubiquitin, to the coated microtiter plate wells. Then, E2 conjugating enzymes under consideration can be 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 E2-E3 partnering activity. **Furthermore, this detection strategy does not require additional non-native tagging or labeling of ubiquitin, which could lead to experimental artifact.**

The E2 Profiling Kit is flexible by design; essentially providing a singular platform for the focused investigation of any E2/E3 enzyme pairs, as well as any particular polyubiquitylation linkage type. In support of this flexible platform, LifeSensors offers the widest array of reagents on the market to meet your research needs. The kit itself can be assembled with any one of more than (25) E2 conjugation enzymes, with a selection representing members from each enzyme class. LifeSensors also offers E2 Selection Kits (Cat.No.UB200) that provides a panel of (4) E2s and E2 profiling Kit-23 that provides a panel of 23 E2s (Cat.No.UC104). The recombinant ubiquitin component of this kit can be wild-type, variants that specifically form K48 linkage types, or variants that form linkages only through one of the seven possible surface exposed lysines.

BENEFITS

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

SUGGESTED USES

1. Testing of E2 conjugating activity with cognate E3 ligase.
2. Demonstration of novel E2 conjugating activity with cognate E3 ligase.
3. High-throughput screening (HTS) of agonist/antagonists of either E2 or E3 activity.
4. Demonstration of novel E3 ligase activity with an E2 enzyme.
5. Measuring Km values for E2::E3 interactions. (See E2::E3 Kinetic Application Note)

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

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(s)

Size: Each x 25 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 140 µl (200X)

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. Poly-Ubiquitin Linear Chain (Control)

Size: 1 x 200 µl (3X)

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

7. 96-Well Microtiter Plate (modular)

Plates are pre-coated with LifeSensors' proprietary polyubiquitin capture reagent, prior to shipment in a storage solution. Manipulation of modular plate strips most easily achieved while storage solution is frozen. This solution must be removed prior to assay.

**ADDITIONAL ITEMS
REQUIRED**

1. Assay Buffer Components

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

MgCl₂, 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. Luminescence capable plate reader

4. Streptavidin Secondary Detection Reagent

Streptavidin-Horse Radish Peroxidase (HRP) conjugates tested:

Anaspec (catalog # 60668)

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

Rockland, Inc. (catalog # S000-03)

Sigma-Aldrich (catalog # S-2438)

Enhanced Chemiluminescent Reagent

Millipore Western Immobilon ECL is recommended.

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

WBKLS0500 (500ml)

5. Adenosine triphosphate (ATP)

Recommended: Stock of 0.1M.

6. 1.5 ml snap cap tubes

7. 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 MgCl₂, 2mM β-Mercaptoethanol (or 0.2mM DTT).

Enzyme Cocktail (4x), 250µl

1. To a final volume 250 µl in **Assay Buffer** add;
2. Add 2.5 µl **E1 activating enzyme** for a (4x) concentration of 20nM.
3. Add optimized volume of **E3 Ligase enzyme** for a (4x) concentration.
(See E₃LITE Customizable Ubiquitin Ligase Kit Manual, Cat No. UC101)
4. Add 5 µl supplied **recombinant human ubiquitin**.

E2 Conjugating Solution (4x), 250µl

In a final volume of 250µl, add 2.5 µl **E2 conjugating enzyme** for a (4x) concentration of 400nM.

Control E1-E2-E3 Solution (2x), 100µl

1. Add 5 µl of supplied **Control E1-E2-E3 Solution** to 45 µl of **assay Buffer** for a final concentration of (2X).

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 8.**

Streptavidin Secondary Solution, 1 ml

Dilute **Streptavidin Secondary Detection Reagent** into 1 ml of PBST with BSA **immediately before use, Step 10.**

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

E3 Ligase Activity Assay (Suggested Protocol)

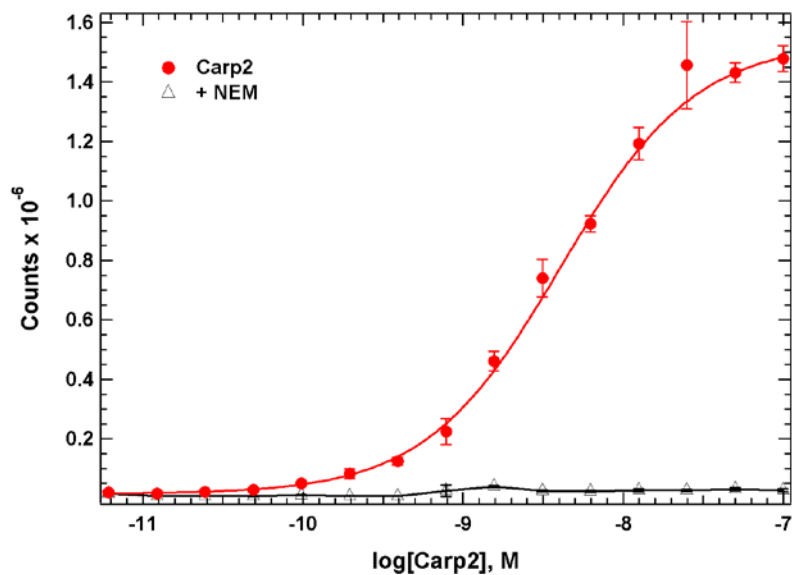
1. As directed in previous sections, prepare all reagents, standards, and samples.
2. Determine the number of **coated microplate wells** required (based upon the number of reactions to be run), cut and remove aluminum seal from wells and transfer strips to user plate frame. **Transfer of strips may 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 2-3 times. Wash by filling each well with PBS (200 μ l) 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 μ l of (4x) **E1/E3 enzyme cocktail** to each well.
5. Add 25 μ l of (4x) **E2 conjugating** solution to each well containing enzyme cocktail. If desired, add 25 μ l assay Buffer (containing no E2 enzyme) for background signal.
6. Add 50 μ l of (2) **Control E1-E2-E3 Solution** to separate well(s) as a positive control.
7. To start the enzymatic reaction, add 50 μ l 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 three times. Wash each well by filling each well with **PBST** (200 μ l) using a multichannel pipet, manifold dispenser or autowasher.
9. Add 100 μ l of **Detection Solution 1** to each well. Incubate for 1 hour at room temperature.
10. Repeat the removal/wash as in step 8.
11. Add 100 μ l of **Streptavidin Secondary Solution** to each well. Incubate for 1 hour at room temperature.
12. Remove and discard well contents and wash each well **four** times
13. For Streptavidin-HRP detection, add 100 μ l 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 100 μ l 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 8 through 13 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 (\bullet) 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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