MANUAL

Gel-Based *In Vitro* E3 Ligase Activity Kit (Alexa647)

Catalog Number UC201

LifeSensors, Inc., 271 Great Valley Parkway, Malvern PA 19355 • (p) 610.644.8845 (f) 610.644.8616
techsupport@lifesensors.com • www.lifesensors.com • sales@lifesensors.com

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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 *In vitro* ubiquitination kit (UC201) has been developed to be simple, fast, and easy to use. This E3 ligase assay is carried out using fluorescently labeled Ubiquitin-Alexa647 and separated on SDS-PAGE allowing researchers to directly visualize and quantify their gels saving precious time and reagents. This assay kit circumvents the more laborious and time-consuming western blot-based E3 assays.

BENEFITS

1. Save time and reagents by directly visualizing ubiquitin and ubiquitin conjugations in your gel without the need to transfer to a membrane and probing with antibodies.
2. Avoid introducing variability by reducing the number of experimental steps.
3. Use this kit as an orthogonal assay to confirm results from high throughput screening campaigns.

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SUGGESTED USES

1. Testing E3 ligases for Autoubiquitylation activity.
2. Testing different E2s to determine the best E2 combination for your E3 ligase.
3. Test compounds for E3 activity inhibition or activation.

COMPONENTS

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

1. **10x Assay Buffer**

   Size: 1 vial, 1.0 ml

   Note: Dilute 10x assay buffer in ultrapure deionized water to 1x and add fresh β-mercaptoethanol to a final concentration of 1mM in 1x assay buffer.
2. **Your E3 of choice**

   Quantity and concentration product dependent
3. **Your E2 of choice**

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

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

   Size: 1 vial, 250 µl
6. **ATP (100mM)**

   Size: 1 vial, 100 µl
7. **Positive Control E3 with E2 (Carp2 with UBE2D3, 20x)**
   Size: 1 vial, 20 µl
8. **6x SDS Gel loading buffer**
   Size: 1 vial, 1ml

ADDITIONAL ITEMS REQUIRED BUT NOT PROVIDED

1. **Fluorescent gel imager capable of detecting Alexa647 (Excitation 620nm, Emission 671nm) such as LiCor Odyssey Fc with 700nm channel filter.**
2. **β-mercaptoethanol**
3. **Polyacrylamide gel and gel running apparatus.**
   - **10% Gel or 4%-12% gradient gel is appropriate**
4. **Microcentrifuge tubes or 96-well Polypropylene plate and plate seal**
5. **Pipets appropriate for 25 µl and 10 µl volumes**
6. **Heating block (Dry bath) or water bath set to 95 ºC**

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Protocol Overview

1. Prepare 1x assay buffer by diluting 10X assay buffer.
2. Prepare Mix A (E3 mix) at a 2x concentration and dispense to microtube or 96-well polypropylene plate; Dispense 25 µl per sample.
3. Prepare Mix B (E1, E2, Ubiquitin Mix, ATP) and dispense 25 µl per sample.
4. Allow E3 ligase reaction to proceed.
5. Stop reaction with 10 µL 6x SDS Gel Loading buffer.
6. Heat samples for 5 minutes
7. Run polyacrylamide gel
8. Image gel

Suggested Protocol

Volumes listed below are sufficient for one 15-lane gel

1. Dilute 125 µl10x Assay buffer in 1125 µl Ultra pure water and add fresh β-mercaptoethanol to final concentration of 1mM in 1X assay buffer.
   - If performing a serial dilution of your E3, prepare a larger volume of 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 2.4 µl DMSO/compound to microtubes or polypropylene plate.
   - DMSO tolerance of individual E3 ligases should be determined by the end user.
3. Prepare 500 µl 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 25 µl Mix A per sample to microtubes or 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 25 µl 1x assay buffer for this control.
4. Prepare 500 µl Mix B at a 2x concentration in assay buffer; Dilute E2 to 200nM, E1 to 2x, Ubiquitin mix to 2x, and ATP to 800 µM into assay buffer. Dispense 25 µl Mix B per sample. (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.
5. Briefly centrifuge samples. If using a plate for sample preparation seal the plate.
6. Incubate samples at room temperature for the duration of your reaction.
   - E3 ligases have varying levels of activity; appropriate E3 concentration and reaction time for your E3 may need to be empirically determined.
7. Stop the ligase reaction by dispensing 10 µl 6x SDS gel loading buffer to all samples.
   - At this point samples can be stored at -20 °C or -80 °C until gel loading.
8. Heat samples in a 95oC heating block for 5 minutes.
9. Load 25 µl of samples into polyacrylamide gel and run under standard conditions. Using a 10% gel or a 4-12% gradient gel is appropriate.
   - Ubiquitin-Alexa647’s molecular weight is 9563 Da and runs closer to 10KD. If you would like to visualize unconjugated ubiquitin, do not run it off the gel.
   - Extremely long poly-ubiquitin chains may fail to enter the resolving gel. Using a gradient gel may help.

10. Carefully remove gel from casing and place on imaging tray. Image gel for 2-10mins on imaging system capable of detecting Alexa 647(Excitation 620nm, Emission 671nm) such as LiCor Odyssey Fc with 700 channel filter.

- It is not necessary to wash or rinse the gel prior to imaging.
- The signal intensity of polyubiqutin smear can be quantified using LiCor Odyssey software, exported to excel and plotted on Graphpad prism or other software.

Table 1. Example Volume Calculations for 1 Gel

	Final Concentration	2x Concentration	Volume Required
500 uL Mix A			
Example E3 (20 μM)	100nM	200nM	5 μl
1x Assay buffer	-	-	495 μl
25 uL Mix A Positive Control E3			
Positive Control E3 with E2 (Carp2 with UBE2D3)(20x)	1x	2x	1.25 μl
1x Assay buffer	-	-	23.75 μl
500 uL Mix B			
Example E2 (40μM)	100nM	200 nM	2.5 μl
E1 (20x)	1x	2x	50 μl
Ubiquitin Mix (20x)	1x	2x	50 μl
ATP (100 mM)	400 μM	800 μM	4 μl
1x Assay buffer	-	-	393.5 μl

Example Data

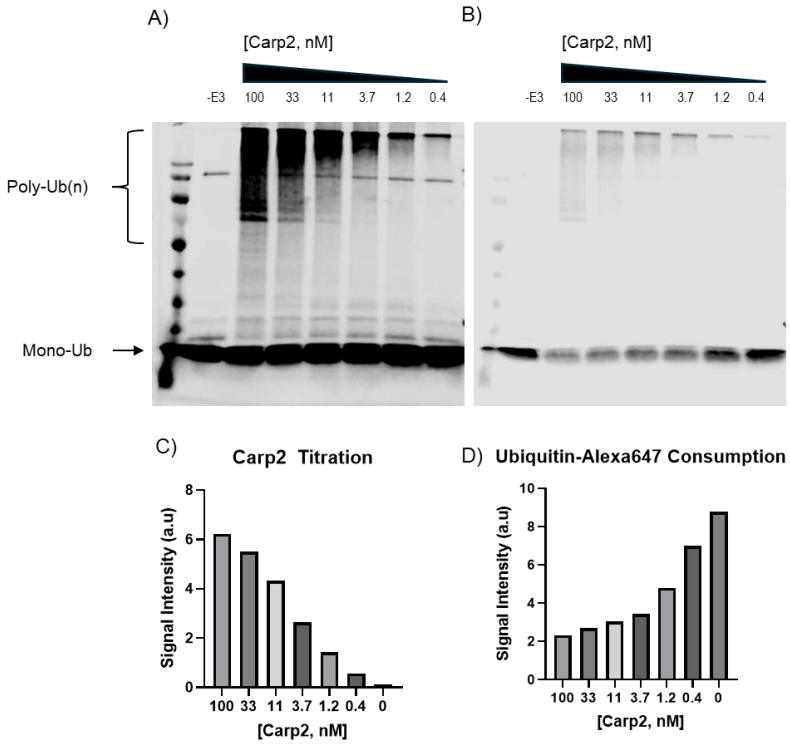

Figure 1. **Carp2 dose response in Gel-Based In Vitro E3 Ligase Activity Kit (Alexa647)**. Carp2 was serially diluted in a 6 point 3-fold dilution series starting at 100nM and ending at 0.4nM. E3 ligase reaction proceeded for 60minutes before stopping. Samples were run in 10% SDS-PAGE and images were acquired on LiCor Odessey Fc using 700 nM filter for 10 minutes.
(A) High contrast image shows Carp2 activity in a dose dependent manner. Carp2 activity is evident even at 0.4nM.
(B) Low contrast image of gel shows the consumption of Ubiquitin-Alex647 by Carp2 in a dose response manner.
(C) Poly-Ubiquitin smear was quantified from gel image using LiCor's Image Studio software. Quantification box was drawn from the top of the resolving gel down to ~20 kDa. See Table 2.
(D) Mono-Ubiquitin-Alexa647 was quantified from gel image LiCor's Image Studio software.

Table 2. Quantification of CARP2 autoubiquitination signal

[Carp2, nM]	Signal	S/B
100	6.23	50.35
33.3	5.51	44.55
11.1	4.32	34.88
3.7	2.63	21.28
1.2	1.42	11.51
0.4	0.56	4.53
0	0.12	1

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