MANUAL

Gel-Based *In Vitro* E3 Ligase Activity Kit (VU1HRP)

Catalog Number UC202

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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 has been developed to be simple, fast, and easy to use. E3 ligase assay is carried out using human Ubiquitin and separated on SDS-PAGE followed by traditional Western blotting and probing with the anti-Ubiquitin antibody VU1-HRP.

BENEFITS

1. Traditional Western blot analysis remains the gold standard.
2. Utilizing anti-ubiquitin antibody directly conjugated to HRP saves time and reagents.
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 (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
9. **50% Glutaraldehyde solution**
   Size: 1 vial, 600 µl
10. **Anti-Ubiquitin Antibody VU1-HRP**
    Size: 1 vial, 12.5 µg

ADDITIONAL ITEMS REQUIRED BUT NOT PROVIDED

1. **Gel imager capable of detecting luminescence such as LiCor Odyssey**
2. **β-mercaptoethanol**
3. **Polyacrylamide gel, gel running apparatus, and Gel transfer system**
   - **10% Gel or 4%-12% gradient gel is appropriate**
4. **PVDF or Nitrocellulose membrane**
5. **Microcentrifuge tubes or 96-well Polypropylene plate and plate seal**
6. **Pipets appropriate for 25 µl and 10 µl volumes**
7. **Heating block (Dry bath) or water bath set to 95 °C**

8. **Orbital shaker**
9. **Phosphate Buffered Saline (PBS)**
10. **Tris-buffered saline with 0.1% Tween20 (TBST)**
11. **Blocking buffer: 5% non-fat milk/TBS/0.1%Tween (TBST)**
12. **Electrochemiluminescence (ECL) reagent of choice such as Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore cat# WBKLS0500)**

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, 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 and transfer gel to membrane
8. Treat membrane with 0.5%glutaraldehyde solution for 20 minutes
9. Block membrane for 30 minutes
10. Probe with VU1-HRP antibody at 1:1000 for at least 2 hours
11. Image membrane

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 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 95 °C 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's molecular weight is 8,564.9 Da. 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. Transfer onto PVDF or nitrocellulose membrane using typical conditions
11. Wash membrane with PBS or water three times for 2 minutes each.
12. Dilute 100 µl 50% Glutaraldehyde into 10 ml PBS to create a 0.5% solution. Incubate membrane with 0.5% glutaraldehyde/PBS solution for 20 min.
    - Important Note: DO NOT USE Tris-HCl containing buffer since glutaraldehyde is amine reactive.
13. Wash membrane with PBS three times for 10 minutes each.
14. Block membrane with 10-20 ml Blocking buffer (5% non-fat milk in TBST) for 30 min at room temperature on an orbital shaker.
15. Dilute 10 µl VU-1 HRP into 10 ml Blocking buffer (5% non-fat milk in TBST), apply to membrane, and incubate for 2 hours at room temperature or overnight at 4 °C on orbital shaker.
16. Wash membrane with TBST three times for 10 minutes each.
17. Develop blot using ECL of choice. Tested with Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore cat# WBKLS0500).
    - The signal intensity of polyubiquitin smear can be quantified using LiCor Odyssey software Image Studio, 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 (Carp2 with UBE2D3)	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 (20x)	1x	2x	50 µl
ATP (100 mM)	400 µM	800 µM	4 µl
1x Assay buffer	-	-	393.5 µl

Example Data

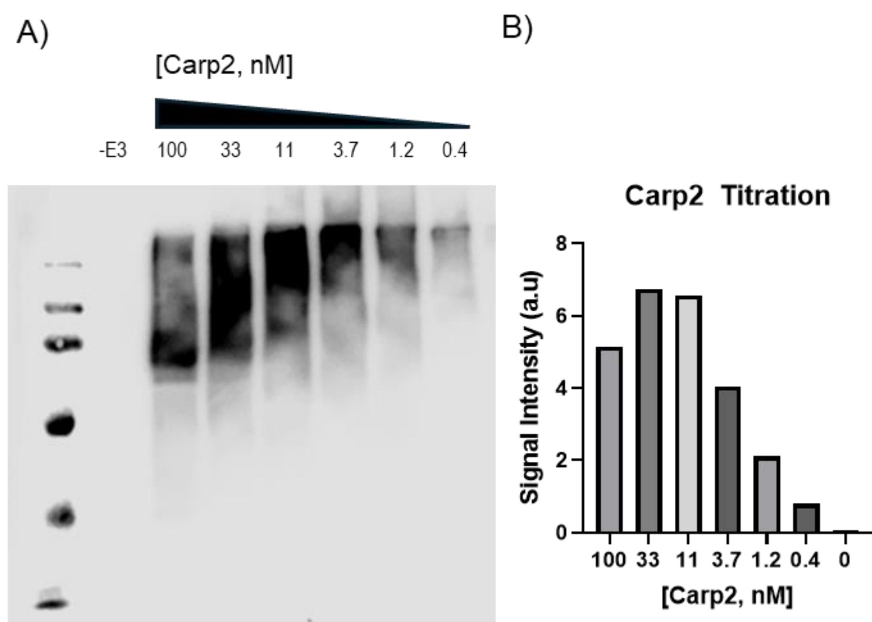

Figure 1. Carp2 dose response in Gel-Based In Vitro E3 Ligase Activity Kit. Carp2 was serially diluted in a 6 point 3-fold dilution series starting at 100nM and ending at 0.4nM. E3 ligase reaction proceeded for 60minutes before stopping. Samples loaded on 10% polyacrylamide gel, transferred to PVDF membrane using Bio-Rad Trans Turbo transfer system, developed using Immobilon ECL, and imaged using LiCor Odessey Fc for 2 minutes.
(A) Image of blot shows Carp2 activity in a dose dependent manner.
(B) Poly-Ubiquitin chains were quantified from image using LiCor's Image Studio software. Quantification box was drawn from the top of the resolving gel down to ~20KD.

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.
5. Liu N, Lin M, and Wang Y. The Emerging Roles of E3 Ligases and DUBs in Neurodegenerative Diseases. Mol Neurobiol. 2023; 60(1): 247–263.
6. Jeong Y *et al*. Targeting E3 ubiquitin ligases and their adaptors as a therapeutic strategy for metabolic diseases. Experimental & Molecular Medicine. 2023. 55, pages2097–2104.

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