Enzo

Product Manual

Auto-ubiquitinylation Kit

Catalog #BML-UW0970-0001

For assessment of protein Ub E3 ligase activity

* New antibody included.

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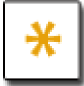
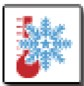


Please read entire booklet before proceeding with the assay.



Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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BACKGROUND

The covalent attachment of ubiquitin to proteins (ubiquitinylation) plays a fundamental role in the regulation of cellular function through biological events involving cell cycle, differentiation, immune responses, DNA repair, chromatin structure, and apoptosis[1,2,3,4].

Ubiquitinylation is achieved through three enzymatic steps. In an ATP-dependent process, the ubiquitin activating enzyme (E1) catalyzes the formation of a reactive thioester bond with ubiquitin, followed by its subsequent transfer to the active site cysteine of a ubiquitin carrier protein (E2). The selectivity of the ubiquitin cascade for a particular substrate protein relies on the interaction between the E2 conjugating enzyme (of which a cell contains relatively few) and a ubiquitin-protein ligase (E3), of which over 600 have been identified to date[2,5].

The E3s are a large, diverse group of proteins, characterized by one of several defining motifs. These include a HECT (homologous to E6-associated protein C-terminus), RING (really interesting new gene) or U-box (a modified RING motif without the full complement of Zn^{2+}-binding ligands) domain. Whereas HECT E3s have a direct role in catalysis during ubiquitinylation, RING and U-box E3s facilitate protein ubiquitinylation. These latter two E3 types act as adaptor-like molecules. They bring an E2 and a substrate into sufficiently close proximity to promote the substrate's ubiquitinylation. Although many RING-type E3s, such as MDM2 and c-Cbl, can apparently act alone, others are found as components of much larger multi-protein complexes, such as the anaphase-promoting complex.

Taken together, these multifaceted properties and interactions enable E3s to provide a powerful, and specific, mechanism for protein clearance within all cells of eukaryotic organisms utilizing the ubiquitin-proteasome system. The importance of E3s is highlighted by the number of normal cellular processes they regulate, and the number of diseases associated with their loss of function or inappropriate targeting.

E3 ligases also undergo auto-ubiquitinylation, through modification of specific lysine residues within an individual ligase, providing a mechanism thought to be responsible for the regulation of the E3 enzyme itself[5,6].

KIT DESCRIPTION

This E3 ligase auto-ubiquitinylation kit enables proteins to be tested for ubiquitin E3 ligase activity through assessment of their ability to undergo auto-ubiquitinylation. Utilizing the first three steps in the ubiquitin cascade the kit facilitates ubiquitinylation of known or putative E3 ligase enzymes followed by Western blot analysis using the highly sensitive reagents provided or using antibodies to the specific protein of interest (user supplied). A high integrity ubiquitin E3 ligase enzyme is also provided for use as a positive control.

The Kit provides sufficient material for approximately 10 auto-ubiquitinylation assays.

SUGGESTED APPLICATION

1. Qualitative assessment of an Ub E3 ligase enzyme's activity through its ability to auto-ubiquitinylate.
2. Testing of proteins for auto-ubiquitinylation activity allowing their identification as putative ubiquitin E3 ligases.
3. Ubiquitinylation of substrate proteins (user provided) specific to a particular ubiquitin E3 ligase.

Note: Protocol provided covers applications 1-2. Assay set-up can be readily modified for alternative applications by inclusion, omission or substitution of specific components.

KIT COMPONENTS

1. **Ubiquitin Activating Enzyme Solution, E1 (20X):**

   20X Ub Activating E1
   Ubiquitin activating enzyme E1 (human), (recombinant) (His-tag) (BML-UW9410-0025).
   Use 2.5µL per 50µL reaction.
   25µL provided, sufficient for 10 x 50µL reactions.

2. **UbcH5a (h)(rec.) (His-tag) (20X):**

   20X Ub Conjugating E2
   UbcH5a (human), (recombinant) (His-tag) (BML-UW9050-0025).
   Use 2.5µL per 50µL reaction.
   25µL provided, sufficient for 10 x 50µL reactions.

3. **Ubiquitin (10X):**

   10X Ubiquitin Solution
   Ubiquitin (BML-UW8795-0050).
   Use 5µL per 50µL reaction.
   50µL provided, sufficient for 10 x 50µL reactions.

4. **Hdm2 (h) (rec.) (catalytic RING domain) (GST-tag) (20X):**

   20X Control Ub Ligase E3
   Hdm2 (catalytic RING domain) (human), (recombinant) (GST-tag) (BML-UW0200-9090).
   Use 2.5µL per 50µL reaction.
   25µL provided, sufficient for 10 x 50µL reactions.

5. **Mg^{2+}-ATP Solution (20X):**

   20X Mg^{2+}-ATP Solution
   Mg^{2+}/ATP (BML-EW9805-0025).
   Use 2.5µL per 50µL reaction.
   25µL provided, sufficient for 10 x 50µL reactions.
   Note: Ensure Mg^{2+}-ATP is fully dissolved by warming to room temperature and mixing by vortex prior to use.

6. **Ub E3 Ligase Buffer (10X):**

   10X Ub E3 Ligase Buffer (BML-KW0965-0050)
   Use 5µL per 50µL reaction
   50µL provided, sufficient for 10 x 50µL reactions

7. **Ubiquitin mAb (P4G7-H11):**

   Ubiquitin Antibody Solution

   Ubiquitin, mouse monoclonal antibody (ADI-SPA-203-0025)

25µL provided. Dilution of at least 1:500-1:1000 recommended for Western blotting.

STORAGE

All kit components should be stored at –80°C to ensure stability and activity. Components with storage temperatures other than -80°C can be stored at the temperature listed on the label OR at -80°C. Avoid multiple freeze/thawing.

OTHER MATERIALS NEEDED

1. Eppendorf tubes (0.5 mL)
2. 2x SDS-PAGE gel loading buffer (*e.g.* 0.25M Tris-Cl, pH 6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.01% bromophenol blue).
3. DTT (Dithiothreitol) solution (50mM in dH20)
4. Inorganic pyrophosphatase solution (IPP) (100U/mL in 20mM Tris-HCl, pH7.5) (e.g. pyrophosphatase, inorganic, Baker's Yeast, Sigma, I1643)
5. 10X PBS solution (VWR, J373) or 10X TBS solution
6. Tween 20 solution (Sigma, P-5927)

AUTO-UBIQUINYLATION ASSAY

The protocol set out in this section describes the running of reactions to assess the auto-ubiquitinylation activity of the control Ub E3 ligase enzyme provided and user supplied proteins for subsequent analysis by Western blotting.

Hdm2 RING domain (BML-UW0200) is provided as a control ubiquitin E3 ligase for use in auto-ubiquitinylation assays.

Assay protocol

Note: recommended total reaction volume = 50µL.

* Adjust dH2O volume in accordance with available Ub E3 ligase protein concentration. A final assay concentration of 150-300nM is recommended as a starting point for Ub E3 ligase auto-ubiquitinylation (e.g. use 2.5µL of 6µM Ub E3 ligase protein solution).

** Inorganic pyrophosphatase solution (IPP) is recommended to be included in auto-ubiquitinylation reaction but it is not absolutely necessary. Adjust dH2O volume if IPP is not included in reaction.

Component	Sample E3-Ub	Sample E3 (–ve control)	Hdm2-Ub (+ve control)	Hdm2 (–ve control)
	Volume/µL			
dH2O	21.5*	24.0*	19.0	21.5
10x Ub E3 ligase buffer	5.0	5.0	5.0	5.0
20x Ub E1	2.5	2.5	2.5	2.5
20x E2	2.5	2.5	2.5	2.5
10x Ubiquitin	5.0	5.0	5.0	5.0
20x E3 control (6µM)	-	-	2.5	2.5
*Sample E3 protein	X	X	-	-
50mM DTT	1.0	1.0	1.0	1.0
20x Mg^{2+}-ATP	2.5	-	2.5	-
IPP ** (100U/mL)	10	10	10	10

Negative control reactions omitting Mg^{2+}-ATP (BML-EW9805-0025) cofactors demonstrate formation of auto-ubiquitinylated proteins is ATP-dependent (required for E1 activation, also known as one of the critical characters of ubiquitin activating enzyme E1) and hence derived from the ubiquitin cascade.

Set-up assays/controls required as follows:

1. Add assay components to 0.5 mL Eppendorf tube(s) in order shown in table above. Keep all enzymes on ice throughout.
2. Mix tube contents gently.
3. Incubate at 37°C for 60 minutes.
4. Quench assays by addition of 50µL 2x SDS-PAGE gel loading buffer followed by heating to 95°C for 5 minutes or 70°C for 10 minutes.
   Note: This step removes all Ub thioester linked species (Ub-E1/Ub-E2) so only isopeptide linked Ub-E3 species are detected using ubiquitin antibody/Western blotting.
5. Proceed directly to “Analysis by Western blotting” or store at –20°C until ready.

ANALYSIS BY WESTERN BLOTTING

Summary of analysis steps

1. Separate proteins by SDS-PAGE.
2. Western transfer to nitrocellulose or PVDF membrane.

Note: Western blotting conditions appropriate for the transfer of large proteins may be required to ensure good transfer of Ubiquitinylated-E3 protein to PVDF membrane. For example, use BSN transfer buffer 48mM Tris, pH9.2, 39mM glycine with 10% MeOH and 0.0375% SDS.

3. Block membrane with BSA/PBS-T solution.
4. Probe blot with either:
   a) ubiquitin antibody supplied or
   b) appropriate target protein specific primary antibody in conjunction with suitable secondary antibodies.
5. Develop with Western blotting detection reagents.

Note: Do NOT use milk in blocking/antibody binding solutions. Please use 5% BSA in PBS-Tween or TBS-Tween instead.

Materials Needed

1. SDS-PAGE gels - User prepared (10% standard / 4-15% linear gradient)
2. Pre-stained SDS-PAGE molecular weight markers (e.g. See Blue™ Plus 2, Thermo Fisher Scientific, LC5925)
3. PVDF membrane or nitrocellulose membrance (e.g. EMD Millipore, Immobilon-P or GE Healthcare, 10600002)
4. Anti-mouse IgG secondary antibody (HRP linked) (e.g. Goat anti-mouse IgG $F(ab')_2$, pAb (HRP conjugate), Prod. No. ADI-SAB-100).
5. (If required) Target protein specific primary antibody (user supplied) and appropriate secondary antibody-HRP conjugate.
6. Western blotting detection reagents (e.g. ECL Reagent).
7. PBS solution 1X PBS.
8. PBS-T solution 1X PBS containing 0.2% Tween 20.
9. BSA/PBS-T blocking solution PBS-T containing 5% bovine serum albumin (BSA).

Note: TBS-T can be used as an alternative to PBS-T if required.

EXAMPLE PROCEDURE FOR WESTERN BLOTTING

1. Apply 10 - 20µL of each quenched reaction to the SDS-PAGE gel alongside selected molecular weight markers, electrophorese, and transfer protein to nitrocellulose or PVDF membrane according to standard procedures.
2. Remove membrane from the transfer unit and block with BSA/PBS-T blocking buffer for 1 hour at room temperature on a rotor mixer.

   Note: Drying PVDF membrane prior to blocking, as per Manufacturers' instructions, may considerably enhance results.
3. Check for completion of transfer as indicated by bands of pre-stained protein standard evident on membrane.

Ubiquitin-conjugate detection

4. Dilute supplied ubiquitin antibody 1:500 or 1:1000 in BSA/PBS-T.
5. Incubate membrane with ubiquitin antibody solution at room temperature for 1-2 hours or overnight at 4°C on a rotor mixer.
6. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
7. Dilute appropriate anti-mouse IgG secondary antibody (HRP-linked) according to the manufacturer's instructions (e.g. Prod. No. ADI-SAB-100, 1:3000 in BSA/PBS-T).
8. Incubate membrane with secondary antibody solution for 1 hour at room temperature on a rocking platform, or as specified by the manufacturer.
9. Wash membrane for 4 x 10mins with PBS-T on a rocking platform.
10. (optional) Wash membrane for 1x10mins with 1X PBS (without Tween) on a rocking platform.
11. Proceed to **step 20**.

Specific target protein detection (if required)

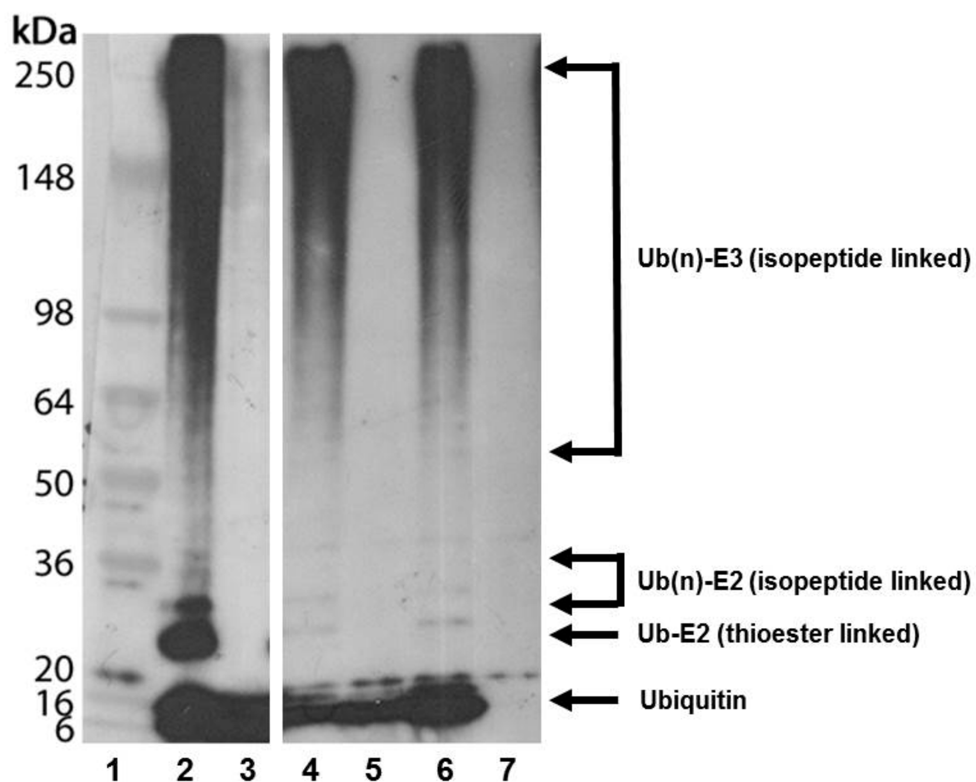
12. Dilute appropriate target protein specific primary antibody according to manufacturer's instructions.
13. Incubate membrane with target protein specific primary antibody solution overnight at 4°C on a rotor mixer.
14. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.

15. Dilute appropriate secondary antibody according to the manufacturer's instructions in BSA/PBS-T.
16. Incubate membrane with secondary antibody solution for 1 hour at room temperature on a rocking platform, or as specified by the manufacturer.
17. Wash membrane for 4 x 10mins with PBS-T on a rocking platform.
18. (optional) Wash membrane for 1x10mins with 1X PBS (without Tween) on a rocking platform.
19. Proceed to **step 20**.

Analysis

20. Prepare western blotting detection reagent according to the manufacturer's instructions.
21. Incubate membrane with detection reagent for appropriate time.
22. Detect emitted signal by luminography or CCD imaging instrument.

EXAMPLE RESULTS FOR WESTERN BLOTTING



Lane 1	**Pre-stained Protein Standard**
Lane 2	**Hdm2-Ub, (+) control, (non-reduced)**
Lane 3	**Hdm2, (-) control (no Mg-ATP), (non-reduced)**
Lane 4	**Hdm2-Ub, (+) control, (reduced)**
Lane 5	**Hdm2, (-) control (no Mg-ATP), (reduced)**
Lane 6	**Hdm2-Ub, (+) control, (reduced)**
Lane 7	**Hdm2, (-) control (no Ubiquitin), (reduced)**

Figure: Western blot analysis of control Ub E3 ligase Hdm2 RING domain auto-ubiquitinylation assays. Auto-ubiquitinylation assays set-up and run as described in "Assay protocol". Ubiquitinylated E3 ligase species were detected by Western blotting as described in "Analysis by western blotting", using the provided ubiquitin antibody (ADI-SPA-203) at a dilution of 1:1000 dilution.

Results demonstrate auto-ubiquitinylation of the control Hdm2 RING domain ligase under the given assay conditions. Please note E2-Ub (thioester linked) is visible in non-reduced condition (left, lane 2), in which β-ME and DTT were omitted from SDS-PAGE gel loading buffer.

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NOTES

NOTES

GLOBAL HEADQUARTERS
Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free:1.800.942.0430
Phone:631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE
Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone:+41/0 61 926 89 89
Fax:+41/0 61 926 89 79
info-ch@enzolifesciences.com

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