

**ab139469**

# **E3 Ligase Auto- Ubiquitylation Assay Kit**

## **Instructions for Use**

For testing ubiquitin E3 ligase activity through assessment of their ability to undergo auto-ubiquitylation

This product is for research use only and is not intended for diagnostic use.

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# 1. Background

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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.

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.

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<sup>2+</sup>-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

ubiquitylation. 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 utilising 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-ubiquitylation, 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.

## 2. Principle of the Assay

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Abcam E3 Ligase Auto-Ubiquitylation Assay Kit (ab139469) enables proteins to be tested for ubiquitin E3 ligase activity through assessment of their ability to undergo auto-ubiquitylation. Utilizing the first three steps in the ubiquitin cascade the kit facilitates ubiquitylation 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-ubiquitylation assays.

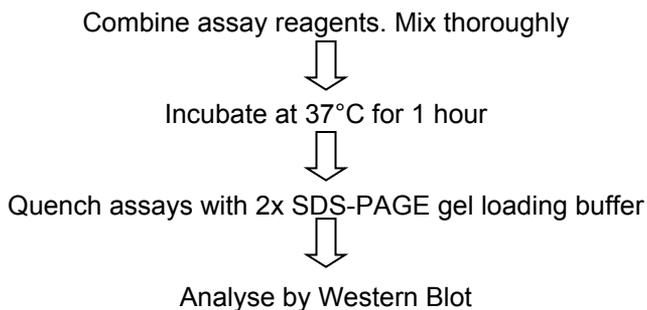
Suggested uses for this kit include:

- 1) Qualitative assessment of an Ub E3 ligase enzyme's activity through its ability to auto-ubiquitylate.
- 2) Testing of proteins for auto-ubiquitylation activity allowing their identification as putative ubiquitin E3 ligases.
- 3) Ubiquitylation 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.

### 3. Protocol Summary

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## 4. Materials Supplied

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Item	Quantity	Storage
20X Ubiquitin Activating Enzyme Solution (E1)	1 x 25 $\mu$ L	-80°C
20X UbcH5a (Human, Recombinant)	1 x 25 $\mu$ L	-80°C
10X Ubiquitin	1 x 50 $\mu$ L	-80°C
20X Hdm2 (Human, Recombinant)	1 x 25 $\mu$ L	-80°C
20X Mg-ATP Solution	1 x 25 $\mu$ L	-80°C
10X Ub E3 Ligase Buffer	1 x 50 $\mu$ L	-80°C
Ubiquitin Antibody Solution (pAb)	1 x 25 $\mu$ L	-80°C

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## 5. Storage and Stability

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- All kit components should be stored at -80°C to ensure stability and activity. Avoid multiple freeze/thawing.

## 6. Materials Required, Not Supplied

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- Eppendorf tubes

- 2x SDS-PAGE gel loading buffer (e.g. 0.25M Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 2%  $\beta$ -mercaptoethanol, 0.01% bromophenol blue)
- DTT (Dithiothreitol) solution (50mM in dH<sub>2</sub>O)
- Inorganic pyrophosphatase solution (IPP) (100U/mL in 20mM Tris-HCl, pH7.5) (e.g. pyrophosphatase, inorganic, Baker's Yeast, Sigma, I1643)

### **For Western Blot Analysis**

- SDS-PAGE Gels (User prepared (10% standard / 4-15% linear gradient) or pre-formed.
- Pre-stained SDS-PAGE molecular weight markers
- PVDF membrane
- Anti-mouse IgG secondary antibody (HRP linked)  
e.g. ab205719 or ab6789
- (If required) Target protein specific primary antibody (user supplied) and appropriate secondary antibody-HRP conjugate.
- Western blotting detection reagents
- PBS Solution. 1x PBS.
- PBS-T Solution. PBS containing 0.2% Tween 20
- BSA/PBS-T Blocking Solution. PBS-T containing 1% Bovine Serum Albumin (BSA)

## 7. Assay Protocol

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### A. Auto-Ubiquitylation Assay

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

Hdm2 RING domain is provided as a control ubiquitin E3 ligase for use in auto-ubiquitylation assays.

### Assay Protocol

Note: recommended total reaction volume = 50  $\mu$ L. \*Adjust dH<sub>2</sub>O volume in accordance with available Ub E3 ligase protein concentration. \*\*Inorganic pyrophosphatase solution (IPP) is recommended to be included in auto-ubiquitylation reaction but it is not absolutely necessary. Adjust dH<sub>2</sub>O volume if IPP is not included in reaction. A final assay concentration of 150-300nM is recommended as a starting point for Ub E3 ligase auto-ubiquitylation (e.g. use 2.5  $\mu$ L of 6  $\mu$ M Ub E3 ligase protein solution)

Negative control reactions omitting Mg-ATP cofactors demonstrate formation of auto-ubiquitylated proteins is ATP dependent (required for E1 activation) and hence derived from the ubiquitin cascade.

<b>Component</b>	<b>Sample E3-Ub</b>	<b>Sample E3 (-ve control)</b>	<b>Hdm2-Ub (+ve control)</b>	<b>Hdm2 (-ve control)</b>
	<b>Volume (μL)</b>			
dH <sub>2</sub> O	21.5*	24.0*	19.0	21.5
10X Ub E3 ligase buffer	5	5	5	5
20X Ubiquitin Activating Enzyme Solution (E1)	2.5	2.5	2.5	2.5
20X Ubch5a (Human, Recombinant)	2.5	2.5	2.5	2.5
10X Ubiquitin	5	5	5	5
20X Hdm2 (Human, Recombinant)	-	-	2.5	2.5
*Sample E3 protein	X	X	-	-
50mM DTT	1.0	1.0	1.0	1.0
20X Mg-ATP	2.5	-	2.5	-
IPP** (100U/mL)	10	10	10	10

**Set-up assays/controls required (keep all enzymes on ice throughout)**

1. Add assay components to 0.5 mL Eppendorf tube(s) in order shown above.
2. Mix tube contents gently.
3. Incubate at 37°C for 1 hour.
4. Quench assays by addition of 50  $\mu$ L 2x SDS-PAGE gel loading buffer followed by heating to 95°C for 5 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 “Western Blot Analysis” or store at -20°C until ready.

## **B. Western Blot Analysis**

### **Summary of analysis steps**

1. Separate proteins by SDS-PAGE.
2. Western Transfer to nitrocellulose/PVDF membrane.

**Note:** Western blotting conditions appropriate for the transfer of large proteins may be required to ensure good transfer of Ubiquitinated-E3 protein to PVDF membrane. For example, use BSN transfer buffer 48 mM Tris, pH 9.2, 39 mM glycine with 10% MeOH and 0.0375% SDS.

3. Block membrane with BSA/TBS-T solution.
4. Probe with either:
  - a) Ubiquitin Antibody Solution (pAb) 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 1% BSA in PBS or TBS Tween instead.

## **Example procedure for Western blotting**

Note: This protocol has been optimized using the materials indicated above. Using materials other than those listed may require additional optimization.

1. Apply ~20  $\mu$ L of each quenched assay solution to the gel, alongside selected molecular weight markers, electrophorese and transfer protein to membrane according to standard procedures.
2. Remove membrane from the transfer unit and block membrane with BSA/PBS-T blocking solution for 1 hour at room temperature on a rocking platform, or overnight at 4°C.

**Note:** Drying PVDF membrane prior to blocking, as per Manufacturers' instructions, may considerably enhance results.

3. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
4. Dilute Ubiquitin Antibody Solution 1/500 or 1/1000 in BSA/PBST.
5. Incubate membrane with Ubiquitin Antibody Solution overnight at 4°C on a rocking platform.
6. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
7. Dilute selected anti-mouse IgG secondary antibody according to the manufacturer's instructions.

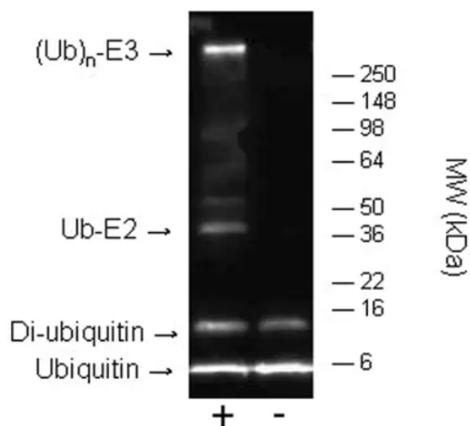
**Note:** We recommend using ab205719 or ab6789

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 6 x 10 mins with PBS-T on a rocking platform.
10. Prepare Western blotting detection reagent according to the manufacturer's instructions. (e.g. Pierce ECL Western Blotting Substrate: Mix equal amounts of Reagent A and B and allow to stand for 1 minute).
11. Incubate membrane with Western blotting detection reagent for appropriate time.
12. Detect emitted signal by Luminography or CCD imaging instrument.

## 8. Data Analysis

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### Example results for Western blotting



**Figure 1:** Western blot analysis of control Ub E3 ligase Hdm2 RING domain auto-ubiquitylation assays. Auto-ubiquitylation assays set-up and run as described in “Assay protocol”. Ubiquitylated E3 ligase species were detected by Western blotting as described in “Western Blot Analysis”, using the provided Ubiquitin Antibody Solution (pAb) at a dilution of 1/1000 dilution.

## Technical Support

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