

**ab139467**

# **Ubiquitylation Assay Kit**

## **Instructions for Use**

For the activation of ubiquitin for use in  
ubiquitylation experiments

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 (ubiquitylation) and their subsequent proteasomal degradation 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.

Ubiquitylation 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, in the presence of a  $Mg^{2+}$  cofactor, followed by its subsequent transfer to the active site cysteine of a ubiquitin carrier protein (E2). The specificity of ubiquitin ligation arises from the subsequent association of the E2-ubiquitin thioester with a substrate specific ubiquitin-protein isopeptide ligase (E3), which facilitates the formation of the isopeptide linkage between ubiquitin and its target protein.

## 2. Principle of the Assay

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Abcam Ubiquitylation Assay Kit (ab139467) provides the means of generating thioester linked, activated ubiquitin-E1 conjugates, utilizing the first step in the ubiquitin cascade, for investigation of ubiquitin activation, subsequent ubiquitin transfer to/interaction with E2 conjugating enzymes and their use in the ubiquitinylation of E3 ligases and target substrate proteins. The reagents supplied are intended to be used in conjunction with user supplied wild type or mutant E2 enzymes in E1 initiated/mediated reactions. Kit is supplied with a highly sensitive ubiquitin antibody for detection of ubiquitin and ubiquitin conjugates. Kit provides sufficient material for 20 x 50 $\mu$ L reactions.

Suggested uses for this kit include:

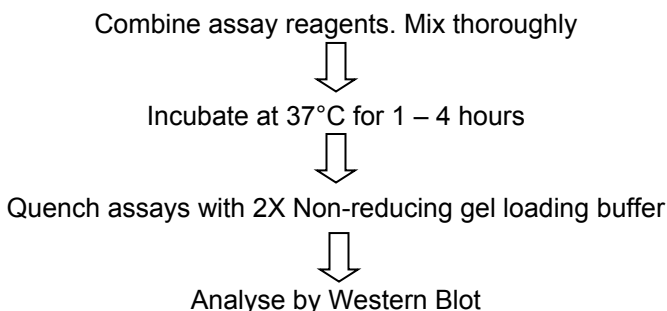
- 1) Activation of ubiquitin for conjugation to wild type or mutant E2 enzymes (user supplied) via thioester bond formation.
- 2) Use of Ub-E2 conjugates produced in the subsequent ubiquitin modification of specific target proteins in presence of a dedicated ubiquitin E3 ligase.
- 3) Investigation of ubiquitin activation by E1 activating enzyme.

- 4) Substitution of the wild type ubiquitin provided with ubiquitin mutants or derivatives (e.g. biotinylated-ubiquitin) allowing their activation and subsequent utility in the ubiquitin cascade.

Note: Protocols provided for applications 1 and 3. Assay set-up can be readily modified for alternative applications by inclusion, omission or substitution of specific enzyme components

### 3. Protocol Summary

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## 4. Materials Supplied

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Item	Quantity	Storage
20X Ubiquitin Activating Enzyme Solution (E1) (2 $\mu$ M)	1 x 50 $\mu$ L	-80°C
20X Ubiquitin Solution (Ub) (50 $\mu$ M)	1 x 50 $\mu$ L	-80°C
Ubiquitin Antibody Solution	1 x 25 $\mu$ L	-80°C
20X Mg-ATP Solution (0.1 M)	1 x 50 $\mu$ L	-80°C
2X Non-reducing Gel Loading Buffer	1 x 1.25 mL	-80°C
10X Ubiquitinylation Buffer	1 x 100 $\mu$ L	-80°C

## 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.
- If precipitation observed upon thawing 2x Non-reducing Gel Loading Buffer, warm tube at 37°C for 5-10mins until solution clears.

## 6. Materials Required, Not Supplied

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- Eppendorf tubes
- EDTA solution (50mM in 20mM Tris-Cl, pH 7.5)
- Inorganic pyrophosphatase solution (100U/mL in 20mM Tris-HCl, pH 7.5)
- DTT (Dithiothreitol) solution (50mM in 20mM Tris-Cl, pH 7.5)

### For Western Blot Analysis

- SDS-PAGE Gels (User prepared (12% 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. Goat Anti-Mouse IgG H&L (HRP) (ab6789)
- Western blotting detection reagents
- PBS Solution. 1x PBS.
- PBS-T Solution. PBS containing 0.1% Tween
- BSA/PBS-T Blocking Solution. PBS-T containing 1% Bovine Serum Albumin (BSA)



## 7. Assay Protocol

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### A. Ubiquitylation Assay

The reaction described is the basic assay setup:

1. E1 activation of ubiquitin (thioester linked)
2. Ubiquitin-E2 conjugate formation (thioester linked)

Note: Assay set-up can be readily modified for alternative applications (as outlined previously) by inclusion, omission or substitution of specific enzyme components.

#### Standard assay set-up

Note: Suggested E1/E2 protein concentrations are given as a starting point for such reactions and may require optimization for specific enzymes/combinations.

Component	Concentration	Notes
Ub	2.5 $\mu$ M	Supplied as 50 $\mu$ M (20x) solution
E1	100 nM	Supplied as 2 $\mu$ M (20x) solution
E2	2.5 $\mu$ M	User supplied, 25 $\mu$ M (10x) solution suggested
Mg-ATP	5 mM	Supplied as 100 mM (20x)

		solution
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## Assay Protocol

Note: recommended total reaction volume = 50µL

Component	E1-Ub	E1-Ub –ve Control	E2-Ub	E2-Ub –ve Control
	volume / µL			
dH <sub>2</sub> O	26.5	24	21.5	19
10X Ubiquitylation Buffer	5	5	5	5
IPP (100 U/mL)	10	10	10	10
DTT (50 mM)	1	1	1	1
Mg-ATP (0.1 M)	2.5	-	2.5	-
EDTA (50 mM)	-	5	-	5
20X E1 (2 µM)	2.5	2.5	2.5	2.5
10X E2 (25 µM)	-	-	5	5
20X Ub (50 µM)	2.5	2.5	2.5	2.5

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

1. Add assay components to 0.5mL Eppendorf tube(s) in order shown above.
2. Mix tube contents gently.
3. Incubate at 37°C for 1 – 4 hours.
4. Quench assays by addition of 50µL 2X Non-reducing gel loading buffer.
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.
3. Block membrane with BSA/TBS-T solution.
4. Probe with Ubiquitin Antibody Solution provided and secondary antibody conjugate.
5. Develop with western blotting detection reagents.

### **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.
3. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
4. Dilute Ubiquitin Antibody Solution 1/1000 in BSA/PBST.
5. Incubate membrane with Ubiquitin Antibody Solution for 1 hour at room temperature 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.

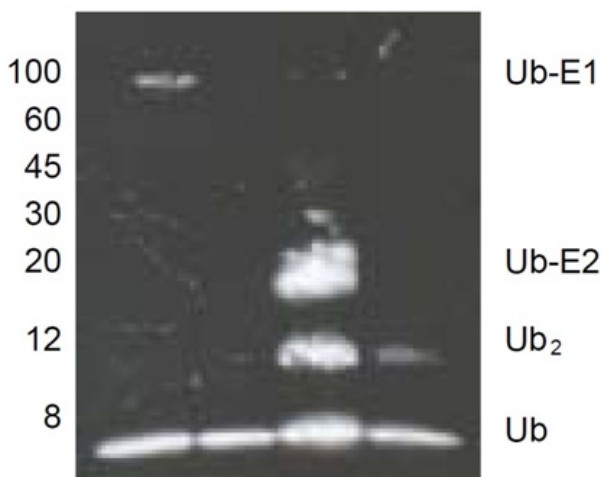
NOTE: We recommend using Goat Anti-Mouse IgG H&L (HRP) (ab6789).

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.
11. Incubate membrane with Western blotting detection reagent for 1 minute.
12. Detect emitted signal by Luminography or CCD imaging instrument.

## 8. Data Analysis

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### Example results for Western blotting



Western blot of ubiquitin thioester assays for the E1 activating enzyme provided and an example E2, UbcH5a (user supplied). Procedures as described in Assay Protocol section. Ubiquitin-enzyme conjugates were detected by Western blotting of E1-Ub (**A/B**) or E2-Ub (**C/D**) thioester assays in the presence (**A/C**) or absence (**B/D**) of Mg-ATP co-factor, using the Ubiquitin Antibody Solution provided, as described in “Western Blot Analysis” section. Results demonstrate the formation of ubiquitin thioester linked E1 and/or E2 conjugates in all +Mg-ATP reactions. The absence of such conjugates in negative control reactions demonstrates that their formation is Mg-ATP dependent (required for E1 activation) and hence derived from the ubiquitin cascade.



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