Version 3 Last updated 3 February 2017

# ab139472 E2-Ubiquitin Conjugation Kit

For the assessment of formation of thioester-linked ubiquitin conjugated E2 enzymes.

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

# Table of Contents

# 1. Overview

E2-Ubiquitin Conjugation Kit (ab139472) provides the tools necessary for generating a range of thioester-linked ubiquitin-conjugated E2 enzymes for use in ubiquitinylation experiments. Biotinylated ubiquitin (provided in the kit) can be detected with streptavidin-linked enzymes via SDS PAGE and western blotting.

This product provides sufficient material for a total of 50 reactions: 4 reactions with each included E2 enzyme.

Uses for this kit include:

1) Ubiquitinylation of target proteins in presence of dedicated E3 ligase. Panel of E2s provided for generation of E2-Ub thioester conjugates for testing vs. specific E3/target combinations. For example: ubiquitinylation of p53 in the presence of mdm2 (E3) and UbcH5b (E2).

2) Activation of Ub for thioester conjugation to novel E2 enzymes (substituted like for like with kit E2s, under directly comparable conditions).

Ubiquitinylation, the covalent attachment of ubiquitin to proteins, 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 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. Protocol Summary

Combine assay reagents and target protein

Incubate at 37°C for 30 – 60 minutes

Quench assays with 2X Non-reducing gel loading buffer

Analyze by Western Blot

## 3. Precautions

**Please read these instructions carefully prior to beginning the assay.**

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

## 4. Storage and Stability

**Store kit at -80°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

## 5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

## 6. Materials Supplied

| Item | Quantity | Storage Condition (Before prep) | Storage Condition (After prep) |
|---|---|---|---|
| 20X Ubiquitin Activating Enzyme Solution (E1) | 125 µL | -80°C | -80°C |
| 20X Biotinylated Ubiquitin Solution | 125 µL | -80°C | -80°C |
| 10X UbcH1-His tagged (hu, recombinant) | 20 µL | -80°C | -80°C |
| 10X UbcH2-His tagged (hu, recombinant) | 20 µL | -80°C | -80°C |
| 10X UbcH3-His tagged (hu, recombinant) | 20 µL | -80°C | -80°C |
| 10X UbcH5a-His tagged (hu, recombinant) | 20 µL | -80°C | -80°C |
| 10X UbcH5b-His tagged (hu, recombinant) | 20 µL | -80°C | -80°C |
| 10X UbcH6-His tagged (hu, recombinant) | 20 µL | -80°C | -80°C |
| 10X UbcH7-His tagged (hu, recombinant) | 20 µL | -80°C | -80°C |
| 10X UbcH8-His tagged (hu, recombinant) | 20 µL | -80°C | -80°C |
| 10X UbcH10 (hu, recombinant) | 20 µL | -80°C | -80°C |
| 10X UbcH13/Mms2-His tagged (hu, recombinant) | 20 µL | -80°C | -80°C |
| 20X Mg-ATP Solution (0.1 M) | 125 µL | -80°C | -80°C |
| 10X Ubiquitinylation Buffer | 250 µL | -80°C | -20°C |
| 2X Non-reducing gel loading buffer | 2 x 1.25 mL | -80°C | -20°C |

## 7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- MilliQ water or other type of double distilled water ($ddH_2O$)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Ubiquitin E3 ligase of interest
- Target protein for ubiquitinylation
- EDTA solution 50 mM stock solution (in 20 mM tris-Cl, pH 7.5)
- Inorganic pyrophosphatase solution (100 U/mL in 20 mM Tris-Cl, pH 7.5)
- DTT solution (50 mM in 20 mM tris-Cl, pH 7.5)

For Western blot analysis:

- Polyacrylamide gel for protein separation (12% standard/ 4 – 15% linear gradient SDS-PAGE)
- Standard instruments for SDS-PAGE and western blot analysis systems
- PVDF or nitrocellulose membrane
- Biotinylated/pre-stained SDS-PAGE molecular weight marker
- Streptavidin-HRP conjugate protein detection system
- TBS Solution 1X TBS
- TBST Solution (TBS + 0.1% Tween)
- BSA/TBST Blocking Solution (TBST + 1% BSA)
- ECL Detection Reagent: we recommend Optiblot ECL Detect Kit (ab133406)

## 8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.

# 9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

## 9.1 20X Ubiquitin Activating Enzyme (E1) Solution:

Ready to use as supplied. Keep on ice while in use. Aliquot so that you have enough volume to perform the desired number of assays. Avoid multiple freeze/thaw cycles. Store at -80°C.

## 9.2 20X Biotinylated Ubiquitin Solution:

Ready to use as supplied. Keep on ice while in use. Aliquot so that you have enough volume to perform the desired number of assays. Avoid multiple freeze/thaw cycles. Store at -80°C.

## 9.3 10X Ubiquitin Conjugating Enzyme (E2) Solutions- 11 vials [UbcH1, UbcH2, UbcH3, UbcH5a, UbcH5b, UbcH6, UbcH7, UbcH8, UbcH10, UbcH13]:

Ready to use as supplied. Keep on ice while in use. Aliquot so that you have enough volume to perform the desired number of assays. Avoid multiple freeze/thaw cycles. Store at -80°C.

## 9.4 20X Mg-ATP Solution (0.1 M):

Ready to use as supplied. Keep on ice while in use. Aliquot so that you have enough volume to perform the desired number of assays. Avoid multiple freeze/thaw cycles. Store at -80°C.

## 9.5 2X Non-reducing gel loading buffer:

Ready to use as supplied. Store at -20°C.

## 9.6 10X Ubiquitinylation Buffer:

Ready to use as supplied. Store at -20°C.

## 10. Assay Procedure

- We recommend that you perform the assay with appropriate controls at least twice to confirm results.
- Two types of reactions are described in this procedure, using the same basic assay set-up:
  1. E3 mediated ubiquitinylation of target/substrate proteins
  2. Ubiquitin-E2 thioester (TE) bond formation (essential control for assay 1)

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

| Component | End concentration | Notes |
|---|---|---|
| Ubiquitin | 2.5 µM | Supplied as 50 µM (20X; 0.45 mg/mL) solution |
| E1 | 100 nM | Supplied as 2 µM (20X) solution |
| E2 | 2.5 µM | Supplied as 0.5 mg/mL (10X) solution |
| Mg-ATP | 5 mM | Supplied as 100 mM (20X) solution |
| E3 | 100 nM | User supplied – ideally available as 2 µM (20X) solution |
| Target | 1 µM | User supplied – ideally available as 5 µM (10X) or 10 µM (5X) solution |

## 10.1 Assay set up:

10.1.1 Set up controls and assay reactions in 0.5 mL microcentrifuge tubes as described in the table below.

| Component | Target Ub | Target Ub (-)ve control | TE (+)ve control | TE (-)ve control |
|---|---|---|---|---|
| 10X Ubiquitinylation Buffer | 5 µL | 5 µL | 5 µL | 5 µL |
| 20X Bt-Ub | 2.5 µL | 2.5 µL | 2.5 µL | 2.5 µL |
| IPP (100 U/mL) | 10 µL | 10 µL | 10 µL | 10 µL |
| DTT (50 mM)* | 1 µL | 1 µL | 1 µL | 1 µL |
| Mg-ATP | 2.5 µL | - | 2.5 µL | - |
| EDTA (50 mM) | - | 5 µL | - | 5 µL |
| 20X E1 | 2.5 µL | 2.5 µL | 2.5 µL | 2.5 µL |
| 10X E2 | 5 µL | 5 µL | 5 µL | 5 µL |
| 20X E3 | 2.5 µL | 2.5 µL | - | - |
| 10X Target protein | 5 µL | 5 µL | - | - |
| dd$H_2O$ | 14 µL | 11.5 µL | 21.5 µL | 19 µL |
| **TOTAL** | **50 µL** | **50 µL** | **50 µL** | **50 µL** |

*UbcH2 is sensitive to reducing agents. Do not use DTT with UbcH2.

10.1.2 Mix tube contents gently by pipetting up and down.

10.1.3 Incubate at 37°C for 30 – 60 minutes. For enhances results, samples may be incubated at 37°C for 4 – 8 hours.

10.1.4 Quench assays by adding 50 µL of 2X Non-reducing gel loading buffer.

**Δ Note:** Quenched reactions can be stored at -20°C for short period until ready to perform Western blotting analysis.

## 10.2 Analysis by Western blotting:

10.2.1 Add 15 µL of each quenched assay solution to the gel, alongside MW marker.

10.2.2 Perform SDS-PAGE as per manufacturer's instructions.

10.2.3 Transfer the gel protein to a PVDF or nitrocellulose membrane as per manufacturer's instructions.

**Δ Note:** PVDF membrane needs to be activated by immersing PVDF membrane in 100% methanol for 15 seconds prior soaking in transfer buffer.

10.2.4 Following transfer step, block the membrane with 5% non-fat dry milk in TBST for 1 hour at RT (or overnight at 4°C) with constant agitation.

10.2.5 Wash membrane three times with TBST, 10 minutes each time, at RT with constant agitation.

10.2.6 Prepare Streptavidin-HRP solution as per manufacturer's instructions (dilute streptavidin-HRP solution in BSA/TBST blocking solution prior use).

10.2.7 Incubate membrane with Streptavidin-HRP solution for 1 hour at RT with constant agitation

10.2.8 Wash membrane six times with TBST, 10 minutes each time, at RT with constant agitation.

10.2.9 Prepare ECL detection reagent as per manufacturer's instructions.

10.2.10 Incubate membrane with ECL detection reagent for 1 minute.

10.2.11 Detect emitted signal by luminography or CCD imaging instrument.

# 11. Typical Data

Data provided **for demonstration purposes only**.

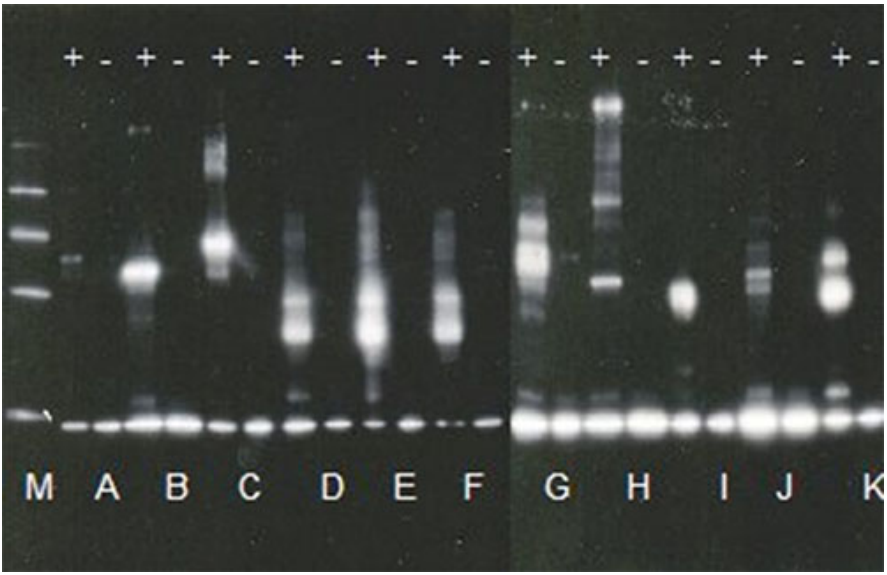


**Figure 1**. Western blot analysis of Thioester assays (TE (+)ve controls) for all E2 conjugating enzymes provided. Biotinylated-ubiquitin/enzyme conjugates were detected by WB on thioester assays containing UbcH1 (A), UbcH2 (B), UbcH3 (C), UbcH5a (D), UbcH5b (E), UbcH5c (F), UbcH6 (G), UbcH7 (H), UbcH8 (I), UbcH10 (J), Ubc13/MM2 (K), respectively. M: biotinylated SDS molecular weight marker.


**Figure 2.** Western blot analysis of Thioester assays (TE (+)ve/(-)ve control) for E2 conjugating enzyme UbcH2. Procedure as described in the assay set up section (without DTT), and ubiquitin conjugate detected by streptavidin AP. The absence of conjugate in TE (-)ve control control demonstrate that formation of TE is ATP dependent (required for E1 activation).

# 12. Notes

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