Product Manual

Ubiquitin Activating Kit

Catalog #: BML-UW0400A

For activation of ubiquitin for use in ubiquitinylation experiments

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Product Manual

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) 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[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, 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.

KIT DESCRIPTION

This kit provides the means of generating thioeseter 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 ubiquitin monoclonal antibody for detection of ubiquitin and ubiquitin conjugates. Kit provides sufficient material for 20 x 50µl reactions.

SUGGESTED USES / APPLICATIONS

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, Cat. #BML-UW8705) 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.

MATERIALS SUPPLIED

1. **20x Ubiquitin Activating Enzyme Solution (E1): 1 vial**

   Ubiquitin E1 (BML-KW9410-0050).
   2µM, use 2.5µl per 50µl reaction.
   50µl provided, sufficient for 20 x 50µl reactions.

2. **20x Ubiquitin Solution (Ub): 1 vial**

   Ubiquitin (BML-KW8795-0050).
   50µM, use 2.5µl per 50µl reaction.
   50µl provided, sufficient for 20 x 50µl reactions.
   Note: For detection using supplied ubiquitin antibody ADI-SPA-203.

3. **Ubiquitin Antibody Solution: 1 vial**

   Ubiquitin, mouse mAb (P4G7-H11)(ADI-SPA-203-0025).
   25µl provided. Dilution of at least 1:1000 recommended for Western blotting.

4. **20x Mg-ATP Solution: 1 vial**

   Mg-ATP (BML-KW9805-0050).
   0.1M, use 2.5µl per 50µl reaction.
   50µl provided, sufficient for 20 x 50µl reactions.

5. **2x Non-reducing Gel Loading Buffer: 1 vial**

   Use 50µl per 50µl reaction (BML-KW9880-1250).
   1.25mL provided, sufficient for at least 20 x 50µl reactions.
   Note: If precipitation is observed upon thawing warm tube at 37ºC for 5-10mins until solution clears.

6. **10x Ubiquitinylation Buffer: 1 vial**

   Use 5µl per 50µl reaction (BML-KW9885-0100).
   100µl provided, sufficient for 20 x 50µl reactions.

For further information on individual components please visit our website at www.enzolifesciences.com.

STORAGE

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

OTHER MATERIALS NEEDED

1. Eppendorf tubes
2. EDTA solution (50mM in 20mM Tris-Cl, pH7.5) (e.g. EDTA tetrasodium salt, Sigma, E5391)
3. Inorganic pyrophophatase solution (100U/mL in 20mM Tris-Cl, pH7.5) (e.g. pyrophosphatase, inorganic, Baker's Yeast, Fluka, 83205)
4. DTT (Dithiothreitol) solution (50mM in 20mM Tris-Cl, pH7.5) (e.g. dithiothreitol, Melford, MB1015)

UBIQUITINYLATION ASSAY

Overview

The reaction described is the basic assay setup for Ubiquitin-E2 conjugate formation (thioester linked) with associated control reactions.

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	2.5µM	Supplied as 50µM (20x) solution
E1	100nM	Supplied as 2µM (20×) solution
E2	2.5µM	User supplied, 25µM (10x) solution suggested
Mg-ATP	5mM	Supplied as 100mM (20x) solution

Assay protocol

Note: recommended total reaction volume = 50µl.

Component	E2-Ub	ATP -ve control	Ub –ve control	E1 -ve control
dH2O	21.5	19	24	24
10x Ubiquitinylation Buffer	5	5	5	5
IPP (100U/mL)	10	10	10	10
DTT (50mM)	1	1	1	1
Mg-ATP (0.1M)	2.5	-	2.5	2.5
EDTA (50mM)	-	5	-	-
20x E1 (2µM)	2.5	2.5	2.5	-
10x E2 (25µM)	5	5	5	5
20x Ub (50µM)	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 37ºC for 1-4 hours.
4. Quench assays by addition of 50µl 2x Non-reducing gel loading buffer.
5. Proceed directly to “Analysis by Western Blotting” or store at –20ºC until ready.

ANALYSIS BY WESTERN BLOTTING

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 provided and secondary antibody conjugate.
5. Develop with western blotting detection reagents.

Materials required

1. SDS-PAGE Gels (User prepared (12% Standard / 4-15% Linear Gradient) or preformed (e.g. ReadyGel, 4-15% Linear Gradient, BioRad, 161-1104)).
2. Pre-stained SDS-PAGE molecular weight markers (e.g. See Blue Plus 2, pre-stained SDS-PAGE markers, Invitrogen, LC5925).
3. PVDF membrane (e.g. Immobilon-P PVDF Membrane (0.45µm, 26.5cm (w)), Millipore, IPVH00010).
4. Anti-mouse IgG secondary antibody (HRP linked) (e.g. Prod No. ADI-SAB-100).
5. Western blotting detection reagents (e.g. Pierce ECL Western Blotting Substrate, Pierce, 32106).
6. PBS Solution. 1x PBS.
7. PBS-T Solution. PBS containing 0.1% Tween 20 (e.g. Sigma, P1379).
8. BSA/PBS-T Blocking Solution. PBS-T containing 1% Bovine Serum Albumin (BSA) (e.g. Albumin [bovine serum], Sigma, A7906).

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 ~20µl 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 4ºC.
3. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
4. Dilute ubiquitin antibody (ADI-SPA-203) 1:1000 in BSA/PBS-T.
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.
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 3 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 1 minute.
12. Detect emitted signal by Luminography or CCD imaging instrument.

Example results for Western blotting

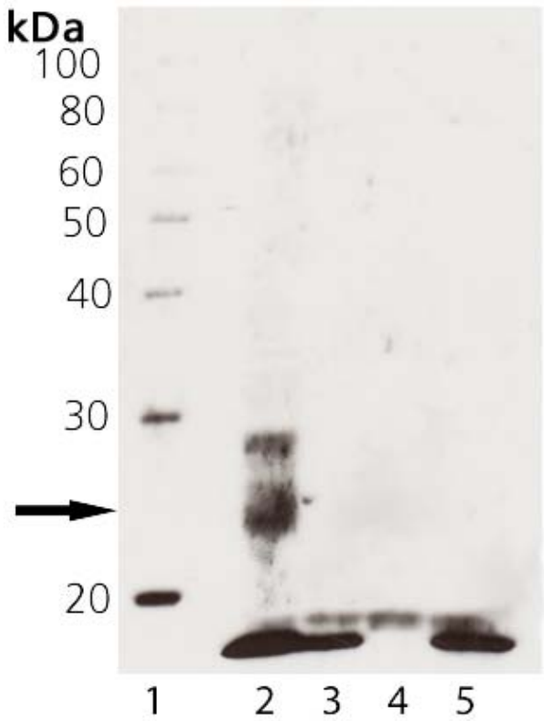

Figure 1: Western blot of ubiquitin thioester assays for the E1 activating enzyme provided and an example E2, UbcH5a (user supplied). Procedures as described in “Ubiquitinylation Assay” section. Ubiquitin-enzyme conjugates were detected by Western blotting using Ubiquitin monoclonal antibody (Prod No.ADI-SPA-203). Lane 1: MW Marker, Lane 2: 15 µL reaction E2-Ub, Lane 3: 15 µL reaction ATP –ve control, Lane 4: 15 µL Ub –ve control, Lane 5: 15 ul E1 –ve control. Results demonstrate the necessity of all reaction components (E1 enzyme, Mg-ATP, and ubiquitin) to successfully transfer Ubiquitin to E2 enzymes *in vitro*.

REFERENCES

1. Haas, A.L. and Siepmann, T. J. Pathways of ubiquitin conjugation. FASEB J. 11, 1257-1268 (1997)
2. Hershko, A. and Ciechanover, A. The ubiquitin system. Annu.Rev.Biochem. 67, 425-479 (1998)
3. Pickart, C.M. Mechanisms underlying ubiquitination. Annu.Rev.Biochem. 70, 503-533 (2001)
4. Strous, G.J. and Govers, R. The ubiquitin-proteasome system and endocytosis. J.Cell Sci. 112 (Pt 10), 1417-1423 (1999)

ALSO AVAILABLE FROM ENZO LIFE SCIENCES

Components supplied in the Enzo Life Sciences Ubiquitin Activating Kit are available separately.

Catalog #	Description	Quantity
UW9410	Ubiquitin-activating Enzyme E1, His6-tagged (human, recombinant)	50µg
UW8795	Ubiquitin	5mg
UW8705	Ubiquitin, biotinylated	100µg
UW8555	Ubiquitin, methylated	1mg
UW8615	[K48R]Ubiquitin, untagged (human, recombinant)	1mg
EW9805	Mg-ATP, 0.1M	100µl
SPA-203	Ubiquitin , mAb (P4GH7-H11)	50/200µg
PW8805	Polyubiquitinylated conjugates, mouse mAb [clone FK1]	500µg
PW8810	Mono- and polyubiquitinylated conjugates, mouse mAb [clone FK2]	500µg
UW9020	UbcH1, His6-tagged (human, recombinant)	100µg
UW9025	UbcH2, His6-tagged (human, recombinant)	100µg
UW8730	UbcH3, His6-tagged (human, recombinant)	100µg
UW9050	UbcH5a, His6-tagged (human, recombinant)	100µg
UW9060	UbcH5b, His6-tagged (human, recombinant)	100µg
UW9070	UbcH5c, His6-tagged (human, recombinant)	100µg
UW8710	UbcH6, His6-tagged (human, recombinant)	100µg
UW9080	UbcH7, His6-tagged (human, recombinant)	100µg
UW9135	UbcH8, His6-tagged (human, recombinant)	100µg
UW9565	UbcH13/Mms2 (human, recombinant)	100µg

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/ASIA
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

For local distributors and detailed product information visit us online:
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