Enzo

Product Manual

Ubiquitinylation kit

Catalog #: BML-UW9920

For assessment of thioester-linked ubiquitin conjugated E2 enzymes.

NOTE: This version contains a change to the suggested assay set-up table on page 5.

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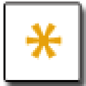
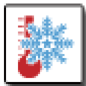


Please read entire booklet before proceeding with the assay.



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) 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[1-4].

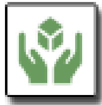
Ubiquitinylation is achieved through three enzymatic steps. In an ATP-dependent process, the ubiquitin activating enzyme (E1) catalyzes the formation of a reactive thioester bond with ubiquitin, followed by its subsequent transfer to the active site cysteine of a ubiquitin carrier protein (E2). The specificity 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.

An excellent example of the importance of the ubiquitinylation process is the role that the oncoprotein mdm2 plays in regulating cellular concentration and function of the p53 tumour repressor protein[5–7]. Perturbation in concentration and/or function of p53 is one of the most common features associated with human cancers[8, 9]. Mdm2 act as a ubiquitin ligase (E3), catalysing the ubiquitylation of p53, thus promoting p53 degradation through the ubiquitin-proteasome pathway.

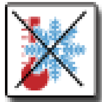
KIT DESCRIPTION

This kit provides the means of generating a range of thioester-linked ubiquitin-conjugated E2 enzymes, utilizing the first two steps in the ubiquitin cascade, for use in the ubiquitinylation of E3 ligases and target substrate proteins. The reagents supplied also allow for the thioester formation and detection of E1-Ub and/or E2-Ub and the use of alternative (user supplied) E2 enzymes in E1 initiated/mediated reactions. Biotinylated ubiquitin is provided for sensitive detection with streptavidin-linked enzymes via SDS-PAGE and western blotting. Kit provides sufficient material for 4 reactions with each included E2 enzyme.

SUGGESTED USES/APPLICATIONS


Handle with care

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)[10].
2. Activation of Ub for thioester conjugation to novel E2 enzymes (substituted like for like with kit E2s, under directly comparable conditions).
3. Use of cell lysate or crude fractions/preparations as source of E3 ligases to facilitate ubiquitinylation of purified target proteins in the presence of ubiquitinylation kit components.
4. Substrate (target) independent in vitro ubiquitinylation reactions. Determine ubiquitin ligase activity/specificity of proposed E3 enzymes and/or their catalytic domains/fragments[11].


Avoid freeze / thaw cycles

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

KIT COMPONENTS

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

   E1 (human) (recombinant) (His-tag) (BML-UW9410-0125)

   Use 2.5 µL per 50 µL reaction.
   125 µL provided, sufficient for 50 x 50 µL reactions

2. **10x Ubiquitin Conjugating Enzyme Solutions (E2): 11 vials:**

   UbcH1 (human), (recombinant) (His-tag) (BML-UW9020-0020)
   UbcH2 (human), (recombinant) (His-tag) (BML-UW9025-0020)*
   UbcH3 (human), (recombinant) (His-tag) (BML-UW8730-0020)
   UbcH5a (human), (recombinant) (His-tag) (BML-UW9050-0020)
   UbcH5b (human), (recombinant) (His-tag) (BML-UW9060-0020)
   UbcH5c (human), (recombinant) (His-tag) (BML-UW9070-0020)
   UbcH6 (human), (recombinant) (His-tag) (BML-UW8710-0020)
   UbcH7 (human), (recombinant) (His-tag) (BML-UW9080-0020)
   UbcH8 (human), (recombinant) (His-tag) (BML-UW9135-0020)
   UbcH10 (human), (recombinant) (untagged) (BML-UW0960-0020)
   UbcH13/Mms2 (human), (recombinant) (His-tag) (BML-UW9565-0020)
   Use 5 µL per 50 µL reaction.
   20 µL of each E2 provided, sufficient for 4 x 50 µL reactions.

***Note**: UbcH2 is sensitive to reducing agents such as DTT and BME. Do not use reducing agents with this enzyme. Assays performed with this enzyme must use only non-reducing buffers.

3. **20x Biotinylated Ubiquitin Solution (Bt-Ub): 1 vial:**

   Biotinylated-ubiquitin (BML-UW8705-0125)
   Use 2.5 µL per 50 µL reaction
   125 µL provided, sufficient for 50 x 50 µL reactions

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

   Mg-ATP (BML-EW9805-0125)
   Use 2.5 µL per 50 µL reaction
   125 µL provided, sufficient for 50 x 50 µL reactions.

5. **2x Non-reducing Gel Loading Buffer: 2 vials of 1.25 mL:**

   BML-KW9880-1250

   Use 50 µL per 50 µL reaction
   2.5 mL provided, sufficient for 50 x 50µL reactions

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

   BML-KW9885-0250

   Use 5 µL per 50 µL reaction

   250 µL provided, sufficient for 50 x 50 µL reactions

STORAGE

All kit components should be stored at –70ºC/–80ºC to ensure stability and activity. After opening, individual component can be stored at temperature as labeled. Avoid multiple freeze/thawing.

OTHER MATERIALS REQUIRED

1. Eppendorf tubes
2. EDTA solution (50mM in 20mM Tris-HCl, pH7.5) (e.g. EDTA tetrasodium salt, Sigma, E5391)
3. Inorganic pyrophophatase solution (100U/mL in 20mM Tris-HCl, pH7.5) (e.g. Pyrophosphatase, Inorganic from baker's yeast *(S. cerevisiae)*, Sigma, 83205)
4. DTT (Dithiothreitol) solution* (50mM in 20mM Tris-Cl, pH7.5) (e.g. DL-Dithiothreitol, Siamg, D9779)

*Please see note concerning sensitivity of UbcH2 (BML-UW9025) to reducing agents.

UBIQUITINYLATION ASSAY

A. Overview

Two types of reaction described, using 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)

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

B. Standard assay set-up

Note: Suggested E2/E3/target protein concentrations are given as a starting point for such reactions and will require optimisation for specific enzymes/combinations.

Component	Concentration	Notes
Ub	2.5µM	Supplied as a 50µM (0.45 mg/mL; 20x) solution
E1	100nM	Supplied as 2µM (20x) solution
E2	~1µM-2.5µM	Supplied as a (10x) solution
Mg-ATP	5mM	Supplied as 100mM (20x) solution
E3	100nM	Ideally available as 2µM (20x) solution
Target	1µM*	10µM (10x) solution

* Target protein concentration can vary in range of 0.5-2µM. Concentration in table is a suggested initial starting concentration and is subject to be optimized based on target protein.

C. Assay protocol

Note: recommended total reaction volume = 50 µL.

Note: UbcH2 (BML-UW9025) is sensitive to reducing agents. Do not use DTT with UbcH2 (BML-UW9025).

Component	Target-Ub	Target Ubiquitin –ve control	TE +ve control	TE –ve control
	volume / µL			
dH_2O	14	11.5	21.5	19
10x Ubiquitinylation Buffer	5	5	5	5
IPP (100U/mL)	10	10	10	10
DTT (50mM)	1	1	1	1
Mg-ATP	2.5	-	2.5	-
EDTA (50mM)	-	5	-	5
20x E1	2.5	2.5	2.5	2.5
10x E2	5	5	5	5
20x E3	2.5	2.5	-	-
10x Target protein*	5	5	-	-
20x Bt-Ub	2.5	2.5	2.5	2.5

* Volume of target protein is subject to adjustment depending on desired final concentration in reaction mix.

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

3. Add assay components to 0.5 mL Eppendorf tube(s) in order shown above.
4. Mix tube contents gently.
5. Incubate at 37ºC for 30-60 minutes. For enhanced results, samples may be incubated for 4-8 hours at 37ºC.
6. Quench assays by addition of 50 µL 2x Non-reducing gel loading buffer.
7. Proceed directly to “Analysis by Western Blotting” or store at –20ºC until ready.

ANALYSIS BY WESTERN BLOTTING

a) 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 HRP-Streptavidin detection system.
5. Develop with western blotting detection reagents.

b) Materials Required

1. SDS-PAGE Gels (user prepared (12% Standard / 4-15% Linear Gradient) or precast (*e.g*. Criterion™, 4-15% Linear Gradient, Bio-Rad, 3450029)).
2. Biotinylated/pre-stained SDS-PAGE molecular weight markers (*e.g*. Biotinylated SDS molecular weight markers, Sigma, SDS-6B. / SeeBlue Plus2, Pre-stained Protein Standard, ThermoFisher, LC5925).
3. Nitrocellulose or PVDF membrane (e.g. Nitrocellulose Membrane (0.45µm, 20x20cm), BioRad, 162-0113) (*e.g*. Immobilon-P PVDF Membrane (0.45µm, 26.5cm (w)), Millipore, IPVH00010).
4. Streptavidin-HRP conjugate (*e.g*. Jackson ImmunoResearch, 016-030-084).
5. Western blotting detection reagents (*e.g*. Clarity™ and Clarity Max™ ECL Western Blotting Substrates, Bio-Rad).
6. TBS Solution. (1x TBS diluted from TBS Buffer, 20X liquid, VWR, J640-4L).
7. TBS-T Solution. TBS containing 0.1% Tween 20 (*e.g*. Sigma, P1379).
8. BSA/TBS-T Blocking Solution. TBS-T containing 1% Bovine Serum Albumin (BSA) (*e.g*. Albumin [bovine serum], Sigma, A7906).

c) 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 15 µL of each quenched assay solution to the 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 membrane with BSA/TBS-T blocking buffer for 1 hour at room temperature on a rocking platform, or overnight at 4ºC.
3. Wash membrane for 3 x 10mins with TBS-T on a rocking platform.
4. Prepare Streptavidin-HRP solution according to the manufacturer's instructions. (recommended working concentration is between 100ng/mL – 1µg/mL).
5. Incubate membrane with Streptavidin-HRP solution for 1 hour at room temperature on a rocking platform.
6. Wash membrane for 6 x 10mins with TBS-T on a rocking platform.
7. Prepare Western blotting detection reagent according to the manufacturer's instructions.
8. Incubate membrane with Western blotting detection reagent for 1 minute.
9. Detect emitted signal by Luminography or CCD imaging instrument.

d) Example results for Western blotting

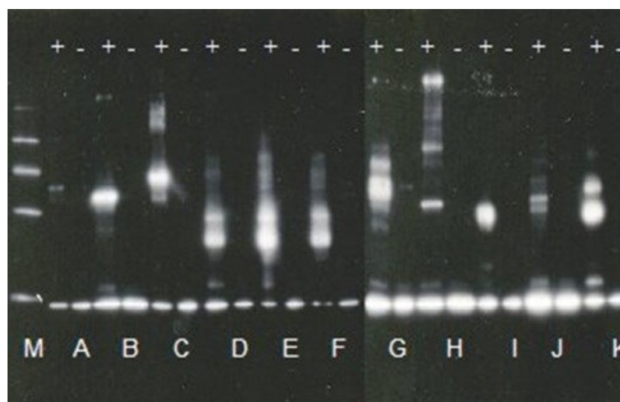


Figure 1: Western Blot of Thioester Assays (TE +ve/-ve controls) for all E2 conjugating enzymes provided. Procedures as described in "Assay Protocol" section. Biotinylated-ubiquitin-enzyme conjugates were detected by Western Blotting on thioester assays containing **A:** UbcH1 (BML-UW9020), **B:** UbcH2 (BML-UW9025), **C:** UbcH3 (BML-UW8730), **D:** UbcH5a (BML-UW9050), **E:** UbcH5b (BML-UW9060), **F:** UbcH5c (BML-UW9070), **G:** UbcH6 (BML-UW8710), **H:** UbcH7 (BML-UW9080), **I:** UbcH8 (BML-UW9135), **J:** UbcH10 (BML-UW0960), **K:** Ubc13/MMS2 (BML-UW9565) respectively, using Streptavidin-HRP detection system as described in "Analysis by Western Blotting" section. **M:** Biotinylated SDS molecular weight markers (Sigma, SDS-6B) from bottom: 20.1, 29.0, 39.8, 58.1kDa.

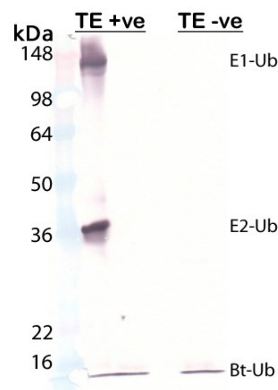


Figure 2: Western Blot of Thioester assay (TE +ve/-ve control) for E2 conjugating enzyme UbcH2 (BML-UW9025). Procedure as described in Assay protocol section with the noted absence of DTT, and that detected by Streptavidin-AP.

Results demonstrate the formation of ubiquitin thioester linked E2 conjugates of the expected size in all TE +ve control reactions. The absence of such conjugates in TE –ve control reactions demonstrates that their formation is ATP dependent (required for E1 activation) and hence derived from the ubiquitin cascade.

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