



Ubiquitylation kit

Catalog #: BML-UW9920

For assessment of thioester-linked ubiquitin conjugated E2 enzymes.



Product Manual

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Please read entire booklet before proceeding with the assay.



Carefully note the handling and storage conditions of each kit component.



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BACKGROUND

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 apoptosis¹⁻⁴.

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, 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 ubiquitylation process is the role that the oncoprotein mdm2 plays in regulating cellular concentration and function of the p53 tumour repressor protein⁵⁻⁷. 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 ubiquitylation 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



Avoid
freeze /
thaw cycles

1. Ubiquitylation 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: ubiquitylation 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 ubiquitylation of purified target proteins in the presence of ubiquitylation kit components.
4. Substrate (target) independent in vitro ubiquitylation reactions. Determine ubiquitin ligase activity/specificity of proposed E3 enzymes and/or their catalytic domains/fragments¹¹.

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 2.5µL per 50µL reaction.
125µL provided, sufficient for 50 x 50µL reactions
2. **10x Ubiquitin Conjugating Enzyme Solutions (E2): 11 vials:**
UbcH1 (h) (rec.) (His-tag) (BML-UW9020-0020); teal cap
UbcH2 (h) (rec.) (His-tag) (BML-UW9025-0020)*; gold cap
UbcH3 (h) (rec.) (His-tag) (BML-UW8730-0020); natural cap
UbcH5a (h) (rec.) (His-tag) (BML-UW9050-0020); purple cap
UbcH5b (h) (rec.) (His-tag) (BML-UW9060-0020); orange cap
UbcH5c (h) (rec.) (His-tag) (BML-UW9070-0020); green cap
UbcH6 (h) (rec.) (His-tag) (BML-UW8710-0020); yellow cap
UbcH7 (h) (rec.) (His-tag) (BML-UW9080-0020); red cap
UbcH8 (h) (rec.) (His-tag) (BML-UW9135-0020); blue cap
UbcH10 (h) (rec.) (untagged) (BML-UW0960-0020); solid blue cap
UbcH13/Mms2 (h) (rec.) (His-tag) (BML-UW9565-0020); black cap
Use 5µL per 50µL reaction.
20µL of each E2 provided, sufficient for 4 x 50µL 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 μ L per 50 μ L reaction

125 μ L provided, sufficient for 50 x 50 μ L reactions

Note: For use with streptavidin-conjugated detection systems (e.g. Vectastain Elite ABC Kit, Vector Labs)

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

Mg-ATP (BML-EW9805-0125)

Use 2.5 μ L per 50 μ L reaction

125 μ L provided, sufficient for 50 x 50 μ L reactions.

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

BML-KW9880-1250

Use 50 μ L per 50 μ L reaction

2.5mL provided, sufficient for 50 x 50 μ L reactions

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

BML-KW9885-0250

Use 5 μ L per 50 μ L reaction

250 μ L provided, sufficient for 50 x 50 μ L reactions

STORAGE

All kit components should be stored at -70°C to ensure stability and activity. Avoid multiple freeze/thawing.

OTHER MATERIALS REQUIRED

1. Eppendorf tubes
2. EDTA solution (50mM in 20mM Tris-Cl, pH7.5) (e.g. EDTA tetrasodium salt, Sigma, E5391)
3. Inorganic pyrophosphatase 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)

* Please see note concerning sensitivity of Ubch2 (BML-UW9025) to reducing agents on page 4.

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	2.5 μ M	Supplied as a 50 μ M (0.45 mg/mL; 20x) solution
E1	100nM	Supplied as 2 μ M (20x) solution
E2	~1 μ M-2.5 μ M	Supplied as a (10x) solution
Mg-ATP	5mM	Supplied as 100mM (20x) solution
E3	100nM	Ideally available as 2 μ M (20x) solution
Target	1 μ M	Ideally as 5 μ M (10x) or 10 μ M (5x) solution

C. Assay protocol

Note: recommended total reaction volume = 50 μ L.

Note: Ubch2 (UW9025) is sensitive to reducing agents. Do not use DTT with Ubch2 (UW9025).

Component	Target-Ub	Target Ubiquitin – ve control	TE +ve control	TE – ve control
			volume / μ L	
dH ₂ O	14	11.5	21.5	19
10x Ubiquitylation 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

D. 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 30-60 minutes. For enhanced results, samples may be incubated for 4-8 hours at 37°C.
4. Quench assays by addition of 50 μ L 2x Non-reducing gel loading buffer.
5. Proceed directly to “Analysis by Western Blotting” or store at –20°C 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 preformed. (e.g. ReadyGel, 4-15% Linear Gradient, BioRad, 161-1104)
2. Biotinylated/pre-stained SDS-PAGE molecular weight markers (e.g. Biotinylated SDS molecular weight markers, Sigma, SDS-6B. See Blue Plus 2, pre-stained SDS-PAGE markers, Invitrogen, LC5925).
3. Nitrocellulose or PVDF membrane (e.g. Nitrocellulose Membrane (0.45 μ m, 20x20cm), BioRad, 162-0113) (e.g. Immobilon-P PVDF Membrane (0.45 μ m, 26.5cm (w)), Millipore, IPVH00010).
4. Streptavidin-HRP conjugate protein detection system (e.g. Vectastain Elite ABC Kit (Standard), Vector Labs, PK-6100).
5. Western blotting detection reagents (e.g. ECL Reagent, Amersham, RPN2209).
6. TBS Solution. 1x TBS.
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 optimised using the materials indicated above. Using materials other than those listed may require additional optimization.

1. Apply 15 μ L 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 4°C.
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. (eg. Vectastain ABC Elite Kit: Add 2 drops of Reagents A and B to 5mL TBS, mix and allow to stand for 30 minutes at room temperature. Prior to use dilute Streptavidin-HRP solution with BSA/TBS-T blocking solution, (100µL per 3mL buffer)).
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. (eg. ECL Reagent: Mix equal amounts of Reagent A and B and allow to stand for 1 minute).
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

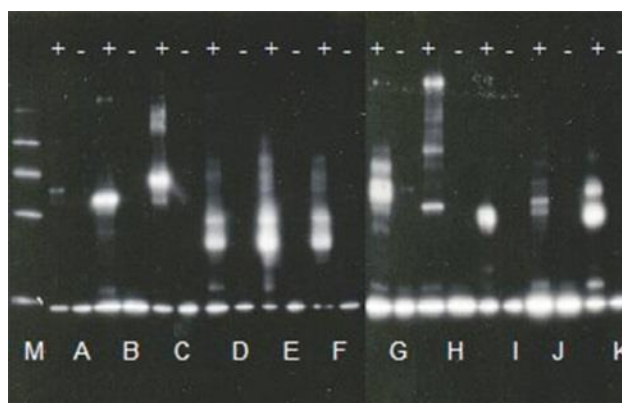


Figure 1: Western Blot of Thioester Assays (TE +ve/-ve controls) for all E2 conjugating enzymes provided. Procedures as described in “Assay Protocol” section. Biotinylated-ubiquitin-enzyme conjugates were detected by Western Blotting on thioester assays containing **A:** Ubch1 (KW9020), **B:** Ubch2 (KW9025), **C:** Ubch3 (KW9030), **D:** Ubch5a (KW9050), **E:** Ubch5b (KW9060), **F:** Ubch5c (KW9070), **G:** Ubch6 (KW8710), **H:** Ubch7 (KW9080), **I:** Ubch8 (KW9135), **J:** Ubch10 (KW8715), **K:** Ubch13/MMS2 (KW9565) 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.

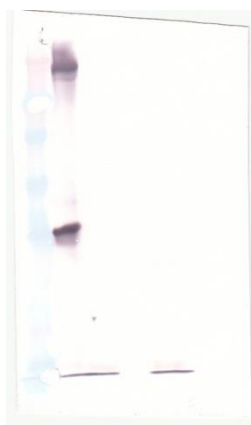


Figure 2: Western Blot of Thioester assay (TE +ve/-ve control) for E2 conjugating enzyme Ubch2 (UW9025). Procedure as described in Assay protocol section with the noted absence of DTT, and that detected by Streptavidin AP.

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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Product Manual

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