Product Manual

Ubiquitin Conjugating Kit

(HeLa lysate-based)

Catalog # BML-UW9915

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TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

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 in the form of K^{48}-linked polyubiquitin chains (of at least four subunits in length) 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]. Attachment of polyubiquitin chains via linkage at alternative lysine residues has been implicated in a wide range of processes including DNA repair, translation, IκB kinase activation and endocytosis[5]. In addition, the attachment of mono-ubiquitin at single or multiple sites on target proteins, or of short ubiquitin chains, has been shown to regulate the location and activity of a diverse range of cellular proteins[6].

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. The HeLa S100 cytosolic lysate (Prod. No. BML-SW8750) provided contains the full HeLa cell compliment of E1, E2s, 20S and 26S proteasome, deubiquitinylating, and E3 enzymes. This lysate is ideal for demonstrating ubiquitin-proteasome mediated conjugation/ degradation of radiolabelled or immunodetectable substrates[7-9]. Addition of deubiquitinylating enzyme inhibitors such as ubiquitin aldehyde (Prod. No. BML-UW8450), and proteasome inhibitor, such as epoxomicin (Prod. No. BML-PI127), help to facilitate the accumulation of ubiquitin-protein conjugates.

KIT DESCRIPTION

This kit provides the means of generating ubiquitin-conjugated lysate proteins. The HeLa S100 lysate facilitates controlled ubiquitin conjugation of endogenously-contained substrate proteins of interest through the ubiquitin cascade. Conjugate formation can be detected and monitored by Western blotting using the ubiquitin-conjugate specific antibody supplied and antibodies for specific target proteins. Modified proteins can then be subjected to further purification prior to their use in subsequent experiments if required. Proteasome/ deubiquitinylating enzyme inhibitor is provided in order to enhance yields of ubiquitin-conjugated proteins. Kit provides sufficient material for 20x50µl reactions.

SUGGESTED APPLICATION

1. Ubiquitinylation of specific endogenous HeLa lysate proteins, followed by their immediate detection/analysis using antibodies to the protein(s) of interest, indicating a particular protein is a substrate for the ubiquitin-proteasome pathway.
2. Ubiquitinylation of proteins of interest contained in exogenously added expression culture/cell extracts or tissue lysates/extracts, followed by their immediate detection/analysis or isolation/purification for use in subsequent experiments.
3. Modification of proteins using ubiquitin derivatives or mutants for improved detection/analysis or investigation of alternative (non-proteasomal) ubiquitin related pathways in subsequent experiments. For example, biotinylated-ubiquitin (Prod. No. BML-UW8705), for sensitive detection of low level ubiquitin-conjugates or methylated-ubiquitin (Prod. No. BML-UW8555) for mono-ubiquitin conjugate formation.

   Note: The HeLa S100 fraction contains a wide range of Ub E3 ligases that may catalyze ubiquitin modification of the full range of available lysine residues of a specific protein of interest. Primary antibodies selected by the user for target protein detection should therefore not be specific for epitopes contain or are neighboring such modification sites.

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

MATERIALS SUPPLIED

1. **HeLa S100 fraction (S100)**, Catalog No. BML-SW8750-0100
    - Use 10 µL per 50 µL reaction
    - 200 µL provided (2 x 100 µL vials)
2. **10× Ubiquitin solution (Ub**), Catalog No. BML-UW8795-0050
    - Use 5 µL per 50 µL reaction
    - 100 µL provided (2 x 50 µL vials)
3. **10× ATP-(Energy) Regeneration Solution (ATP-ERS),**

    Catalog No. BML-EW9810-0100
    - Use 5 µL per 50 µL reaction
    - 100 µL provided (1 x 100 µL vial)
4. **10× Ubiquitinylation Buffer**, Catalog No. BML-KW9885-0100
    - Use 5 µL per 50 µL reaction
    - 100 µL provided (1 x 100 µL vial)
5. **Ubiquitin aldehyde** (inhibitor), Catalog No. BML-UW8450-0050
    - Use 1 µL per 50 µL reaction
    - 50 µL provided (1 x 50 µL vial)
6. **Ubiquitin-conjugate specific antibody solution (FK2)**,

Catalog No. BML-PW8810-0010
    - Mono- and polyubiquitinylated conjugates mouse mAb (FK2)
    - 10 µL of provided (1 x 10 µL vial)
    - Dilution of 1:100 to 1:1000 for Western blotting.

STORAGE

Store all components at -80°C for the highest stability. Components labeled with storage temperature other than -80°C can be stored at the temperature listed OR at -80°C. Avoid multiple freeze/thawing.

OTHER MATERIALS NEEDED

1. Microcentrifuge tubes
2. Dimethyl sulfoxide (DMSO)
3. Dithiothreitol (DTT) solution (50 mM in 20 mM Tris-HCl, pH7.5)
4. 2 × SDS-PAGE gel loading buffer For example: 0.25M Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.01% bromophenol blue.

UBIQUINTINYLATION ASSAY

Two types of reaction are described, using the same basic assay set-up:

1) Ubiquitin modification of endogenous (HeLa S100 lysate) proteins
2) Ubiquitin modification of specific, exogenously-added target proteins

Ubiquitin modified proteins can then be analyzed immediately subjected to additional purification and/or used in subsequent experiments.

Assay protocol

Note: recommended total reaction volume = 50 µL.

Component	Lysate Protein-Ub Volume (µL)	Lysate Protein-Ub (–ve control) Volume (µL)
dH2O	22	27
10x Ubiquitinylation Buffer	5.0	5.0
HeLa S100 fraction	10	10
DTT (50mM)	2.0	2.0
Ubiquitin-aldehyde	1.0	1.0
10x Ubiquitin solution	5.0	5.0
10x ATP-ERS	5.0	-

Negative control reactions omitting ATP-ERS cofactor demonstrates that formation of ubiquitinylated target protein/increased levels of endogenous ubiquitinylated proteins is ATP dependent (required for E1 activation) and, hence, derived from the ubiquitin cascade.

Note: Suggested concentrations and assay incubation times are given as a starting point for such reactions and will require optimization by end-user.

Note: Incubation times will require optimization for ubiquitinylation of specific target proteins. It is recommended to run a time course as an initial experiment and analyze with FK2 Ub-conjugate (BML-PW8810)/target protein specific antibodies as described in “Analysis by Western Blotting” to identify optimal incubation times.

Set-up assays/controls required as follows:

1. Add assay components to 0.5 mL tube(s) in order shown in table above. Keep all enzymes on ice throughout.
2. Mix tube contents gently.
3. Incubate at 37°C for 0.5-4 hours.
4. Store assay solutions at –20°C prior to additional processing/use in subsequent experiments.

For analysis of all/part of the crude assay solution by Western blotting:

5. Quench assays by addition of 50 µL 2x SDS-PAGE gel loading buffer followed by heating to 95°C for 10 minutes.
6. Proceed directly to “Analysis by Western blotting” or store at –20°C until ready

Note: DO NOT quench whole assay solution if additional purification or subsequent use of active/native ubiquitin-modified proteins is required.

ANALYSIS BY WESTERN BLOTTING

Summary of analysis steps

1. Separate proteins by SDS-PAGE.
2. Western transfer to PVDF membrane.
3. Block membrane with BSA/PBS-Tween solution.
4. Probe blot with either
   i) FK2 ubiquitin-conjugate specific antibody supplied (BML-PW8810), or
   ii) Appropriate target protein specific primary antibody, followed by a suitable secondary antibody.
5. Develop with Western Blotting detection reagents.

Note: Do not use milk in blocking/antibody binding solutions. 1-2% BSA in PBS or PBS-Tween is recommended.

Materials Needed

1. SDS-PAGE gels - User prepared or precast polyacrylamide gel (12% standard / 4-15% gradient).
2. Pre-stained SDS-PAGE molecular weight markers (e.g., SeeBlue® Plus2 Pre-stained Protein Standard)
3. PVDF membrane (e.g., Immobilon-P)
4. Target protein specific primary antibody.
5. Anti-mouse secondary antibody (HRP linked) for use with Ub-conjugate primary antibody FK2 (e.g., Goat anti-mouse IgG F(ab')2, polyclonal antibody (HRP conjugate), Prod. No. ADI-SAB-100, or Anti-Mouse Polyvalent Immunoglobulins (G,A,M)−Peroxidase antibody produced in goat, Sigma, A0412).
6. Appropriate secondary antibody-HRP conjugate for use with chosen target protein specific primary antibody.
7. Western blotting detection reagents (e.g., ECL Reagent).
8. PBS solution 1X PBS.
9. PBS-Tween solution 1X PBS containing 0.2% Tween 20.
10. BSA/PBS-T blocking solution PBS-T containing 1-2% bovine serum albumin (BSA).

Note: TBS-T can be used as an alternative to PBS-T if required.

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 ~10 µL of each quenched reaction to the SDS-PAGE gel alongside selected molecular weight markers, electrophorese, and transfer protein to PVDF membrane according to standard procedures.
2. Remove membrane from the transfer unit and block with BSA/PBS-T blocking buffer for 1 hour at room temperature on a rotor mixer.

   Note: Drying PVDF membrane prior to blocking, as per manufacturers' instructions, may considerably enhance results.
3. Wash membrane for 3 x 10mins with PBS-T on a rocking platform at room temperature.

Detection

4. Dilute supplied FK2 ubiquitin-conjugate reactive primary antibody 1:100-1:1000 in BSA/PBS-T. (Alternatively dilute appropriate target protein specific primary antibody according to manufacturer's instructions (e.g., p53, monoclonal antibody (DO-1), at 1:1000)).
5. Incubate membrane with primary antibody solution for 1 hour at room temperature on a rocking platform, or overnight at 4°C.
6. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
7. Dilute appropriate secondary antibody according to the manufacturer's instructions (e.g., Anti-Mouse Polyvalent Immunoglobulins (G,A,M)−Peroxidase antibody produced in goat (Sigma, A0412) diluted 1:1000-1:5000 in BSA/PBS-T, following Ub-conjugate specific primary antibody)
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 10mins with PBS-T on a rocking platform.
10. Proceed to **step 17**.

Specific target protein detection (if required)

11. Dilute appropriate target protein specific primary antibody according to manufacturer's instructions.
12. Incubate membrane with target protein specific primary antibody solution overnight at 4°C on a rotor mixer.
13. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
14. Dilute appropriate secondary antibody in BSA/PBS-T according to the manufacturer's instructions.
15. Incubate membrane with secondary antibody solution for 1 hour at room temperature on a rocking platform, or as specified by the manufacturer.
16. Wash membrane for 6 x 10mins with PBS-T on a rocking platform.

Analysis

17. Prepare Western Blotting detection reagent according to the manufacturer's instructions.
18. Incubate membrane with detection reagent for appropriate time.
19. Detect emitted signal by luminography or CCD imaging instrument.

EXAMPLE RESULTS FOR WESTERN BLOTTING

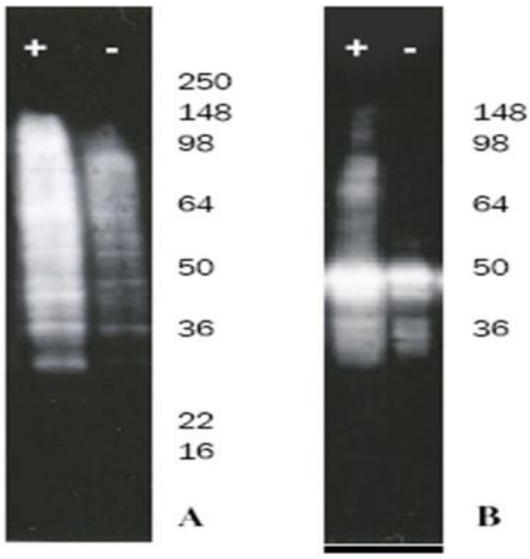

Figure: Western Blot of S100 ubiquitin conjugation assays of both endogenous lysate and exogenously added p53 proteins. Assays set-up and run as described in “Assay protocol” section. Ubiquitin-protein conjugate formation was detected by Western blotting of assays for A: General ubiquitinylation of endogenous HeLa S100 lysate proteins using the supplied FK2 ubiquitin-conjugate specific antibody (BML-PW8810) or B: specific modification of p53 present in HeLa S100 lysate using p53 specific monoclonal antibody DO-7 (BML-PW1095) as described in “Analysis by Western blotting”. Results demonstrate the utility of the HeLa conjugation kit for both the ubiquitin modification of endogenous HeLa S100 lysate proteins in general and of specific endogenous proteins of interest, such as p53. The elevated level (**A**) or formation (**B**) of ubiquitin modified proteins can be clearly seen in the +ve (ATP containing) assays. The lower level (**A**) or absence (**B**) of ubiquitin conjugated proteins in –ve control reactions (-ATP) demonstrates that their formation is ATP-dependent (required for E1 activation) and, hence, derived from the ubiquitin cascade.

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NOTES

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