ab139471

Ubiquitylation Assay Kit (HeLa lysate-based)

Instructions for Use

For the generation of ubiquitin-conjugated lysate proteins

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

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1. 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 biologicalevents involving cell cycle, differentiation, immune responses, DNA repair, chromatin structure, and apoptosis. 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. 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.

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 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. Addition of deubiquitinylating enzyme inhibitors such as ubiquitin aldehyde, and proteasome inhibitor, such as epoxomicin, help to facilitate the accumulation of ubiquitin-protein conjugates.

2. Principle of the Assay

Abcam Ubiquitylation Assay Kit (HeLa lysate-based) (ab139471) provides the means of generating ubiquitin-conjugated lysate proteins. The HeLa S100 Fraction 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 20 X 50µL reactions.

Suggested uses for this kit include:

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

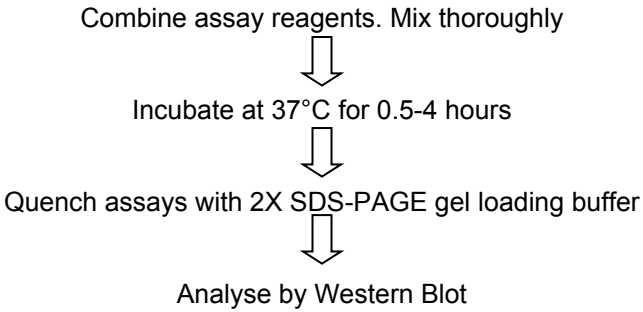
detection/analysis or isolation/purification for use in subsequent experiments.

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, for sensitive detection of low level ubiquitin-conjugates or methylated-ubiquitin for mono-ubiquitin conjugate formation.

Note: The HeLa S100 Fraction contains a wide range of Ub E3 ligases that may catalyse 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.

3. Protocol Summary



4. Materials Supplied

Item	Quantity	Storage
HeLa S100 Fraction (6 mg/ml)	2 x 100 µL	-80°C
10X Ubiquitin (5 mg/ml)	2 x 50 µL	-80°C
10X ATP Regeneration Solution	1 x 100 µL	-80°C
10X Ubiquitinylation Buffer	1 x 100 µL	-80°C
Ubiquitin Aldehyde (1 mg/ml)	1 x 50 µL	-80°C
Ubiquitin Antibody Solution (mAb)	1 x 10 µL	-80°C

5. Storage and Stability

- All kit components should be stored at -80°C to ensure stability and activity. Avoid multiple freeze/thawing.
- Inhibitors are stable for up to 3 months at -20ºC when dissolved in DMSO.

6. Materials Required, Not Supplied

- Eppendorf tubes.
- Dimethyl sulfoxide (DMSO).
- Dithiothreitol (DTT) solution (50mM in 20mM Tris-Cl, pH 7.5).
- 2 X 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.

For Western Blot Analysis

- SDS-PAGE gels - user prepared (12% standard / 4-15% linear gradient) or pre-formed.
- Pre-stained SDS-PAGE molecular weight markers.
- PVDF membrane.
- Target protein specific primary antibody.
- Anti-mouse secondary antibody (HRP linked) for use with Ubiquitin Antibody Solution (mAb) (Goat anti-Mouse Polyvalent Immunoglobulins (G, A, M)-Peroxidase antibody).
  e.g. Goat polyclonal Secondary Antibody to Mouse IgG - H&L (HRP) (ab97023)
- Appropriate secondary antibody-HRP conjugate for use with chosen target protein specific primary antibody.
- Western blotting detection reagents.
- PBS Solution 1X PBS.
- PBS-T Solution (1X PBS containing 0.2% Tween 20).

- BSA/PBS-T blocking Solution (PBS-T containing 1% Bovine Serum Albumin (BSA)).
  Note: TBS-T can be used as an alternative to PBS-T if required.

7. Assay Protocol

A. Ubiquitinylation Assay

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

1. Ubiquitin modification of endogenous (HeLa S100 Fraction) 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	Lysate Protein-Ub (–ve control)
	Volume/µL	
dH_2O	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	5.0	5.0
10X ATP Regeneration Solution	5.0	-

Negative control reactions omitting 10X ATP Regeneration Solution 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 Ubiquitin Antibody Solution (mAb) /target protein specific antibodies as described in "Western Blot Analysis" to identify optimal incubation times.

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

1. Add assay components to 0.5 mL Eppendorf tube(s) in order shown above. Keep all enzymes on ice throughout.
2. Mix tube contents gently.
3. Incubate at 37ºC for 0.5-4 hours.
4. Store assay solutions at –20ºC 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 95ºC for 10 minutes.
6. Proceed directly to "Western Blot Analysis" or store at -20ºC until ready.

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

B. Western Blot Analysis

Summary of analysis steps:

1. Separate proteins by SDS-PAGE.
2. Western transfer to PVDF membrane.
3. Block membrane with BSA/TBS-T solution.
4. Probe blot with either
   a) Ubiquitin Antibody Solution (mAb) supplied or
   b) 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% BSA in PBS or TBS Tween is recommended.

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 10minutes with PBS-T on a rocking platform at room temperature.
4. Dilute supplied Ubiquitin Antibody Solution (mAb) primary antibody 1:1000 in BSA/PBS-T.

   (Alternatively, dilute appropriate target protein specific primary antibody according to manufacturer's instructions).
5. Incubate membrane with primary antibody solution for 1 hour at room temperature on a rocking platform, or overnight at 4ºC.
6. Wash membrane for 3 x 10 minutes with PBS-T on a rocking platform.
7. Dilute appropriate secondary antibody according to the manufacturer's instructions.

Note: We recommend using Goat polyclonal Secondary Antibody to Mouse IgG - H&L (HRP) (ab97023).

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 10 minutes with PBS-T on a rocking platform.
10. Proceed to Analysis.

Specific target protein detection (if required)

1. Dilute appropriate target protein specific primary antibody according to manufacturer's instructions.
2. Incubate membrane with target protein specific primary antibody solution overnight at 4ºC on a rotor mixer.
3. Wash membrane for 3 x 10 minutes with PBS-T on a rocking platform.
4. Dilute appropriate secondary antibody in BSA/PBS-T according to the manufacturer's instructions.
5. Incubate membrane with secondary antibody solution for 1 hour at room temperature on a rocking platform, or as specified by the manufacturer.
6. Wash membrane for 6 x 10 minutes with PBS-T on a rocking platform.

Analysis

1. Prepare western blotting detection reagent according to the manufacturer's instructions.
2. Incubate membrane with detection reagent for appropriate time.
3. Detect emitted signal by luminography or CCD imaging instrument.

8. Data Analysis

Example results for Western blotting

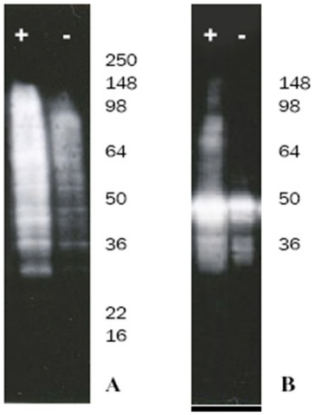


Figure 1: Western blot of Ubiquitin-protein conjugate formation assays of both endogenous lysate and exogenously added p53 proteins. Assays were perfomed as described. A: General ubiquitinylation of endogenous HeLa S100 lysate proteins using the supplied Ubiquitin Antibody Solution (mAb) or B: specific modification of p53 present in HeLa S100 lysate using p53 specific monoclonal antibody.

Results demonstrate the utility of the Ubiquitylation Assay Kit (HeLa lysate-based) for both the ubiquitin modification of endogenous HeLa S100 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.

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)