

ab139468

Neddylation Assay Kit

Instructions for Use

For generating a thioester-linked NEDD8-conjugated E2 enzyme for use in the NEDDylation of E3 ligases and target substrate proteins.

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

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1. Background

NEDD8 (neural precursor cell expressed developmentally downregulated gene 8) is an ubiquitin-like protein, with approximately 60% identity to ubiquitin (the highest homology of any ubiquitin-like protein). Conjugation of mature NEDD8 (via its exposed C-terminal glycine⁷⁶ residue) to specific lysine residues on a limited number of cellular target proteins via isopeptide bonds, allows NEDD8 to play a critical regulatory role in cell proliferation and development.

The NEDD8 conjugation pathway proceeds by a mechanism analogous to that of the ubiquitin conjugation cascade, consisting of a dedicated NEDD8 E1 activating enzyme (a heterodimeric complex consisting of a regulatory subunit APP-BP1 and a catalytic subunit Uba3), E2 conjugating enzyme (UbcH12) and substrate specific E3 ligases.

An increasing number of substrates for NEDDylation, including the tumor suppressors p53 and VHL, and EGFR have been identified in addition to members of the well-characterized cullin family of proteins, that play a structural role in ubiquitin E3 ligase complexes such as SCF.

Cullin NEDDylation in SCF complexes is facilitated by the RING domain containing Rbx1 (Roc1) SCF subunit, which is thought to act as a NEDD8 E3 ligase, and enhances the complex's ubiquitin E3 ligase activity. NEDDylation of p53 is mediated by its ubiquitin E3 ligase Mdm2, providing an additional control mechanism for inhibition of its transcriptional activity. E3 independent NEDDylation of p53 has also been demonstrated *in vitro*.

Reconstitution of the NEDD8 conjugation pathway *in vitro* using recombinant proteins is readily achieved and has been used to investigate a number of NEDD8 modification systems.

2. Principle of the Assay

Abcam's Neddylation Assay Kit (ab139468) provides the means of generating a thioester-linked NEDD8-conjugated E2 enzyme, utilizing the first two steps in the NEDD8 cascade, for use in the NEDDylation of E3 ligases and target substrate proteins (user supplied). A NEDD8-specific antibody is provided for detection of NEDDylated proteins via SDS-PAGE and western blotting. This kit provides sufficient material for 20 x 20 µL reactions.

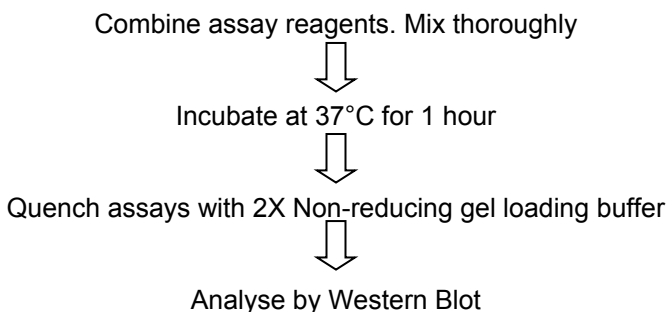
Suggested uses for this kit include:

- 1) Generate NEDD8-E2 thioesters for use in the *in vitro* NEDDylation of target proteins in the presence of a dedicated (user supplied) NEDD8 E3 ligase.
- 2) Demonstrate novel proteins are potential targets for NEDDylation under *in vitro* conditions (E3 ligase may be required).
- 3) Study of NEDD8 activation (E1) and conjugation (E2) steps in NEDDylation cascade.
- 4) Generate substrates for deNEDDylating enzymes, such as NEDP1.
- 5) Test proteins for NEDD8 E3 ligase activity.

- 6) Use of cell lysate or crude fractions/preparations as source of NEDD8 E3 ligases to facilitate NEDDylation of purified target proteins in the presence of NEDDylation kit components.

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

3. Protocol Summary



4. Materials Supplied

Item	Quantity	Storage
10x NEDD8 Activating Enzyme Solution	1 x 40 μ L	-80°C
20X UbcH12 Solution (E2)	1 x 20 μ L	-80°C
10X NEDD8 Solution	1 x 40 μ L	-80°C
10X NEDDylation Buffer	1 x 40 μ L	-80°C
20X Mg-ATP Solution	1 x 25 μ L	-80°C
NEDD8 Antibody Solution	1 x 25 μ L	-80°C
2X Non-reducing Gel Loading Buffer	1 x 400 μ L	-80°C

5. Storage and Stability

- All kit components should be stored at -80°C to ensure stability and activity. Avoid multiple freeze/thawing.
- If precipitation observed upon thawing 2x Non-reducing Gel Loading Buffer, warm tube at 37°C for 5-10mins until solution clears.

6. Materials Required, Not Supplied

- Eppendorf tubes
- Dithiothreitol (1M in 20 mM Tris-Cl, pH7.5)
- Inorganic pyrophosphatase solution (100U/mL in 20mM Tris-HCl, pH7.5)
- Double distilled water (dH₂O)

For Western Blot Analysis

- SDS-PAGE Gels (User prepared (13.5% Standard / 4-15% Linear Gradient) or pre-formed.
- Pre-stained SDS-PAGE molecular weight markers
- PVDF membrane
- Anti-rabbit IgG secondary antibody (HRP linked)
e.g. Goat polyclonal secondary antibody to rabbit IgG-H&L (HRP) (ab97051)
- Western blotting detection reagents
- PBS Solution. 1x PBS.
- PBS-T Solution. PBS containing 0.2% Tween
- BSA/PBS-T Blocking Solution. PBS-T containing 1% Bovine Serum Albumin (BSA)

7. Assay Protocol

A. NEDDylation Assay

The reaction for the *in vitro* generation of NEDD8-E2 thioesters. The primary function of this kit is to provide a means of generating NEDD8-E2 thioesters for use in conjunction with NEDD8 E3 ligases/target proteins (**user supplied**).

Note: Assay conditions may require optimisation when used in conjunction with specific NEDD8 E3 ligase/target proteins.

Assay Protocol

Note: recommended total reaction volume = 20 μ L.

Negative control reaction omitting Mg-ATP cofactors demonstrates formation of NEDDylated proteins is ATP dependent (required for E1 activation) and hence derived from the NEDD8 cascade.

Component	NEDD8-UbcH12 Thioester (TE)	NEDD8 TE (-ve control)
dH ₂ O	7.6 µL	8.6 µL
10X NEDDylation Buffer	2 µL	2 µL
10X NEDD8 E1	2 µL	2 µL
20X UbcH12	1 µL	1 µL
10X NEDD8	2 µL	2 µL
50mM DTT	0.4 µL	0.4 µL
100U/mL IPP	4 µL	4 µL
20X Mg-ATP	1 µL	-

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.
2. Mix tube contents gently.
3. Incubate at 37°C for 1 hour.
4. Quench assays by addition of 20 μ L 2x Non-reducing gel loading buffer.
5. Proceed directly to “Western Blot Analysis” or store at -20°C until ready.

B. Western Blot Analysis

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 NEDD8 antibody provided and secondary antibody conjugate.
5. Develop with western blotting detection reagents.

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 μ L 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 4°C.
3. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
4. Dilute NEDD8 antibody 1:1000 in BSA/PBS-T.
5. Incubate membrane with NEDD8 Antibody Solution overnight at 4°C on a rocking platform.
6. Wash membrane for 5 x 10mins with PBS-T on a rocking platform.
Dilute selected anti-rabbit IgG secondary antibody according to the manufacturer's instructions.

NOTE: We recommend using Goat polyclonal secondary antibody to rabbit IgG-H&L (HRP) (ab97051).

7. Incubate membrane with secondary antibody solution for 1 hour at room temperature on a rocking platform, or as specified by the manufacturer.
8. Wash membrane for 5 x 10 mins with PBS-T on a rocking platform.
9. 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).
10. Incubate membrane with Western blotting detection reagent for 1 minute.
11. Detect emitted signal by Luminography or CCD imaging instrument.

8. Data Analysis

Example results for Western blotting

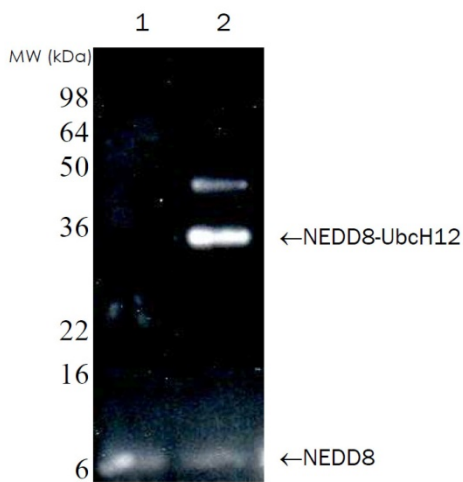


Figure 1: Western Blot of NEDD8-UbcH12 thioester assay. Procedures as described in Section 7.A. “NEDDylation Assay” section. NEDD8 modified proteins were detected by Western Blotting using NEDD8 antibody as described in “Western Blot Analysis”.

Lane 1: absence of Mg-ATP

Lane 2: presence of Mg-ATP

Results demonstrate formation of NEDD8-UbcH12 thioester in the presence of Mg-ATP (2). A second higher molecular weight NEDD8 detectable band (approx. ~44kDa) is sometimes observed, possibly due to NEDD8-NEDD8 isopeptide linkage formation giving (NEDD8)₂-UbcH12.

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