Product Manual

NEDDylation Kit

Catalog #: BML- UW0590

Provides the means of generating a thioester-linked NEDD8-conjugated E2 enzyme

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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

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 glycine[76] 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[1].

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[2,3].

An increasing number of substrates for NEDDylation, including the tumor suppressors p53 and VHL[4,5], 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[6].

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[7,8]. NEDDylation of p53 is mediated by its ubiquitin E3 ligase Mdm2, providing an additional control mechanism for inhibition of its transcriptional activity[9]. E3 independent NEDDylation of p53 has also been demonstrated *in vitro*[9].

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[10].

KIT DESCRIPTION

This kit 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 20µL reactions.

Note: This Kit does not contain any NEDD8 E3 ligases.

SUGGESTED USES

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 (Cat. # UW9770).
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.

KIT COMPONENTS

1. **NEDD8 activating enzyme E1 (h), (rec) (His-tag):**

   ENZ-PRT112-0040

   10x NEDD8 Activating Enzyme Solution (E1)

   Use 2µL per 20µL reaction.

   40µL provided, sufficient for 20x 20µL reactions.

2. **UbcH12 (h) (rec) (His-tag):**

   (BML-UW9145-0020).

   20x UbcH12 Solution (E2)

   Use 1µL per 20µL reaction.

   20µL provided, sufficient for 20x 20µL reactions.

3. **NEDD8 (human), (rec):**

   (BML-UW9225-0040).

   10x NEDD8 Solution

   Use 2µL per 20µL reaction, as required.

   40µL of each provided, sufficient for 20x 20µL reactions.

4. **10x NEDDylation Buffer:**

   BML-KW0750-0040

   Use 2µL per 20µL reaction.

   40µL provided, sufficient for 20x 20µL reactions.

5. **Mg^{2+}/ATP Solution (20x):**

   BML-EW9805-0025

   Use 1µL per 20µL reaction.

   25µL provided, sufficient for 20x 20µL reactions.

6. **NEDD8, pAb:**

   BML-PW9340A-0025

   NEDD8, rabbit polyclonal antibody

   25µL provided. Dilution of 1:1000 recommended for Western blotting.

7. **2x Non-reducing Gel Loading Buffer:**

   BML-KW9880-0400

   Use 20µL per 20µL reaction

   400µL provided. Sufficient for 20x 20µL reactions.

STORAGE

Store all components at -70°C for the highest stability. Components with labeled storage temperatures other than -70°C can be stored at the temperature listed OR at -70°C.Avoid multiple freeze/thawing.

OTHER MATERIALS REQUIRED

1. Eppendorf tubes.
2. Dithiothreitol (1M in 20mM Tris-Cl, pH7.5) e.g. MELFORD MB1015
3. Inorganic pyrophosphatase solution (100U/mL in 20mM Tris-Cl, pH 7.5) e.g. pyrophosphatase, inorganic, bakers yeast, Fluka, 83205.
4. Double distilled water (ddH2O)

NEDDYLATION ASSAY

Overview

Assay described 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 optimization when used in conjunction with specific NEDD8 E3 ligase/target proteins.

Assay protocol

Recommended total reaction volume = 20µL.

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 (neg control)
dH2O	7.6µL	8.6µL
10x NEDDylation Buffer	2.0µL	2.0µL
10x NEDD8 E1	2.0µL	2.0µL
20x UbcH12	1.0µL	1.0µL
10x NEDD8	2.0µL	2.0µL
50mM DTT	0.4µL	0.4µL
100U/mL IPP	4.0µL	4.0µL
20x Mg-ATP	1.0µL	0µL

Set-up/run assay and control reactions as follows:

1. Add assay components to 0.5mL Eppendorf tube(s) in order shown in table above. Keep all enzymes on ice throughout.
2. Mix tube contents gently.
3. Incubate at 37ºC for 60 minutes.

Processing reactions:

4. Quench assays by addition of 20µL 2x non-reducing SDS-PAGE Gel Loading Buffer. DO NOT HEAT/BOIL
5. Proceed directly to “Analysis by Western Blotting” or store at –20ºC until ready.

Note: If active/native NEDD8-UbcH12 thioester is required for use in subsequent experiments either take a 5µL sample from the reaction to test for thioester formation (add 5µL 2x non-reducing SDS-PAGE Gel Loading Buffer to quench) or repeat reaction without quenching once successful thioester formation has been confirmed by Western blotting.

ANALYSIS OF NEDD8-UBCH12 THIOESTER FORMATION 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-T solution.
4. Probe with NEDD8 antibody.

Develop with Western blotting detection reagents.

Materials required:

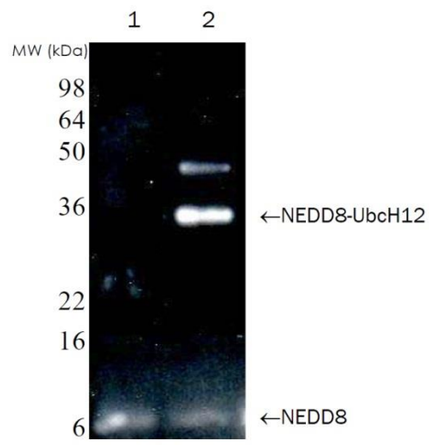
1. SDS-PAGE Gels (13.5% Standard / 4-15% Linear Gradient).
2. Pre-stained SDS-PAGE molecular weight markers (e.g. See Blue Plus 2, pre-stained SDS-PAGE markers, Invitrogen, LC5925).
3. PVDF membrane (e.g. Immobilon-P PVDF Membrane (0.45µm, 26.5cm (w)), Millipore, IPVH00010).
4. Anti-rabbit IgG secondary antibody (HRP linked) (e.g. Goat Anti-Rabbit IgG-peroxidase antibody, Sigma, A0545).
5. Western blotting detection reagents (e.g. Clarity™ Western ECL substrate, Bio-Rad).
6. PBS Solution. 1x PBS.
7. PBS-T Solution. 1x PBS containing 0.2% Tween 20 (e.g. Sigma, P1379).
8. BSA/PBS-T Blocking Solution. PBS-T containing 1% Bovine Serum Albumin (BSA) (e.g. Albumin [bovine serum], Sigma, A7906).

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 10µL of each quenched assay solution to the 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 membrane with BSA/PBS-T blocking buffer for 1 hour at room temperature on a rocking platform.
3. Wash membrane for 3x10mins 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.
6. Wash membrane for 5x10mins with PBS-T on a rocking platform.
7. Dilute selected anti-rabbit IgG secondary antibody according to the manufacturer's instructions (e.g. Sigma Goat Anti-Rabbit IgG-peroxidase antibody (A0545) diluted 1:5000 in BSA/PBS-T).
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 5x10mins with PBS-T on a rocking platform.
10. Prepare Western blotting detection reagent according to the manufacturer's instructions.
11. Incubate membrane with Western blotting detection reagent for 1 minute.
12. Detect emitted signal by luminography or CCD imaging instrument.

Example result for Western blotting



Western Blot of NEDD8-UbcH12 thioester assay. Reactions set-up and run in the absence (1) and presence (2) of Mg^{2+}-ATP as described in “NEDDylation Assay”. NEDD8 modified proteins were detected by Western Blotting using NEDD8 antibody as described in “Analysis of NEDD8-UbcH12 thioester formation by Western Blotting”.

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.

REFERENCES

1. Pan, Z.Q., Kentsis, A., Dias, D. C., Yamoah, K., and Wu, K. Nedd8 on cullin: building an expressway to protein destruction. Oncogene. 23, 1985-1997 (2004)
2. Gong, L. and Yeh, E. T. Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway. J.Biol.Chem. 274, 12036-12042 (1999)
3. Huang, D.T., Walden, H., Duda, D., and Schulman, B. A. Ubiquitin-like protein activation. Oncogene. 23, 1958-1971 (2004)
4. Harper, J.W. Neddylating the guardian; Mdm2 catalyzed conjugation of Nedd8 to p53. Cell. 118, 2-4 (2004)
5. Stickle, N.H., Chung, J., Klco, J. M., Hill, R. P., Kaelin, W. G., Jr., and Ohh, M. pVHL modification by NEDD8 is required for fibronectin matrix assembly and suppression of tumor development. Mol.Cell Biol. 24, 3251-3261 (2004)
6. Willems, A.R., Schwab, M., and Tyers, M. A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. Biochim.Biophys.Acta. 1695, 133-170 (2004)
7. Morimoto, M., Nishida, T., Nagayama, Y., and Yasuda, H. Nedd8-modification of Cul1 is promoted by Roc1 as a Nedd8-E3 ligase and regulates its stability. Biochem.Biophys.Res.Commun. 301, 392-398 (2003)
8. Read, M.A., Brownell, J. E., Gladysheva, T. B., Hottelet, M., Parent, L. A., Coggins, M. B., Pierce, J. W., Podust, V. N., Luo, R. S., Chau, V., and Palombella, V. J. Nedd8 modification of cul-1 activates SCF(beta(TrCP))-dependent ubiquitination of IkappaBalpha. Mol.Cell Biol. 20, 2326-2333 (2000)
9. Xirodimas, D.P., Saville, M. K., Bourdon, J. C., Hay, R. T., and Lane, D. P. Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell. 118, 83-97 (2004)
10. Chiba, T. *In vitro* systems for NEDD8 conjugation by Ubc12. Methods Enzymol. 398, 68-73 (2005)

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