

MATERIAL DATA SHEET**NEDD8 Conjugation Kit****Cat. # K-800**

The ubiquitin-like NEDD8 is conjugated to a variety of proteins in the presence of Ubc12 and the NEDD8 E1 activating enzyme (UBA3/NAE1 in humans). The heterodimeric UBA3/NAE1 complex (52 and 60 kDa respectively) uses ATP to adenylate the C-terminal glycine residue of a NEDD8 protein; in a second step a thioester bond is formed between NEDD8 and a cysteine residue within the UBA3 subunit. Following this activation, NEDD8 is transferred to Cys111 of Ubc12. Ubc12 (also known as UBE2M) is a member of the E2 family and interacts with Rbx1 to conjugate NEDD8 to a variety of target proteins, most notably Cullin family members CUL1, CUL2, CUL3 and CUL4.

NOTE: Kit contains reagents sufficient for 10 x 20 µl reactions.

Reagents Provided in Kit	
<u>Component</u>	<u>Volume</u>
1. 10X NEDD8 E1 enzyme	20 µl
2. 10X NEDD8	20 µl
3. 10X Ubc12 (UBE2M)	20 µl
4. 10X Reaction Buffer	20 µl
5. 10X Mg ²⁺ -ATP Solution	20 µl

Storage: Store protein components at -80°C. Avoid multiple freeze/thaw cycles.

Reagents to be Provided by Investigator

The following reagents and materials need to be obtained by the investigator prior to using this kit.

Waterbath or heating block	37°C
Reaction tubes	0.5 ml or 1.5 ml polypropylene, microcentrifuge compatible
dH ₂ O	Sterile
EDTA	0.5 M solution (optional)
Dithiothreitol (DTT)	1M in dH ₂ O (Pierce #20290) or similar (optional)
SDS-PAGE Sample Buffer	(optional)

Assay Considerations

The included protocol is provided as a general guideline for a 20 µl volume reaction. The recommended substrate volume of 5 µl may be altered to accommodate experimental design, and the total reaction size may be scaled up or down depending on individual requirements. Individual results may vary with different conditions and substrates—it is up to the end-user to optimize the reaction. The kit protocol suggests that reactions be terminated with SDS-PAGE Sample Buffer; therefore, proteins are denatured and typically not suitable for further enzymatic. If NEDDylated samples are to be used in downstream applications the reactions may be terminated by adding EDTA and DTT (10-20 mM final) if compatible with experimental design.

When complex samples (such as lysates or partially purified proteins from eukaryotic cells) are used as a source of substrate, the user may want to consider adding NEDD8-Vinyl Sulfone (UL-802) to reactions to counteract the presence of Nedd8-specific proteases that may be present. In such instances the suggested starting concentration of inhibitor is 1-5 µM final, and the volume of water in the reactions should be adjusted to compensate for the added inhibitors.

Further information is available via techsupport@bostonbiochem.com

Recommended Assay Protocol (20 µl volume)

1. Reagent Preparation
 - a. Quickly thaw all protein reagents and buffers by gently and continuously swirling tubes in a lukewarm water bath ($\leq 30^{\circ}\text{C}$). Alternatively, tubes may be thawed with a rapid back-and-forth rolling motion between palms of hands. Do not heat tubes for an extended period of time. Do not vortex or shake vigorously.
 - b. When completely thawed, *gently* tap tubes to make sure components are well mixed and then briefly spin in a microcentrifuge (5 seconds) to collect contents in bottom of tubes.
 - c. Immediately ice components. Entire process from steps 1a-1b should be accomplished in ≤ 5 minutes.
 - d. It is ***strongly recommended*** that each reagent be divided into smaller aliquots to minimize the number of freeze-thaw cycles of kit components. Avoid multiple freeze-thaw cycles to maximize kit performance. Rapidly re-freeze any aliquoted materials in dry ice bath.
2. Reaction Assembly
 - a. Prepare reactions on ice in 0.5 or 1.5 ml polypropylene tubes using the following volumes and order of addition:
 - i. 5 µl dH₂O
 - ii. 2 µl 10X Reaction Buffer. Mix gently following addition.
 - iii. 5 µl Substrate protein and E3 ligase (such as Rbx1) at a suggested final concentration of 2.5-10 µM each
 - iv. 2 µl 10X NEDD8 E1 enzyme
 - v. 2 µl 10X Ubc12/UBE2M (E2) enzyme
 - vi. 2 µl 10X NEDD8
 - b. Any pre-reaction incubations may be done at this point (e.g. treatment of complex lysates with SENP inhibitors). Addition of Mg²⁺-ATP in the next step will start the reaction.
 - c. Add 2 µl of 10X Mg²⁺-ATP solution. Mix by gently pipetting up and down 2-3 times. For negative control reactions, omit ATP addition and replace with 2 µl dH₂O.
 - d. Spin tubes to collect contents and place reactions in 37°C water bath.
 - e. After 30-60 minutes, terminate reactions with addition of 5 µl 5X Loading Buffer (SDS-PAGE sample buffer) and 1 µl 1M DTT. (An initial time course is recommended to determine the optimal incubation time for efficient substrate conjugation.)
 - f. Heat reactions to 90°C for 5 minutes.

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Literature

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For help with this kit, e-mail: techsupport@bostonbiochem.com

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Rev: 08/03/2015

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