Lot # XXXXX

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MATERIAL DATA SHEET

Ubiquitin Conjugation Initiation Kit
Cat. # K-995

This kit is designed for the purpose of charging (activating) Ubiquitin with an E1 Ubiquitin Activating Enzyme, the first reaction in the Ubiquitin conjugation cascade. This E1-Ubiquitin thioester can be subsequently used for the initiation of E1-mediated *in vitro* reactions including E2-Ubiquitin thioester formation and the ubiquitination of target substrate proteins with the addition of appropriate E2 and E3 Ubiquitin Ligase enzymes (not supplied).

NOTE: Kit contains reagents sufficient for 12 x 20 µl reactions.

Reagents Provided in Kit

Component	Volume
1. 20X E1 Enzyme	15 µl
2. 10X Mg^{2+}-ATP Solution	25 µl
3. 10X Ubiquitin Solution	25 µl
4. 10X Reaction Buffer	25 µl
5. 5X Loading Buffer (non-reducing)	80 µl

Storage: Store proteins, Reaction Buffer and ATP solution at -20°C or -80°C. Avoid multiple freeze/thaw cycles. 5X Loading Buffer may be stored at room temperature.

Reagents to be Provided by Investigator

The following reagents and materials are not supplied with the kit and may need to be obtained by the investigator depending on experimental design.

Waterbath	37°C
Reaction tubes	Polypropylene, microcentrifuge compatible
EDTA	0.5 M solution, sterile (optional)
Dithiothereitol (DTT)	1M in dH_2O (Pierce #20290) or similar (optional)
E2 Conjugating Enzyme	Choices available from Boston Biochem (optional)
E3 Ubiquitin Ligase	Choices available from Boston Biochem (optional)
Substrate Protein	Choices available from Boston Biochem (optional)

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Assay Considerations

The included protocol is provided as a general guideline for a 20 µl reaction. Reaction volume may be scaled accordingly depending on individual requirements. Variations (+/- 10-20%) in the final concentration of kit components in reactions are typically well-tolerated. Individual results may vary with different conditions and substrates—it is up to the end-user to optimize the reaction.

The kit protocol suggests terminating reactions with EDTA and DTT. Either compound *may* interfere with downstream applications depending on experimental design. Examples include the analysis of Ubiquitin-E2 or Ubiquitin-E3 thioesters (which are DTT-labile), and proteasomal degradation assays (which may be inhibited by EDTA). In such instances different means of halting the conjugation reaction—including purification of the final product(s)—may be required.

This kit may be used in a wide variety of experimental designs. If added, we suggest the following starting concentrations of user-supplied components:
E2 conjugating enzymes: 0.1-2 µM; E3 Ubiquitin Ligases: 0.2-5 µM;
Substrates for E3 Ligases: 0.1-10 µM.

Further information is available via techsupport@bostonbiochem.com.

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Example 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 (≤ 30°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 or invert gently 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. Steps 1a-1b should be accomplished in approximately 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 a pre-reaction mix in 0.5 or 1.5 ml polypropylene tubes using the following volumes:
        7 µl dH_2O
        2 µl 10X Reaction Buffer
        2 µl 10X Ubiquitin
        1 µl 20X E1 Enzyme
        2 µl 10X E2 conjugating enzyme (user supplied, optional)
        2 µl 10X E3 Ligase enzyme (user supplied, optional)
        2 µl 10X Ligase substrate (user supplied, optional)

        dH_2O may be substituted for any components that are not present in the investigators experimental design.
    b. Initiate conjugation reaction by adding 2 µl of 10X Mg^{2+}-ATP solution. Mix by pipetting or gently flicking tube.
    c. Spin tube briefly in microcentrifuge to collect contents. Incubate for desired period of time in 37°C water bath depending on individual conditions and requirements.
    d. Reaction may be terminated with EDTA or DTT, 10 mM final.
    e. Reaction may be analyzed via SDS-PAGE gel. Western blotting with an anti-substrate antibody may be used to determine conjugate formation, which may appear as either a high molecular weight smear, or discrete banding pattern.

For Laboratory Research Use Only, Not For Use in Humans

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