Ubiquitin Conjugation Kit

Catalog Number K-960

This kit is for the formation of ubiquitinated substrate proteins.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

This kit is for the formation of ubiquitinated substrate proteins. The enzymes supplied represent the full complement of purified conjugation enzymes (E1, E2s, and E3s) that are found in Fraction II (F-360 or F-370). Conjugation Fraction A contains many E1 and E2 enzymes, while Conjugation Fraction B contains E3 and deubiquitinating enzymes. The enzymes have been tested and shown to work with typical [^{125}I]-labeled substrate proteins such as lysozyme and β-lactoglobulin. This conjugation fraction also contains ubiquitin C-terminal Hydrolases and therefore the addition of Ubiquitin Aldehyde (U-201) or Ubiquitin Vinyl Sulfone (U-202) is recommended for the inhibition of UCHs to improve overall conjugate yield. The supplied fractions do not contain 20S or 26S proteasome-mediated protein degradation activity. If the substrate being conjugated requires E2 or E3 enzymes not found in Fraction II, the reaction can be supplemented with purified recombinant enzymes, or enzyme mixes found in Fraction I (F-375).

TECHNICAL HINTS

- The included protocol is provided as a general guideline for a 100 µL volume reaction. Reaction size may be increased or decreased depending on individual requirements. Slight variations (+/- 10%) in final volume are acceptable. 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. EDTA may interfere with downstream applications that require Mg^{2+}-ATP, such as proteasomal degradation assays, and therefore may need to be removed by desalting or purification of the final product(s).
- Conjugation fractions are rich in deubiquitinylating enzymes (DUBs) that may rapidly remove ubiquitin from target proteins. The inclusion of deubiquitinase inhibitors (available separately) is highly recommended to maximize ubiquitinated substrate products.
- Further information is available via techsupport@bostonbiochem.com.

MATERIALS PROVIDED & STORAGE CONDITIONS

Kit contains reagents sufficient for 6 x 100 µL reactions.

COMPONENT	VOLUME	STORAGE OF COMPONENTS
2.5X Conjugation Fraction A	250 µL	Store at -80 °C.* Avoid multiple freeze-thaw cycles.
2.5X Conjugation Fraction B	250 µL	
15X Ubiquitin Solution	50 µL	
10X Energy Solution	75 µL	

*Provided this is within the expiration date of the kit.

OTHER MATERIALS REQUIRED

- **Waterbath or heating block:** 37 °C
- **Reaction tubes:** 0.5 mL or 1.5 mL polypropylene, microcentrifuge compatible
- **dH_2O:** Sterile
- **Deubiquitinase Inhibitor:** Ubiquitin-aldehyde, Ubiquitin-Vinyl Sulfone, or Ubiquitin Vinyl Methyl Ester (U-201, U-202, or U-203); optional
- **EDTA:** 0.5 M solution; optional
- **Dithiothreitol (DTT):** 1M in dH_2O (Pierce #20290) or similar

ASSAY PROTOCOL

Reagent Preparation

1. 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.
2. When completely thawed, **gently** tap tubes or invert gently to make sure components are well mixed, then briefly spin in a microcentrifuge (5 seconds) to collect contents in bottom of tubes.
3. Immediately ice components. Steps 1-2 should be accomplished in approximately 5 minutes.
4. 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.

Reaction Assembly

1. Prepare reactions in 0.5 or 1.5 mL polypropylene tubes using the following volumes and order of addition:
    a. 40 µL 2.5X Conjugation Fraction A
    b. 40 µL 2.5X Conjugation Fraction B
    c. 6.7 µL 15X Ubiquitin Solution
2. We strongly recommend the addition of a deubiquitinase inhibitor such as Ubiquitin-aldehyde, Ubiquitin-Vinyl Sulfone, or Ubiquitin Vinyl Methyl Ester (U-201, U-202, or U-203) at this point. Optimally, 1or 2 µL of 250 µM inhibitor in DMSO (2.5-5 µM inhibitor and
1-2% DMSO, final) should be added, followed by gentle mixing and incubation for 5-10 minutes at 37 °C to allow for inhibition of deubiquitinating enzymes (USPs, UCHs).
For best results, do not omit this pre-incubation step.
3. Add 10 µL of 10X Energy Solution.
4. Initiate conjugation reaction by addition of 50-500 ng of substrate protein. Mix by gentle vortexing or pipetting up and down 2-3 times.
5. Spin tubes to collect contents and incubate 0.5-4 hours in 37 °C water bath depending on individual conditions and requirements. Determine optimal incubation times by monitoring 30 or 60 minute time-points during the first experiment. Quench samples with EDTA and DTT, 10 mM final.
6. Analyze reactions for substrate ubiquitination by running an aliquot on an 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.
7. Once optimal conditions have been determined, the reaction may be aliquoted and frozen for future use. If desired, the conjugates may also be purified using standard chromatographic procedures.

REFERENCES

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