CREATIVE BIOMART®
Assay Kit

Ubiquitinylation kit

Product Information

Short Name

Ubiquitinylation kit

Cat.No. KITZ-010

Size

50 x 50 µl reactions

Product Overview

Ubiquitinylation kit provides the means of generating a range of thioester-linked ubiquitin conjugation enzymes (E2s), utilizing the first two steps in the ubiquitin cascade; for use in the transfer of ubiquitin to E3 ligases; and the subsequent ubiquitinylation of target proteins. Biotinylated ubiquitin provided supports thioester formation and high sensitivity detection of ubiquitin conjugates. Suggested uses: Generation of ubiquitin-E2 thioesters for use in ubiquitinylation experiments, Ubiquitinylation of target proteins in the presence of a dedicated E3 ligase, Activation of ubiquitin for thioester conjugation to novel E2 enzymes, Use of cell lysate or crude fractions/preparations as source of E3 ligases to facilitate ubiquitinylation, Substrate (target) independent in vitro ubiquitinylation reactions.

Applications

For generation of ubiquitin-E2 thioesters for use in ubiquitinylation experiments.

Storage

All kit components should be stored at -80°C to ensure stability and activity.

Shipping

Dry ice

Kit Components

1. 20x Ubiquitin Activating Enzyme Solution (E1): 1 vial
E1 (human) (recombinant) (His-tag), Use 2.5 µL per 50 µL reaction. 125 µL provided, sufficient for 50 x

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50 µL reactions.
2. 10x Ubiquitin Conjugating Enzyme Solutions (E2): 11 vials:
UbcH1 (human), (recombinant) (His-tag)
UbcH2 (human), (recombinant) (His-tag)
UbcH3 (human), (recombinant) (His-tag)
UbcH5a (human), (recombinant) (His-tag)
UbcH5b (human), (recombinant) (His-tag)
UbcH5c (human), (recombinant) (His-tag)
UbcH6 (human), (recombinant) (His-tag)
UbcH7 (human), (recombinant) (His-tag)
UbcH8 (human), (recombinant) (His-tag)
UbcH10 (human), (recombinant) (untagged)
UbcH13/Mms2 (human), (recombinant) (His-tag)
Use 5 µL per 50 µL reaction. 20 µL of each E2 provided, sufficient for 4 x 50 µL reactions.
Note: UbcH2 is sensitive to reducing agents such as DTT and BME. Do not use reducing agents with this enzyme. Assays performed with this enzyme must use only nonreducing buffers.
3. 20x Biotinylated Ubiquitin Solution (Bt-Ub): 1 vial:
Biotinylated-ubiquitin. Use 2.5 µL per 50 µL reaction, 125 µL provided, sufficient for 50 x 50 µL reactions.
4. 20x Mg-ATP Solution: 1 vial:
Mg-ATP. Use 2.5 µL per 50 µL reaction, 125 µL provided, sufficient for 50 x 50 µL reactions.
5. 2x Non-reducing Gel Loading Buffer:
2 vials of 1.25 mL, Use 50 µL per 50 µL reaction. 2.5 mL provided, sufficient for 50 x 50µL reactions
6. 10x Ubiquitinylation Buffer: 1 vial:
Use 5 µL per 50 µL reaction, 250 µL provided, sufficient for 50 x 50 µL reactions.

Materials Required but Not Supplied

1. Eppendorf tubes
2. EDTA solution (50mM in 20mM Tris-HCl, pH7.5)
3. Inorganic pyrophophatase solution (100U/mL in 20mM TrisHCl, pH7.5)
4. DTT (Dithiothreitol) solution* (50mM in 20mM Tris-Cl, pH7.5)

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*Please see note concerning sensitivity of UbcH2 (BML-UW9025) to reducing agents.

Assay Protocol

Assay protocol:
A. Overview Two types of reaction described, using same basic assay set-up:
1. E3 mediated ubiquitinylation of target/substrate proteins
2. Ubiquitin-E2 thioester (TE) bond formation (essential control for assay 1)
Note: Assay set-up can be readily modified for alternative applications (as outlined previously) by inclusion, omission or substitution of specific enzyme components.
B. Standard assay set-up
Note: Suggested E2/E3/target protein concentrations are given as a starting point for such reactions and will require optimisation for specific enzymes/combinations.
Component Concentration Notes
Ub 2.5μM Supplied as a 50μM (0.45 mg/mL; 20x) solution
E1 100nM Supplied as 2μM (20x) solution
E2 ~1μM-2.5μM Supplied as a (10x) solution
Mg-ATP 5mM Supplied as 100mM (20x) solution
E3 100nM Ideally available as 2μM (20x) solution
Target 1μM* 10μM (10x) solution
Target protein concentration can vary in range of 0.5-2μM. Concentration in table is a suggested initial starting concentration and is subject to be optimized based on target protein.
C. Assay protocol
Note: recommended total reaction volume = 50 μL.
Note: UbcH2 is sensitive to reducing agents. Do not use DTT with UbcH2
Component Target-Ub Target Ubiquitin –ve control TE +ve control TE –ve control
dH2O 14 11.5 21.5 19
10x Ubiquitinylation Buffer 5 5 5 5
IPP (100U/mL) 10 10 10 10
DTT (50mM) 1 1 1 1
Mg-ATP 2.5 - 2.5 –
EDTA (50mM) - 5 – 5

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20x E1 2.5 2.5 2.5 2.5
10x E2 5 5 5 5
20x E3 2.5 2.5 - -
10x Target protein* 5 5 - -
20x Bt-Ub 2.5 2.5 2.5 2.5
* Volume of target protein is subject to adjustment depending on desired final concentration in reaction mix.
D. Set-up assays/controls required (keep all enzymes on ice throughout)
3. Add assay components to 0.5 mL Eppendorf tube(s) in order shown above.
4. Mix tube contents gently.
5. Incubate at 37°C for 30-60 minutes. For enhanced results, samples may be incubated for 4-8 hours at 37°C.
6. Quench assays by addition of 50 µL 2x Non-reducing gel loading buffer.
7. Proceed directly to "Analysis by Western Blotting" or store at –20°C until ready.
Analysis by western blotting
a) 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 HRP-Streptavidin detection system.
5. Develop with western blotting detection reagents.
b) Materials Required
1. SDS-PAGE Gels (user prepared (12% Standard / 4-15% Linear Gradient) or precast.
2. Biotinylated/pre-stained SDS-PAGE molecular weight markers
3. Nitrocellulose or PVDF membrane
4. Streptavidin-HRP conjugate
5. Western blotting detection reagents
6. TBS Solution.
7. TBS-T Solution. TBS containing 0.1% Tween 20
8. BSA/TBS-T Blocking Solution. TBS-T containing 1% Bovine Serum Albumin (BSA)

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c) 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 15 µL of each quenched assay solution to the gel, alongside selected molecular weight markers, electrophorese and transfer protein to nitrocellulose or PVDF membrane according to standard procedures.
2. Remove membrane from the transfer unit and block membrane with BSA/TBS-T blocking buffer for 1 hour at room temperature on a rocking platform, or overnight at 4°C.
3. Wash membrane for 3 x 10mins with TBS-T on a rocking platform.
4. Prepare Streptavidin-HRP solution according to the manufacturer's instructions. (recommended working concentration is between 100ng/mL – 1µg/mL).
5. Incubate membrane with Streptavidin-HRP solution for 1 hour at room temperature on a rocking platform.
6. Wash membrane for 6 x 10mins with TBS-T on a rocking platform.
7. Prepare Western blotting detection reagent according to the manufacturer's instructions.
8. Incubate membrane with Western blotting detection reagent for 1 minute.
9. Detect emitted signal by Luminography or CCD imaging instrument.

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