CREATIVE BIOMART®
Assay Kit

Auto-ubiquitinylation kit

Product Information

Cat.No. Kit-2589

Product Overview

This E3 ligase auto-ubiquitinylation kit enables proteins to be tested for ubiquitin E3 ligase activity through assessment of their ability to undergo auto-ubiquitinylation. Utilizing the first three steps in the ubiquitin cascade the kit facilitates ubiquitinylation of known or putative E3 ligase enzymes followed by Western blot analysis using the highly sensitive reagents provided or using antibodies to the specific protein of interest (user supplied). A high integrity ubiquitin E3 ligase enzyme is also provided for use as a positive control. The Kit provides sufficient material for approximately 10 autoubiquitinylation assays.

Applications

1. Qualitative assessment of an Ub E3 ligase enzyme's activity through its ability to auto-ubiquitinylate.
2. Testing of proteins for auto-ubiquitinylation activity allowing their identification as putative ubiquitin E3 ligases.
3. Ubiquitinylation of substrate proteins (user provided) specific to a particular ubiquitin E3 ligase.

Storage

-80°C

Shipping

Shipped on Dry Ice

Kit Components

20X Ubiquitin Activating Enzyme Solution (E1), 25µl
20X Ubiquitin Conjugating Enzyme Solution (E2), 25µl
10X Ubiquitin Solution (Ub), 50µl
20X Control Ubiquitin Ligase Solution (E3), 25µl
20X Mg-ATP Solution, 25µl
10X Ub E3 ligase Buffer, 50µl

Tel: 1-631-559-9269 1-516-512-3133
Email:info@creative-biomart.org
Fax:1-631-938-8127
45-1 Ramsey Road, Shirley, NY 11967, USA

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Ubiquitin antibody solution, 25µl

Materials Required but Not Supplied

1. Eppendorf tubes (0.5 mL)
2. 2x SDS-PAGE gel loading buffer (e.g. 0.25M Tris-Cl, pH6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.01% bromophenol blue).
3. DTT (Dithiothreitol) solution (50mM in dH20)
4. Inorganic pyrophosphatase solution (IPP) (100U/mL in 20mM Tris-HCl, pH7.5)
5. 10X PBS solution or 10X TBS solution
6. Tween 20 solution

Assay Protocol

Set-up assays/controls required as follows:
1. Add assay components to 0.5 mL Eppendorf tube(s) in order shown in table bellow. Keep all enzymes on ice throughout.

Component	Sample	E3-Ub Sample	E3 (–ve control)	Hdm2-Ub (+ve control)	Hdm2 (–ve control)
Volume/µL					
dH2O	21.5*	24.0*	19.0	21.5	
10x Ub E3 ligase buffer	5.0	5.0	5.0	5.0	
20x Ub E1	2.5	2.5	2.5	2.5	
20x E2	2.5	2.5	2.5	2.5	
10x Ubiquitin	5.0	5.0	5.0	5.0	
20x E3 control (6µM)	-	-	2.5	2.5	
*Sample E3 protein	X	X	-	-	
50mM DTT	1.0	1.0	1.0	1.0	
20x Mg2+-ATP	2.5	-	2.5	-	
IPP ** (100U/mL)	10	10	10	10	

Note: recommended total reaction volume = 50µL.
* Adjust dH2O volume in accordance with available Ub E3 ligase protein concentration. A final assay concentration of 150-300nM is recommended as a starting point for Ub E3 ligase

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autoubiquitinylation (e.g. use 2.5µL of 6µM Ub E3 ligase protein solution).
** Inorganic pyrophosphatase solution (IPP) is recommended to be included in auto-ubiquitinylation reaction but it is not absolutely necessary. Adjust dH2O volume if IPP is not included in reaction. Negative control reactions omitting Mg2+-ATP cofactors demonstrate formation of auto-ubiquitinylated proteins is ATP-dependent (required for E1 activation, also known as one of the critical characters of ubiquitin activating enzyme E1) and hence derived from the ubiquitin cascade.
2. Mix tube contents gently.
3. Incubate at 37°C for 60 minutes.
4. Quench assays by addition of 50µL 2x SDS-PAGE gel loading buffer followed by heating to 95°C for 5 minutes or 70°C for 10 minutes.
Note: This step removes all Ub thioester linked species (Ub-E1/Ub-E2) so only isopeptide linked Ub-E3 species are detected using ubiquitin antibody/Western blotting.
5. Proceed directly to "Analysis by Western blotting" or store at –20°C until ready.

Analysis

Summary of analysis steps
1. Separate proteins by SDS-PAGE.
2. Western transfer to nitrocellulose or PVDF membrane.
Note: Western blotting conditions appropriate for the transfer of large proteins may be required to ensure good transfer of Ubiquitinylated-E3 protein to PVDF membrane. For example, use BSN transfer buffer 48mM Tris, pH9.2, 39mM glycine with 10% MeOH and 0.0375% SDS.
3. Block membrane with BSA/PBS-T solution.
4. Probe blot with either:
a) ubiquitin antibody supplied
or b) appropriate target protein specific primary antibody in conjunction with suitable secondary antibodies.
5. Develop with Western blotting detection reagents.
Note: Do NOT use milk in blocking/antibody binding solutions. Please use 5% BSA in PBS-Tween or TBS-Tween instead.