E3 Ligase Auto-Ubiquitylation Assay Kit

Cat. No. Kit-5900 **Lot. No.** (See product label)

SPECIFICATION

Product Overview	Ubiquitin E3 ligase (EC 2.3.2.B11) carry out the final step in the ubiquitination cascade, catalyzing transfer of ubiquitin from an E2 enzyme to form a covalent bond with a substrate lysine. The covalent attachment of ubiquitin to proteins (ubiquitylation or ubiquitination) plays a fundamental role in the regulation of cellular function through biological events involving cell cycle, differentiation, immune responses, DNA repair, chromatin structure, and apoptosis. E3 ligases also undergo auto-ubiquitinylation, which can be used to detect ubiquitin E3 ligase activity. Utilizing the first three steps in the ubiquitin cascade, the kit facilitates ubiquitylation 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). Note: The Kit provides sufficient material for approximately 10 auto-ubiquitinylation assays. Hdm2 ubiquitin E3 ligase enzyme is also provided for use as a positive control.
Storage	-80°C
Size	10 Tests
Kit Components	10X Ub E3 Ligase Buffer: 500µ 10X Ubiquitin: 500µ 20X Mg-ATP Solution: 250µ

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	20X Ubiquitin Activating Enzyme Solution (E1): 250µ Hdm2 (Human, Recombinant): 250µ UbcH5a (Human, Recombinant): 250µ Ubiquitin Antibody Solution: 250µ
Materials Required but Not Supplied	1. Eppendorf tubes 2. 2x SDS-PAGE gel loading buffer (e.g. 0.25M Tris-Cl, pH 6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.01% bromophenol blue) 3. DTT (Dithiothreitol) solution (50mM in dH20) 4. Inorganic pyrophophatase solution (IPP) (100U/mL in 20mM Tris-Cl, pH7.5) (e.g. pyrophosphatase, inorganic) For Western Blot Analysis 1. SDS-PAGE Gels (User prepared (10% standard / 4-15% linear gradient) or pre-formed. 2. Pre-stained SDS-PAGE molecular weight markers 3. PVDF membrane 4. Anti-rabbit IgG secondary antibody (HRP linked) e.g. Goat polyclonal secondary antibody to rabbit IgG-H&L (HRP) 5. (If required) Target protein specific primary antibody (user supplied) and appropriate secondary antibody-HRP conjugate. 6. Western blotting detection reagents 7. PBS Solution. 1x PBS. 8. PBS-T Solution. PBS containing 0.2% Tween 20 9. BSA/PBS-T Blocking Solution. PBS-T containing 1% Bovine Serum Albumin (BSA)
Detection method	Western Blot
Assay Protocol	Note: recommended total reaction volume = 50 µL. 1. Adjust dH2O volume in accordance with available Ub E3 ligase protein concentration.

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2. 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.
A final assay concentration of 150-300nM is recommended as a starting point for Ub E3 ligase autoubiquitinylation (e.g. use 2.5 µL of 6 µM Ub E3 ligase protein solution)
Negative control reactions omitting Mg-ATP cofactors demonstrate formation of auto-ubiquitinylated proteins is ATP dependent (required for E1 activation) and hence derived from the ubiquitin cascade.

Set-up assays/controls required (keep all enzymes on ice throughout)
1. Add assay components to 0.5 mL Eppendorf tube(s).
2. Mix tube contents gently.
3. Incubate at 37ºC for 1 hour.
4. Quench assays by addition of 50 µL 2x SDS-PAGE gel loading buffer followed by heating to 95ºC for 5 minutes.
Note: This step removes all Ub thioester linked species (UbE1/Ub-E2) so only isopeptide linked Ub-E3 species are detected using ubiquitin antibody/Western blotting.
5. Proceed directly to “Western Blot Analysis” or store at -20ºC until ready

B. Western Blot Analysis
1. Separate proteins by SDS-PAGE.
2. Western Transfer to nitrocellulose/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 48 mM Tris, pH 9.2, 39 mM glycine with 10% MeOH and 0.0375% SDS.
3. Block membrane with BSA/TBS-T solution.
4. Probe with either:

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	a) Ubiquitin Antibody Solution (pAb) 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 1% BSA in PBS or TBS Tween instead.

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