SUMOlink™
in vitro SUMOylation Kits

(version B1)

SUMOlink™ SUMO-1 Kit (Catalog No. 40120)
SUMOlink™ SUMO-2/3 Kit (Catalog No. 40220)

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Overview

The pathway of SUMO (small ubiquitin-like modifier) conjugation affects many biological processes including chromosomal organization and function, DNA repair, nuclear transport and signal transduction pathways. Many SUMO substrates are transcription factors, where SUMO modification is commonly associated with decreased transcriptional activity[1, 2]. However, in some cases SUMOylation may actually augment transcription factor activity[3]. In vertebrates three paralogs, SUMO-1, SUMO-2 and SUMO-3 are expressed.

Active Motif's SUMOlink™ Kits provide a simple method for generating SUMOylated proteins *in vitro*. Using the SUMOylation enzyme cascade, covalent linkage of the carboxy-terminal SUMO-1, -2 or -3 peptide substrates to specific lysine residues on the target protein occurs via isopeptide bonds. SUMOlink Kits provide all the reagents required for performing *in vitro* SUMOylation of target proteins. Applications of the kit include the investigation of SUMOylation on enzyme function, understanding the role of SUMOylation in the regulation of cellular processes and the identification of novel proteins as targets for SUMO.

product	format	catalog no.
SUMOlink™ SUMO-1 Kit	20 rxns*	40120
SUMOlink™ SUMO-2/3 Kit	20 rxns*	40220

*SUMOlink Kits provide reagents to perform 20 *in vitro* SUMOylations with the WT SUMO protein and 20 *in vitro* SUMOylations with the Mutated SUMO protein.

Note: Upon receiving this kit, please store the individual components at the temperatures listed in the Kit Contents section on page 4.

Introduction

Post-translational modifications can alter protein function through modifying activity or intracellular localization. An example is the modification of proteins by covalent attachment of the small protein ubiquitin. In addition to its well-known function to earmark proteins for degradation, modification by ubiquitin (ubiquitylation) serves a variety of different functions (*e.g.* in protein sorting, transcriptional activation or DNA repair)[4].

Modification of proteins by covalent attachment of other proteins is also common. Since 1997, several "ubiquitin like modifiers" (UBLs) have been discovered, with different degrees of sequence identity with ubiquitin. Among these, SUMO (small ubiquitin-like modifier) has received the widest attention[5]. This is because modification by SUMO (SUMOylation) is rather prevalent and several known SUMOylation targets are proteins of singular interest (*e.g.*, p53, IκB and PCNA).

UBLs are conjugated to target proteins by an enzymatic cascade involving a Ubl activating enzyme (E1), a UBL-conjugating enzyme (E2), and typically a UBL protein ligase (E3)[5]. SUMO conjugation involves the same set of enzymatic steps. The SUMO activating enzyme is a heterodimer containing SAE1 and SAE2 subunits (known as Aos1 and Uba2 in yeast)[6].

SUMO is distantly related to ubiquitin (20% identity) and was first identified in mammals, where it was found to be covalently linked to the GTPase activating protein RanGAP1[4, 7]. The pathway of SUMO conjugation affects many biological processes including chromosomal organization and function, DNA repair, nuclear transport and signal transduction pathways. It is also required for cell viability in yeast, nematodes and higher eukaryotes[3, 5, 7]. In vertebrates three paralogs, designated SUMO-1 (also known in humans as Smt3c, PIC1, GMP1, sentrin and Ubl1), SUMO-2 (also known as Smt3a and Sentrin3) and SUMO-3 (also known as Smt3b and Sentrin2) are expressed. The conjugated forms of SUMO-2 and SUMO-3 differ by only three N-terminal residues and have yet to be functionally differentiated. They form a distinct subfamily known as SUMO-2/3 and are 50% identical in sequence to SUMO-1.

SUMO modification of proteins occurs on lysine residues generally found within a short consensus sequence containing the yKXE motif. This motif consists of y which represents a large hydrophobic amino acid (isoleucine, leucine or valine), K is the lysine that becomes modified, X is any residue and E is glutamic acid. The glutamic acid is the most highly conserved position other than the lysine. SUMO-2/3 each have exposed SUMO modification consensus motifs that can be utilized to form polymeric SUMO chains[8]. SUMO-1 has not been observed to form polymers.

SUMO modifications have also been reported as a ubiquitin antagonist because lysine residues act as acceptors for all UBLs and are also sites for methylation and acetylation[9]. Thus, it is possible that SUMO conjugation may block other lysine dependent modifications such as ubiquitination. For example, in unstimulated cells NFκB is maintained in an inactive state by IκB inhibitor proteins. In response to activation signals, IκB undergoes polyubiquitination at lysines 21 and 22, targeting the protein for proteasome-mediated degradation and release of active NFκB. However, IκB, which shuttles between the nucleus and cytoplasm, is also modified by SUMO-1 on lysine 21, thus blocking ubiquitination and stabilizing the protein[6, 9].

Kit Performance

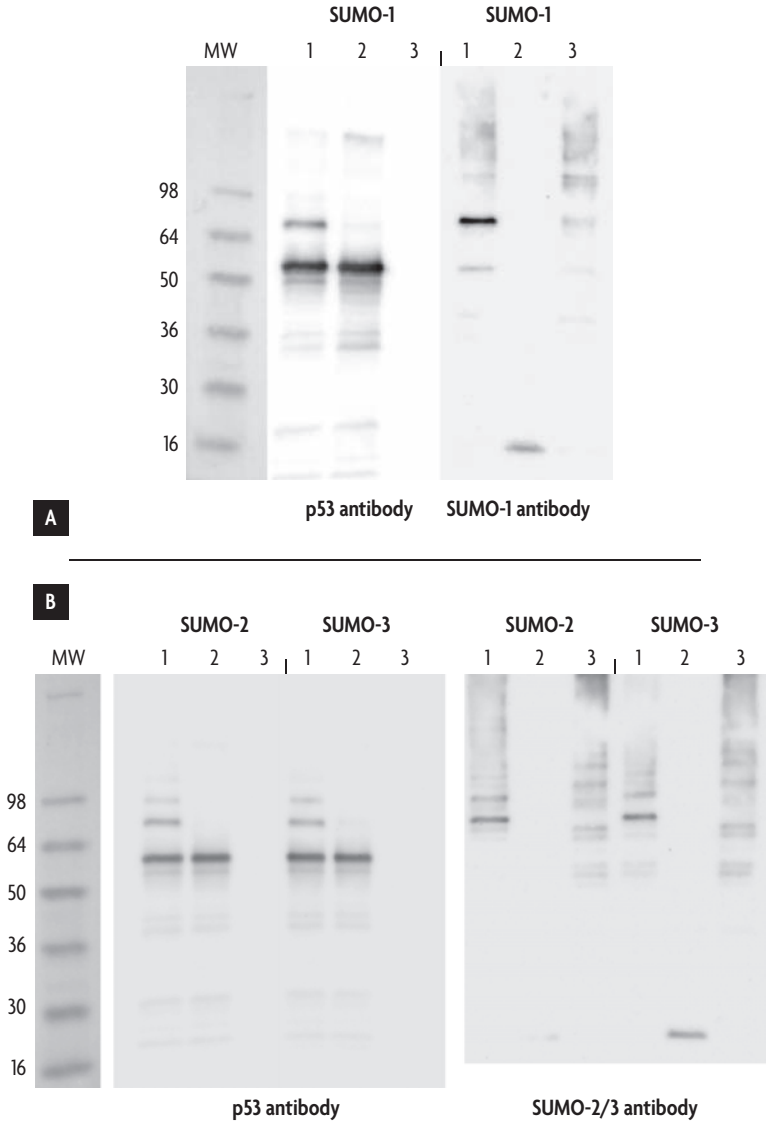

Figure 1: SUMOylation of p53 with SUMO-1, SUMO-2 and SUMO -3 wild-type (WT) isoforms and mutated isoforms.

A. SUMO-1 analysis: Western Blots incubated with p53 antibody (1:5000 dilution), and SUMO-1 antibody (1 :4000 dilution).
B: SUMO-2/3 analysis: Western Blots incubated with p53 antibody (1:5000 dilution) and SUMO-2/3 antibody (1:1000 dilution).

Lane 1: Wild-type SUMO protein conjugation reaction
Lane 2: Mutated SUMO protein conjugation reaction
Lane 3: No p53 control protein used in the conjugation reaction.

Kit Components and Storage

SUMOlink Kits provide enough reagents to perform a total of 40 *in vitro* SUMOylation reactions (20 WT and 20 Mutated). Positive control p53 protein and antibody are supplied for 8 reactions (4 WT and 4 Mutated). Enough SUMO-1 or SUMO-2/3 antibody is provided for 20 Western blots.

Note: SUMOlink Kits are shipped on dry ice. Upon receipt, please store the individual components at the temperatures indicated below. We recommend the E1 activating enzyme, the E2 conjugating enzyme and SUMO proteins be aliquoted upon first use and stored at -80°C. The 5X SUMOylation Buffer should also be aliquoted upon first use and stored at -20°C.

SUMOlink™ SUMO-1 Kit

Reagents	Quantity	Storage / Stability
E1 activating enzyme	40 µl	-80°C for 6 months
E2 conjugating enzyme	40 µl	-80°C for 6 months
SUMO-1 protein	20 µl	-80°C for 6 months
SUMO-1 mutant protein	20 µl	-80°C for 6 months
5X SUMOylation Buffer	160 µl	-20°C for 6 months
Protein Buffer	40 µl	-20°C for 6 months
p53 protein (positive control)	8 µl	-80°C for 6 months
SUMO-1 rabbit antibody	25 µl	-20°C for 6 months
p53 rabbit antibody	10 µl	-20°C for 6 months

SUMOlink™ SUMO-2/3 Kit

Reagents	Quantity	Storage / Stability
E1 activating enzyme	40 µl	-80°C for 6 months
E2 conjugating enzyme	40 µl	-80°C for 6 months
SUMO-2 protein	20 µl	-80°C for 6 months
SUMO-3 protein	20 µl	-80°C for 6 months
SUMO-2 mutant protein	20 µl	-80°C for 6 months
SUMO-3 mutant protein	20 µl	-80°C for 6 months
5X SUMOylation Buffer	160 µl	-20°C for 6 months
Protein Buffer	40 µl	-20°C for 6 months
p53 protein (positive control)	8 µl	-80°C for 6 months
SUMO-2/3 rabbit antibody	100 µl	-20°C for 6 months
p53 rabbit antibody	10 µl	-20°C for 6 months

Additional materials required

- Sterile Water
- Microcentrifuge tubes
- 30°C incubator/waterbath
- 90°C heating block
- Reagents and apparatus for performing Western blotting

Protocols

PLEASE READ ALL PROTOCOLS ENTIRELY BEFORE STARTING

Active Motif's SUMOlink Kits provide reagents to perform 40 *in vitro* SUMOylations (20 WT and 20 Mutated) including 8 optional positive control reactions with p53 (4 WT and 4 Mutated).

SUMOylation Assay

1. Add the assay components to microcentrifuge tubes in the order listed in the tables below. Keep the reaction components on ice. The p53 control is provided for performing 4 WT and 4 Mutated reactions. In addition, you can perform a no protein negative control, if desired.

   Note: We recommend using between 0.2 - 1 µg of protein per reaction. It is possible to perform the reaction with more or less POI (protein of interest), but the efficiency of the reaction may be affected. Adjust the final amount of H_2O to account for the increased/reduced amount of protein used.

2. Mix the tube contents gently and incubate the reactions at 30°C for 3 hours.

   Note: A 3 hour incubation at 30°C has been found to be the optimal temperature and time for the *in vitro* SUMOylation reaction. However, shorter reaction times (30 min. - 1 hour) may also be used, though with reduced efficiency.

3. Stop the reactions by adding an equal volume of 2X SDS-PAGE Loading Buffer (130 mM Tris pH 6.8, 4% SDS, 0.02% Bromophenol blue, 20% glycerol, 100 mM DTT).

   Note: The reactions may also be stopped by the addition of 5 mM EDTA, which will chelate the free Mg^{++} that is required for the ATP-dependent reaction.

4. Analyze by Western blot or store at -20°C.

SUMO-1 Conjugation Reaction

(POI = Protein of Interest)

Component	POI SUMO-1	POI SUMO-1 mutant	Control p53 SUMO-1	Control p53 SUMO-1 mutant	Optional: No protein
Sterile H_2O*	11 µl	11 µl	11 µl	11 µl	12 µl
Protein Buffer	1 µl	1 µl	1 µl	1 µl	1 µl
5X SUMOylation Buffer	4 µl	4 µl	4 µl	4 µl	4 µl
POI (Protein of Interest) (0.5 µg/µl)	1 µl	1 µl	0	0	0
p53 protein	0	0	1 µl	1 µl	0
E1 activating enzyme	1 µl	1 µl	1 µl	1 µl	1 µl
E2 conjugating enzyme	1 µl	1 µl	1 µl	1 µl	1 µl
SUMO-1 protein	1 µl	0	1 µl	0	1 µl
SUMO-1 mutant protein	0	1 µl	0	1 µl	0
Total Volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**

*Adjust the final H_2O amount to account for increased/reduced protein usage

SUMO-2 Conjugation Reaction

(POI = Protein of Interest)

Component	POI SUMO-2	POI SUMO-2 mutant	Control p53 SUMO-2	Control p53 SUMO-2 mutant	Optional: No protein
Sterile H_2O*	11 µl	11 µl	11 µl	11 µl	12 µl
Protein Buffer	1 µl	1 µl	1 µl	1 µl	1 µl
5X SUMOylation Buffer	4 µl	4 µl	4 µl	4 µl	4 µl
POI (Protein of Interest) (0.5 µg/µl)	1 µl	1 µl	0	0	0
p53 protein	0	0	1 µl	1 µl	0
E1 activating enzyme	1 µl	1 µl	1 µl	1 µl	1 µl
E2 conjugating enzyme	1 µl	1 µl	1 µl	1 µl	1 µl
SUMO-2 protein	1 µl	0	1 µl	0	1 µl
SUMO-2 mutant protein	0	1 µl	0	1 µl	0
Total Volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**

*Adjust the final H_2O amount to account for increased/reduced protein usage

SUMO-3 Conjugation Reaction

(POI = Protein of Interest)

Component	POI SUMO-3	POI SUMO-3 mutant	Control p53 SUMO-3	Control p53 SUMO-3 mutant	Optional: No protein
Sterile H_2O*	11 µl	11 µl	11 µl	11 µl	12 µl
Protein Buffer	1 µl	1 µl	1 µl	1 µl	1 µl
5X SUMOylation Buffer	4 µl	4 µl	4 µl	4 µl	4 µl
POI (Protein of Interest) (0.5 µg/µl)	1 µl	1 µl	0	0	0
p53 protein	0	0	1 µl	1 µl	0
E1 activating enzyme	1 µl	1 µl	1 µl	1 µl	1 µl
E2 conjugating enzyme	1 µl	1 µl	1 µl	1 µl	1 µl
SUMO-3 protein	1 µl	0	1 µl	0	1 µl
SUMO-3 mutant protein	0	1 µl	0	1 µl	0
Total Volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**

* Adjust the final H_2O amount to account for increased/reduced protein usage.

Western Blotting Protocol

For your convenience, a Western blotting protocol is provided below. When performing Western blot, individual optimization of antigen and antibody quantities may be required in order to detect the protein of interest. Commercially available reagents can be used for the SDS-PAGE and Western Blot analysis. Enough SUMO-1 or SUMO-2/3 antibody is provided to perform 20 Western blots The SUMO-1, SUMO-2/3 and p53 antibodies are all rabbit polyclonal antibodies.

Electrophoresis

1. Heat the reactions at 90°C for 3 minutes. Centrifuge briefly to collect the samples at the bottom of the tubes.
2. Load 10 µl of each sample, alongside selected molecular weight markers, onto an appropriate SDS-PAGE gel and run until migration front reaches bottom of gel.
3. Store the remainder of the samples at -20°C.

Transfer

4. Transfer protein to polyvinylidene difluoride (PVDF) membranes at 30V for 3 hours using Transfer Buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 15% methanol).

Blocking

5. Block membranes by incubating overnight at 4°C (or room temperature for 1 hour) with shaking in 5ml 5% Blotto (5% dry, non-fat skim milk powder in PBS (pH 7.4)). The addition of 0.1% Tween 20 to all Blotto and PBS solutions may increase the antibody specificity and decrease the background.

Incubation with Primary Antibody:

6. Rinse membranes with PBS (pH 7.4). Dilute the antibody (at the appropriate dilution) in 5 ml 5% Blotto. Incubate for 1 hour at 37°C, 2 hours at RT (room temperature) or overnight at 4°C with agitation. For SUMO-1 we recommend a 1:4000 antibody dilution and for SUMO 2/3 we recommend a 1:1000 antibody dilution. For the p53 antibody we recommend a 1:5000 dilution.
7. Decant the antibody solution. Wash the membrane 5 times for 5-10 minutes at RT in PBS. Washing should be performed with vigorous agitation over a minimum 30-minute period.

Incubation with Secondary Antibody

8. Incubate membranes with secondary HRP conjugate diluted in 5% Blotto for 1 hour at RT with gentle shaking. The dilution of the secondary antibody conjugate will vary according to manufacturer's specifications.
9. Repeat step 7.
10. Wash membrane with PBS for 5 minutes with agitation before proceeding to the chemiluminescence reaction.

Chemiluminescence Reaction

11. Prepare and use the chemiluminescent substrate according to the manufacturer's instructions.

References

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4. Matunis, M.J. *et al.*, (1996) *J. Cell. Biol.* 135: 1457-1470.
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Appendix

Section A. Section A. Troubleshooting Guide

Problem/question	Possible Cause	Recommendation
No or low signal of SUMOylated proteins by Western blot	Insufficient incubation time for the reaction.	3 hours is the optimal incubation time for the SUMOylation reaction. Less time will reduce the reaction efficiency.
	Reaction incubated at the incorrect temperature.	Incubate the reaction for 3 hours at 30°C. The reaction can also occur at 37°C but the length of the incubation may have to be optimized.
	Too much SUMO protein in the *in vitro* SUMOylation reaction	Increasing the amount of SUMO protein in the reaction may reduce the efficiency of the reaction. Use only recommended amounts of the SUMO protein.
	The 5X SUMO Buffer may have lost its buffering capacity due to multiple freeze/thaws.	The 5X SUMOylation Buffer contains ATP. Avoid repeated freeze/thaw cycles.
	Insufficient primary antibody or insufficient secondary antibody.	Increase amount of SUMO antibodies to 1:2000. For p53 samples, use 1:3000. Alternatively, use more secondary antibody.
	Insufficient target protein in the *in vitro* SUMOylation reaction.	The efficiency of the reaction may depend on the specific target. 0.2-1 mg of protein is recommended.
Multiple bands seen in the SUMO-2/3 Western blots as compared to the SUMO- 1 Westerns.	SUMO-2/3 can produce poly-SUMO conjugates while SUMO-1 can produce only mono-SUMO conjugates.	No action necessary; this result is possible.
SUMOylated proteins are present in the sample without any target protein.	These are E1 and E2 enzyme SUMO conjugates.	No action necessary. The E1 and E2 SUMO conjugates can be distinguished from the target SUMO conjugates by comparing the Western blot with the target antibody and the Western blot with SUMO-1 or SUMO-2/3 antibodies. The specific SUMO-target conjugates should be appear in both Western blots.
Observe band in Western blot of approximately 16 kDa.	This is the free SUMO protein.	No action necessary.
What are the species reactivities of the kit reagents?		The SUMO-1, SUMO-2/3 and p53 antibodies are reactive with samples from human, mouse and rat origin. Other species reactivities have not been determined.
Do the SUMO proteins posses a His tag?		Yes. The human SUMO-1, SUMO-2 and SUMO-3 proteins are His-tagged and have been expressed in bacteria. The accession numbers are: SUMO-1 (U67122.1), SUMO-2 (X99584.1) and SUMO-3 (X99585.1).

Section B. Related Products

ChIP-IT™ Kits	Format	Catalog No.
ChIP-IT™ Express	25 rxns	53008
ChIP-IT™ Express Enzymatic	25 rxns	53009
ChIP-IT™ Protein G Magnetic Beads	25 rxns	53014
ChIP-IT™	25 rxns	53001
ChIP-IT™ w/o controls	25 rxns	53004
ChIP-IT™ Shearing Kit	10 rxns	53002
ChIP-IT™ Enzymatic	25 rxns	53006
ChIP-IT™ Enzymatic w/o controls	25 rxns	53007
Enzymatic Shearing Kit	10 rxns	53005
Salmon Sperm DNA/Protein G agarose	25 rxns	53003
ChIP-IT™ Control Kit – Human	5 rxns	53010
ChIP-IT™ Control Kit – Mouse	5 rxns	53011
ChIP-IT™ Control Kit – Rat	5 rxns	53012
Ready-to-ChIP HeLa Chromatin	10 rxns	53015

Chromatin Assembly	Format	Catalog No.
Chromatin Assembly Kit	10 rxns	53500

DNA Methylation	Format	Catalog No.
MethylDetector™	50 rxns	55001
MethylCollector™	25 rxns	55002

Histone Research	Format	Catalog No.
Histone Purification Kit	10 rxns	40025
HAT Assay Kit (Fluorescent)	1 x 96 rxns	56100
HDAC Assay Kit (Colorimetric)	1 x 96 rxns	56210
HDAC Assay Kit (Fluorescent)	1 x 96 rxns	56200

Transcription Factor ELISAs	Format	Catalog No.
TransAM™ AP-1 Family	2 x 96-well plates	44296
TransAM™ GATA Family	2 x 96-well plates	48296
TransAM™ HNF Family	2 x 96-well plates	46296
TransAM™ MAPK Family	2 x 96-well plates	47296
TransAM™ NFκB Family	2 x 96-well plates	43296
TransAM™ Flexi NFκB Family	2 x 96-well plates	43298
TransAM™ STAT Family	2 x 96-well plates	42296

Universal Co-IP Kit	Format	Catalog No.
Universal Magnetic Co-IP Kit	25 rxns	54002

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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