Lot # XXXXX

BostonBiochem

MATERIAL DATA SHEET

SUMO-Detect Protein SUMOylation Detection Kit
Cat. # K-425

Small ubiquitin-related modifiers (SUMOs) are ubiquitin-like proteins that are covalently attached to (or detached from) other proteins in cells to modify their function. In a manner that is analogous to ubiquitination, SUMO proteins are ligated to target proteins via an enzymatic process referred to as SUMOylation. In contrast to ubiquitin, SUMO is not used to tag proteins for degradation. SUMOylation is a post-translational modification that is clearly established as a key regulatory protein modification in eukaryotic cells. More than 1000 proteins involved in cellular processes including nuclear-cytosolic transport, transcriptional regulation, apoptosis, DNA damage repair, protein stability, and cell cycle progression are subject to reversible SUMOylation.

The SUMO-Detect Protein SUMOylation Detection Kit is a practical tool for helping the investigator answer a straightforward question: is a protein of interest SUMOylated? This kit provides reagents and a protocol that may be used to isolate a protein (or protein complex) by immunoprecipitation, and then analyze the complex for the presence of SUMO. Samples from cell lysates or other complex mixtures may be analyzed.

NOTE: Kit contains reagents sufficient for preparing 12 lysates from 6-well plates or T25 tissue culture flasks, and 2 Western Blots (small or medium sized gel format).

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Technical Hints

It is essential that the investigator read the kit instructions before initiating any experiments.

SUMO-Detect Protein SUMOylation Detection Kit data output consists of SDS-PAGE gel-based detection of SUMO via Western Blot. The SUMOylation of a protein is potentially influenced by a large number of factors depending on the identity of the protein. Some proteins are SUMOylated in response to changes in the cell-cycle, activation of cell-surface receptors, DNA damage, etc. It is not in the scope of this protocol to guide the investigator on how and when a protein of interest may be SUMOylated. It is assumed that a set of experimental conditions has been defined by the investigator in which the (potential) SUMOylation of a protein will be examined.

The reagents and protocol provided in this kit may result in the isolation of protein complexes via immunoprecipitation ("co-IP"). In some cases the protein of interest might not be SUMOylated, but is part of a co-IP'd multi-protein complex containing one or more SUMOylated proteins. This could lead to an errant conclusion about the SUMOylation of the target protein. There are multiple techniques to address this issue—contact techsupport@bostonbiochem.com for more details.

Complex lysates from eukaryotic cells contain deSUMOylases ("SENPs") that can quickly remove SUMO from a target protein of interest. The DUB-Stop Protease Inhibitor Mix included with this kit contains fast-acting compounds that provide excellent protection against SENPs. For additional protection, consider adding 2-10 µM SUMO-aldehyde or SUMO-vinyl sulfone to help prevent unwanted SENP activity.

The ability to detect SUMOylation of low-abundance proteins may be enhanced by inhibiting proteasome activity for 4-12 hours prior to generating cellular lysates. The use of cell-permeable proteasome inhibitors such as epoxomicin may be employed in these instances.

SDS-PAGE gels, PVDF membrane, blocking reagent, and protocols provided or referenced in this kit have been chosen following careful testing at Boston Biochem. Modifications to the protocol or selection of alternative reagents may require assay optimization by the end-user. More information is available at techsupport@bostonbiochem.com.

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Materials Provided and Storage Conditions

	Component	Volume
1.	10X Ubl Lyse Reagent	2.4 ml
2.	100X DUB-Stop Protease Inhibitor Mix	240 µl
3.	5X HS Wash Buffer	4.8 ml
4.	10X LS Wash Buffer	1.2 ml
5.	α-SUMO antibody, biotinylated	80 µl
6.	SUMOylated Positive Control Sample	36 µl

Storage: Store components at -20°C. Avoid multiple freeze/thaw cycles.

Other Materials Required

The investigator will need to obtain the following reagents prior to using this kit.

dH_2O	Sterile
SDS-PAGE Gels	Refer to Protocols for guidance.
SDS-PAGE Sample Buffer	Compatible with SDS-PAGE gel.
Dithiothreitol (DTT)	1M in dH_2O (Pierce #20290) or similar
IP antibody	User-validated antibody used to immunoprecipitate ("IP") target protein of interest
PBST	1X PBS (Calbiochem #524650) + 0.05% Tween-20 (Sigma #P1379) or similar
BSA	OmniPur BSA (EMD-Millipore #2910) or similar
Towbin Buffer	25 mM Tris Base, 192 mM glycine, 20% methanol (pH ~ 8.3. Do not adjust pH)
PVDF Membrane	Immun-Blot PVDF Membrane (BioRad #162-0177) or similar. Nitrocellulose membrane may also be used.
ECL Reagents	SuperSignal West Pico Chemiluminescent Substrate (Pierce #34080) or similar
NeutrAvidin-HRP	"High Sensitivity" (Pierce #31030).
Immunoprecipitation Kit (optional)	(Pierce #26146) or equivalent. Alternatively, commonly used IP reagents may be used.
Epoxomicin (optional)	(Boston Biochem # I-110) Cell-permeable proteasome inhibitor.

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Sample Preparation

It is not in the scope of this protocol to guide the investigator on how and when a protein of interest may be SUMOylated. It is assumed that a set of experimental conditions (including how many cells to use) has been defined by the investigator in which the (potential) SUMOylation of a protein will be examined.

1. Prepare cultured cells of choice by seeding either 6-well plates or T25 flasks as appropriate. Continue growth of cells in appropriate medium/conditions as previously established.
2. (Optional) At 4-12 hours prior to harvesting cells, add epoxomicin (or other cell-permeable proteasome inhibitor) to growth media at a final concentration of 5 µM.
3. At 45 minutes prior to harvesting cells, thaw the 10X Ubl Lyse Reagent and the 100X DUB-Stop Protease Inhibitor Mix 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. Once completely thawed, briefly centrifuge to collect contents at bottom of tubes. Do not store 100X DUB-Stop Mix on ice—it contains DMSO and will solidify if stored below room temperature. Wear gloves to prevent accidental exposure to DMSO.
4. For each cell lysis that will be done, prepare 1 ml of Lysis Buffer by combining the following in order (mix after each addition):
   i. 890 ul dH_2O
   ii. 100 ul 10X Ubl Lyse Reagent
   iii. 10 ul 100X DUB-Stop Mix

   We recommend using 1 ml of Lysis Buffer per well of a 6-well tissue culture plate. Scale volumes appropriately for the number of lysates required and total amount of cells harvested. If more than 1 ml of Lysis Buffer is required, a 15 ml flip-top conical tube (such as a Falcon 2059) is convenient for mixing components. Vortex gently to mix components (avoid frothing), then chill the Lysis Buffer on ice for at least 20 minutes.
5. Harvest cells by aspirating culture media and rinsing once or twice with PBS. Cells may then been collected by standard trypsinization procedures to generate small cell pellets in centrifuge tubes. We recommend omitting the trypsinization process by directly adding 1 ml of the ice-cold Lysis Buffer to the rinsed, aspirated cells on the plates or flasks.

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6. Use gentle, mechanical agitation to aid disruption of cells with the Lysis Buffer. Tapping the plates/flasks or repeatedly pipetting the Lysis Buffer over the surface of the cells helps to thoroughly solubilize the samples. The sample may become viscous due to release of genomic DNA. We recommend the use of 1 ml wide-bore pipette tips (made by cutting off the ends of the pipette tips with a sharp razor blade) to facilitate handling of the samples. Carefully transfer the samples to 1.5 ml microcentrifuge tube.
7. (Optional) Viscous lysates are not optimal for IP techniques. Genomic DNA may be sheered by a number of techniques in order to lower sample viscosity. We suggest using one of the following:
    i. Sonication using a microtip. Care must be taken to avoid sample heating. Use multiple 10-15 second treatments at 50% duty, with icing for 1-2 minutes in between treatments to cool sample(s). 4-6 total cycles is effective in reducing viscosity. Be certain to rinse microtip between samples to avoid cross-contamination.
    ii. Pass sample through a Qiashredder (Qiagen #79656) one or more times to reduce viscosity—see manufacturer's recommendations and protocols.
8. Clear insoluble material from samples by centrifugation at 4°C for 15-20 minutes at ≥ 15000 x*g*. Transfer supernatants to clean microfuge tubes and place on ice.
9. Samples are now ready for IP.

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Immunoprecipitation of Target Protein

It is left to the investigator to determine how best to perform IP reactions. We recommend using a polyclonal antibody generated using a recombinant form of the target protein of interest. While monoclonal antibodies can also be used in the IP, the potential exists that the epitope recognized by the antibody may be a site of SUMOylation on the target protein. If so, then SUMOylated target protein may not be IP'd, leading to a false-negative result. The efficacy of the antibody in the IP of the target protein should be previously validated by the investigator.

General suggested guidelines for target protein IP are as follows:

1. Transfer 400-1000 µl of cell lysate(s) (obtained in Sample Preparation) to labeled, chilled 1.5 ml microcentrifuge tubes. Adjust the volume of lysate(s) used depending on the abundance of the target protein as previously determined by the investigator.
2. Add an appropriate amount of antibody to the sample and incubate for 1-4 hours at 4ºC. We routinely begin with 20 µg of polyclonal antibody.
3. Add 10-20 µl (packed volume) of Protein A/G Sepharose ("resin") to the sample and place on rocker for 1-2 hours at 4ºC (overnight is acceptable). For convenience, resin may be added as 20-40 µl of a 50% slurry.
4. Place tubes containing 5X HS Wash Buffer and 10X LS Wash Buffer in a water bath (≤ 30°C) or incubator until completely thawed. Gently vortex tubes to ensure solutions are well mixed. The 5X HS Buffer may be cloudy at room temperature.
5. For each IP reaction, make 2 ml of HS Wash Buffer by diluting 400 µl of 5X stock into 1.6 ml of dH_2O. Make 1 ml of LS Wash Buffer by diluting 100 µl of 10X stock into 0.9 ml of dH_2O. Scale as needed to generate enough of each wash buffer for all samples.
6. Chill both Wash Buffers on ice for at least 20 minutes.
7. Pellet resins from Step 3 by brief, low-speed centrifugation (1-2 minutes at 5000 x*g*). Carefully remove supernatant(s) by aspiration or pipetting.
8. Wash resin(s) by adding 1 ml ice-cold 1X HS Wash Buffer. Briefly vortex to suspend resin, then place tubes on a rocker or end-over-end mixer for 5 minutes at 4ºC. Briefly centrifuge (1-2 minutes at 1000 x*g*) to pellet resin, then carefully aspirate wash supernatant. Repeat once for a total of two washes in 1X HS Wash Buffer.
9. Wash resin(s) twice with 0.5 ml ice-cold 1X LS Wash Buffer using the procedures listed in Step 8. After the last wash, carefully remove all supernatant by aspiration.
10. Add 30µl of 2X SDS-PAGE sample buffer and 4 µl 1M DTT. Mix well and heat reactions to 80-90°C for 5 minutes. Centrifuge briefly to pellet resin.

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SDS-PAGE and Western Blot Analysis

The choice of SDS-PAGE gel that is used for separating the target protein of interest will depend on the size of the target protein. A constant percentage or gradient gel may be used. We routinely use BioRad's *Criterion* gels and buffering systems which are available in many formats and yield consistent Western Blot results.

General suggested guidelines for SDS-PAGE/Western Blot of target protein IP are as follows:

1. Assemble SDS-PAGE gel according to manufacturer's instructions.
2. Thaw the SUMOylated Positive Control sample included with the kit. Add 4 µl of 1M DTT, mix, and heat at 37-40 °C for 10 minutes. (Enough Positive Control is provided for approximately 4 lanes worth of analysis. Only add DTT to the Positive Control the first time it is opened. Freeze down any unused Positive Control sample at -80°C in useful aliquot sizes for future use.)
3. Load 10 µl of Positive Control into the appropriate gel well(s).
4. Load 10-30 µl of each sample (from Step 10 in Immunoprecipitation of Target Protein section) into appropriate wells. The volume loaded will depend on your gel format.
5. Run the gel until the dye-front just reaches bottom of gel (approximately 1 hour at 180V using the Criterion gel system—adjust run times and voltage accordingly for your system).
6. Carefully disassemble gel and transfer to a small tray containing 50-100 ml of Towbin Buffer. Soak the gel at room temperature for 20 minutes with gentle rocking. Some gels tend to float on top of the buffer—this should be minimized with gentle agitation.
7. Prepare PVDF membrane by wetting in a small amount of 100% methanol for 1-2 minutes. When membrane is completely wetted, transfer to 20-30 ml of Towbin Buffer for 5 minutes at room temperature with gentle rocking.
8. Assemble transfer "sandwich" using pre-wetted blotting paper, gel, PVDF membrane, and any other components for electroblotting according to system manufacturer's suggested protocol. (We utilize the BioRad Trans·Blot SD Semi Dry Transfer Cell)
9. Electroblot gel contents to PVDF at 15V (constant) for 45 minutes at room temperature (settings will depend on your system).
10. Disassemble gel sandwich and prepare for Blocking/Antibody Detection.

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11. Prepare Blocking Solution by dissolving 3 grams solid Fraction V BSA in 100 ml PBST solution (PBST+3% w/v BSA). The Blocking Solution may be filter sterilized and stored at 4°C for up to 5 days, but we suggest preparing it fresh when needed.
12. Soak PVDF membrane in 50-100 ml Blocking Solution 1 hour at room temperature (or overnight at 4°C) with gentle rocking in a covered container or sealable bag.
13. Decant Blocking Solution and rinse membrane for 10-15 minutes in 50 ml PBST.
14. Prepare antibody solution by diluting 40 µl of biotinylated α-SUMO antibody (supplied) in 4 ml of PBST + 0.5% w/v BSA.
15. Using a sealable plastic bag, incubate membrane in antibody solution for 2 hours at room temperature (or overnight at 4°C) with rocking or shaking. Make certain that the entire PVDF membrane surface is uniformly exposed to antibody solution.
16. Remove membrane from antibody solution and wash with 50-100 ml PBST for 15 minutes at room temperature using rocking or shaking. Decant wash solution. Repeat twice for a total of three membrane washes.
17. Prepare avidin-HRP solution by diluting 4 µl of Pierce High Sensitivity NeutrAvidin HRP (or equivalent) in 20 ml of PBST + 0.5% BSA.
18. Incubate membrane in avidin-HRP solution for 60 minutes at room temperature (longer incubations may lead to higher background signals) with rocking or shaking. Make certain that the entire PVDF membrane surface is uniformly exposed to solution.
19. Remove membrane from avidin-HRP solution and wash with 50-100 ml PBST for 15 minutes at room temperature using rocking or shaking. Decant wash solution. Repeat twice for a total of three washes.
20. Membrane is now ready for ECL detection procedure. Chemiluminescence reagents for detecting products are available from a number of sources. We recommend using SuperSignal West Pico Chemiluminescent Substrate from Pierce. Typical film exposure times range from 5 seconds to 2 minutes. Using the protocol listed above, the Positive Control is typically observed with film exposures of 60 seconds or less.

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Example Data

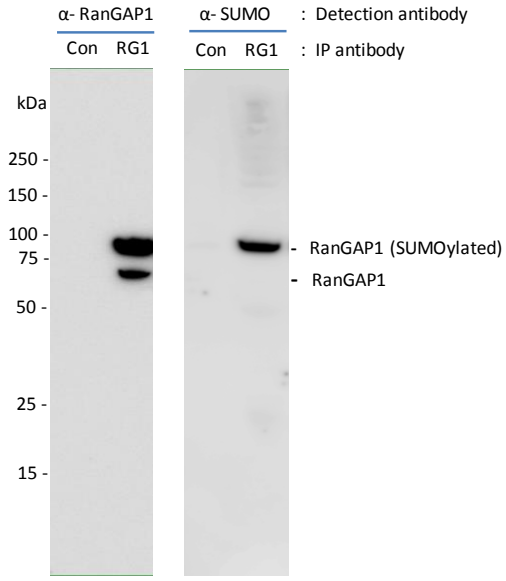

Figure 1. SUMO-Detect Analysis of RanGAP1

Cellular lysates were generated from HCT-116 cells using methods described in this protocol (more details available upon request). A rabbit polyclonal anti-RanGAP1 ("RG1", Bethyl Laboratories A302-027A) or non-specific control antibody ("Con") was used to immunoprecipitate RanGAP1 protein. IP'd complexes were resolved on 4-20% SDS-PAGE gels and then blotted to PVDF membranes. Left Panel: membrane was developed using anti-RanGAP sheep pAb (R&D Systems # AF7834) at 0.5 mg/ml and anti-sheep HRP (R&D Systems # HAF016) at 1/2000 dilution. IP'd RanGAP1 is detected as two discrete bands. Right Panel: membrane was developed as described in the protocol. The upper band is confirmed as SUMOylated RanGAP1.

Literature

References: Wang Y. & Dasso M. (2009) J of Cell Science **122**: 4249
Flotho A. & Melchior F. (2013) Ann. Rev. Biochem. **82**: 357

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