Product Manual

SUMO-QAPTURE-T® Kit

Catalog #: BML-UW1000A

For the isolation and enrichment of SUMOylated proteins

NOTE: Kit component BML-UW0130A Control SUMOylated-protein lysate has been replaced by three recombinant protein standards

SUMO-1 Control (Prod. No. BML-UW9195-0125), 500 ng (125 µL), 4 ng/µL in 20 mM HEPES pH 7.5, 50 mM NaCl

SUMO-2 Control (Prod. No. BML-UW9205-0125), 500 ng (125 µL), 4 ng/µL in 20 mM HEPES pH 7.5, 50 mM NaCl

SUMO-3 Control (Prod. No. BML-UW9215-0125), 500 ng (125 µL), 4 ng/µL in 20 mM HEPES pH 7.5, 50 mM NaCl

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Product Manual

Please read entire booklet before proceeding with the assay.

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Carefully note the handling and storage conditions of each kit component.

The unopened kit should be stored at -80ºC upon receipt. After thawing, SUMO-QAPTURE-T® matrix should be stored at 4°C. SUMO-1, SUMO-2 and SUMO-3 controls should be stored at -80°C. SUMO antibody solutions can be stored at -20°C.

Please contact Enzo Life Sciences Technical Support if necessary.

BACKGROUND

Small Ubiquitin-like Modifier (SUMO) is involved in diverse biological processes ranging from transcriptional regulation to defining sub-cellular localization[1]. Vertebrates express three SUMO paralogs, SUMO-1, SUMO-2 and SUMO-3. While processed SUMO-2 and SUMO-3 are 98% identical, they each share only 45% identity with SUMO-1.

SUMO-2 and SUMO-3 each contain an acceptor lysine (K11) that allows the formation of polySUMO chains[2] by canonical means. Under appropriate conditions, SUMO-1 may also form polySUMO chains at sites not comprising a recognized SUMOylation motif.

Mono-SUMOylation of target proteins is involved in a range of cellular processes such as nuclear protein targeting, formation of sub-nuclear complexes, regulation of transcriptional activities, and control of protein stability.

In addition to mono-SUMOylation, a number of studies have implicated important biological functions for SUMO polymers[3], including roles in chromosome segregation, recovery from checkpoint arrest, DNA damage response and meiosis[4,5]. Exposure of HeLa cells to heat-shock and other stresses has been shown to enhance general SUMO-2/3 conjugation[6] and polySUMO chain formation[7], particularly of proteins involved in checkpoint response and DNA repair. The few polySUMO conjugates identified include hypoxia induced factor 1α (HIF1α)[8], PARP1[9], Topoisomerase 2 (TOP2)[10], proliferating cell nuclear antigen (PCNA)[11] and the promyelocytic leukemia protein (PML)[12].

The functional consequences of mono- or poly-SUMO modification are mediated by recruitment of effector proteins that contain one or more SUMO interaction motifs (SIM). These SIMs are composed of short stretches of hydrophobic residues that engage with a shallow hydrophobic depression on one face of the SUMO molecule[13].

KIT DESCRIPTION

The SUMO-QAPTURE-T® Kit is an efficient tool for the selective isolation of SUMOylated proteins. The Kit facilitates the affinity purification of SUMOylated proteins from cell extracts and tissue lysates using a high-binding SIM-containing affinity matrix. Captured proteins are eluted under denaturing conditions followed by analysis by Western blotting, using the SUMO antibodies provided or antibodies to specific proteins of interest, or potential substrate identification by proteomic methods.

SUMO-conjugate-containing samples are prepared in native form in the presence of protease inhibitors to prevent loss through the action of deSUMOylating enzymes. Optimization of binding permits complete isolation of the full range of SUMO-protein conjugates from a specific lysate. The Kit is also supplied with high quality recombinant SUMO proteins for use as a positive control in SUMO Qapture assays and qualitative analysis of yield.

The Kit provides sufficient material for approximately 10 binding assays.

SUGGESTED APPLICATION

1. Capture and isolation of SUMO-protein conjugates from specific cell/tissue lysates of interest with subsequent detection and analysis by Western Blotting.
2. Identification of SUMO-modified protein substrates by proteomic analysis methods following release of free SUMOylated proteins in denatured form.
3. Selective purification/pull down of SUMOylated proteins from *in vitro* SUMOylation assays.

Note: Protocol provided covers application 1. Assay set-up can be readily modified for alternative applications by inclusion, omission or substitution of specific components.

MATERIALS SUPPLIED

1. **SUMO-QAPTURE-T® matrix, BML-UW0980-0200**:

   50% suspension, use 40 µL per binding assay.
   200 µL settled resin provided, sufficient for approx. 10 binding assays.

2. **SUMO-1 Control, BML-UW9195-0125**

   500 ng (125 µL), 4 ng/µL in 20 mM HEPES pH 7.5, 50 mM NaCl

3. **SUMO-2 Control, BML-UW9205-0125**

   500 ng (125 µL), 4 ng/µL in 20 mM HEPES pH 7.5, 50 mM NaCl

4. **SUMO-3 Control, BML-UW9215 -0125**

   500 ng (125 µL), 4 ng/µL in 20 mM HEPES pH 7.5, 50 mM NaCl

5. **SUMO antibody solutions:**

   SUMO-1, rabbit polyclonal antibody (BML-PW0505A-0010)
   SUMO-2, rabbit polyclonal antibody (BML-PW0510A-0010)

   10µg of each provided. Dilution of at least 1:500-1:1000 recommended for Western blotting.

Reagents require separate storage conditions.

STORAGE

Unopened kit should be stored at -80°C upon receipt. After thawing, SUMO-QAPTURE-T® matrix should be stored at 4°C. Avoid a second freeze/thaw cycle. SUMO-1, SUMO-2 and SUMO-3 controls should be stored at -80°C. SUMO antibody solutions can be stored at -20°C. Avoid multiple freeze/thaw cycles of components to ensure stability and activity.

OTHER MATERIALS NEEDED

5x SDS-PAGE gel loading buffer
(e.g., 0.25M Tris-HCl, pH 6.8, 15% SDS, 50% glycerol, 25% β-mercaptoethanol, 0.01% bromophenol blue).

Binding Buffer
50 mM Tris-HCl, pH 7.5 (include 1 mg/mL BSA if working with purified proteins)

Lysis Buffer (Suggested)
50 mM Tris-HCl, pH 7.5, 150mM NaCl, 200 mM iodoacetamide

CAPTURE/ENRICHMENT OF SUMOYLATED PROTEINS

The protocol set out in this section is designed for capture and elution of SUMO-protein conjugates for subsequent analysis by Western blotting.

These conditions should be used as a starting point for isolation of SUMO-protein conjugates from a specific lysate/solution and may require optimization to ensure complete pull down/capture of particular SUMOylated proteins of interest.

Lysate sample preparation

Samples should be prepared under native conditions in order to allow efficient binding of SUMO modified proteins to the SUMO-QAPTURE-T® matrix.

In order to prevent deSUMOylation of SUMO conjugates by SUMO proteases (SENPs) appropriate protease inhibitors should be included when preparing and storing lysate samples. Iodoacetamide can be used for this purpose

- Include protease inhibitors in any subsequent buffers used during lysate preparation, e.g., for resuspension of nuclei after centrifugation.

Note: Sample lysis buffers containing components that cause protein denaturation, particularly chaotropes such as urea, should be avoided. The use of reducing agents (e.g., DTT) and detergents should be minimized. High salt levels may affect SUMO-conjugate binding (>500 mM).

A total sample protein content of ~100 µg (maximum sample volume: 100 µL) is recommended for initial SUMO-protein conjugate capture experiments. Additional lysate material or serial dilution of lysate samples may be necessary for optimization of the SUMO-Qapture process.

The binding capacity of the SUMO-QAPTURE-T® matrix is estimated to be greater than 2 µg SUMOylated protein/20 µL matrix suspension.

Note: Centrifugation of SUMO-QAPTURE-T® matrix containing solutions MUST NOT be performed at greater than 5000 x g, to prevent damage to the SUMO-QAPTURE-T® matrix beads.

SUMO-Qapture protocol

Keep all components on ice throughout.

1. Dilute 50µL of the desired control SUMO protein with 50 µL of Binding Buffer at 4°C in a clean Eppendorf tube to give the control binding solution (100 µL final volume, 200 ng total protein). If desired, multiple control proteins may be bound simultaneously. If all three are used, increase binding reaction volume of subsequent samples to 150 µL.
2. Using 100 µg total protein content as a starting point, prepare lysate samples for SUMO enrichment in clean 0.5 mL screw capped tubes, using either neat lysate or stock lysate solution diluted in Binding Buffer, to give a final sample binding solution volume of 100 µL (total protein concentration 1 mg/mL).
3. To allow comparison between captured SUMOylated species and original lysate samples (Starting Material), retain an equivalent amount of each sample.
   - Prepare 'Starting Material' samples for subsequent Western blot analysis by addition of 25 µL 5X SDS-PAGE gel loading buffer to each 100 µL 'Starting Material' lysate solution, followed by heating to 95°C for 10 minutes. Store at -20°C until required.
   - Addition of reducing buffer to iodoacetamide containing solutions will lower pH giving a yellow solution
4. Resuspend the SUMO-QAPTURE-T® matrix by gently inverting the tube several times.
5. Using a wide-bore pipette tip, aliquot 40 µL of matrix suspension (20 µL settled resin) to a fresh tube for each of the samples to be analyzed.
6. Add 200 µL Binding Buffer to each tube. Mix gently by inversion and centrifuge for 30 seconds at 5000xg. Carefully remove the buffer so as not to disturb the matrix pellet by aspiration using a gel-loading pipette tip or equivalent to avoid disturbing the surface tension.
7. Wash resin with a further 200 µL Binding Buffer, centrifuge, and remove supernatant.
8. Add sample/control binding solution (100 µL) to tubes containing washed SUMO-QAPTURE-T® matrix and resuspend gently by inversion.
9. Bind SUMOylated protein conjugates to the affinity matrix overnight at 4°C on a horizontal rotor mixer

Note: Binding assays can be run with two hours incubation at room temperature if required but are less likely to be optimal.

10. Centrifuge samples for 30 seconds at a speed of 5000xg to collect the SUMO-QAPTURE-T® matrix.
11. Carefully remove the unbound supernatant to a fresh tube.

    • (Optional) Label as ‘Unbound Fraction’ (approximately 100 µL) and Prepare for Western blot analysis by addition of 25 µL 5X SDS-PAGE gel loading buffer followed by heating to 95°C for 10 minutes. Store at -20°C until required.

12. Wash matrix with 200 µL Binding Buffer. Mix by gently inverting tube.
13. Centrifuge samples for 30 seconds at a speed of 5000xg to collect the SUMO-QAPTURE-T® matrix.
14. Carefully remove the supernatant and discard.
15. Repeat Wash twice more (steps 12-14).
16. Elute SUMO-protein conjugates by addition of 100 µL Binding Buffer and 25 µL 5X SDS-PAGE gel loading buffer to each sample.
17. Quench by mixing at room temperature for 5 minutes, followed by heating to 95°C for 10 minutes. Label as ‘Elution Fraction’.
    - Elution Fraction should then be clarified by centrifugation prior to analysis.
    - Proceed directly to “Analysis by Western Blotting” or store at -20°C until ready.

Alternative elution method

If Elution Fraction samples are to be analyzed by methods not requiring separation by SDS-PAGE (e.g., LC-MS) appropriate buffers including a denaturant, such as 8 M urea, can be used as an alternative to the elution buffer (Binding Buffer + 5X SDS-PAGE gel loading buffer) used in Step 16, followed by mixing/incubation at 4°C and clarification of Elution Fraction by centrifugation.

ANALYSIS BY WESTERN BLOTTING

Comparison of equivalent amounts (15-20%) of Starting Material and Elution Fraction (and Unbound Fraction, if required) by SDS-PAGE/Western blotting is recommended to allow binding efficiency to assessed.

Note: Higher loading of Elution Fraction may be required if specific SUMO modified proteins (e.g., SUMO-RanGAP1) are to be detected using protein specific antibodies.

Summary of analysis steps

1. Separate proteins by SDS-PAGE.
2. Western transfer to PVDF membrane.

Note: Western Blotting conditions appropriate for the transfer of large proteins may be required to ensure good transfer of SUMOylated proteins to PVDF membrane. For example, use BSN transfer buffer 48 mM Tris, pH9.2, 39 mM glycine with 10% MeOH and 0.0375% SDS.

3. Block membrane with BSA/PBS-T solution.
4. Probe blot with either:
   a. SUMO-1 antibody supplied
   b. SUMO-2 antibody supplied, or
   c. Appropriate target protein specific primary antibody in conjunction with suitable secondary antibodies.
5. Develop with Western Blotting detection reagents.

Materials required

1. SDS-PAGE gels - User prepared (12% standard / 4-15% linear gradient)
2. Pre-stained SDS-PAGE molecular weight markers (e.g., SeeBlue Plus2)
3. PVDF membrane (e.g., Immobilon-P)
4. Anti-rabbit IgG secondary antibody (HRP-linked) – for use with SUMO antibodies provided (e.g., Goat anti-rabbit IgG, polyclonal antibody (HRP conjugate), Prod. No. ADI-SAB-300)
5. Target protein specific primary antibody (user supplied) and appropriate secondary antibody-HRP conjugate (if required).
6. Western blotting detection reagents (e.g., ECL Reagent).
7. PBS solution 1X PBS.
8. PBS-T solution 1X PBS containing 0.2% Tween 20.
9. BSA/PBS-T blocking solution PBS-T containing 1% bovine serum albumin (BSA)

EXAMPLE PROCEDURE FOR WESTERN BLOTTING

1. Apply 20 µL of the Starting Material and Elution Fraction samples (and Unbound Fraction if required) to the SDS-PAGE gel alongside selected molecular weight markers, electrophorese, and transfer protein to PVDF membrane according to standard procedures.

2. Remove membrane from the transfer unit and block with BSA/PBS-T blocking buffer for 1 hour at room temperature on a rotor mixer.

Note: Drying PVDF membrane prior to blocking, as per manufacturers' instructions, may considerably enhance results.

3. Wash membrane for 3 x 10mins with PBS-T on a rocking platform at room temperature.

SUMO-conjugate detection

4. Dilute supplied SUMO-1 and/or SUMO-2 antibodies provided 1:500 to 1:1000 in BSA/PBS-T.
5. Incubate membrane with SUMO antibody solution overnight at 4°C on a rotor mixer.
6. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
7. Dilute appropriate anti-rabbit IgG secondary antibody (HRP-linked) according to the manufacturer's instructions (e.g., 1:5000 in BSA/PBS-T).
8. Incubate membrane with secondary antibody solution for 1 hour at room temperature on a rocking platform, or as specified by the manufacturer.
9. Wash membrane for 6 x 10mins with PBS-T on a rocking platform.
10. Proceed to step 17.

Specific target protein detection (if required)

11. Dilute appropriate target protein specific primary antibody according to manufacturer's instructions.
12. Incubate membrane with target protein specific primary antibody solution overnight at 4°C on a rotor mixer.
13. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
14. Dilute appropriate secondary antibody according to the manufacturer's instructions.

15. Incubate membrane with secondary antibody solution for 1 hour at room temperature on a rocking platform, or as specified by the manufacturer.
16. Wash membrane for 6 x 10mins with PBS-T on a rocking platform.

Analysis

17. Prepare western blotting detection reagent according to the manufacturer's instructions (e.g., ECL reagent: Mix equal amounts of Reagent A and B and allow to stand for 1 minute).
18. Incubate membrane with ECL reagent for 1 minute.
19. Detect emitted signal by luminography or CCD imaging instrument.

EXAMPLE RESULTS FOR WESTERN BLOTTING

SUMO-Qapture experiment set-up and run as described in “Capture / enrichment of SUMOylated proteins”. SUMO-1 and SUMO-2/3 conjugates present in Starting Material and Elution Fraction were detected by Western blotting as described in “Analysis by Western blotting”, using the provided SUMO-1 (BML-PW0505A-0010) and SUMO-2 (BML-PW0510A-0010) antibodies at a dilution of 1:1000 for each antibody.

Capture of SUMO-protein conjugates from HeLa lysate S100 fraction.

Results demonstrate capture/enrichment and subsequent detection of SUMOylated proteins (general and specific) from a cell lysate using the SUMO-QAPTURE-T® kit.

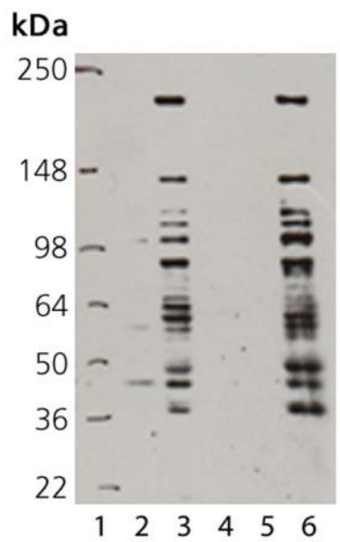

Figure 1: Western blot analysis of SUMO-Qapture enrichment of lysate derived SUMO-1 modified proteins. Lane 1: MWM, Lane 2: Unbound lysate, Lane 3: Elution, Lane 4. Wash 1, Lane 5. Wash 2, Lane 6: Starting material

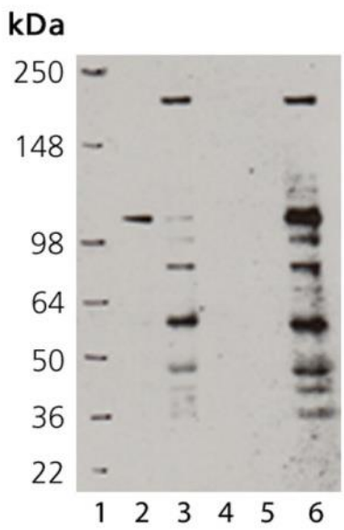

Figure 2: Western blot analysis of SUMO-Qapture enrichment of lysate derived SUMO-2/3 modified proteins. Lane 1: MWM, Lane 2: Unbound lysate, Lane 3: Elution, Lane 4: Wash 1, Lane 5: Wash 2, Lane 6: Starting material.

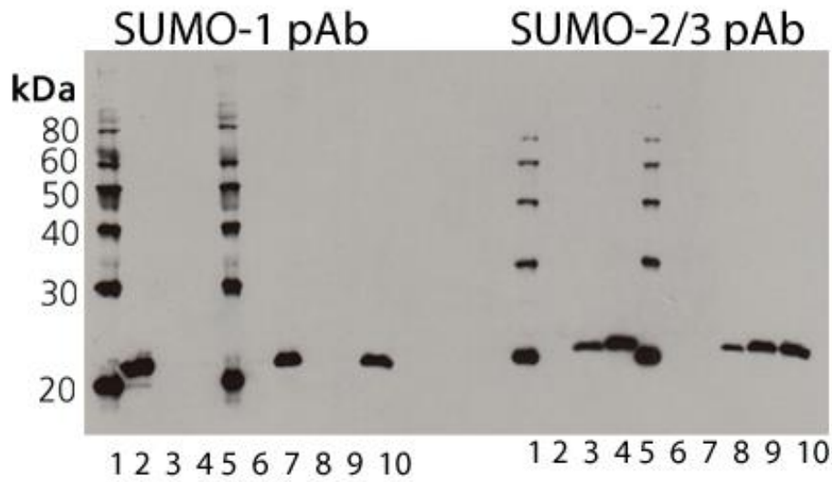

Figure 3: Western blot analysis of SUMO-Qapture binding of recombinant SUMO protein controls. 200 ng of each recombinant control were bound alone or simultaneously using the described protocol. Lane 1: MWM, Lane 2: 40 ng SUMO-1, Lane 3: 40 ng SUMO-2, Lane 4: 40 ng SUMO-3, Lane 5: MWM, Lane 6: SUMO 1/2/3 final wash fraction, Lane 7: SUMO-1 elution, Lane 8: SUMO-2 elution, Lane 9: SUMO-3 elution, Lane 10: SUMO 1/2/3 elution. Left probed with SUMO-1 pAb, Right probed with SUMO 2/3 pAb.

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