Lot # XXXXX

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MATERIAL DATA SHEET

RNF4 Ubiquitin Ligase Kit - di-SUMO3 Substrate
Cat. # K-220

RNF4 (SNURF) is a RING-finger ubiquitin E3 ligase that ubiquitinates and mediates the proteasomal destruction of PML/RARα, a fusion protein that is a hallmark of acute promyelocytic leukemia (APL). APL can sometimes be treated effectively with arsenic trioxide, which induces PML/RARα modification by small ubiquitin-like modifiers (SUMO). RNF4 subsequently binds to and ubiquitinates the poly-SUMOylated PML/RARα, thereby targeting it to the proteasome.

RNF4 contains four SUMO-interacting motifs (SIMs) that function to recruit this ligase to a variety of poly-sumoylated substrates. RNF4 will autoubiquitinate *in vitro*, and will also ubiquitinate poly-SUMO chains. This kit is designed for *in vitro* RNF4-mediated ubiquitination of user-supplied, poly-SUMOylated substrates such as PML, PEA3, CENP1, and PARP1. The resulting proteins can be used in downstream applications, or analyzed by Western blot using antibodies specific for the target protein. A control substrate, di-SUMO3 (**ULC-300)** and detection antibody (**MAB2959**) are included in the kit.

NOTE: Kit contains reagents sufficient for 10 x 30 µl reactions and 5 Western Blots (mini-gel format).

Reagents Provided in Kit

Component	Volume
1. 10X E1 enzyme	30 µl
2. 10X E2 enzyme (UBE2D1)	30 µl
3. 10X RNF4	30 µl
4. 10X di-SUMO3 control substrate	30 µl
5. 10X ubiquitin	30 µl
6. 10X Reaction Buffer	50 µl
7. 10X Mg^{2+}-ATP	30 µl
8. α-SUMO primary antibody	50 µl
9. 5X Loading Buffer	1 ml

Storage: Store protein components at -80°C. Avoid multiple freeze/thaw cycles.
Loading Buffer may be stored at room temperature.
Mg^{2+}-ATP and α-SUMO antibody may be stored at -20°C.

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Reagents to be Provided by Investigator

The following reagents and materials need to be obtained by the investigator prior to using this kit.

dH_2O	Sterile
Dithiothreitol (DTT)	1M in dH_2O (Pierce #20290) or similar
PBST	1X PBS (Calbiochem #524650) + 0.05% Tween-20 (Sigma #P1379) or similar
Non-fat milk	Dry/powdered (CARNATION® Instant Nonfat Dry Milk or similar)
Towbin Buffer	25 mM Tris Base, 192 mM glycine, 20% methanol (pH ~ 8.3. *Do not adjust pH*)
SDS-PAGE Gels	*Criterion* 10% SDS-PAGE Gel (BioRad #345-0010) or similar
PVDF Membrane	*Immun-Blot* PVDF Membrane (BioRad #162-0177) or similar
HRP-α-mouse 2° Ab	(R&D Systems #HAF007) or similar
ECL Reagents	*SuperSignal West Pico* Chemiluminescent Substrate (Pierce #34080) or similar

Assay Considerations

RNF4 displays a robust *in vitro* autoubiquitination activity that may deplete the free ubiquitin pool quickly in some experimental conditions. If altering the source of ubiquitin from that supplied in the kit, please consider adding ubiquitin to a final concentration of at least 20 µM. RNF4 ubiquitinates target proteins that are polySUMOylated (including unanchored SUMO chains), with little or no ligase activity against proteins that are monoSUMOylated.

The kit protocol is designed for reaction termination with SDS-PAGE Sample Buffer; therefore, proteins are denatured and typically not suitable for further enzymatic manipulation. If ubiquitinated protein is to be utilized in further reactions prior to SDS-PAGE analysis, reactions may be terminated by the addition of EDTA (10 mM final) plus DTT or βME (5-10 mM final) if compatible with downstream experimental protocols.

SDS-PAGE gels, PVDF membrane, blocking reagent, antibodies 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 (particularly substrate protein) may require assay optimization by the end-user. Further information available at techsupport@bostonbiochem.com.

Recommended Assay Protocol (30 µl volume)

1. Reagent Preparation
    a. Quickly thaw all protein reagents and buffers 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. Do not vortex or shake vigorously.
    b. When completely thawed, *gently* tap tubes to make sure components are well mixed (SDS-PAGE Sample Buffer may be inverted to mix), then briefly spin in a microcentrifuge (5 seconds) to collect contents in bottom of tubes.
    c. Immediately ice components. (SDS-PAGE Sample Buffer may be kept at room temperature.) Entire process from steps 1a-1c should be accomplished in approximately 5 minutes.
    d. It is ***strongly recommended*** that each reagent be divided into smaller aliquots to minimize the number of freeze-thaw cycles of kit components. Avoid multiple freeze-thaw cycles to maximize kit performance. Rapidly re-freeze any aliquoted materials in dry ice bath. SDS-PAGE Sample Buffer may be stored at room temperature.

2. Reaction Assembly
    a. Prepare 30 µl reactions on ice in 0.5 or 1.5 ml polypropylene tubes using the following volumes and order of addition:
        i. 9 µl dH_2O.
        ii. 3 µl 10X Reaction Buffer. Mix gently following addition.
        iii. 3 µl 10X di-SUMO3 substrate protein (or substrate supplied by user)
        iv. 3 µl 10X E1 enzyme.
        v. 3 µl 10X E2 enzyme.
        vi. 3 µl 10X RNF4 enzyme.
        vii. 3 µl 10X Mg^{2+}-ATP solution. For negative control reactions, omit ATP addition and replace with 3 µl dH_2O.
    b. At this point, reactions are ready to initiate—addition of ubiquitin in the next step will start the reaction.
    c. Add 3 µl of 10X ubiquitin solution. Mix by gently pipetting up and down 2-3 times.
    d. Spin tubes to collect contents and place reactions in 37°C water bath.
    e. After 60 minutes, terminate reactions with addition of 8 µl 5X Loading Buffer (SDS-PAGE sample buffer) and 1 µl 1M DTT. Heat reactions to 90°C for 5 minutes.

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3. SDS-PAGE
    a. Assemble SDS-PAGE gel according to manufacturer's instructions. (We utilize the BioRad *Criterion* gel unit with Tris buffering system.)
    b. Load 13 µl of terminated reaction per well (*Criterion* Precast 10% Tris-HCl, 1.0 mm thickness, 18 well comb, 30 µl/well capacity). Volume loaded per well will depend on your choice of gel.
    c. Run gel until dye-front just reaches bottom of gel (approximately 1 hour at 180V using the *Criterion* gels referenced in step 3b—adjust run times and voltage accordingly for your system).
    d. Carefully disassemble gel and prepare for electro-transfer to blotting membrane.

4. Gel Transfer
    a. Soak gel in 50-100 ml of Towbin Buffer 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.
    b. 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.
    c. Assemble transfer "sandwich" using 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)
    d. Electroblot gel contents to PVDF at 15V (constant) for 45 minutes at room temperature (settings will depend on your system).
    e. Disassemble gel sandwich and prepare for Blocking/Antibody Detection.

5. Membrane Blocking
    a. Prepare Blocking Solution by dissolving 5 grams solid, non-fat milk per 100 ml PBST solution (PBST+5% w/v non-fat milk). 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.
    b. Soak PVDF membrane in 50-100 mls Blocking Solution overnight at 4°C with gentle rocking in a covered container or sealable bag.

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6. Antibody Staining
    a. Prepare Antibody Dilution Buffer by dissolving 0.2 grams of solid, non-fat milk in 40 ml PBST (PBST + 0.5% w/v non-fat milk).
    b. Dilute α-SUMO primary antibody by adding 10 µl antibody to 20 ml Antibody Dilution Buffer (1:2000 dilution).
    c. Decant Blocking Solution from PVDF membrane, then add the 20 ml diluted α-p53 primary antibody solution. There is no need to rinse the PVDF membrane prior to adding antibody as long as the Blocking Solution is completely removed.
    d. Incubate membrane in primary antibody solution for 60 minutes at room temperature with rocking or shaking. Make certain that the entire PVDF membrane surface is exposed to antibody solution uniformly.
    e. Decant primary antibody solution.
    f. Wash membrane with 50-100 ml PBST for 15 minutes at room temperature using rocking or shaking. Decant wash solution.
    g. Repeat step 6f twice more, for three washes total.
    h. Dilute HRP-labeled secondary antibody (R&D Systems #HAF007, sold separately) by adding 4 µl antibody to 20 ml Antibody Dilution Buffer (1:5000 dilution). Add diluted antibody to membrane. If a different secondary antibody is utilized, follow manufacturer's guide for appropriate dilution.
    i. Incubate membrane in secondary antibody solution for 60 minutes at room temperature with rocking or shaking. Make certain that the entire PVDF membrane surface is exposed to antibody solution uniformly.
    j. Wash membrane with 50-100 ml PBST for 15 minutes at room temperature using rocking or shaking. Decant wash solution.
    k. Repeat step 6j twice more, for three washes total.
    l. Membrane is now ready for ECL detection.

7. ECL Detection

    Chemiluminescence reagents for detecting reaction 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, mono-ubiquitination of di-SUMO3 is easily observed with film exposures of 10 seconds or less.

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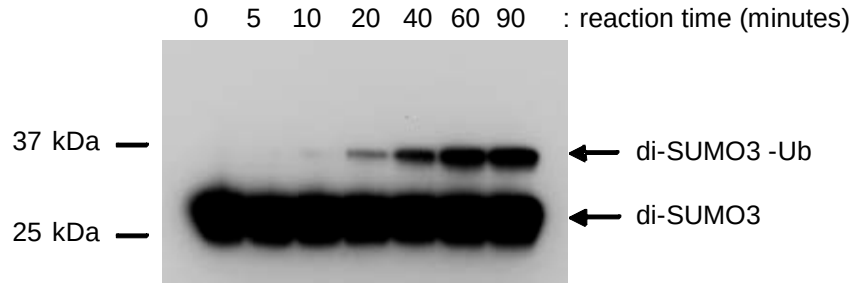
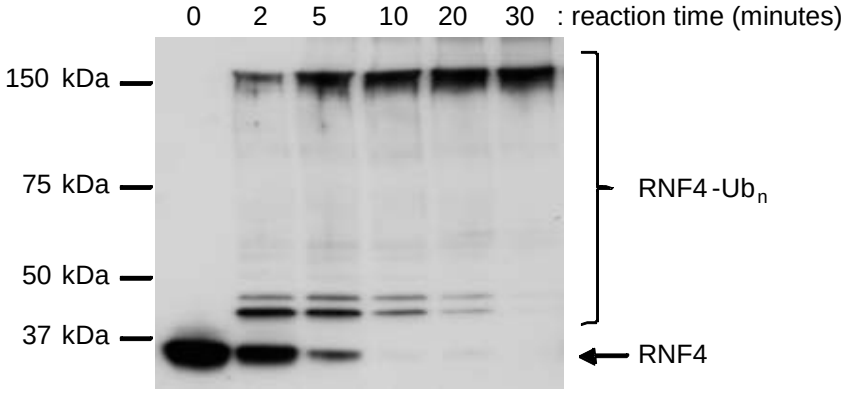
Sample Data



Ubiquitination of di-SUMO3 by the RNF4 E3 ligase

Reaction contained all components as described in the protocol, and was initiated by the addition of ubiquitin. At indicated times, an aliquot of the reaction was removed and terminated with Loading Buffer + DTT. Western blotting with α-SUMO-specific antibody was performed as described in the protocol—film exposure time was 10 seconds.



Autoubiquitination of RNF4 E3 ligase

Reaction contained all components as described in the protocol except di-SUMO3 substrate, and was initiated by the addition of ubiquitin. At indicated times, an aliquot of the reaction was removed and terminated with Loading Buffer + DTT. Western blotting with α-RNF4-specific antibody was performed—film exposure time was 30 seconds.

Literature

References:

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Hakli M., *et al.* (2004) FEBS Lett. **560:** 56-62
Tatham M.H., *et al.* (2008) Nat. Cell Biol. **10**: 538-546
Vertegaal A.C.O. (2011) Chem. Rev. **111**: 7923–7940

For help with this kit, e-mail: techsupport@bostonbiochem.com

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